iPS Cell Induction from Human Non-T, B Cells from Peripheral Blood

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[Abstract] The generation of iPS cells gives an opportunity to use patient-specific somatic cells which are a valuable source for disease modeling and drug discovery. To promote these studies, it is important to make iPS cells from easily accessible and less invasive tissues like blood. Here, we describe the basic method to generate human iPS cells from adult peripheral blood. After the isolation of mononuclear cells, a combination of cytokines stimulates the expansion of hematopoietic stem/progenitor population, which is the main target of this protocol. The cells are transduced with plasmid mixture encoding reprogramming factors. In most cases, the plasmids are lost during the establishment of iPS clones.

Materials and Reagents

1. Fresh anti-coagulated blood (~10 ml)
2. PBS without Ca^{2+} and Mg^{2+} (Nacalai tesque, catalog number: 14249-95)
3. Ficoll-paque Plus (GE Healthcare, catalog number: 17-1440-02)
4. StemSpan H3000 (STEMCELL Technologies, catalog number: 09800)
5. Recombinant human Interleukin (IL)-6 (100 μg/ml) (PeproTech, catalog number: AF-200-06)
6. Recombinant human Stem Cell Factor (SCF) (300 μg/ml) (PeproTech, catalog number: AF-300-07)
7. Recombinant human Thrombopoietin (TPO) (300 μg/ml) (PeproTech, catalog number: AF-300-18)
8. Recombinant human Flt3 ligand (300 μg/ml) (PeproTech, catalog number: AF-300-19)
9. Recombinant human Interleukin (IL)-3 (10 μg/ml) (PeproTech, catalog number: AF-200-03)
10. Recombinant human basic Fibroblast Growth Factor (bFGF) (10 μg/ml) (Wako, catalog number: 064-04541)
11. Amaxa Human $CD34^+$ cell Nucleofector Kit (Lonza, catalog number: VPA-1003)
12. MEF feeder (Repro Cell, catalog number: RCHEFC003)
13. Matrigel growth factor reduced (BD Biosciences, catalog number: 356231)

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14. Primate ES Cell Medium (Repro Cell, catalog number: RCHEMD001)
15. Essential 6 (Life Technologies, catalog number: A1516401)
16. Gelatin (Sigma-Aldrich, catalog number: G1890)
17. Dulbecco's Modified Eagle's Medium (DMEM) High glucose with stable L-glutamine (Nacalai tesque, catalog number: 08459-35)
18. Fetal Bovine Serum (FBS) (Life Technologies, catalog number: 10437-028)
19. Plasmid set 2 (Life Technologies, catalog number: A15960)
20. Blood medium (see Recipes)
21. Plasmid mixture (see Recipes)
22. Transfection mixture (see Recipes)
23. iPS medium 1 (see Recipes)
24. iPS medium 2 (see Recipes)
25. 0.1% Gelatin solution (see Recipes)
26. MEF medium (see Recipes)

Equipment

1. Nucleofector 2b (Lonza, catalog number: AAB-1001)
2. 6-well tissue culture plate (BD Biosciences, Falcon®, catalog number: 353046)
3. 15 ml conical tube (BD Biosciences, Falcon®, catalog number: 352196)
4. 50 ml conical tube (BD Biosciences, Falcon®, catalog number: 352070)
5. 37 °C 5% CO_2 Cell culture incubator
6. Microscope
7. Centrifuge

Procedure

Day 0

1. Culture medium preparation:
   a. Add 2 ml of blood medium containing cytokines to a well of 6-well plate.
   b. Add 2 ml of PBS in the remaining wells of the plate to prevent excess evaporation of the medium and store the plate at 37 °C, 5% CO_2.
2. Purification of mononuclear cells:
   a. To 10 ml of anti-coagulated blood (EDTA), add 10 ml of PBS.
   b. In two 15 ml tubes, add 5 ml Ficoll-Paque and gently add 10 ml of blood + PBS.
   c. Spin tubes at 400 *x g* for 30 min at 18 °C. Use slow acceleration and slow brake.
   d. Remove the plasma (around 2 ml) for the top fraction without disrupting the

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mononuclear cells at the interface.

e. Transfer the cells at interface (1-2 ml) to a 15 ml tube and mix well with 12 ml of PBS.
f. Spin at 200 *x g* for 10 min at 18 °C (slow brake).
g. Resuspend cells in 3 ml of H3000 medium and count cells.
h. Prepare aliquots of 3 x 10^6 cells in 1.5 ml tube.
i. Spin at 200 *x g* for 10 min at 18 °C (slow brake).
j. Aspirate supernatant.

