Title	iPSC derivation from fibroblast in chemically defined medium
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❖ Introduction:

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have the potential to become the source materials for cell-based therapy, so the quality of the stem cells has great impact on how the cells could be utilized in future applications. Derivation and maintenance conditions have critical role determining the iPSC quality, mainly due to the involvement of animal products and feeder cells. We developed a simple procedure to derive and maintain hiPSCs in chemically defined media.

Here we describe how to derive hiPSCs in E8 based growth conditions. This method has been successfully used on fibroblast, pre-adipocyte and HUVEC. The procedure can be adapted to most common reprogramming methods, such as Lentivirus, Episomal DNA and Sendai Virus. In companion with E8 medium and vitronectin (or synthetic surface), human pluripotent stem cells could be maintained in an enzyme-free, xeno-fee and chemically defined environment. To simplify the description, we use Sendai Virus to reprogram fibroblast, and use matrigel as the coating surface for cell culture in this protocol.

❖ Protocol:

A. Plate coating.

1. Pour cold 12 ml of DMEM/F12 in conical tube, and use 1.5 ml to resuspend 2mg frozen matrigel with 5ml pipet. Matrigel should be in freezer right before the experiment.
2. Rinse the matrigel tube again with the same media.
3. Mix the matrigel well, plate 1ml in each well, and shake well to cover all the surface.

4. Leave the plate at room temperature or 37°C for at least 30 minutes. Or coat overnight at 4°C.

B. EDTA dissociation buffer: for iPSCs

1. Add 500ul 0.5M EDTA and 0.9g NaCl into 500ml Calcium/Magnesium free PBS (Invitrogen # 14190).
2. Sterilize by filtration, and store at 4°C.

D. Reprogramming with Sendai Virus.

1- **Day 0** - Take low passage # fibroblast culture and plate in 1 well of a 6 well dish so that cells will be about 80% confluent next day (100K or 150K cells/well) in fibroblast media (usually 10% FBS and Pen/Strep,L-glut, NEAA in DMEM).

2- **Day 1** – Thaw the four Sendai Viruses on ice, mix together, and drop-wisely add them to the cells. Incubate at 37°C.

3- **Day 2** - If cells look good in the morning and nearly confluent, prepare plates for splitting. Coat 6 well plates with Matrigel.

4- **Still Day 2** - Pass reprogramming well using TryPLE (Note, some lines do not come off plate well with TryPLE, with those lines, you may want to wash plate 2x with EDTA before adding TryPLE). Incubate 5 min in incubator, then wash off plate and dilute with fibroblast media.

5- Spin cells down and resuspend in fibroblast media. Plate on Matrigel (1 well of infected cells into 2x6 well plates coated with matrigel), also in Reprogramming Medium I.

6- Keep cells in Reprogramming Medium I, feeding them every other day for 3-5 days.

7- **Day 5 or 7 (approximately)** - change media to Reprogramming Medium II, and 100 μM Sodium Butyrate can be added to improve reprogramming efficiency.

8- Continue feeding every other day.

9- Depending on cell density and the appearance of any iPS colonies in the reprogramming plates, cells may need to be passed with EDTA at some point in the next 2 weeks. Also, it may be necessary to start feeding E8 medium without sodium butyrate.

10- **Around Day 20-25**, colonies should be ready to try picking. Around this time original reprogramming plates should begin being fed normal E8 media (with TGFβ1) daily, if they haven't been switched to this already.

11- For picking---prep a 24 well plate by coating with Matrigel. After coating, add E8 (TGFβ1 media) with 1x Rock inhibitor added to each well. Spray microscope and surrounding area as well as pipet and box of tips with alcohol. Wear face mask. Find colonies under scope with 4X objective. Using

P20 with tip, circle around colony until it is loosened from surrounding cells. Also using tips, cross hatch the colony so it will come off plate in smaller pieces. Then use pipet to push colony off plate and suck it into pipette. Transfer colony pieces into 1 well on the 24 well plate. Repeat with other colonies. The day after picking, change media to E8 (TGFb1) without rock inhibitor. Feed daily until colony is big enough to pass.

