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Reprogramming Peripheral Blood Mononuclear Cells (PBMCs) with the CytoTune®-iPS 2.0 Reprogramming Kit under feeder-free conditions

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Introduction

This protocol supplements the CytoTune®-iPS 2.0 Sendai Reprogramming Kit User Guide (Pub. part no. MAN0009378). Refer to the user guide for general descriptions of the CytoTune®-iPS 2.0 Sendai Reprogramming Kit and associated methodologies.

Materials Needed

- CytoTune® -iPS 2.0 Sendai Reprogramming Kit (Cat. nos. A16517 or A16518)

  Note: For successful reprogramming, you need all three reprogramming vectors supplied with the kit.
- Peripheral blood mononuclear cells (PBMCs) to reprogram

  Note: You can use PBMCs extracted from blood by a conventional method (i.e., Ficoll-Paque® purification) or using frozen PBMCs.
- StemPro®-34 SFM Medium[1] (Cat. no. 10639-011)
- L-Glutamine (Cat. no. 25030)
- SCF (c-kit Ligand), Recombinant Human (Cat. no. PHC2111)
- FLT-3 Ligand, Recombinant Human (Cat. no. PHC9414)
- IL-3, Recombinant Human (Cat. no. PHC0034)
- IL-6, Recombinant Human (Cat. no. PHC0065)
- Gibco® Mouse Embryonic Fibroblasts (Irradiated) (Cat. no. S1520-100)
- Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. no. A14700)
- Geltrex® LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Cat. no.A1413301) (optional)
- DPBS Without Calcium or Magnesium (Cat. no. 14190-144)
- Sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereo microscope
- Inverted microscope
- Incubator set at 37°C, 5% CO_2
- Water bath set at 37°C
- Sterile serological pipettes (5-mL, 10-mL)
- Centrifuge
- 15-mL centrifuge tubes
- 60-mm and 100-mm tissue culture-treated dishes
- 6-well tissue culture-treated plates
- 25-gauge 1½-inch needle

[1] StemPro®-34 SFM Medium: For human ex vivo tissue and cell culture processing applications. CAUTION: When used as a medical device, Federal Law restricts this device to sale by or on the order of a physician.

Guidelines for Reprogramming

- To maintain sterile culture conditions, carry out all of the procedures using sterile laboratory practices in a laminar flow hood.
- For successful reprogramming, transduce your cells using all three reprogramming vectors supplied with the kit.

  Note: For successful reprogramming, all four Yamanaka factors (i.e., Oct4, Sox2, Klf4, and c-Myc) need to be expressed in your host cell.
- The titer of each CytoTune®-iPS 2.0 Sendai reprogramming vector is lot-dependent. For the specific titer of your vectors, refer to the Certificate of Analysis (CoA) available on our website. Go to **www.lifetechnologies.com/cytotune** and search for the CoA by product lot number, which is printed on the vial.
- Viral titers can decrease dramatically with each freeze/thaw cycle. Avoid repeated freezing and thawing of your reprogramming vectors. Viral titer is not guaranteed for kits that have been refrozen or thawed.
- Prior to starting, ensure that the media are equilibrated to 37°C and appropriately gassed.
- For positive control, we recommend performing a reprogramming experiment with human neonatal foreskin fibroblast cells (strain BJ; ATCC no. CRL2522). Note that experimental conditions may vary among target cells and need to be optimized for each cell type. The example given in the following protocol does not guarantee the generation of iPSCs for all cell types.

Prepare Media

SCF (c-kit Ligand), FLT-3 Ligand, IL-3, and IL-6 Stock Solutions

SCF (c-kit Ligand), FLT-3 Ligand, IL-3, and IL-6 are supplied lyophilized. Prepare stock solutions as described in their specific product inserts and store small aliquots frozen. Thaw at time of use.

PBMC Medium (for 500 mL complete medium)

PBMC medium consists of complete StemPro®-34 medium supplemented with the appropriate cytokines. Follow the procedure below to prepare 500 mL of complete PBMC medium.

1. Thaw the frozen StemPro®-34 Nutrient Supplement at 4°C overnight.
2. After thawing, mix the supplement well by gently inverting the vial a couple of times, and then aseptically transfer the entire contents of the vial to the bottle of StemPro®-34 SFM. Swirl the bottle to mix and to obtain a homogenous complete medium.
3. Aseptically add L-Glutamine to a final concentration of 2 mM (5 mL of 200 mM L-Glutamine to 500 mL of medium).
4. Add the following growth factors to the indicated final concentration:

Cytokine	Final concentration
SCF	100 ng/mL
FLT-3	100 ng/mL
IL-3	20 ng/mL
IL-6	10 ng/mL

5. The complete medium has a shelf life of 30 days when stored at 2–8°C, in the dark.

Essential 8® Medium (for 500 mL complete medium)

