Dec 16, 2024

Blood into iPSCs reprogramming protocol using Sendai virus

DOI
https://dx.doi.org/10.17504/protocols.io.8epv52z8jv1b/v1

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Protocol Citation: yuchae lee 2024. Blood into iPSCs reprogramming protocol using Sendai virus. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv52z8jv1b/v1

Protocol status: Working
We use this protocol and it's working

Created: November 26, 2024

Last Modified: December 16, 2024

Protocol Integer ID: 112805

Keywords: sendai virus this protocol, using sendai virus, coated culture dish, reprogramming protocol, protocol, free on vitronectin, culture dish, vitronectin, blood, virus

Abstract

This protocol is based on feeder free on vitronectin-coated culture dish.

Materials

- Cytotune 2.0 sendai reprogramming vectors
- PBMCs or fresh blood
- StemPro™-34 SFM Medium
- L-Glutamine
- DMEMwith GlutaMAX™-I (High Glucose)
- KnockOut™ DMEM/F-12
- Fetal Bovine Serum (FBS), ES Cell-Qualified
- SCF (C-Kit Ligand), Recombinant Human
- FLT-3 Ligand, Recombinant Human
- IL-3, Recombinant Human
- IL-6, Recombinant Human
- Optional: Penicillin-Streptomycin, Liquid
- Optional: Polybrene Hexadimethrine Bromide
- Dulbecco's PBS (DPBS) without Calcium and Magnesium
- Essential 8™ Medium
- Vitronectin, truncated recombinant human (VTN-N)
- Nunc™ 14 mL Round-Bottom Tubes

Before start

This protocol is based on feeder free on vitronectin-coated culture dish.

0. Collect PBMCs via Ficoll-paque purification

1 **Before You Begin**: Ensure all reagents are at room temperature (15 - 25°C).
Dilute the blood sample to a 1:1 volume ratio with the appropriate culture medium or PBS + 2% FBS.

2 Add a volume of density gradient medium to a fresh tube according to the specifications of that density gradient medium.

Table 1. Recommended Volumes and Tube Sizes for Density Gradient Centrifugation using Lymphoprep™

Blood (mL)	PBS + 2% FBS (mL)	Lymphoprep™ (mL)	Tube Size (mL)
1	1	1.5	5
2	2	3	14
3	3	3	14
4	4	4	14
5	5	10	50
10	10	15	50
15	15	15	50

3 Gently layer the diluted blood on top of the density gradient medium. Take care not to mix the two layers.

4 Centrifuge at 800 x *g* for 20 - 30 minutes with the brake OFF.

5 Carefully harvest the cells by inserting the pipette directly through the upper plasma layer to the mononuclear cells at the interface. Alternatively, you can first remove the upper layer and then collect the cells.

6 Wash the harvested cells twice in the appropriate buffer. Cells are now ready for downstream applications.

7 Cryopreservation in 10% DMSO with 90% FBS
Before You Begin: Ensure all media is cold prior to starting this protocol.

7.1

1. Prepare 20% DMSO in FBS. Keep on ice. Do not put 100% DMSO on ice or it will form crystals. Use a glass pipette for DMSO.
2. Ensure PBMCs are in a single-cell suspension. Centrifuge cells at 300 x *g* for 10 minutes to obtain a cell pellet.
3. Carefully remove the supernatant with a pipette, leaving a small amount of medium to ensure the cell pellet is not disturbed.
4. Resuspend PBMCs in cold FBS to a concentration of 1 - 20 × 10^6 cells/mL. Keep on ice.
5. Mix cells gently with 20% DMSO in FBS at a ratio of 1:1. The final cell suspension will be in 10% DMSO and 90% FBS. The final cell concentration will be between 0.5 - 10 × 10^6 cells/mL. Rapidly transfer 1 mL of cell suspension to each cryovial.
   Note: You should try freezing the cells at multiple concentrations to determine which concentration gives the desired viability, recovery, and functionality upon thaw.
6. Place cryogenic vials immediately into an isopropanol freezing container (CoolCell). Place the container in a -80°C freezer overnight.
   Note: Do not let cells sit in cryopreservation medium at room temperature. Keep on ice and transfer rapidly.
7. For long-term storage, transfer the vials of frozen PBMCs to vapor phase liquid nitrogen (below -135°C). Minimize exposure to room temperature by placing vials on dry ice during transfer from the -80°C freezer to liquid nitrogen. Long-term storage at -80°C is not recommended.

1. Day -4 : Seed PBMCs

8 Four days before transduction, remove vial(s) of PBMCs from liquid nitrogen storage. Thaw the vial quickly in 37°C water bath. When only a small ice crystal remains in the vial, remove it from the water bath.

9 Centrifuge the cell suspension at 200 × g for 10 minutes, discard the supernatant, and resuspend the cells in complete PBMC medium to 5 × 105 cells/mL.

10 Add 1 mL per well to the middle section of a 24-well plate to prevent excessive evaporation of the medium during incubation.
Note: Use at least 4 wells to ensure a sufficient number of cells on Day 0.

