Title	**Human fibroblast reprogramming: Lentiviral method (hSTEMCCA)**
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Adapted from -	Somers et al. *Stem Cells*. 2010
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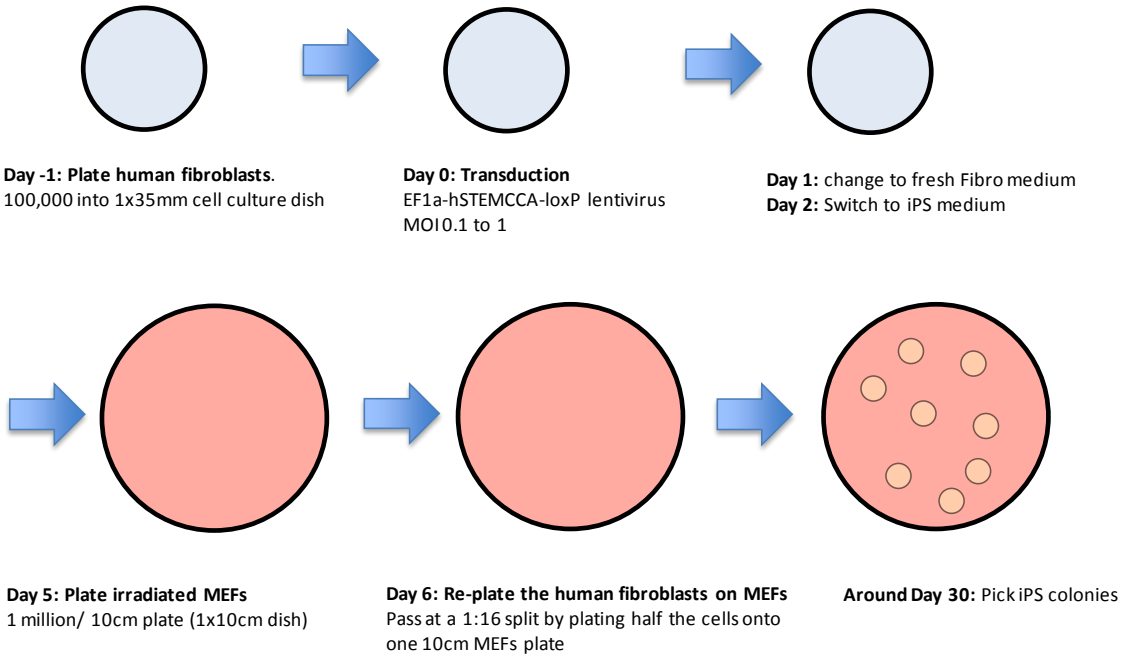
- Introduction: Induced pluripotent stem cells can be derived by over-expressing the so-called Yamanaka's factors (Oct4, Sox2, Klf4 and c-Myc) in somatic cells. In this protocol, a polycistronic lentiviral vector is used, allowing a lower number of integrations into the genome compared to the standard 4 viruses. This vector also contains loxP sites permitting the excision of the provirus after the generation of iPS lines.

- Flow Chart:



- Protocol:
  - Media Preparation

    -Media component details

bFGF	100ng/ul
Polybrene	10ug/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 plating the cells for transduction, 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.
    - Count cells

- Plate fibroblasts for transduction:

    - For each sample, plate 100,000 human dermal fibroblast cells in fibroblast medium on a gelatin coated 35mm plastic culture dish. For fresh adult cells, passage 3-4 is best and reprogramming efficiency declines with each passage.

- Transduction: VIRUS HANDLING: Follow biosafety recommendations
    - The next day, change to 1ml of fibroblast medium adding polybrene to the medium (5ug/ml)

- Add EF1a-hSTEMCCA-loxP lentivirus (4 factors, excisable vector) at a multiplicity of infection (MOI)=0.1-1.
- Example: for MOI 1, you need to add 100,000 viral particles to 100,000 cells. If your titer is $1x10^8$ particles/ml, add 1ul of virus to the cells.
- Transduce overnight (around 16 hours)
- The next day (day 1) change to fresh fibroblast medium without polybrene.
- On day 2, change medium to iPS medium.

➢ Plating MEFs, on day 5:
- Prepare 1x10cm plate for each 35mm plastic culture dish used for transduction
- 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 6:

- Remove Fibroblast medium from each well
- Wash once with PBS
- Add 1ml of 0.25% trypsin/EDTA
- Place in the incubator for 4 minutes
- Add 1 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
- Pass at a 1:16 split by plating half the cells onto one 10cm MEFs plate. Discard the other half of cells (or keep to passage if more plates/colonies are needed).

➢ Feeding:

- Refeed the cells every 2 days or so and observe occasionally to watch the reprogrammed cells changing morphology as early colonies form. Resist the urge to pick colonies prior to day 30. If dense overgrowth and too many colonies are observed then repeat the reprogramming from step 1, but passage 1:40 onto MEFs on day 6 (by discarding 80% of the fibroblasts on day 6 and passing the remainder onto a 10cm plate of feeders).

- Picking colonies:

    - Around day 30, mechanically pick colonies of good morphology with a P200 pipette. An inverted microscope placed in a sterile culture hood is recommended to visualize colonies for picking.
    - The picked colonies are placed in 1 well of a 24 or 96 well plate, pre-plated with inactivated MEF feeders on gelatin. The picked colony should be gently mechanically broken up by pipetting but kept in chunks. Do not trypsinize.
    - Each colony will be one line.
    - After outgrowth of the picked colony, passage the well to a new 24 well plate and then expand as usual.

❖ Materials:

Reagents and plastics:

Irradiated MEFs. GlobalStem cat# 6001G	1 million/10cm plate
0.1% gelatin: Millipore cat# ES-006-B	4ml/10cm plate
EF1a-hSTEMCCA-loxP lentivirus	
T25 flask	1 per sample
35mm cell culture dish	1 per fibroblast sample
10cm dish	1 per fibroblast sample
15 ml tubes	
50 ml tubes	
Pipets	

MEDIA 1: <u>MEF Media (500 ml)</u>

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

MEDIA 2: <u>Standard hiPS Media (500 ml)</u>

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:
  -In this protocol, low MOI is recommended to keep the number of integrations into the genome low and facilitate the excision of the provirus to generate transgene free iPS lines. If no changes are observed, increase the MOI.
  -It is important to split the fibroblasts 1:16 or more onto MEFs. Fibroblasts overgrowth will be observed if the dilution is incorrect.
  -Many colonies should appear on the plates 4 weeks after transduction, it is recommended to select the fully reprogrammed iPS colonies by using a TRA-1-60 Live staining.

- References:
  - Somers et al. *Stem Cells*. 2010

- Acknowledgements:
  - Dr. Gustavo Mostoslavsky for the plasmid

- Comments