

Salk Stem Cell Core Protocol for Reprogramming Fibroblasts using Sendai Virus

Purpose: To reprogram human dermal fibroblasts with Sendai Virus.

Materials:

TrypLE

Fibroblast Media (a.k.a. 20/20) (recipe at end)

CytoTune 2.0 Sendai virus kit (Life Tech: A16517)

Matrigel/Cultrex

DMEM/F12

iPS Brew with supplement (Miltenyi 130-104-368)

sodium butyrate (StemCell Technologies 72242; 0.5M in DMSO=1000x)

WNT inhibitor, XAV939 (R&D Systems 3748/10)

Rock Inhibitor, Y-27632 in DMSO

DPBS -/-

CellStripper (Corning 25-056-CI) or Versene (Life Tech 15040-066)

Procedure:

Day -1:

- Split cells using TrypLE. Make sure cells are 80% or more confluent before splitting (easiest to do this as primary fibroblasts are banked)
- Seed 125K fibroblasts in each well of a 12-well plate coated with 0.1% gelatin (1mL gelatin per 12-well for 1 hour). (2 wells/line)
- Culture cells at 37°C overnight

Day 0:

- Prepare SeV aliquots according to the production lot spec sheet (for Lot #1304456A: [\(1304456A.pdf\)](#)
 - SeV factor volumes indicated on spec sheet are typically used to infect 4 fibroblast lines (500K cells at an MOI of 3), but we use 125k cells per line and the number of lines is determined by the titer of the lot, keeping the MOI at 5:5:3.
 - Freeze aliquots at -80°C in microcentrifuge tubes, can usually prepare about 18 aliquots per kit
- Aspirate media from fibroblasts.
- Add 500µL fibroblast media (20/20) to each frozen aliquot to thaw and mix well; add to fibroblasts in well (1 aliquot/well)
- Incubate at 37°C for exactly 24 hours.
- Bleach all tubes and pipette tips that have come into contact with virus (10% bleach; minimum 30 min) before discarding to dry waste

(10/17/13 - modified from Harvard Stem Cell Core's protocol)

(8/19/16 – Edited CD)

(Updated 12/15/23 J. Page)

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Day 1

- Refresh culture with fibroblast media. DO NOT aspirate media containing virus; pipette off carefully and discard to 10% bleach.
- Replace with 1.5mL/well fibroblast media (20/20)

Day 2 -

- Supplement 20/20 with sodium butyrate (NaB) at 0.5 μ M final concentration
 - NaB is an inhibitor of histone deacetylases and facilitates chromatin remodeling during reprogramming¹
- If cells reach below ~50% confluency, consider increasing the FBS to 20% and/or increasing the FGF2 to 20ng/mL.

Day ~5-8

- Once cells have gone through a die-off phase (does not always happen), a morphology change (less elongated, more diamond shaped) and repopulated the well to a dense confluency (~100%), TrypLE-dissociate transduced fibroblasts and seed them onto 60mm plates coated with Cultrex at a density of 60k-120k cells/plate.
- Supplement iPS Brew with NaB at same final concentration.
 - **[NOTE: it is better to wait to passage cells until they are very confluent than passage too early while there are fewer cells].**
- A range of densities is advised; 60K, 90K, and 120K. Easiest to control by counting cells, spin, then resuspend to 10⁶/mL. Serial dilute 1:5 twice for final density of 40K cells/mL. Seed as previously stated in 5mL iPS Brew + NaB/plate

Day 8-11

- Replenish with iPS Brew + NaB every 48 hours.

Day 12-21+

- Replenish with iPS Brew (no NaB) every 48 hours
- If colonies begin to get very large and use up the media (on the 120K plate mostly), feed daily

Day 21+

Authentic colonies will emerge after day 21. Therefore, pick colonies during the 4th week post infection.

