

iPSC generation from fibroblasts using episomal plasmids

Notes

For episomal iPSC generation the PSCF uses the reagents reported by the Yamanaka lab: Okita et al. (2011). "A more efficient method to generate integration-free human iPS cells." Nat Methods 8(5): 409-412. This system consists of 4 plasmids that separately express reprogramming factors as follows:

Name	Genes expressed	Addgene cat #
pCXLE-hOCT4-shp53-F	Oct4, p53shRNA	27077
pCXLE-hSK	Sox2, Klf4	27078
pCXLE-hUL	LIN28, L-myc	27080
pCXLE-EGFP*	GFP	27082

*We include the GFP plasmid to monitor transfection efficiency and to allow visual assessment of the loss of episomal DNA in iPSCs.

- Cultured fibroblasts should be healthy, mycoplasma-free and at ~90% confluence on the day of nucleofection for optimal nucleofection and iPSC generation.
- Fibroblasts should also be proliferating robustly for optimal iPSC generation. This can be achieved by ensuring the cells are passaged after reaching 80-90% confluency and before becoming 100% confluent. We have found that replating primary fibroblasts at relatively high density (1:3 split ratio) is important to keep proliferation robust. If you replate too sparsely, you can stress the cells and they may exit cell cycle.
- If nucleofection efficiency is low you may want to repeat the nucleofection using fresh cells. You could also try plating more cells on Day 6/step 43 of the protocol so that you are seeding more nucleofected cells. Alternatively, you could attempt to optimize nucleofection conditions using Amaxa™ 4D-Nucleofector™ Optimization Protocol for Cell Lines.
- Using the Amaxa Nucleofector 4D device with X unit, you can nucleofect 1, 2 (cuvette) or 16 (strips) samples at once. The details below are for processing 1 or 2 cuvettes at a time and for nucleofection of human fibroblasts ONLY. If you wish to nucleofect other cell types or use the strip format for 16 samples at once please read the device instructions/reagent manuals.

Materials

Material	Supplier; Catalog #
Patient-derived fibroblasts for iPSC generation	N/A
P2 Primary Cell 4D-Nucleofector X kit	Lonza; V4XP-2024
Amaxa 4D Nucleofector instrument	
pCXLE-hOCT4-shp53-F plasmid DNA	Addgene; 27077
pCXLE-hSK plasmid DNA	Addgene; 27078
pCXLE-hUL plasmid DNA	Addgene; 27080
pCXLE-EGFP plasmid DNA	Addgene; 27082

0.05% Trypsin/EDTA	Life Technologies; 25300-054
MEF media	See below
10cm well tissue culture-treated dish	Fisher; 08-772E
6 well tissue culture dishes	Fisher; 14-832-11
0.1% gelatin in water	Stem Cell Technologies; 7903
Irradiated CF-1 MEFs (0.5×10^6 per cryovial)	Globalstem; GSC-6301G
hESC media + bFGF	See below

Recipes

MEF media (250mL)

Material	Supplier; Cat #	Volume (mL)	Final conc.
DMEM (+glucose/+L-Glu/-Na-pyr)	Invitrogen; 11965-092	200mL	-
Defined fetal bovine serum	Hyclone; SH30070.02	50mL	20%
100x non-essential amino acids (NEAA)	Invitrogen; 11140-050	1.25mL	1mM

* Filter sterilize and store at 4°C. Use within 2 weeks of preparation.

hESC media + bFGF (250mL)

Material	Supplier; Cat #	Volume (mL)	Final conc.
DMEM-F12 50/50 media	Life Technologies; 11330-032	200mL	-
KO replacement serum (KO-SR)	Life Technologies; 10828-028	50mL	20%
200mM L-glutamine	Life Technologies; 25030-081	1.25mL	1mM
55mM β -mercaptoethanol	Life Technologies; 21985-023	0.455mL	0.1mM
100x non-essential amino acids	Life Technologies; 11140-050	2.5mL	1x
2ug/mL Basic-FGF	Life Technologies; 13256-029	0.5mL	4ng/mL

*Filter sterilize and store at 4°C. Use within 2 weeks of preparation.

