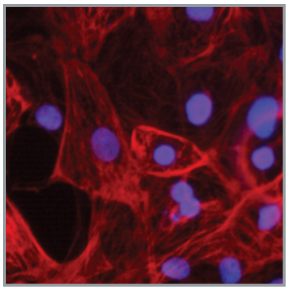


Stem Cell Application Protocol

Reprogramming peripheral blood mononuclear cells (PBMCs)

Using the Cellartis® DEF-CS™ 500 Culture System

that's GOOD science!

I. Introduction

Induced pluripotent stem (iPS) cells originate from adult cells that have been reprogrammed with key transcription factors to exhibit pluripotency. PBMCs are a popular source of adult cells for reprogramming due to routine blood collection methods and the presence of a wide variety of banked blood samples. Once the reprogramming factors have been delivered to PBMCs, the cells can be transferred to the DEF-CS system to maximize the number of emerging colonies and for the robust expansion into stable iPS cell lines.

This protocol has been developed using Sendai viruses for delivery of the transcription factors. Optimization may be necessary if using other delivery methods.

II. Materials required

- Cellartis DEF-CS 500 Culture System (Takara Bio, Cat. #Y30010) (includes COAT-1, Basal Medium, GF-1, GF-2, and GF-3)
- Stem Cell Cutting Tools (Vitrolife, Cat. # 14601)
- Transfer Pipettes (Vitrolife, Cat. # 14319)
- PBS Dulbecco's with Ca^{2+} & Mg^{2+} (D-PBS +/+)
- PBS Dulbecco's w/o Ca^{2+} & Mg^{2+} (D-PBS –/–)
- TrypLE Select Enzyme (1X), no phenol red (Thermo Fisher Scientific, Cat. # 12563011)
- Cell culture vessels, tissue-culture-treated polystyrene surface

III. Preparing medium and coating cell culture vessels

A. Maintenance medium for human iPS cells

Prepare an appropriate volume of Cellartis DEF-CS Medium for Maintenance by adding GF-1 (dilute 1:333) and GF-2 (dilute 1:1,000) to Cellartis DEF-CS Basal Medium.

B. Passaging medium for human iPS cells

Prepare an appropriate volume of Cellartis DEF-CS Medium for Passaging by adding GF-1 (dilute 1:333), GF-2 (dilute 1:1,000), **and GF-3** (dilute 1:1,000) to Cellartis DEF-CS Basal Medium.

