Protocol

Human iPS cell culture under feeder-free conditions

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Culture of blood-derived mononuclear cells

【Preparation】

Cells: Peripheral or cord blood-derived mononuclear cells (MNCs) (1.2 x 10^7 cells)

Reagents: StemFit without C medium* (AJINOMOTO)
IL-6 (20 µg, 098-06041, Wako)
SCF (10 µg, 197-15511, Wako)
TPO (10 µg, 207-17581, Wako)
Flt-3L (10 µg, 061-05391, Wako)
IL-3 (10 µg, 090-05761, Wako)
G-CSF (10 µg, 072-06101, Wako)
PBS (-) (Nacalai Tesque, 14249-24)
Trypan blue stain (0.4%) for use with the Countess automatic cell counter (Life Technologies, T10282)

Materials: 24-well plates
15/50 mL conical tubes
Plastic pipettes (5, 10 mL)
Countess automatic cell counter (Life Technologies, C10227)
Countess® cell-counting chamber slides (Life Technologies, C10228) (or hemocytometer)

【Procedure】

(1) Preparation of MNC culture medium

Mix the indicated cytokines with StemFit not containing C (=StemFit without C) medium to the final concentrations shown below.

- IL-6: 50 ng/mL
- SCF: 50 ng/mL
- TPO: 10 ng/mL
- Flt-3L: 20 ng/mL
- IL-3: 20 ng/mL
- G-CSF: 10 ng/mL

＊ It is also possible to use the following media instead of StemFit without C medium as the MNC culture medium:

- StemSpan-ACF (STEMCELL Technologies, ST-09805/09855)

- X-VIVO 10 (Lonza, 04-743Q)

(2) Culture of MNCs

1. Prepare the MNCs in a 15 mL tube.
2. Centrifuge for 10 min at 200 x g at 18˚C (stop time setting: slow).
3. Remove the supernatant.
4. Break the pellet by tapping and suspend in 4 mL of MNC culture medium.
5. Dispense 1 mL into the four central wells of the 24-well plate.
6. To prevent evaporation, the other wells should be filled with 1 mL of distilled water or PBS(-).
7. Culture for 7 days (37˚C, 5% CO_2) in an incubator.

Generation of iPS cells

【Preparation】

Cells: Cultured MNCs

Reagents: Episomal vectors (pCXLE or pCE set)
P3 Primary Cell 4D-Nucleofector X Kit L (Lonza, V4XP-3012)
MNC culture medium (StemFit without C + 6 cytokines)
iMatrix-511 (Nippi, 892001/892002)
StemFit (AJINOMOTO)
Trypan blue stain (0.4%) for use with the Countess automatic cell counter (Life Technologies, T10282)
PBS (-) (Nacalai Tesque, 14249-24)

Materials: 6-well plates
15/50 mL conical tubes
Plastic pipettes
Countess automatic cell counter (Life Technologies, C10227)
Countess® cell-counting chamber slides (Life Technologies, C10228)
(or hemocytometer)

【Procedure】

(1) Preparation of gene transfer reagents (pCXLE set)

1. Mix the indicated plasmids.
   - pCXLE-hOCT3/4-shp53-F (1 mg/mL) 2.76 µL
   - pCXLE-hSK (1 mg/mL) 2.76 µL
   - pCXLE-hUL (1 mg/mL) 2.76 µL
   - pCXWB-EBNA1 (1 mg/mL) 1.72 µL

2. Prepare the gene transfer reagents as shown below.
   - P3 solution 164 µL
   - Supplement solution 36 µL
   - pCXLE mix 10 µL

(2) Preparation of gene transfer reagents (pCE set)

1. Mix the indicated plasmids.
   - pCE-hOCT3/4 (1 mg/mL) 2.1 µL
   - pCE-mp53DD (1 mg/mL) 2.1 µL

- pCE-hSK (1 mg/mL) 2.1 µL
- pCE-hUL (1 mg/mL) 2.1 µL
- pCXB-EBNA1 (1 mg/mL) 1.6 µL

2. Prepare the gene transfer reagents as shown below.
   - P3 solution 164 µL
   - Supplement solution 36 µL
   - pCE mix 10 µL

