Title	**iPSC Reprogramming From Human Peripheral Blood Using Sendai Virus Mediated Gene Transfer**
Date Submitted	April 26, 2012
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Adapted from -	UPenn iPSC Core and CHOP iPSC Core in-house protocols
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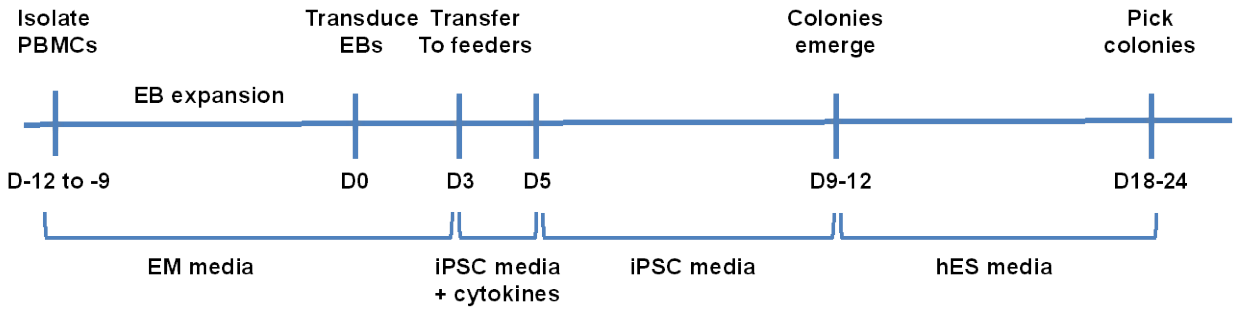
Introduction

This protocol allows efficient generation of integration-free iPS cells from a small amount of peripheral blood (less than 1 ml). Peripheral blood mononuclear cells (PBMCs) are briefly cultured to expand the erythroblast (EB) population. They are then used to derive iPS cells using four recombinant Sendai viral vectors, expressing the four reprogramming factors Oct4, Sox2, Kfl4 and c-Myc.

Flow Chart



Materials and Preparation

Reagents	**Supplier**	**Catalogue number**
QBSF-60	Quality Biologicals	160-204-101
Primocin	Invivogen	ant-pm-1
Pen/Strep	Life Technologies	15140-155
DMEM (high glucose)	Life Technologies	11965-118
Non-Essential Amino Acid (MEM-NEAA)	Life Technologies	11140-050
L-Glutamine	Life Technologies	25030-156
Cytotune™- iPS Sendai Reprogramming kit		A1378001
DMEM/F12	Life Technologies	11330

2-Mercaptoetheranol	Sigma	M7522 -100ml
b-FGF	Life Technologies	PHG0021
Knockout Serum Replacement (KOSR)	Life Technologies	10828
FBS	Life Technologies	16000-044
Define FBS	Hyclone	SH30070.01
MEF feeders	Global Stem	6001G
0.1% geletin	Millipore	ES-006-B
Recombinant human EPO (tissue culture grade)	R&D Systems	287-TC-500
Recombinant human IL-3, CF	R&D Systems	203-IL-010/CF
Recombinant human IGF-1, CF	R&D Systems	291-G1-200
Recombinant human SCF, CF	R&D Systems	255-SC-010/CF
Dexamethasone	Sigma	D8893-1MG
L-Ascorbic Acid	Sigma	A4544-25G

Expansion Medium (EM)*	**Stock conc**	**Final Conc**	**Volume**
QBSF-60 (serum free medium)			10 mL
(Antibiotics)			
Primocin	500x	100 μg /mL	20 μL
Pen/Strep	100x	1%	100 μL
Ascorbic Acid (AA)	10 mg/mL	50 μg /mL	50 μL
(Growth factors)			
SCF	50 μg/mL	50 ng/mL	10 μL
IL-3	10 μg/mL	10 ng/mL	10 μL
EPO	2 U/μL	2 U/mL	10 μL
IGF-1	100 μg/mL	40 ng/mL	4 μL
Dexamethasone**	1mM	1 μM	10 μL

*EM = QBSF (base media) + AA + growth factors
EM+P/S = QBSF (base media) + P/S + AA + growth factors
EM+Primocin = QBSF + primocin + AA + growth factors
**Keep dexamethasone and doxycycline protected from light (discard every 2 weeks)

MEF media (500 ml)
DMEM (high glucose): 450 ml
FBS: 50 ml
NEAA: 5 ml
L-glutamine
Pen/Strep: 5 ml

iPSC Media (500 ml)
DMEM/F12: 450 ml
Define FBS: 50 ml
NEAA: 5 ml
L-glutamine: 5ml