- Pick each single iPSC colony into 1 Cultrex coated well of a 12 well plate with iPS Brew + Rock Inhibitor.
 - Prior to picking, change media in 60mm plate to iPS Brew+ ROCKi
 - Use P20 set to maximum volume to gently break up colonies to smaller clumps; distribute evenly in 1w/12w
 - Change media in 60mm plates back to regular iPS Brew when finished; retain plates until clones have been frozen in case additional colonies are needed
 - Typically, more than 12 clones is unnecessary; 1 full 12-well plate

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- Leave colonies in ROCKi 48 hours before changing media to allow clumps to attach; less time in ROCKi results in slower outgrowth

At this point, switch over to iPSC QC protocol

First split

- Approximately 1 week after picking, the clones will be ready to split (varies by clone)
- Prepare 6-well plates coated with Cultrex, 1mL/well (need 2 plates/line)
 - Use marking objective to circle any areas of differentiation in the clone
 - Remove differentiation by aspiration while removing media
 - Wash wells with 1mL each DPBS -/-
 - Add 0.5mL/well CellStripper or Versene. Incubate 37°C 5 min. Versene may take longer; CellStripper is fast but gentle
 - Aspirate CellStripper; avoid colonies
 - Pick up clones (requires gentle scraping with pipette) in 1mL iPS Brew Supplemented with ROCKi and WNTi (a.k.a. XY-PS Brew), transfer to prepared 1w/6w also containing 1mL XY-PS Brew
 - WNTi XAV939 helps prevent spontaneous differentiation²
 - Clones are P1
- 24 hours after split, change media to iPS Brew without ROCKi or WNTi
- When 80-90% confluent, split to 1 new well of a 6-well plate each; adjusting cell seeding to standardize cell density (P2)
- When 80-90% confluent, freeze each clone to 3 vials and split 20% to 1 well/6-well
 - For KT: also split ¼ of these to new 1w/6w (P2). Wash and dissociate clone with CellStripper. After incubation, collect cells in 1mL iPS Brew (no inhibitors needed). Transfer 0.25mL cells to new 1w/6w in iPS Brew (P2); continue culturing to P4
 - To remaining 0.75mL, add 0.75mL 40% iPS Brew+ 60% KOSR (final is 30% KOSR)
 - Add 1.5mL 2x iPSC Freezing media (50% iPS Brew+ 30% KOSR+20% DMSO) (final is 60% iPS Brew+30%KOSR+10%DMSO)
 - Mix gently and transfer to 3 cryovials
- When P4 clones are confluent, split to 1w/6w and 1w/12w each (scale all volumes accordingly).

Troubleshooting and Expansion:

- Be sure to carefully mark and aspirate differentiation before all passages but be particularly vigilant when passaging out of the 12 well plate. Differentiation not removed during that passage tends to make cleaning up the cell line later much more difficult.
- Clones may be split with ReLeSR (Stem Cell Technologies, cat# 100-0483) to remove patches of differentiation during splitting. (It may not work at 1st split as the colonies

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typically require manual scraping to lift off bottom of well.) If using ReLeSR, wash with PBS as usual, then add ReLeSR at same volume as with Versene or CellStripper. Immediately aspirate ReLeSR and incubate at RT ~5 min. Firmly knock plate on bench to help dissociate colonies. Carefully wash colonies off plate with fresh media. Differentiated cells will remain adhered to the plate and only undifferentiated cells are passaged. If you let it go too long, differentiated cells will lift too. Optimal incubation time may vary by cell line.

Fibroblast Media (a.k.a. 20/20)-for 1L

DMEM 821mL

FBS 150mL

NEAA 10mL

GlutaMax 10mL

Anti-anti 10mL

FGF-2 (50µg/mL) 400µL

Filter through 0.1µm pore; aliquot to 100mL bottles, freeze as needed

References

1. Mali, P. *et al.* Butyrate greatly enhances derivation of human induced pluripotent stem cells by promoting epigenetic remodeling and the expression of pluripotency-associated genes. *Stem Cells* **28**, 713–720 (2010).
2. Roodgar, M. *et al.* Chimpanzee and pig-tailed macaque iPSCs: Improved culture and generation of primate cross-species embryos. *Cell Rep* **40**, (2022).