1. Thaw frozen Essential 8® Supplement at 2–8°C overnight before using it to prepare complete medium. **Do not thaw the frozen supplement at 37°C.**
2. Mix the thawed supplement by gently inverting the vial a couple of times, remove 10 mL from the bottle of Essential 8® Basal Medium, and then aseptically transfer the entire contents of the Essential 8® Supplement to the bottle of Essential 8® Basal Medium. Swirl the bottle to mix and to obtain 500 mL of homogenous complete medium.
3. Complete Essential 8® Medium can be stored at 2–8°C for up to 2 weeks. Before use, warm complete medium required for that day at room temperature until it is no longer cool to the touch. **Do not warm the medium at 37°C.**

Reprogram PBMCs

The following protocol has been optimized for peripheral blood mononuclear cells (PBMCs) isolated by centrifugation through a Ficoll-Paque® density gradient and frozen in FBS and DMSO-containing medium. We recommend that you optimize the protocol for your cell type.

Day –4: Seed PBMCs

1. 4 days before transduction, thaw PBMCs and gently transfer the cells into a 15-mL conical tube. Slowly (dropwise) add 5–10 mL pre-warmed complete PBMC medium (complete StemPro®-34 serum-free medium containing the appropriate cytokines; aliquot the cytokines and add fresh daily) to the cell suspension. Remove an aliquot of cells to count and determine cell viability.
2. Centrifuge the cell suspension at 200 × *g* for 10 minutes, discard the supernatant, and resuspend the cells in complete PBMC medium to 500,000 cells/mL.
3. Add 1 mL of the cell suspension per well to the middle section of a 24-well plate to prevent excessive evaporation of the medium during incubation.
4. Incubate the cells overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.

Day –3 to –1: Observe cells and add fresh medium

5. Count the cells gently, remove 0.5 mL of the medium, and add 0.5 mL of fresh complete PBMC medium without disturbing the cells. If cells are removed in the 0.5 mL, centrifuge the cell suspension for 10 minutes at 200 × *g*, discard the supernatant, and resuspend the cells in 0.5 mL fresh medium.

   Note: Some cell death is generally observed the first day after the thaw. Some cells may adhere to the surface of the tissue culture plate. Proceed with the cells in suspension. Cells will not proliferate, but should maintain stable cell numbers after the first day.

Day 0: Count cells and perform transduction

6. Count the number of cells using a hemocytometer or the Countess® Automated Cell Counter to determine the viability and total number of cells.

   Note: Cells may have decreased in number. Proceed with transduction using the **live** cell count.
7. Harvest the cells and bring the cell concentration to 2.5×10^6–5×10^6 cells/mL for transduction. We generally recommend transducing 300,000 cells per sample. Add the appropriate volume of cells to be transduced to a sterile, round-bottom centrifuge tube.
8. Remove one set of CytoTune® 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.

9. Transduce the cells by adding the Klf4, Oct4, and Sox2 (KOS) virus and the c-Myc virus at an MOI of 5 (KOS MOI=5, hc-Myc MOI=5) and the Klf4 virus at an MOI of 3 (hKlf4 MOI=3) in a total volume of 1 mL. Transduction and reprogramming efficiencies are greatly increased by a centrifugation step at this point. Place the cap on the centrifuge tube and wrap with Parafilm® sealing film. Centrifuge at 2250 rpm for 30 minutes at room temperature. When the centrifugation is complete, resuspend the pellet in the same medium containing the virus and add an additional 1 mL of complete PBMC media to the well and plate in a 12-well tissue culture plate and incubate overnight at 37°C.

   Note: We recommend initially performing the transductions with MOIs of 5, 5, and 3 as described above. This MOI can be optimized for your specific application and experimental conditions.

Day 1: Remove CytoTune® Sendai virus and culture cells

10. 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.
11. Remove the CytoTune® Sendai viruses by centrifuging the cells at 200 × *g* for 10 minutes, aspirating the supernatant, and resuspending the cells in 0.5 mL of complete PBMC medium in the 24-well plate.

    Note: The cells may have drastic cell death (>60%); continue with the protocol using **live** cell count. For the first 48 hours, view the cells under the microscope for changes in cell morphology as a validation of transduction. Expect large, aggregated cells.
12. Culture the cells at 37°C for 2 days.

    Note: While the cells are incubating, prepare at least two wells of a 6-well plate with either Geltrex®matrix or vitronectin coating for each well of transduced cells. Higher reprogramming efficiencies have been observed on Geltrex® matrix-coated plates, but following this protocol we have obtained reprogramming efficiencies of 0.8% on vitronectin-coated plates. See **Coating Culture Vessels with Vitronectin** and **Coating Culture Vessels with Geltrex® Matrix** instructions in the Appendix.

