

Fibroblasts reprogramming to iPSCs	Revision Number: 1
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1. PURPOSE

To generate iPSC from fibroblasts

2. SUPPLIES

- 2.1. Fibroblast
- 2.2. Lonza P2 Kit (Lonza: V4XP-2012)
- 2.3. Gelatin (STEMCELL Technologies, NC1620050)
- 2.4. 10cm dishes
- 2.5. 6 well plates
- 2.6. 24 well plates
- 2.7. T25 flasks
- 2.8. ROCKi (S1049, Selleck Chemicals)
- 2.9. GFR Matrigel (356231, Corning)
- 2.10. mTeSR plus or Essential 8 media
- 2.11. Fibroblast media (see below for composition)
- 2.12. Lonza 4D nucleofector
- 2.13. pCXLE-hOCT3/4-shp53-F (27077, Addgene)
- 2.14. pCXLE-hSK (27078, Addgene)
- 2.15. pCXLE-hUL (27080, Addgene)
- 2.16. pCXWB-EBNA1 (37624, Addgene)
- 2.17. DMEM, high glucose (Gibco, 10564011)
- 2.18. MEM Non-Essential Amino Acids (Gibco, 11140050)
- 2.19. Glutamax (Gibco, 35-050-061)
- 2.20. Pen/Strep (Hyclone, SV30010)
- 2.21. Fetal Bovine Serum (Gibco, A5256801)
- 2.22. ReLeSR (STEMCELL Technologies, 05872)
- 2.23. Accutase (STEMCELL Technologies, 07922)
- 2.24. PBS (Gibco, 10010023)

1. SCOPE

This procedure applies to fibroblasts to be reprogrammed into iPSCs using nucleofection of the episomal vectors OCT3/4, shRNA against p53, SOX2, KLF4, L-MYC, LIN28, and EBNA1.

2. PROCEDURE

- 2.1. Coat T25 flasks with gelatin and incubate at 37C for at least 15 minutes at room temperature
- 2.2. Prepare 100ml of fibroblast media (good for two weeks)

Reagent	Final Concentration	Volume
DMEM		87ml
Glutamax	1x	1ml
Pen/Strep	1x	1ml
Non-essential amino acid	1x	1ml
FBS	10%	10ml

- 2.3. After 15 minutes of incubating T25 flask, aspirate the gelatin and add 3ml of fibroblast media
- 2.4. Thaw fibroblast and transfer into a 15ml conical tube with 3ml of fibroblast media
- 2.5. Centrifuge conical tube at 300g for 5 minutes
- 2.6. Aspirate supernatant and resuspend cell pellet with 1ml of fibroblast media using a P1000
- 2.7. Transfer the 1ml of cell suspension to the T25 flask. Rock back and forth, side to side, before placing in incubator.
- 2.8. Change media daily and passage twice before transfecting
- 2.9. After the second passage, culture fibroblasts until 70% confluency
- 2.10. On the day of transfection (Day 0), coat one well of a 6 well plate with gelatin and incubate at room temperature for at least 15 minutes
- 2.11. Prepare nucleofection buffer (Cat# PBP2-00675) for each condition

Reagents	Volume (for one condition)
P2 buffer	82 uL
Supplement 1	18 uL

- 2.12. Add 2 ug of each reprogramming plasmid keeping the total volume of plasmids under 5ul into the 100ul of nucleofection buffer
- 2.13. Turn on nucleofector
- 2.14. Disassociate fibroblasts by adding Accutase and incubate for 5 minutes
- 2.15. Add equal volume of media or PBS and transfer cell suspension into 15ml conical
- 2.16. Centrifuge at 300g for 5 minutes
- 2.17. Remove supernatant and resuspend in 4ml media fibroblasts media.
- 2.18. Count cells and transfer suspension containing 500,000 cells into an Eppendorf
- 2.19. Centrifuge at 300g for 5 minutes
- 2.20. Remove supernatant and resuspend cell pellet in nucleofection buffer with plasmids
- 2.21. Transfer cell suspension into P2 cuvette (Cat# V4XP-2012)
- 2.22. Set up nucleofector by putting cuvette as the vessel, P2 as the buffer, and DT-130 as the program
- 2.23. After nucleofecting the cells, transfer fibroblasts into a 6 well plate with fibroblasts media.
- 2.24. Rock the dish back and forth, side to side then place in incubator at 37C overnight
- 2.25. Change media with fibroblast media every day for the next 6 days
- 2.26. **If using feeders:** On day 5 seed 3e6 feeder cells per 10cm dish (gelatin coated) in fibroblast media.
- 2.27. **If using feeder free conditions:** On day 6 from nucleofection (Day 0), coat a 10cm dish with GFR Matrigel.
- 2.28. On day 6 from nucleofection (Day 0), prepare 1:1 media with half fibroblast media and half mTeSR Plus with ROCKi.

- 2.29.** On day 6 after nucleofection (Day 0), disassociate cells from a well of the 6 well plate using Accutase, count and seed 10,000 cells into one 10cm dish either coated with GFR Matrigel or into the 10cm dish with feeders in half fibroblasts and half mTeSR Plus media with Rocki. Make sure the cells are single cell before plating.
- 2.30.** Following day change media to complete mTeSR Plus media.
- 2.31.** Continue feeding every day or every other day with mTeSR Plus media until colonies become visible.
- 2.32.** Around Day 14 from nucleofection, colonies become visible
- 2.33.** When colonies are ready to be picked, coat a 24 well plate with GFR Matrigel 1 hour prior to picking
- 2.34.** When plate have been coated for at least an hour, remove GFR Matrigel and add mTeSR Plus with ROCKi (10uM final concentration) to each of the wells
- 2.35.** Set P200 to 50ul and pick one colony and transfer into one well of 24 well plate. We pick 24 colonies.
- 2.36.** Place the 24 well plate into the incubator at 37C overnight
- 2.37.** Change the media the next day with mTeSR plus without ROCKi
- 2.38.** Change media every day or every other day until colony are ready to passage.
- 2.39.** When colonies are ready to be clump passaged, coat a 12 well plate with GFR Matrigel 1 hour prior to clump passaging
- 2.40.** When plate have been coated for at least an hour, remove GFR Matrigel and add mTeSR Plus with ROCKi (10uM final concentration) to each of the wells in a 12 well plate that are coated with GFR Matrigel
- 2.41.** Select 12 wells that have colonies with good morphology and wash with PBS then aspirate
- 2.42.** Add ReLeSR into each of the 12 wells selected then aspirate immediately
- 2.43.** Incubate the plate at 37C for 5 minutes
- 2.44.** Add mTeSR plus media with ROCKi (10uM final concentration) to each of the selected wells
- 2.45.** Using a P1000, pipet cell suspension up and down to break up the clumps into smaller clumps and transfer the selected wells from the 24 well plate into the 12 well plate
- 2.46.** Place plates back into the incubator at 37C overnight
- 2.47.** Change media for the 12 well plate the next day with mTeSR plus without ROCKi.
- 2.48.** Change media every day or every other day
- 2.49.** Continue culturing until 70% confluency and passage into a 6 well plate.
- 2.50.** Passage iPSCs at least 5 times before freezing

Notes:

1. Always choose colonies with good morphology for picking
2. If there are differentiated cells between colonies of good morphology perform repicking of colonies to get rid of differentiated cells.
3. When cells are plated in 10cm dishes for colony picking ensure that cells are single cells
4. After iPSC derivation make sure to characterize iPSCs for pluripotent status and genomic integrity.