(3) Plate Coating

1. Into each well of the 6-well plate to be coated, dispense 1.5 mL of PBS.
2. Add 4.8 µg/well of laminin-511 E8 and mix well immediately (coating: 0.5 µg/cm^2).
3. Incubate at 37˚C, CO_2 5%, for at least 60 min (plates can be left overnight at 37˚C).
4. Remove from the incubator after 60 min.
5. Add 0.75 mL/well of StemFit without C medium and mix (this prevents drying in the next step).
6. Aspirate the medium and laminin suspension. DO NOT allow the plate to dry, or the coating will become ineffective!
7. Add 1.5 mL/well of MNC culture medium and return the plates to the incubator.

(4) Gene transfer (day 0)

1. Warm 1 mL of MNC culture medium in the incubator.
2. Turn on the power of the Nucleofector 4D and wait for the self-check to complete without abnormality.
3. Select the X unit and select transfer with a 100 µL cuvette as the gene transduction mode.
4. Set to the EO-117 gene transfer program.
5. Collect and place the cultured MNCs in a 15 mL tube.
6. Stain the cells with trypan blue and count the number of living cells with the Countess.
7. Transfer 2.5 x 10^6 live cells into a 15 mL tube.
8. Centrifuge for 10 min at 200 x g at 18˚C (stop time setting: slow).
9. Remove the pre-warmed MNC culture medium and laminin-coated plates from the incubator during centrifugation.
10. Remove as much supernatant as possible with a micropipette.
11. Suspend the cell pellets in 100 µL of gene transfer reagent, avoiding frothing.

12. Transfer the suspension to a cuvette. Do not allow to froth.
13. Place the cuvette in the Nucleofector 4D X unit and start the program.
14. Add 450 µL of MNC culture medium immediately to each cuvette.
15. Collect the gene-transferred cells with medium in a 1.5 mL tube.
16. Dispense 35 µL of the cell suspension into each well of the laminin-coated plates.
17. Rock the plates to achieve uniform cell density and start culture in an incubator.

(5) Medium addition (days 3, 5, and 7)

1. Add 1 mL of StemFit medium to each well.

(6) Medium change (day 9-)

1. Change the medium to 2 mL/well of fresh StemFit every two days.
2. Continue medium change until the size of the iPS cell colonies exceeds 1 mm.

Colony picking

【Preparation】

Cells: Generated iPSC colonies (day 20-30)

Reagents: iMatrix-511 (laminin-511 E8) (Nippi, 892001/892002)
10 mM Y-27632 (Wako, 253-00511)
PBS (-) (Nacalai Tesque, 14249-24)
TrypLE Select (Life Technologies, A12859-01)
Culture medium (StemFit medium) (includes bFGF; penicillin-streptomycin not used) (AJINOMOTO)

Materials: 12/96-well plates
15/50 mL conical tubes
Cell scrapers
Plastic pipettes

【Procedure】

(1) Medium Preparation

To the required amount of culture medium, add 1/1000 of 10 mM Y-27632 (final: 10 µM)

(2) Plate Coating

1. Into each well of the 12-well plate to be coated, dispense 0.6 mL of PBS.
2. Add 1.9 µg/well of iMatrix-511 and mix well immediately (coating: 0.5 µg/cm^2).
3. Incubate at 37˚C, CO_2 5%, for at least 60 min (plates can be left overnight at 37˚C).
4. Remove from the incubator after 60 min.
5. Add 0.3 mL/well of StemFit medium and mix (this prevents drying in the next step).
6. Aspirate the medium and laminin suspension. DO NOT allow the plate to dry, or the coating will become ineffective!
7. Add 0.6 mL/well of culture medium containing Y-27632 and return the plates to the incubator.

(3) Colony picking

1. Prepare the equipment to be used for picking (e.g. stereoscopic microscope with camera system).
2. Change to fresh medium.
3. Collect a colony and transfer to the 96-well plate with a micropipette (10 µL size).
4. Add 10 µL of TrypLE Select to each well.