Day 3: Plate cells on MEF culture dishes

13. Count the cells using the desired method (e.g., Countess® Automated Cell Counter) and seed the Geltrex® matrix- or vitronectin-coated culture dishes with 10,000 and 50,000 **live** cells per dish in 2 mL of complete StemPro®-34 medium without the cytokines. Incubate the cells at 37°C. Plate any excess cells onto a Geltrex® matrix- or vitronectin-coated culture dish or harvest for later RNA analysis as necessary.

Day 3–6: Replace spent medium

14. 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.

Day 7: Start transition to human iPSC medium

15. Prepare 500 mL of complete Essential 8® medium as described above.
16. Remove 1 mL (half) of StemPro®-34 medium from the cells and replace it with 1 mL of complete Essential 8® medium to start the transition of the cells to the new culture medium.

Day 8 to 28: Feed and monitor the cells

17. 24 hours later (day 8), change the full volume of medium to complete Essential 8® medium, and replace the spent medium everyday thereafter.
18. Starting on day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of transformed cells (see Figure 1).
19. Day 15 to 20 after transduction, colonies should have grown to an appropriate size for transfer. Manually pick colonies and transfer them onto prepared 12- or 24-well Vitronectin- or Geltrex® matrix-coated culture plates.

APPENDIX

Coating Culture Vessels with Vitronectin

The instructions for coating a 6-well culture plate with vitronectin at a coating concentration of 0.5 µg/cm^2 are provided below.

1. Upon receipt, thaw the vial of vitronectin at room temperature and prepare 60-µL aliquots of vitronectin in polypropylene tubes. Freeze the aliquots at –80°C or use immediately.
2. To coat the wells of a 6-well plate, remove a 60-µL aliquot of vitronectin from –80°C storage and thaw at room temperature. You will need one 60-µL aliquot per 6-well plate.

   Note: For coating other vessel sizes, refer to Table 1, below.
3. Add 60 µL of thawed vitronectin into a 15-mL conical tube containing 6 mL of sterile DPBS without Calcium and Magnesium (Cat. no. 14190) at room temperature. Gently resuspend by pipetting the vitronectin dilution up and down.

   Note: This results in a working concentration of 5 µg/mL (*i.e.*, a 1:100 dilution).
4. Add 1 mL of the diluted vitronectin solution to each well of a 6-well plate (refer to the table below for the recommended volumes for other culture vessels). When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final concentration will be 0.5 µg/cm^2.
5. Incubate the coated plates at room temperature for 1 hour.

   Note: You can use the coated culture vessel immediately or store it at 2–8°C wrapped in laboratory film for up to a week. Do not allow the vessel to dry. Prior to use, pre-warm the culture vessel to room temperature for at least 1 hour.
6. Before use, aspirate the vitronectin solution from the coated vessels and discard. It is not necessary to rinse off the culture vessel after the removal of the vitronectin solution. You can plate the cells directly onto the vitronectin-coated culture vessels.

Table 1 Volume of diluted vitronectin solution needed to coat various culture vessels.

Vessel size	Growth area	Volume of diluted vitronectin solution
24-well plate	2 cm^2/well	0.2 mL/well
12-well plate	4 cm^2/well	0.4 mL/well
6-well plate	10 cm^2/well	1.0 mL/well
35-mm dish	10 cm^2	1.0 mL
60-mm dish	20 cm^2	2.0 mL
100-mm dish	60 cm^2	6.0 mL

Coating Culture Vessels with Geltrex® Matrix

1. Thaw a 5-mL bottle of Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix at 2–8°C overnight.
2. To create working stocks, dilute an aliquot of Geltrex® matrix solution 1:50 with cold DMEM on ice.

   Note: An optimal dilution of the Geltrex® matrix solution may need to be determined for each cell line. Try various dilutions from 1:30 to 1:100.
3. Quickly cover the whole surface of each culture dish with the Geltrex® matrix solution (see Table 2, below).
4. Incubate the dishes in a 37°C, 5% CO_2 incubator for 1 hour.
5. Geltrex® matrix-coated culture dishes can now be used or stored at 2–8°C for up to a week. Do not allow dishes to dry.
6. Aspirate the diluted Geltrex® matrix solution from the culture dish and discard. You do not need to rinse off the Geltrex® matrix solution from the culture dish after removal. You can plate the cells directly onto the Geltrex® matrix-coated culture dish.

Table 2 Volume of diluted Geltrex® matrix solution needed to coat various culture vessels.

Vessel size	Growth area	Volume of diluted Geltrex® matrix solution
24-well plate	2 cm^2/well	0.35 mL/well
12-well plate	4 cm^2/well	0.75 mL/well
6-well plate	10 cm^2/well	1.5 mL/well
35-mm dish	10 cm^2	1.5 mL
60-mm dish	20 cm^2	3.0 mL
100-mm dish	60 cm^2	6.0 mL

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