Procedure

Day 0

Pre-Nucleofection

- For each donor fibroblast sample being nucleofected place 1 x 10cm dish containing 10mL of MEF media in the incubator. It is important that the media that the nucleofected cells are plated in has had time to equilibrate to the temperature and conditions of the incubator (37°C/5% CO₂)
- Combine 1.25ug of each of the four episomal reprogramming plasmids in a sterile eppendorf tube and set aside for later (5ug total)
 - pCXLE-GFP
 - pCXLE-Oct4/p53shRNA
 - pCXLE-Sox2/Klf4
 - pCXLE-Lmyc/Lin28
- Remove Nucleofector solution and Nucleofector supplement from 4°C storage and warm to room temperature before use

Nucleofection

1. Remove donor cells to be nucleofected from the incubator and trypsinize to produce a single cell suspension. NOTE: sometimes using 0.25% trypsin/EDTA is necessary to lift all cells
2. Inactivate trypsin and count cells using a hemacytometer.
3. Add 1×10^6 cells to a 15mL Falcon tube and centrifuge at 100xg for 10min at RT.
4. In a sterile eppendorf tube combine 82uL of room temperature Nucleofector solution and 18uL of room temperature Nucleofector supplement per donor sample.
5. Carefully aspirate media from cell pellet.
6. Resuspend cell pellet in the 100uL of mixed Nucleofector solution prepared in step 4 and transfer the entire volume to the eppendorf tube containing the episomal plasmid DNA mix prepared earlier.
7. Pipette up and down twice to mix and transfer the entire volume to a cuvette supplied with the Nucleofection kit. Place the cap on the cuvette and gently tap the bottom against the bench to ensure all contents are at the bottom.

Setting up and using the Amaxa 4D Nucleofector:

8. Turn on the power to the device (on switch on back next to power cord).
9. Push touch screen down to elevate.
10. Wait for device to power up.
11. When you see *Choose a device* – touch “X”.
12. When you see *Choose a vessel* – touch the picture of the cuvettes (#1 or #2).
13. On the next screen, touch the *Choose experiment* bar.
14. On the next screen, touch *Fibroblast demo 100uL*.
15. Touch OK.
16. Touch OK again – the tray will open.
17. Place cuvette in the Amaxa 4D nucleofection device. You can nucleofect up to 2 cuvettes at once. NOTE: they will both be subjected to the same program that is suitable for fibroblasts only. Putting only one cuvette in the device is OK.
18. Press “start”. The tray will close and nucleofection is performed. This only takes 1-2 seconds. The program used in this protocol is DT -130.
19. Once complete the tray will eject.
20. Remove cuvette and allow cells to incubate in the cuvette for 10min at RT.
21. After 10min, remove the 10cm dish from incubator and transfer 500uL of equilibrated media to the cuvette.
22. Using a disposable pipette included in the nucleofection kit, *carefully* collect media and cells and transfer dropwise to the 10cm dish containing the remainder of pre-warmed media.
23. Mix by gentle swirling, and place in incubator

Post-Nucleofection

Days 1-3

24. Monitor nucleofection efficiency by fluorescence microscopy. We routinely include a GFP expressing plasmids in our nucleofections, which allows us to monitor transfection efficiency.

GFP expression should be evident on the first day post-nucleofection and is usually >75% under optimal conditions.

Day 3

25. Aspirate media from 10cm dish and replace with fresh, prewarmed MEF media.

Day 4

26. Add 2mL of 0.1% gelatin to 2 wells of a 6 well tissue culture dish per donor. We always use a separate 6 well dish for each donor.
27. Return dish to incubator.

Day 5

28. Aspirate gelatin from the 6 well dish.
29. Add 2mL of prewarmed MEF media to each well.
30. Rapidly thaw a vial of frozen MEFs.
31. Transfer thawed MEFs to a 15mL Falcon tube containing 5mL of warm MEF media.
32. Centrifuge MEFs at 200xg for 5 mins.
33. Aspirate media being careful not to disturb the pellet.
34. Add 5 mL fresh media, resuspend the cell pellet and determine the cell number using a hemacytometer.
35. Add 187,500 MEFs to both of the gelatin-coated wells in the 6 well dish.
36. Swirl the plate to evenly distribute the MEFs and return to the incubator.

Day 6

37. Aspirate media from the 10cm dish of nucleofected cells and add 10mL PBS.
38. Aspirate PBS and prepare a single cell suspension by trypsinization.
39. Inactivate trypsin by adding 5mL MEF media.
40. Centrifuge at 200xg for 5 mins.
41. Aspirate media and resuspend pellet in 5mL fresh media.
42. Count cell number using a hemacytometer.
43. Replate 0.2×10^5 to 2×10^5 nucleofected cells per well in each MEF coated well of the 6 well dish. Cells should be replated in MEF media. See NOTES about cell number.

Day 7

44. Aspirate MEF media.
45. Add 2.5mL hESC media + bFGF to wells and return to incubator.

Days 8 – Day 30

46. Aspirate spent media daily and replace with 2.5mL hESC media + bFGF.
47. Monitor cultures for colony formation.
48. When putative iPSC colonies are identified, excise colonies, expand as discrete iPSC lines and characterize. *NB: We manually passage putative iPSCs directly into mTeSR1 media in 24-well TC dishes coated with hESC-qualified matrigel (BD Biosciences; 354277)*