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Article

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The Developmental Potential of iPSCs Is Greatly Influenced by Reprogramming Factor Selection

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Generation of SNEL-iPSCs

Important points

1. The starting cells must be in their log phase of proliferation. We are using only freshly isolated (Passage 0 or 1) cells.
2. This protocol is calibrated for mouse embryonic fibroblasts (MEFs) only.
3. The cells must express high levels of the dox trans-activator M2rtTA.
4. The vectors used to generate these cells can be found in Addgene. https://www.addgene.org/browse/article/5925/

Day 1- Transfection

1. For transfection, prepare two 10cm plates of HEK293T cells at a density of 70%. It is advisable to seed the HEK293T cells on gelatin (0.2%). This will allow them to adhere better to the plate.
2. Warm "Mirus" reagent to room temp and vortex gently (you can use any transfection reagent that you like as long as it works well for HEK293T cells).
3. Take two sterile eppendorf tubes and place 1ml of RPMI-1640 in each tube.
4. Add 40µl "Mirus" reagent to each tube.
5. Pipet gently to mix completely.

6. Add the following dox-inducible lenti-viral vectors to the diluted "Mirus" reagent according to the below concentrations and ratios (each tube will eventually get a total of 20µg of DNA):
   1st tube: 7µg of FUW-tetO-Sall4 and 3µg FUW-tetO-Lin28.
   2nd tube: 5µg of each FUW-tetO-Nanog, FUW-tetO-Esrrb.
   To each tube add 10µg of virus packaging proteins: 7.5µg psPAX2 and 2.5µg pGDM.2. Pipette gently to mix completely.
7. Incubate at room temperature for 15-30 minutes. In the meantime, aspirate medium from the 10cm HEK293T plates, and **carefully** put new 9ml of 10%FBS DMEM medium.
8. Add "Mirus"/DNA mixture drop by drop into the HEK293T plates.
9. Place the plates in the incubator at 37ºC and 5% CO_2 overnight.
10. Thaw freshly isolated MEFs, at passage 0, into two 10cm plates (the cells should be around 50-60% confluency in each plate).

1 Day post transfection:

1. Remove the media from the HEK293T plates and replace it with 10ml regular DMEM 10%FBS medium to each plate.
2. Monitor the MEFs that you thawed yesterday to make sure they are at good confluency ~70% (in order to reach about 90-100% confluency at the end of the infections).

2 Days post transfection:

1. Collect the Virus-supernatant from the two HEK293T plates into one tube (total 20ml).
2. Add Polybrene at a concentration of 8µg/ml.
3. Filter the supernatant through a 0.45um filter (this is a very important step in order to get rid of any floating HEK293T cells).
4. Add new 10ml of 10%FBS DMEM medium gently to the same plates of HEK293T cells and place back in the incubator.
5. Remove the current medium from the MEFs, and replace it with the filtered Virus- supernatant (each plate receive 10ml of filtered medium).
6. Wait at least 8 hours, and then do the same procedure of infection. Incubate the cells at 37ºC and 5% CO_2 overnight.

3 Days post transfection:

1. Collect the Virus-supernatant from the two HEK293T plates into one tube (total 20ml).
2. Add Polybrene at a concentration of 8µg/ml.

3. Filter the supernatant through a 0.45um filter.
5. Remove the current medium from the MEFs, and replace it with the filtered Virus-supernatant (each plate receive 10ml of filtered medium).
6. Wait at least 8 hours, and then remove the Virus-supernatant and add fresh 10% FBS medium to each plate. Incubate the cells at 37ºC and 5% CO2 overnight.

- **In total, the starting cells should be infected with fresh viruses 3 times.**

4 Days post transfection:

1. Replace the medium of the infected MEFs to ESC medium containing dox (2µg/ml, see list of media below).
2. Change medium **every second day** to allow proper reprogramming process.

Three to five weeks later:

Watch the cells over the next 3 weeks.

The vast majority of the cells **should not** exhibit any/robust morphological change. Keep in mind that this is a very inefficient process (much weaker than OSK reprogramming).

Very Important point- **In some cases you can observe a nice colony/ies in the plate after three weeks of dox addition, but in many other cases the plate will look empty. Following 3-5 weeks (even if you do not see any colony in the plate) remove the ESC containing dox medium and replace it with fresh ESC medium (without dox). Wait 7-10 days and scan thoroughly the plate again for colonies. In 90% of the cases 1-5 colonies will develop in the plate during the 10 days after dox removal.**

Picking Colonies:

1. Mark the colonies under light microscope.
2. Take 96-well plate and add 100µl of trypsin to each well (the amount of wells is corresponding to the amount of colonies you would like to isolate).
3. Pick the marked colonies under the Binocular using 10µl pippetor.
4. Transfer each colony into 1 well of 96-well plate containing trypsin.
5. Incubate 5 minutes in the incubator.
6. Neutralize trypsin by adding 100µl of ESC medium.
7. Pipette up and down and then transfer into one well of 6-well plate containing ESC medium and feeder.

ESC medium

DMEM

15% FBS

1% L-glutamine

1% Pen/Strep

1% Non-essential amino acids

LIF 1:1000 (we are using homemade LIF, for the exact units see the manuscript)

During reprogramming add 2 μg/ml doxycycline.

DMEM 10%FBS

DMEM

10% FBS

1% L-glutamine

1% Pen/Strep

Troubleshooting

Massive cell death is seen after the infection:

- Your HEK293 cells produce too much of viruses, infect fewer times or seed the MEFs at a higher confluency (preferable).

The conversion process is not initiated:

- As mentioned above, do not expect to see dramatic changes in the plate (this is not OSKM reprogramming).

I do not get colonies:

- Make sure your cells contain the M2rtTA cassette.
- Make sure your cells are still in their proliferation stage.
- Examine the levels of the reprogramming factors. They should be highly expressed.
- Following dox removal, one can replace the ESC containing dox medium with 2i medium (instead of regular ESC medium). This medium aids in supporting pluripotency (however, the quality of the cells was examined (in the paper) only for colonies that were developed with regular ESC medium.
- Following three weeks of dox addition, one can trypsinize the entire plate and seed all the cells on a new

plate containing feeder. This step aids as well in acquiring pluripotency. However, once again, the quality of the cells was examined (in the paper) only for colonies that were developed without this step.

Please feel free to contact our lab for further information and help.