11 Incubate the cells in a 37°C incubator with a humidified atmosphere of 5% CO2.

2. Day -3 to -1 : Observe cells and add fresh medium

12 Feed the cells daily,gently remove 0.5 mL of the medium from each well, and replace it with 0.5 mL of fresh complete PBMC medium, trying not to disturb the cells.If cells are

present in 0.5 mL removed from the wells, centrifuge the cell suspension at 200 × g for 10 minutes, discard the supernatant, and resuspend the cells in 0.5 mL fresh PBMC medium before adding them back to the plate.

3. Day 0 : Count cells and perform transduction

13 Count the cells using the desired method, and calculate the volume of each virus needed to reach the target MOI using the live cell count and the titer information on the CoA.

14 For each transduction, pipette 2.5 × 10^5 - 5 × 10^5 cells into a round bottom tube.

15 Remove CytoTune™ 2.0 Sendai tubes from the –80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.

16 Add the calculated volumes of each of the three CytoTune™ 2.0 Sendai tubes to 1 mL of PBMC medium, pre-warmed to 37°C. Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 minutes.

17 Add the reprogramming virus mixture prepared in Step 16 to the round bottom tube containing PBMCs prepared in Step 14. Total volume should now be between 1 –1.5 mL. Place cap tightly onto the tube, and wrap with
Parafilm. Centrifuge the cells and virus at 1000 x g for 30 minutes at room temperature. Once the centrifugation is complete, add an additional 1 mL of PBMC medium to the tube, re-suspend the cells, and transfer them to 1 well of
a 12-well plate (total volume should now be between 2 –2.5 mL). Incubate the plate overnight in a 37°C incubator with a humidified atmosphere of 5% CO2.

4. Day 1 : Replace medium and culture cells

18 The next day, remove the cells and medium from the culture plate and transfer to a 15-mL centrifuge tube. Rinse the well gently with 1 mL of medium to ensure most of the cells are harvested.

19 Remove the CytoTune™ 2.0 Sendai viruses by centrifuging the cell suspension at 200 × g for 10 minutes, aspirating the supernatant, and resuspending the cells in 0.5 mL of complete PBMC medium per well of a 24-well plate.
Note: To prevent attachment of any cells prior to plating onto vitronectin, it may be beneficial to use a low attachment 24-well plate.

20 Culture the cells at 37°C in a humidified atmosphere of 5% CO2 for 2 days. No media change is required during this time.

5. Day 3 : Plate cells on vitronectin-coated culture dishes

21 Coat a sufficient number of tissue culture dishes (e.g. 6-well, 60-mm, or 100-mm) with vitronectin. Geltrex™ Membrane Matrix can be substituted for vitronectin.

22 Count the cells using the desired method and seed the 6-well vitronectin-coated culture plates with 1×10^4 - 1×10^5 live cells per well in 2 mL of complete StemPro™-34 medium without the cytokines.

23 Incubate the cells at 37°C in a humidified atmosphere of 5% CO2.

6. Day 4–6: Replace spent medium

24 Every other day, gently remove 1 mL (half) of the spent medium from the cells and replace it with 1 mL of fresh complete StemPro™-34 medium without cytokines and without disturbing cells. Be sure to perform media changes gently during this time.

7. Day 7: Start transitioning cells to Essential 8™ Medium

25 Prepare Essential 8™ Medium. Remove 1 mL (half) of StemPro™-34 medium from the cells and replace it with
1 mL of Essential 8™Mediumto start the adaptationof the cells to the new culture medium.

8. Day 8 to 28: Feed and monitor the cells

26 24 hours later (day 8), change the full volume of the medium to Essential 8™ Medium, and replace the spent medium every day thereafter.

27 Starting on day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells.

28 By day 15 to 21 after transduction, colonies should have grown to an appropriate size for transfer.

29 When colonies are ready for transfer, perform live staining using Tra1-60 or Tra1-81 for selecting reprogrammed colonies if desired.

30 Manually pick colonies and transfer them onto prepared vitronectin-coated 6-or 12-well culture plates.

9. Pick iPSC colonies

31 Place the culture dish containing the reprogrammed cells under an inverted microscope and examine the colonies under 10X magnification. Mark the colony to be picked on the bottom of the culture dish.

32 Transfer the culture dish to a sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope.

33 Using a 25 gauge 1½ inch needle, cut the colony to be picked into 5–6 pieces in a grid-like pattern.

34 Using a 200 µL pipette, transfer the cut pieces onto a vitronectin-coated 12-or 6-well culture plate containing complete Essential 8™ Medium.

35 Incubate the vitronectin-culture plate containing the picked colonies in a 37°C incubator with a humidified atmosphere of 5% CO2.

36 Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh complete Essential 8™ Medium. After that, change the medium every day.

37 When the colonies cover ~85% of the surface area of the culture vessel, they are ready for passaging. Passage the colonies using 0.5 mM EDTA prepared in Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium or magnesium.

38 Continue to culture, expand, and maintain the reporgrammed colonies in complete Essential 8™ Medium until you have frozen cells from two 60-mm plates.

Protocol references

Sendai virus reprogramming kit manual

Acknowledgements

https://www.youtube.com/watch?v=Cl4sTwKFe9k
https://www.stemcell.com/how-to-cryopreserve-pbmcs.html