5. Incubate for 10 min in an incubator, rocking the plates every 3 or 4 minutes.
6. Add 180 µL of StemFit + Y medium to each well.
7. Dissociate colonies into single cells by pipetting 10 times with a 200 µL micropipette (adjust setting to 180 µL).
8. Transfer the cell suspension to one laminin-coated well of a 12-well plate.
9. Immediately distribute the cells evenly over the plate surface to avoid uneven attachment.
10. Culture the plates at 37˚C, CO_2 5%.
11. The next day, change to regular StemFit medium (NOT containing Y-27632).

(4) Medium change

3. Change the medium to 0.6 mL/well of fresh StemFit every other day.
4. Continue medium change until the size of the iPS cell colonies exceeds 1 mm.

Passage and Maintenance

【Preparation】

Cells: 80% confluent human iPS cells (6-well plate; 10 cm^2)
(Scale down/up as required)

Reagents: iMatrix-511 (laminin-511 E8) (Nippi, 892001/892002)
10 mM Y-27632 (Wako, 253-00511)
PBS (-) (Nacalai Tesque, 14249-24)
0.5X TrypLE Select (see Appendix)
Culture medium (StemFit medium) (includes bFGF; penicillin-streptomycin not used) (AJINOMOTO)
Trypan blue stain (0.4%) for use with the Countess automatic cell counter (Life Technologies, T10282)

Materials: 6-well plates
15 mL conical tubes
Cell scrapers
Plastic pipettes
Countess automatic cell counter (Life Technologies, C10227)
Countess® cell-counting chamber slides (Life Technologies, C10228) (or hemocytometer)

【Procedure】

(1) Medium Preparation

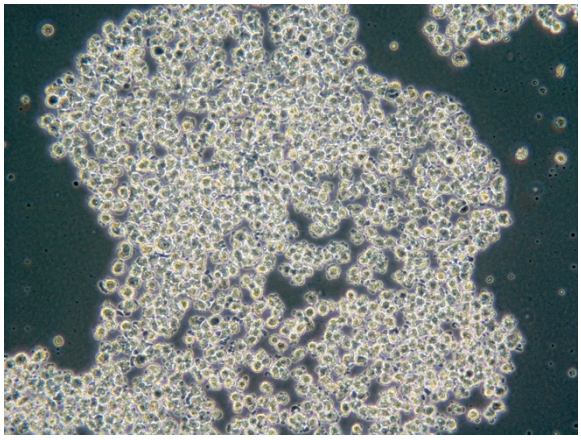
To the required amount of culture medium, add 1/1000 of 10 mM Y-27632 (final: 10 µM).

(2) Plate Coating

1. Into each well of the 6-well plate to be coated, dispense 1.5 mL of PBS.
2. Add 4.8 µg/well of laminin-511 E8 and mix well immediately (coating: 0.5 µg/cm^2).
3. Incubate at 37˚C, CO_2 5%, for at least 60 min (plates can be left overnight at 37˚C).
4. Remove from the incubator after 60 min.
5. Add 0.75 mL/well of culture medium and mix (this prevents drying in the next step).
6. Aspirate the medium and laminin suspension. DO NOT allow the plate to dry, or the coating will become ineffective!
7. Add 1.5 mL/well of culture medium containing Y-27632 and return the plates to the incubator.

(3) Passage

1. Observe the cells by microscopy. If imaging is required, dead cells may be removed by replacing the medium. Culture medium is preferred over PBS as cells tend to detach with extended incubation in PBS.
2. Aspirate the medium.
3. Wash once with 1 mL of PBS and aspirate.
4. Add 300 µL/well of 0.5X TrypLE Select and spread well over the surface.
5. Incubate the plates at 37˚C, CO_2 5%, for 1 min.
6. Remove the plates from the incubator after 1 min and gently re-distribute the TrypLE over the surface.
7. Incubate the plates for another 3 min at 37˚C, CO_2 5%, (total 4 min).
8. Remove the plates from the incubator and observe the cells by microscopy. (The cells should appear separate and rounded. During the 4 min incubation, cell-cell adhesion is destroyed, but the cells remain attached to the matrix (right photo). Caution: Longer incubation will also detach the cells.)