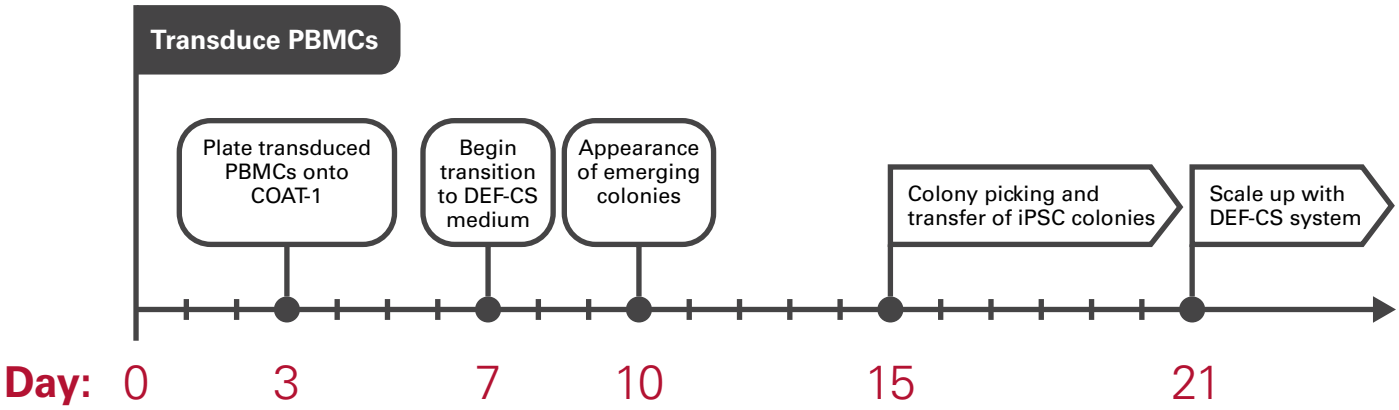
C. Coating cell culture vessels

1. Dilute the required volume of COAT-1 in D-PBS +/+ before use. Make a **1:5** dilution on Day 3, at colony picking, and for the first passage during scale-up. Use a 1:20 dilution for subsequent passages.
2. Mix the diluted COAT-1 solution gently and thoroughly by pipetting up and down.

3. Add the appropriate volume of diluted COAT-1 solution to the cell culture vessel, making sure the entire surface is covered. Use the following volumes of COAT-1 solution: 50 µl/well of a 96-well plate, 200 µl/well of a 48-well plate, 400 µl/well of a 24-well plate, 800 µl/well of a 12-well plate, or 1.5 ml/well of a 6-well plate.
4. Place the cell culture vessel in an incubator for a minimum of 20 min at 37°C ± 1°C, 5% CO_2, and >90% humidity, or for 0.5–3 hr at room temperature (RT, 15–25°C).
5. Aspirate the COAT-1 solution from the cell culture vessel just before use.

IV. Protocol Overview

A



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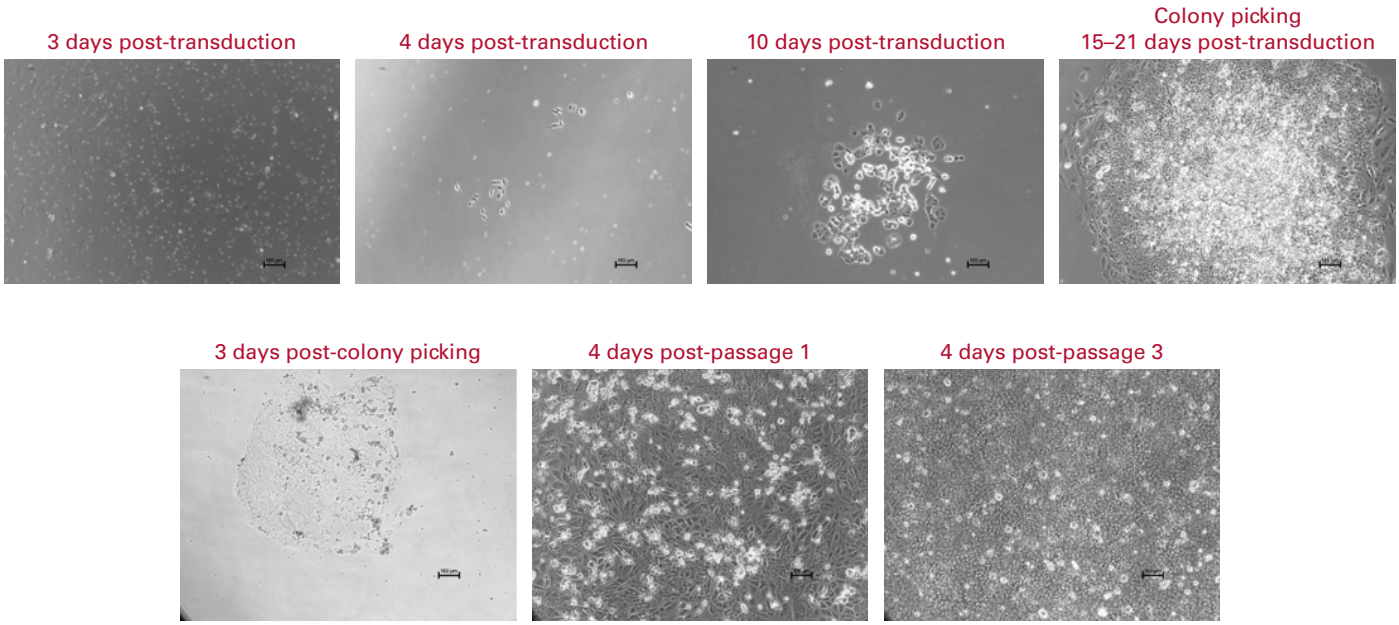

