

iPSC generation from fibroblasts using a polycistronic lentiviral vector

Notes

- Cultured fibroblasts should be healthy, mycoplasma-free and at ~90% confluence on the day of transduction 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.
- Occasionally, we have found that using 0.25% trypsin is necessary to effectively detach fibroblasts from the TC plate.
- Polybrene only needs to be included in MEF media for the initial 3 hour transduction. Do not add it to MEF media at any other time.
- We use standard methods to generate VSV-G pseudotyped lentiviral vector using pRRL.PPT.SF.hOCT4.hKLF4.hSOX2.hcMYC.i2dTomato.pre (reprogramming factor lentiviral plasmid; Voelkel, C., et al., Protein transduction from retroviral Gag precursors. Proc Natl Acad Sci U S A, 2010. 107(17): p. 7805-10), psPAX2 plasmid (Gag/Pol expression; Addgene; 12260, and pMD2.G plasmid (VSV-G expression; Addgene; 12259)
- The following protocol uses a lentiviral vector that co-expresses dTomato. We use this track transduction efficiency and assess silencing of the reprogramming cassette. If your vector does not produce a fluorescent protein you can skip the steps below for assessing successful transduction.
- The successful reprogramming of fibroblasts depends on a number of factors. We have found that a replating density of 0.5×10^5 cells per well can result in overcrowded wells in some instances. You may want to plate fewer transduced cells in the second well (e.g. 0.5×10^5 cells) at step 31.

Materials

Material	Supplier; Catalog #
Patient derived fibroblasts	N/A
MEF media	See recipe
Polybrene (8mg/mL)	Sigma; H9268
0.05% Trypsin/EDTA	Life Technologies; 25300-054
Reprogramming lentivirus	-
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 recipe

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

Preparation

Culture patient-derived fibroblasts in MEF media (or other media previously used to culture these cells in other investigator's lab) in a 6cm TC dish until semi-confluent (~80%)

Day -1: Plate fibroblasts

1. Prepare a single cell suspension of fibroblasts by standard trypsinization.
2. Count cells and plate 2×10^5 fibroblasts per well in 2mL MEF media in 2 wells of a 6 well dish.
3. Carefully shake dish to evenly distribute cells and return dish to incubator.
4. Prepare frozen cell pellets of any excess fibroblasts not plated or needed for additional experiments and store at -80°C. These can be used later for comparison to patient-specific fibroblasts and iPSCs.

Day 0: Transduce cells

5. Remove media from cells and add 1mL fresh MEF media.
6. Add 1uL of 8mg/mL polybrene.
7. Using appropriate biosafety precautions add 20uL of Lenti-OKSM-dTomato to ONE of the 2 wells.
8. Label the transduced well "+OKSM" and the untransduced well "Mock".
9. Gently shake the dish to evenly disperse viral particles and return dish to incubator.
10. Once every 30 minutes, gently shake the dish to ensure even distribution of media.
11. After 3 hours, carefully add 1mL MEF media (w/o) polybrene to each well and return plate to incubator.

Day 1: Feed cells

12. Carefully remove media containing Lenti-OKSM-dTomato from each well and discard in bleach.
13. Add 2mL MEF media and return dish to incubator.

Day 3: Feed cells, check fluorescence and gelatin-coat TC dish

14. Check culture to ensure the cells are proliferating robustly post transduction. This is essential for efficient reprogramming.
15. Remove media from each well and discard in bleach.
16. Add 2mL MEF media.
17. View dish under inverted fluorescence microscope for expression of dTomato. Red fluorescence should be widespread within 3-4 days of transduction.
18. Return dish to incubator.
19. Prepare a 6 well TC dish by adding 2mL 0.1% gelatin to 3 wells.

Day 4: Check fluorescence and plate MEFs

20. View dish for robust proliferation and under inverted fluorescence microscope for expression of dTomato.
21. Return dish to incubator.
22. Rapidly thaw a 0.5×10^6 size vial of Globalstem irradiated MEFs.
23. Gently transfer vial contents into 5mL MEF media in a 15 mL Falcon tube.
24. Centrifuge at $300 \times g$ for 5 minutes to pellet cells.
25. Gently resuspend cells in 2mL MEF media.
26. Determine cell number using hemacytometer.
27. Aspirate gelatin from the 6 well dish and replate irradiated MEFs in MEF media at 1.875×10^5 cells per well into 3 wells. NOTE – you will need 5.625×10^5 irradiated MEFs for 3 wells. There are usually >0.5 million MEFs in one vial of GSC-6301G, so a single vial should be sufficient. If not, you will need 2 vials.
28. Carefully shake dish to evenly distribute cells and return dish to incubator.

Day 5: Replate transduced fibroblasts on MEFs

29. Prepare a single cell suspension of the mock and transduced fibroblasts by standard trypsinization.
30. Determine the cell number of each using a hemacytometer.
31. Replate 0.5×10^5 mock transduced fibroblasts in 2mL MEF media in ONE MEF-coated well and 0.5×10^5 transduced fibroblasts in 2mL MEF media in each of TWO MEF-coated wells of the 6-well MEF dish as follows:

Mock	OKSM	OKSM
empty	empty	empty

32. Carefully shake dish to evenly distribute cells and return dish to incubator.

Day 6: switch to hESC media + bFGF

33. Remove MEF media from cells and replace with 2.5mL hESC media + bFGF per well.

34. Label wells as follows:

Mock	OKSM	OKSM
empty	empty	empty

35. Return dish to incubator.

Days 7 - 28: feed daily with hESC media + bFGF and monitor for colony development

36. Remove ~90% of spent media from each well.

37. Add 2.5mL fresh hESC media + bFGF to each well.

38. Monitor for colony development using both the inverted and dissecting scopes.

39. Once putative iPSC colonies are identified (usually occurs during days 14-28) 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)