3. Plating:
   a. Resuspend the cells in the medium prepared in step 1.
   b. Store the plate at 37 °C, 5% CO_2 for around 6 days. Medium change is not needed during this culture period.

Day 5

1. Gelatin coat:
   a. Add 1 ml/well of 0.1% gelatin solution to a 6-well plate.
   b. Incubate the plate for at least half an hour at 37 °C.
2. Preparation of MEF feeder cells:
   a. Thaw MEF feeder cells at 37 °C and count the cell number.
   b. Aspirate excess gelatin solution prepared above.
   c. Seed the MEF feeder cells at 3 x 10^5 cell/well in MEF medium.

Day 6

1. Culture medium preparation:
   a. Aspirate the medium from MEF feeder cells.
   b. Add 2 ml/well of blood medium containing cytokines to the plate.
2. Harvest the cultured cells:
   a. Resuspend the cells in the medium and count cells. Number of live cells is usually around 1 x 10^6.
   b. Harvest the floating cells into 15 ml tube.
   c. Spin at 200 *x g* for 10 min at 18 °C (slow brake). During spin, prepare transfection mixture.
3. Nucleofection:
   a. Aspirate the supernatant of the cells completely by hand using a pipette.
   b. Add transfection mixture and suspend cells, be careful not to create any bubbles.
   c. Perform nucleofection using program U-008.
4. Plating, 10^5 to 10^4 cells per well:
   a. Immediately following nucleofection, add 800 µl of H3000 to the electroporation

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cuvette, and harvest the cells. Metal ions in the nucleofection solution are toxic to cells!

b. Plate the cells to MEF feeder plate ranging from 5 x 10^5 cells to 5 x 10^4 cells per well.

Day 8, 10, and 12

1. Add additional 1.5 ml of iPS medium 1 per well. Do not aspirate the existing medium.

Day 14-

1. Replace medium with 1.5 ml of iPS medium 1 per well.
   * Medium replacement is performed every 2 days.

Day 25 to 35

1. Pick colonies of about 2 mm diameter.

Notes

1. Frozen peripheral blood mononuclear cells (PBMC) can be used with this protocol. Culture the thawed PBMC directly in blood medium on day 0.
2. iPSCs can be established in non-feeder condition, but the efficiency is low. For non-feeder condition, use Matrigel coated plate and iPS medium 2 instead of MEF feeder and iPS medium 1. Followings are the procedure to make Matrigel coated plate.
   a. Thaw Matrigel at 4 °C and dilute it equivalent to 2 mg of protein in 6 ml of cooled DMEM.
   b. Add 1 ml/well of the solution to a 6-well plate.
   c. Store at RT for 1 h.
   d. Wash the plate once with PBS before cell seeding.

Recipes

1. Blood medium
   StemSpan H3000, containing following cytokines:
   10 ng/ml IL-3
   100 ng/ml IL-6
   300 ng/ml SCF
   300 ng/ml TPO
   300 ng/ml Flt3 ligand
   Use immediately after preparation

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2. Plasmid mixture (Addgene, http://www.addgene.org/Shinya_Yamanaka)
   Use following plasmid mixtures. Set 1 shows higher efficiency. In set 2, we omitted WPRE sequence and replaced shRNA against p53 with dominant negative form of mouse p53, which exist in set 1. Store at -20 °C. Set 2 can be purchased from Life technologies.

Plasmid set 1	pCXLE-hOCT3/4-shp53-F	0.83 g
	pCXLE-hSK	0.83 g
	pCXLE-hUL	0.83 g
	pCXWB-EBNA1	0.5 g
Plasmid set 2	pCE-hOCT3/4	0.63 g
	pCE-hSK	0.63 g
	pCE-hUL	0.63 g
	pCE-mp53DD	0.63 g
	pCXB-EBNA1	0.5 g

3. Transfection mixture
   Amaxa CD34 Solution 81.8 µl
   Supplement 18.2 µl
   Plasmid mixture (set 1 or 2) 3 µl (3 µg)
   Use immediately after preparation
4. iPS medium 1
   Primate ES Cell Medium containing 4 ng/ml bFGF
   Store at 4 °C for 1 week in dark
5. iPS medium 2
   Essential 6 containing 100 ng/ml bFGF
   Store at 4 °C for 1 week in dark
6. 0.1% (w/v) Gelatin solution
   a. Dissolve 1 g gelatin powder in 100 ml dH_2O (1% w/v, 10x concentration), autoclave and store at 4 °C.
   b. To prepare 0.1% (1x) gelatin solution, warm the 10 x gelatin stock at 37 °C, add 50 ml of this to 450 ml dH_2O. Filter the solution with a bottle-top filter (0.22 µm) and store at 4 °C.
7. MEF medium
   DMEM containing 10% FBS

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