9. Aspirate the 0.5X TrypLE Select.
10. Wash once with 2 mL/well PBS. (Add the PBS gently as the cells tend to detach easily.)
11. Add 1 mL of culture medium per well.
12. Detach the cells with a cell scraper.
13. Observe the cells under the microscope to ensure they have detached.
14. Pipette the cells 10 times to fully dissociate. Collect the cells in a 1.5 mL tube (this prevents re-attachment of the cells to the plate surface).
15. Count the cells by trypan blue exclusion using the Countess automatic cell counter (iPSC program - sensitivity: 5, min size: 8, max size: 30, circularity: 75).
16. Plate **13,000 live cells** in one well of a 6-well plate (10 cm^2) coated with laminin. (**Immediately distribute the cells** evenly over the plate surface to avoid uneven attachment.)
17. Culture the plates at 37˚C, CO_2 5%.
18. The next day, change to regular culture medium (NOT containing Y-27632).

- Medium change is performed every other day. If the color of the medium turns orange or yellow, it should be changed every day.

- Should yield approximately 1 x 10^6 cells in 8 days (±1 day, 100-fold expansion).

Freezing Protocol

【Preparation】

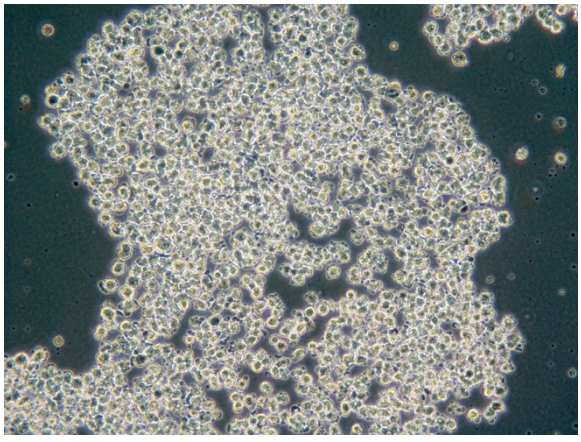
Cells: 80% confluent human iPS cells (6-well plate; 10 cm^2) (Scale down/up as required)

Reagents: STEM-CELLBANKER (Nippon Zenyaku Kogyo, BLC-3S)
PBS (-) (Nacalai Tesque, 14249-24)
0.5X TrypLE Select
Culture medium (StemFit medium) (includes bFGF; penicillin-streptomycin not used)
Trypan blue stain (0.4%) for use with the Countess automatic cell counter (Life Technologies, T10282)

Materials: Cryotubes (2.0 mL)
Cell scrapers
Plastic pipettes (5, 10 mL)
Countess automatic cell counter (Life Technologies, C10227)
Slides for Countess automatic cell counter (Life Technologies, C10228) (or hemocytometer)
PDF-150/250 Programmable freezer (or Bicell, Mr. Frosty)

【Procedure】

1. Observe the cells by microscopy. If imaging is required, dead cells may be removed by replacing the medium. Culture medium is preferred over PBS as cells tend to detach with extended incubation in PBS.
2. Aspirate the medium.
3. Wash once with 1mL PBS and aspirate.
4. Add 300 µL/well of 0.5X TrypLE Select and spread well over the surface.
5. Incubate the plates at 37˚C, CO_2 5%, for 1 min.
6. Remove the plate from the incubator after 1 min and gently re-distribute the TrypLE over the surface.
7. Incubate the plates for another 3 min at 37˚C, CO_2 5% (total 4 min).
8. Remove the plates from the incubator and observe the cells by



microscopy. (The cells should appear separate and rounded. During the 4 min incubation, cell-cell adhesion is destroyed, but the cells remain attached to the matrix (right photo). Caution: Longer incubation will also detach the cells.)