Protocol for the reprogramming of PBMCs into iPS cells generates colonies that show typical monolayer growth when passaged in the Cellartis DEF-CS 500 Culture System. Panel A. Suggested schedule for reprogramming of PBMCs in the DEF-CS system. **Panel B.** Representative photos of the cells during reprogramming and after transfer into the DEF-CS system.

V. Protocol

A. Transduce PBMCs (Day 0)

Deliver the reprogramming factors to your PBMCs using your method of choice.

B. Plate transduced PBMCs onto coated 6-well plates (Day 3)

On Day 3 after transduction, the transduced PBMCs should be transferred to 6-well plates coated with COAT-1. *Do not switch to Cellartis DEF-CS medium at this time.* Continue culturing the transduced PBMCs using the PBMC medium specific to your transduction method of choice.

NOTE: Change pipette tips between wells to prevent cross-contamination.

Preparation

Warm the PBMC medium to 37°C ± 1°C. Coat the appropriate number of wells in the 6-well plate(s) with COAT-1 diluted 1:5.

Passaging

1. Transfer the cell suspension from each well to a separate sterile centrifuge tube.
2. Rinse each well with 500 µl of the PBMC medium and transfer to the corresponding tube.
3. Centrifuge the cells at 200*g* for 10 min.
4. Discard the supernatants and resuspend each cell pellet in 500 µl of the PBMC medium.
5. Count the cells in a hemocytometer or a cell counter (optimized for the specific cell type).
6. Seed 2.5–5 x 10^4 cells/well in 6-well plate(s) coated with COAT-1. Use 2 ml of PBMC medium/well. We recommend using two different seeding densities.

C. PBMC media changes (Days 4 and 6)

For media changes on Days 4 and 6, *do not switch to Cellartis DEF-CS medium. Continue using PBMC medium as on Day 3 (above).*

NOTE: Change pipette tips between wells to prevent cross-contamination.

Preparation

Warm the PBMC medium (1 ml/well of a 6-well plate) to 37°C ± 1°C.

Media change (50% of the volume)

1. Carefully discard 1 ml of media per well.
2. Carefully add 1 ml of PBMC medium per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

D. Begin the transition to DEF-CS medium (Day 7)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (1 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

Media change (50% of the volume)

1. Carefully discard 1 ml of PBMC medium per well.
2. Carefully add 1 ml of Cellartis DEF-CS Medium for Maintenance per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 24 hr.

E. Complete the transition to DEF-CS medium (Day 8)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (2 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

Media change (100% of the volume) *24 hr ± 2 hr after performing the media change on Day 7*

1. Carefully discard all of the media in the wells.
2. Carefully add 2 ml of Cellartis DEF-CS Medium for Maintenance per well.
3. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

F. Daily DEF-CS media changes (Days 9–21)

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance (2 ml/well of a 6-well plate) and warm it to 37°C ± 1°C before use.

Media change (100% of the volume)

1. Examine the cells under a microscope and check for colonies; photo document as necessary.
2. Carefully discard all of the media in the wells.
3. Carefully add 2 ml of Cellartis DEF-CS Medium for Maintenance per well.
4. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

G. Colony picking (during the time span of Days 15–21 post-transduction)

When colonies are 1.5–3 mm in diameter, they are ready to be transferred/picked.

Preparation

Prepare the appropriate volume of Cellartis DEF-CS Medium for Passaging (250 µl/well of a 48-well plate) and warm it to 37°C ± 1°C before use. Coat the appropriate number of wells (one well for each colony to be picked) with 200 µl/well of COAT-1 solution, diluted 1:5.