12- When colony is ready to pass, use EDTA to pass and leave some of the cells in the original well of the 24 well while transferring most to a new Matrigel coated well on a 12 or 6 well plate. (in Rock inhibitor).

13- When expanding each colony, keep track of passage number (picking into 24 well is passage 1). Once you have a well in a 6 well ready to pass, freeze 2 vials down and leave some cells in the well to continue to grow them.

14- My usual order for cells is –expand and freeze 2 vials from first well. Expand well on next passage to 2-3 wells. Freeze 4-6 vials when ready, and continue growing cells. Freeze at least 6 vial, up to 10 for each clone. During one passage, pass a small amount onto 12 well plate for APS staining. After getting enough cells for freezing, continue growing to harvest some for FACS staining.

❖ Materials:

- Cell Materials

Human Fibroblasts
Human iPSCs

- Cell culture media

- Fibroblast medium: 10% Fetal Bovine Serum in DMEM, 1x Non-essential amino acid.
- Basic chemically defined reprogramming medium I: DMEM/F12, 64mg / L L-Ascorbic acid 2-phosphate magnesium salt, 14 µg /L Sodium Selenite, 10.7 mg / L Holo-transferrin 10.7 mg / L , 100 µg / L basic FGF, 20 mg / L Insulin and 1 µM Hydrocortisone. Adjust to pH7.4 with 340 mOsm osmolality.
- Basic chemically defined reprogramming medium II: DMEM/F12, 64mg / L L-Ascorbic acid 2-phosphate magnesium salt, 14 µg /L Sodium Selenite, 10.7 mg / L Holo-transferrin 10.7 mg / L , 100 µg / L basic FGF, and 20 mg / L Insulin and. Adjust to pH7.4 with 340 mOsm osmolality. 100 µM Sodium Butyrate can be added to improve reprogramming efficiency.
- Chemically defined human ESC/iPSC E8 medium: DMEM/F12, 64mg / L L-Ascorbic acid 2-phosphate magnesium salt, 14 µg /L Sodium Selenite,

10.7 mg / L Holo-transferrin 10.7 mg / L , 100 μg / L basic FGF, 1.8 μg / L TGFβ1, 20 mg / L Insulin. Adjust to pH7.4 with 340 mOsm osmolality.

- EDTA/PBS (1000ml)

Ingredient	Amount	Company	Catalog#
PBS	500ml	Life Technology	14190-250
0.5M EDTA	0.5ml	K.D. Biomedical	RGF3130
NaCl	0.9g	Sigma	5886

- Medium Reagents

Ingredient	**Company**	**Catalog#**
DMEM/F12	Life Technology	11330
L-Ascorbic acid 2-phosphate magnesium salt	Sigma	A8960
Sodium Selenite	Sigma	S5261
Sodium Chloride	Sigma	S5886
Holotransferrin	Sigma	T0665
Basic FGF	Peprotech	100-18B
TGFβ1	R&D Systems	240-B/CF
Insulin	Sigma	I9278
Hydrocortisone	Sigma	H0396
Sodium Butyrate	Sigma	B5587

- STUFF

Inverted microscope (i.e., Nikon TE or Olympus IX or Zeiss Promo Vert)
Biosafety cabinet for cell culture
CO_2 incubator with controlling and monitoring system for CO_2, humidity and temperature
Cell culture disposables: Tissue culture dishes, centrifuge tubes, pipettes, pipette tips, cell strainer etc.

❖ Troubleshooting:

- Xeno-free condition for iPSC derivation
  - Matrigel could be replaced with vitronectin recombinant protein.
  - FBS-containing fibroblast medium could be replaced with defined fibroblast medium.
- Low reprogramming efficiency
  - Use sodium butyrate to improve the efficiency.
  - Initial fibroblasts are not actively proliferating or at high passage. Increase the experiment scale, and use more starting cells and viruses.

❖ References:

1. Chen G, Gulbranson D, etc. Chemically defined conditions for human iPS cell derivation and culture. *Nature Methods. 2011 April 10; 8(4):424-429.*