# Protocol for Feeder-Free culturing of human iPS cells

Date Created: August 4, 2021

Date of revision: November 30, 2023

公益財団法人
京都大学iPS細胞研究財団
Public Interest Incorporated Foundation
CiRA Foundation

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## Thawing of iPS cell frozen stock

### ■ Materials

- ☐ Cells: Frozen vial of human iPS cells
- ☐ Reagents: iMatrix-511 (Laminin-511 E8) (Nippi, 892012)
  10 mM Y-27632 (FUJIFILM Wako Pure Chemical Corporation, 036-24023)
  PBS (Nakalai Tesque, 14249-24)
  StemFit AK03N (StemFit medium) (Ajinomoto)
  Trypan blue solution (Sigma-Aldrich, T8154)
- ☐ Plasticware: 6 well plates
  15/50 mL conical tubes
  Plastic Pipettes

### ■ Procedure

#### 1. Preparation of culture medium and reagents

1) Leave the StemFit AK03N medium at room temperature for 30 minutes before starting.
2) Dispense 2 mL of StemFit into a 50 mL tube; Add 2 µL of Y-27632 solution and mix well (final concentration 10 µM). →" StemFit+Y medium."

#### 2. Coating of 6-well plates

1) Prepare the required volume of PBS in 50 mL tubes (1.5 mL x number of wells).
2) Prepare the required volume of iMatrix-511 solution (9.2 µL x number of wells) and add to PBS.
3) Mix well and add 1.5 mL to each well.
4) Immediately shake the plate to spread the solution throughout, then incubate for at least 1 hour at 37°C in 5% $CO_2$ incubator.
5) Remove the plate being coated from the incubator.
6) Add 0.75 mL of StemFit medium to each well, blend well throughout, and remove.
7) Add 1.5 mL of StemFit+Y medium to each coated well. Add 2 mL of PBS to each empty well.
8) Place all plates at 37°C in 5% $CO_2$ incubator.

## 3. Thawing of frozen cells

1) Preheat a water bath (or a melting apparatus) to 37°C.
2) Add 5 mL of StemFit medium into a 50 mL conical tube.
3) Remove the frozen vial of iPS cell from the liquid nitrogen storage container and thaw it in the water bath for about 1 minute until a few ice particles remain.
4) Add the thawed cell suspension in step 3) into StemFit medium in the 50 mL tube prepared in 2).
5) Centrifuge the cells at 160 × g for 5 min at 23°C and remove the supernatant.
6) Add 0.3 mL of StemFit+Y medium and suspend cells by pipetting (6 times).
7) Take 10 µL of the cell suspension, add 10 µL of trypan-blue solution, mix, and count the cells.
8) Seed 65,000 viable cells into each well of a coated 6-well plate. Immediately shake the plate to spread the cells evenly.
9) Check cells using a microscope.
10) Culture the plates at 37°C in 5% $CO_2$ incubator.

## Medium exchange

*The day after cell thawing or cell passage, medium needs to be changed to Y-27632 free StemFit medium.

### ■ Materials

- ☐ Cells: 6-well plate seeded with cells
- ☐ Reagents: StemFit AK03N (StemFit medium) (Ajinomoto)
- ☐ Equipment: Plastic Pipettes

### ■ Procedure

1) Leave StemFit medium at room temperature for 30 minutes prior to use.
2) Check the condition of the cells using a microscope (cell viability, presence of iPS cell-like colonies) and take photographs if necessary.
3) Remove medium and gently add 3 mL of StemFit medium per well (allow it to run down the walls of the well).
4) Incubate at 37°C in 5% $CO_2$ incubator.

*Guideline for medium exchange:

- For passaging on day 6 of culture: Change the medium on day 1 (day 1 after cell seeding), day 4 and day 5.
- For passaging on day 7 of culture: Change the medium on day 1, day 4 and day 6.

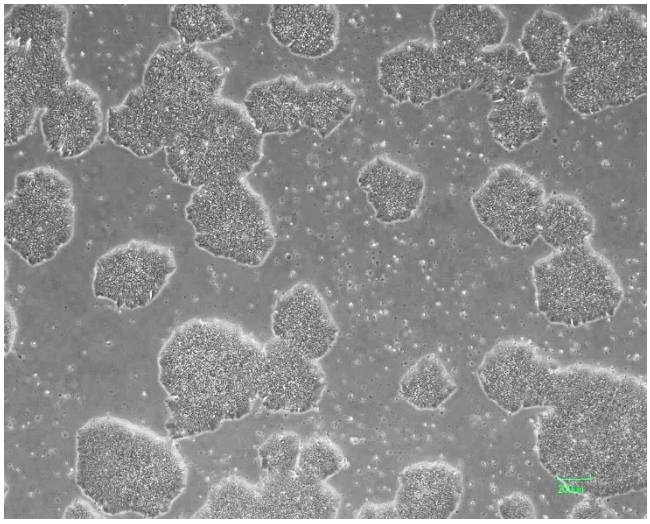
## Passage of iPS cells (from 6-well plate to 6-well plate)

### ■ Materials

☐ Cells: 6-well plate seeded with cells

*Passage is carried out when the colonies have started to grow and are not too large. Usually, we passage cells on day 6 or 7 of culture.