9. Aspirate the 0.5X TrypLE Select.
10. Wash once with 2 mL/well of PBS. (Add the PBS gently as the cells tend to detach easily.)
11. Add 1 mL of culture medium per well.
12. Detach the cells with a cell scraper.
13. Observe the cells under the microscope to ensure they have detached.
14. Pipette the cells 10 times to fully dissociate and collect them in a 1.5 mL tube (this prevents re-attachment of the cells to the plate surface).
15. Count the cells by trypan blue exclusion using the Countess automatic cell counter (iPSC program - sensitivity: 5, min size: 8, max size: 30, circularity: 75).
16. Transfer the required number of cells to a centrifuge tube and spin at 800 rpm (160 X g), 22˚C for 5 min.
17. Remove the supernatant and gently disrupt the pellet.
18. Resuspend the cells at 1 x 10^6 cells/mL in STEM-CELLBANKER freeze solution.
19. Dispense 200 µL (=2 x 10^5 cells) per cryovial.
20. Cool using a programmed freezer (-1˚C/min), or in a Mr. Frosty at -80˚C for at least 3 hrs.
21. Transfer to liquid nitrogen within a few days.

Thawing Protocol

【Preparation】

Cells: Frozen vial of human iPS cells (2 x 10^5 cells) (Scale down/up as required)

Reagents: iMatrix-511 (laminin-511 E8) (Nippi, 892001/892002)
10 mM Y-27632 (Wako, 253-00511)
PBS (-) (Nacalai Tesque, 14249-24)
Culture medium (StemFit medium) (includes bFGF; penicillin-streptomycin not used)
Culture medium containing Y-27632
Trypan blue stain (0.4%) for use with the Countess automatic cell counter (Life Technologies, T10282)

Materials: 6-well plates
15 mL conical tubes
Water bath (37˚C)
Plastic pipettes (5, 10 mL)
Countess automatic cell counter (Life Technologies, C10227)
Slides for Countess automatic cell counter (Life Technologies, C10228) (or hemocytometer)

【Procedure】

(1) Medium Preparation

To the required amount of culture medium, add 1/1000 of 10 mM Y-27632 (final: 10 µM).

(2) Plate Coating

1. Into each well of the 6-well plate to be coated, dispense 1.5 mL of PBS.
2. Add 4.8 µg/well of laminin-511 E8 and mix well immediately (coating: 0.5 µg/cm^2).
3. Incubate at 37˚C, CO_2 5%, for at least 60 min (plates can be left overnight at 37˚C).
4. Remove from the incubator after 60 min.
5. Add 0.75 mL/well of culture medium and mix (this prevents drying in the next step).
6. Aspirate the medium and laminin suspension. DO NOT allow the plate to dry, or the coating will become ineffective!
7. Add 1.5 mL/well of culture medium containing Y-27632 and return the plates to the incubator.

(3) Thawing

1. Warm the water bath to 37˚C and the medium to room temperature.
2. Add 5mL of medium to a 15 mL conical tube.
3. Remove the cells from the liquid nitrogen and thaw immediately in the water bath (~1 min, until only small ice particles remain).
4. Transfer the cell suspension to the conical tube prepared in Step 2 with gentle pipetting (1-2 times).
5. Spin the cells at 800 rpm (160 x g), 22˚C, for 5 min to pellet.
6. Aspirate the supernatant.
7. Tap the pellet gently to break, then resuspend in 1 mL of medium.
8. Count the cells by trypan blue exclusion using the Countess automatic cell counter (iPSC program - sensitivity: 5, min size: 8, max size: 30, circularity: 75).
9. Plate **65,000 live cells** to one well of a laminin-coated 6-well dish.
10. Immediately distribute the cells evenly over the plate surface to avoid uneven attachment.
11. Culture the plates at 37˚C, CO_2 5%.
12. The next day, change to regular culture medium (NOT containing Y-27632).

*Feed the cells the day after thawing, then every other day until day 6 or 7 after thawing. When the cells become more confluent, feed them every day.

iPSC transfer from feeder to laminin culture

【Preparation】

Cells: 80% confluent human iPS cells **on feeders** (6-well plate; 10 cm^2)
(Scale down/up as required)

Reagents: iMatrix-511 (laminin-511 E8) (Nippi, 892001/892002)
10 mM Y-27632 (Wako, 253-00511)
PBS (-) (Nacalai Tesque, 14249-24)
CTK solution (to remove feeder cells)
0.5X TrypLE Select
Culture medium (StemFit medium) (includes bFGF; penicillin-streptomycin not used)
Trypan blue stain (0.4%) for use with the Countess automatic cell counter (Life Technologies, T10282)