Pen/strep: 5 ml
2-mercaptoethonol: 3.5 μl
b-FGF: 10 ng/ml (50 μl of 100 μg/ml stock)
L-Ascorbic Acid: 50 μg/ml –add fresh 10mg/ml stock at each media change

hES Media (500 ml)
DMEM/F12: 400 ml
KOSR: 100 ml
NEAA: 5 ml
L-glutamine: 5ml
Pen/strep: 5 ml
2-mercaptoethonol: 3.5 μl
b-FGF: 10 ng/ml (50 μl of 100 ug/ml stock)
L-Ascorbic Acid: 50 μg/ml –add fresh 10mg/ml stock at each media change

Protocol

D -9 to -12
Collect blood into BD Vacutainer 4 or 8 mL cell preparation tube (CPT) with sodium citrate[1] or into EDTA or heparinized tubes and Ficoll extract PBMCs. Alternatively, thaw frozen PBMCs.

Fresh cells collected into CPT (8 ml)

1. Draw 8 mL of PB into CPT. Invert tube 8-10x and keep upright at room temperature (RT)
2. Centrifuge 30 min at 1,800 RCF at RT (ideally within 2 hrs of collection)
3. Use a sterile transfer pipette to collect buffy coat into sterile 15 mL conical centrifuge tube
4. Bring total volume to 10 mL with sterile 1x PBS, invert several times
5. Centrifuge 15 minutes at 300 RCF and aspirate supernatant
6. Resuspend pellet in 10 mL of sterile 1x PBS and perform cell count (The yield should be ~1-2x10^6 cells/ml of PB)
7. Transfer 1 to 2x10^6 cells into sterile 15 mL conical centrifuge tube and centrifuge at 300 RCF for 10 min
8. Resuspend pellet in **2 mL** of **expansion medium (EM) + primocin** and transfer to 1 well of a 12 well dish
9. Incubate cells at 37C
10. Centrifuge remaining cells at 300 RCF for 10 min and freeze 1 to 2x10^6 cells/vial (Use 90% serum, 10% DMSO for freezing medium).

Frozen cells

11. Thaw 1 vial of PBMCs into 10 mL of QBSF and centrifuge at 300 RCF for 10 min
12. Resuspend pellet in **2 mL** of **EM + primocin** and transfer to 1 well of a 12 well dish, incubate at 37C

D-6 and D-3 (Pre-Transduction)

Switch media to EM (no antibiotics) at D-6 and collect spent media at D-3 for mycoplasma testing. Continue to culture in EM+P/S from D-3 on.

13. Transfer cells to sterile 15 mL conical tube and wash well 1x with 1 mL of QBSF to collect adherent cells

14. Spin cells at 300 RCF for 10 min and resuspend in 2 mL of fresh EM
15. Continue to culture in 1 well of a 12 well dish

D-2 to D0 (FACS for Erythroblast markers)

16. EM media expands the erythroblast population from PBMCs. A 2-fold expansion should occur in about 9-12 days with an initial decrease in cell number. When cells are noticeably dividing and have reached the appropriate density, perform FACS to monitor erythroblast expansion using antibodies to erythroblast cell surface markers (see support protocol). When more than 90% of the cells express CD36 and CD71, you can proceed to transduction.

D0 (Transduction)

4 Sendai viruses (CytoTune^{TM}) each expressing Oct3/4, Sox2, Klf4, c-Myc are used for transduction. We have found that 2.5x10^5 cells and 10 MOI of each virus gives >1% reprogramming efficiency. So less cells and/or lower MOI may be sufficient to generate enough colonies for picking.

17. Transfer cells to sterile 15 mL conical tube and wash well 1x with 1 mL of QBSF to collect adherent cells
18. Count cells
19. Spin down 2.5x10^5 cells in 15 mL conical tube and add 1 mL of fresh EM+P/S plus viruses and transfer to one well of a 12 well plate.
20. Spinnoculation: Spin plate at 2250 rpm at 25C x 90 min.
21. While spinning, divide the remaining cells into two tubes and spin down. Save one tube for RNA and one for DNA.
22. After spin, move plate to incubator with 5% O_2 and culture at this condition from now on
23. At end of day, add an additional 1 mL of fresh EM+P/S to cells (for a total of 2 ml of EM+P/S)

D1 (Wash virus)

24. Collect and spin cells at 300 RCF in a conical tube for 10 min and resuspend in 2 mL of fresh EM+P/S

D2 Plate MEFs

25. Plate MEFs onto 0.1% Gelatin coated 6-well TC plates

D3 (Plate transduced cells)

26. Collect cells into 15 mL conical tube and spin at 300 RCF for 10 min.
27. Resuspend cells in 6 mL of iPSC media plus growth factors as above
28. Plate 1 mL/well into 6 well MEF plate. Add additional 1.5 mL/well of iPSC media plus growth factors for a total of 2.5 mL of media/well
29. Spin plate at 500 rpm at 25C x 30 min