How to pick colonies

1. Aspirate the COAT-1 solution from the cell culture vessel and add 250 µl of Cellartis DEF-CS Medium for Passaging per well.
2. Try to keep the plate at 37°C ± 1°C.
3. Working under a dissection microscope, use a fresh Stem Cell Cutting Tool to microdissect a colony into 2–4 pieces.
4. Use a fresh Transfer Pipette to transfer each piece into a separate well of the 48-well plate.
5. Repeat Steps 3 and 4 until the desired number of colonies has been picked.
6. Place the cells in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity for 48–54 hr. Do not move the plate during this time.

VI. Scale-up using the Cellartis DEF-CS 500 Culture System

A. Changing media during scale-up

Prepare the appropriate volume of Cellartis DEF-CS Medium for Maintenance and warm to 37°C ± 1°C immediately before use.

1. Check cells under a microscope; photo document as necessary.
2. Carefully aspirate the medium and pipet newly warmed medium into the cell culture vessel. *Avoid flushing medium directly onto the cell layer.*
3. Place the cell culture vessel in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

B. Passaging during scale-up

Prepare the appropriate volume of Cellartis DEF-CS Medium for Passaging and warm to 37°C ± 1°C immediately before use. Coat the appropriate number of wells with COAT-1 (1 well per clonal population, in the appropriate format; see **Table 1** below). As a general rule, the area covered by the cells at passage should not be less than 20% of the area of the destination vessel. Passage single and/or small colonies to a new 48-well plate. If a larger area in the well is covered by cells, passage to a 24-well plate.

1. Check the cells under a microscope; photo document as necessary.
2. Aspirate the media from the cell culture vessel and gently wash the cell layer with D-PBS –/–.
3. Add the appropriate volume (Table 1) of TrypLE Select (room temperature) to the cells. Make sure that the entire surface of the well is covered. Incubate for 5 min or until the cells have detached.
4. Resuspend the cells in the appropriate volume (Table 1) of pre-warmed Cellartis DEF-CS Medium for Passaging and transfer all cells from a well to a newly coated well.

   NOTE: Counting the cells is not recommended.

5. Immediately after plating, hold each cell culture vessel in one hand and mix gently using a figure-eight motion, which distributes the cells evenly over the surface. Place in an incubator at 37°C ± 1°C, 5% CO_2, and >90% humidity.

NOTE: When the cells have been scaled up to one T-25 flask per clone, the lines should be cultured according to the Cellartis DEF-CS 500 Culture System User Manual.

Table 1: Suggested schedule for scaling up reprogrammed clones.

Passage number	Starting format	Passage interval*	New format	Dilution of COAT-1	Volume of diluted COAT-1	Volume of TrypLE Select	Volume of Cellartis DEF-CS Medium for Passaging
Colony picking	Colony in 1 well in 48-well plate	5–10 days	1 well in 48-well plate	1:5	200 µl/well in 48-well plate	50 µl/well in 48-well plate	250 µl/clone
1 (start scale-up)	1 well in 48-well plate	2–5 days	1 well in 24-well plate	1:5	400 µl/well in 24-well plate	50 µl/well in 48-well plate	1 ml/clone
2	1 well in 24-well plate	2–5 days	1 well in 12-well plate	1:20	800 µl/well in 12-well plate	100 µl/well in 24-well plate	2 ml/clone
3	1 well in 12-well plate	2–5 days	1 well in 6-well plate	1:20	1.5 ml/well in 6-well plate	200 µl/well in 12-well plate	3 ml/clone
4	1 well in 6-well plate	2–5 days	1 T-25 flask	1:20	2.5 ml per T-25 flask	300 µl/well in 6-well plate	5 ml/clone

* If a clone grows fast and the culture is very dense at passaging, it is possible to expedite the scale-up by skipping some vessels—i.e., passaging from a well of a 24-well plate directly into a well of a 6-well plate.

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