Materials: 6-well plates
15 mL conical tubes
Cell scrapers
Plastic pipettes (5, 10 mL)
Countess automatic cell counter (Life Technologies, C10227)
Slides for Countess automatic cell counter (Life Technologies, C10228) (or hemocytometer)

【Procedure】

(1) Medium Preparation

To the required amount of culture medium, add 1/1000 of 10 mM Y-27632 (final: 10 µM)

(2) Plate Coating

1. Into each well of the 6-well plate to be coated, dispense 1.5 mL of PBS.
2. Add 4.8 µg/well of laminin-511 E8 and mix well immediately (coating: 0.5 µg/cm^2).
3. Incubate at 37˚C, CO_2 5%, for at least 60 min (plates can be left overnight at 37˚C).
4. Remove from the incubator after 60 min.
5. Add 0.75 mL/well of culture medium and mix (this prevents drying in the next step).
6. Aspirate the medium and laminin suspension. DO NOT allow the plate to dry, or the coating will become ineffective!
7. Add 1.5 mL/well of culture medium containing Y-27632 and return the plates to the incubator.

(3) Passage

1. Observe the cells by microscopy. If imaging is required, dead cells may be removed by replacing the medium. Culture medium is preferred over PBS as cells tend to detach with extended incubation in PBS.
2. Aspirate the medium.
3. Wash once with 1 mL PBS and aspirate.
4. Add 600 µL/well of CTK solution and spread well over the surface.
5. Incubate the plates at 37˚C, CO_2 5%, for 2-5 min.
6. Aspirate off the CTK solution, add 1 mL of PBS, then remove the PBS, leaving the iPSCs on the plates.
7. Add 300 µL/well of 0.5X TrypLE Select and spread well over the surface.
8. Incubate the plates at 37˚C, CO_2 5%, for 1 min.
9. Remove the plates from the incubator after 1 min and gently re-distribute the TrypLE over the surface.
10. Incubate the plates for another 3 min at 37˚C, CO_2 5% (total 4 min).
11. Add 700 µL/well of StemFit medium and detach the cells with a cell scraper.
12. Pipette the cells 10 times to fully dissociate and collect them in a 1.5 mL tube.
13. Spin at 800 rpm (160 x g), 22˚C, for 5 min.
14. Resuspend the cells in StemFit medium (volume depends on the pellet).
15. Count the cells by trypan blue exclusion using the Countess automatic cell counter (iPSC program - sensitivity: 5, min size: 8, max size: 30, circularity: 75).
16. Plate **13,000 live cells** in one well of a 6-well plate (10 cm^2) coated with laminin. (**Immediately distribute the cells** evenly over the plate surface to avoid uneven attachment.)
17. Culture the plates at 37˚C, CO_2 5%.
18. The next day, change to regular culture medium (NOT containing Y-27632).

- Medium change is performed every other day. If the color of the medium turns orange or yellow, it should be changed every day.

- Should yield approximately 1 x 10^6 cells in 8 days (±1 day, 100-fold expansion).

Appendix

StemFit medium

【Preparation】

Reagents: StemFit medium (A) 400 mL (4˚C)
StemFit medium (B) 100 mL (-30˚C)
StemFit medium (C) 2 mL (-30˚C)

Materials: Plastic pipettes (5, 10, 25 mL)

【Procedure】

1. Thaw B and C at 4˚C (> 8 hrs).
2. Add the 100 mL of B to A.
3. Add the 2 mL of C to A
4. Seal the lid and mix well.
5. Aliquot 45 mL of StemFit medium into 50 mL conical tubes.
6. Store at -80˚C.

- For the preparation of StemFit without C medium, please skip the Step 3.
- The solution can be stored at -80˚C for up to 6 months. **After thawing, the medium should be used within 2 weeks**.