D5…

30. Feed cells every other day with 2.5 mL of iPSC media.
31. Aspirate and discard floating cells with each feed, add additional MEFs as needed (~1x/wk)

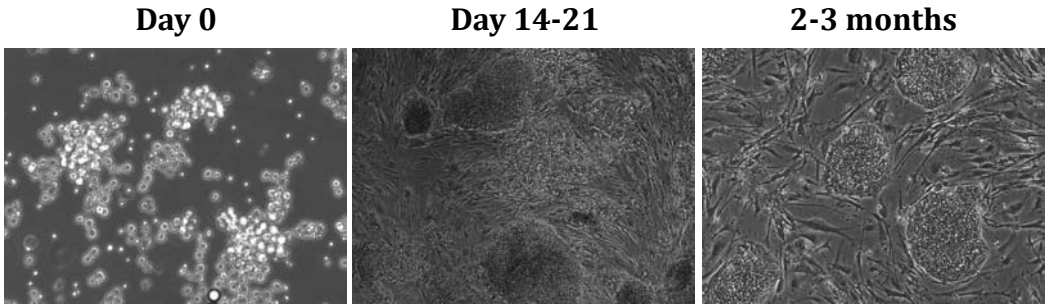
~D9-12 (Small colonies emerge)

32. Once small colonies appear, feed cells daily with 2 mL of hES media**.**

33. Add additional MEFs as needed (~1x/wk)

~D14-21 (Pick colonies)

34. Each colony is picked into one well of a 12-well or 24-well plate with MEFs on gelatin with 1 mL/well of hES media plus 10μM Rock inhibitor.
35. Feed cells two days later and then daily thereafter with 1 mL of hES media. Continue to expand clones for characterization.



Caption: Time course of iPSC generation

Support protocol: FACS analysis of erythroblast surface markers expression

Materials and Preparation

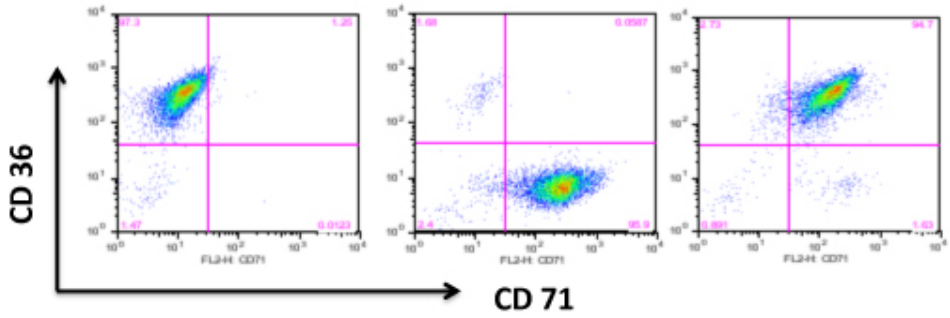
Reagents	Supplier	Catalog #
PE mouse IgG2a isotype control	BD	555575
FITC mouse IgM isotype control	BD	555584
PE mouse anti-human CD71	BD	561938
FITC mouse anti-human CD36	BD	561820
PBS	Life Technologies	14190-136
Round bottom FACS tubes	BD	352054
DMEM/F12	Life Technologies	11330
2-Mercaptoetheranol	Sigma	M7522 -100ml

Staining Buffer: 10% FBS in PBS

Protocol

1. Harvest cells from the 12-well plate in a 15 ml conical tube
2. Centrifuge for 10 min at 400 RCF
3. Discard the supernatant and resuspend cells in 1mL EM media
4. Count cells and transfer 100,000 cells to round bottom tubes with 3 ml of ice-cold PBS

5. Centrifuge cells for 5 min at 400 RCF
6. Discard the PBS and resuspend cells with 100 µL staining buffer.
7. Stain cells with 1µL of each of the antibodies (isotype controls, CD36, CD71, CD36+CD71) at 4°C for 30 min
8. Wash cells with 3 ml ice-cold PBS and centrifuge for 10 min at 0.4 RCF; repeat wash
9. Discard the supernatant by inverting the tube and fix cells with 200 ☐L 1% paraformaldehyde. Proceed with flow cytometry acquisition.



Caption: Surface expression of two erythroblast markers CD36 and CD71. 90% of the cells should be positive for these markers before they can be used for reprogramming.

References

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3. Van den Akker et al. 2010. The majority of the *in vitro* erythroid expansion potential resides in CD34(-) cells, outweighing the contribution of CD34(+) cells and significantly increasing the erythroblast yield from peripheral blood samples. *Haematologica*
4. Leberbauer et al. 2005. Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. *Blood*