0.5X TrypLE Select Solution

【Preparation】

Reagents: TrypLE Select (Life Technologies, A12859-01)
0.5 M EDTA solution (Nacalai Tesque, 689414)
PBS (-) (Nacalai Tesque, 14249-24)

Materials: Plastic pipettes (5, 10 mL)
Micropipette tips (20, 200, 1000 µL)
250 mL medium filtration system (0.2 µm filter)

【Procedure】

1. Aliquot 250 mL of PBS (-) into the medium filtration system.
2. Add 250 µL of 0.5 M EDTA solution.
3. Draw the solution through the filter by vacuum (＝0.5 mM EDTA/PBS(-) solution).
4. Aliquot 7 mL of 0.5 mM EDTA/PBS (-) into 15 mL conical tubes.
5. To the same tubes, add 7 mL of TrypLE Select.
6. Seal the lid and mix well.

- The solution can be stored at room temperature for up to 4 weeks.

Amount of reagent

Laminin Coating

12-well	6-well	60-mm	100-mm	
3.8	9.6	21.29	58.95	cm^2
1.9	4.8	10.6	29.5	µg

Coating at 0.5 µg/cm^2

To prepare multiple wells, prepare a master mix and dispense.

0.5X TrypLE Select Volume

12-well	6-well	60-mm	100-mm	
200	300	600	1,800	µL

iPSC Plating Density

12-well	6-well	60-mm	100-mm	
-	13,000	29,000	80,000	cells

Medium Volume

12-well	6-well	60-mm	100-mm	
0.6	1.5	3.0	9.0	mL

Gene transfer: CytoTune-iPS (Sendai viruses)

【Preparation】

Cells: Cultured MNCs (1 x 10^5 cells)

Reagents: CytoTune-iPS 2.0 (Life Technologies, A16517 (This kit is available from MBL in Japan, DV-0304/A))
MNC culture medium (StemFit without C + 6 cytokines)
iMatrix-511 (Nippi, 892001/892002)
StemFit (AJINOMOTO)
Trypan blue stain (0.4%) for use with the Countess automatic cell counter (Life Technologies, T10282)
PBS (-) (Nacalai Tesque, 14249-24)

Materials: 6/24-well plates
15/50 mL conical tubes
Plastic pipettes
Countess automatic cell counter (Life Technologies, C10227)
Countess® cell-counting chamber slides (Life Technologies, C10228)
(or hemocytometer)

【Procedure】

(1) Preparation of gene transfer reagents

1. Mix the indicated Sendai viruses.
   - CytoTune 2.0 KOS MOI=5
   - CytoTune 2.0 hc-Myc MOI=5
   - CytoTune 2.0 hKlf4 MOI=5
     - Adjust final volume to 1 mL with MNC culture medium.

(2) Gene transfer (day 0)

1. Collect and place the cultured MNCs in a 15 mL tube.
2. Stain the cells with trypan blue and count the number of living cells with the Countess.
3. Transfer 1.0 x 10^5 live cells to a tube.
4. Centrifuge for 10 min at 200 x g at 18˚C (stop time setting: slow).
5. Remove the supernatant.
6. Suspend the cell pellets in 1 mL of gene transfer reagent.
7. Transfer the suspension to one well of a 24-well plate.

8. Culture overnight in an incubator.

(3) Plate Coating (day 1)

1. Into each well of the 6-well plate to be coated, dispense 1.5 mL of PBS.
2. Add 4.8 µg/well of laminin-511 E8 and mix well immediately (coating: 0.5 µg/cm^2).
3. Incubate at 37˚C, CO_2 5%, for at least 60 min (plates can be left overnight at 37˚C).
4. Remove from the incubator after 60 min.
5. Add 0.75 mL/well of StemFit without C medium and mix (this prevents drying in the next step).
6. Aspirate the medium and laminin suspension. DO NOT allow the plate to dry, or the coating will become ineffective!
7. Add 1.5 mL/well of MNC culture medium. Return the plates to the incubator.

(4) Plating (day 1)

1. Collect the gene-transferred cells into a tube.
2. Dispense 35 µL of the cell suspension into each well of laminin-coated 6-well plates.
3. Rock the plates to achieve uniform cell density and start culture in an incubator.

(5) Medium addition (days 4, 6, and 8)

1. Add 1 mL of StemFit medium to each well.

(6) Medium change (day 10-)

1. Change the medium to 2 mL/well of fresh StemFit every two days.
2. Continue medium change until the size of the iPS cell colonies exceeds 1 mm.

Information

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References

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