Cell status before passage



(Scale bar; 200 μm)

☐ Reagents: iMatrix-511 (Laminin-511 E8) (Nippi, 892012)
10 mM Y-27632 (FUJIFILM Wako Pure Chemical Corporation, 036-24023)
PBS (Nakalai Tesque, 14249-24)
StemFit AK03N (StemFit medium) (Ajinomoto)
0.5×TrypLE Select solution
Trypan blue solution (Sigma-Aldrich, T8154)

☐ Plasticware: 6 well plates
15/50 mL conical tubes
Plastic Pipettes
Cell scraper

## ■ Procedure

### 1. Preparation of culture media and reagents

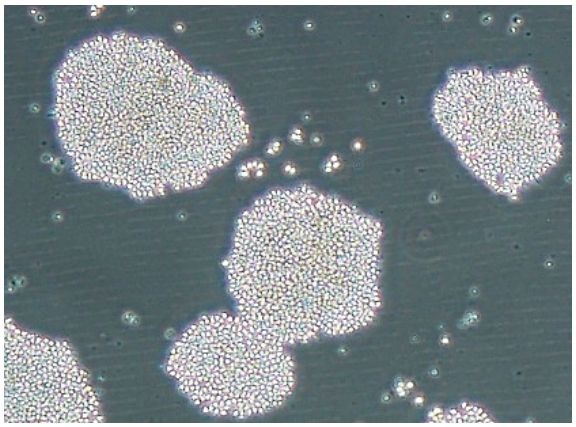
1) Leave the StemFit AK03N medium at room temperature for 30 minutes before starting.
2) Dispense 2 mL of StemFit into a 50 mL tube; Add 2 µL of Y-27632 solution and mix well (final concentration 10 µM). →" StemFit+Y medium."

### 2. Coating of 6-well plate

1) Prepare the required volume of PBS in 50 mL tubes (1.5 mL x number of wells).
2) Prepare the required volume of iMatrix-511 solution (9.2 µL x number of wells) and add to PBS.
3) Mix well and add 1.5 mL to each well.
4) Immediately shake the plate to spread it throughout, then place it in a 37°C, 5% $CO_2$ incubator for at least 1 hour.
5) Remove the plate being coated from the incubator.
6) Add 0.75 mL of StemFit to each well, spread throughout, and remove.
7) Add 1.5 mL of StemFit+Y medium to each coated well. Add 2 mL of PBS to each empty well.
8) Place the plate at 37°C in 5% $CO_2$ incubator.

### 3. Passage

1) Check the condition of the cells using a microscope (cell viability, presence of iPS cell-like colonies) and take photographs if necessary.
2) Remove medium and add 1 mL of PBS. Wash entire cells by shaking the plate, then remove PBS.
3) Add 1 mL of 0.5×TrypLE Select solution and shake the plate to spread the solution throughout.
4) Place 6-well plate in the incubator and leave for up to 10 minutes.
5) Examine the cells using a microscope to see whether cell-cell adhesions are loosened for almost all cells and whether individual cells can be recognized.



6) Remove 0.5 x TrypLE Select solution from each well. Gently add 2 mL of PBS, wash entire cells by shaking the plate, and then remove the PBS.
7) After adding 1 mL of new StemFit medium, scrape the cells with a cell scraper* (If multiple clones or strains are to be passed together, scrape cells in all wellswith cell scrapers before proceeding).
8) Gently pipet the cells to break them apart (10 times) and collect them in a new tube.
9) Take 10 µL of cell suspension, and add 10 µL of Trypan-blue solution, mix and count the cells.
10) Transfer the required number of cells (13000 viable cells per well) to one well of a coated 6-well plate. Immediately shake the plate to spread the cells evenly. For seeding with whole cells, add 1/1000 volume of Y-27632 to the cell suspension.
11) Check the cells in each well using a microscope. Incubate at 37°C in 5% $CO_2$ incubator.
12) The day after passages, the medium is replaced (see "Medium Replacement, page 5").

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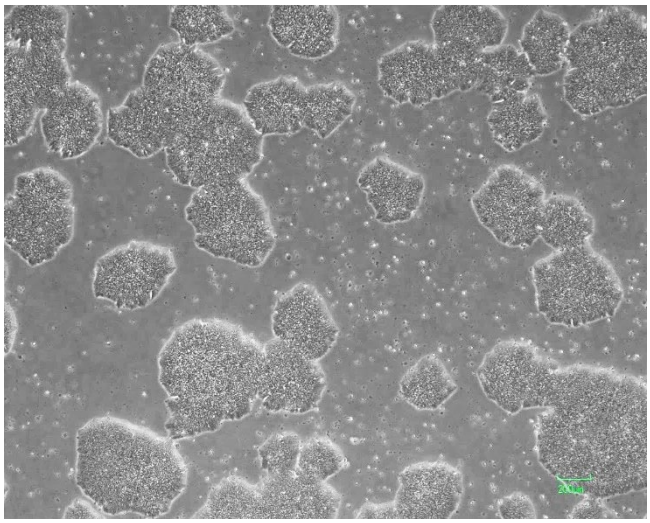
Note: In our internal validation, the method of adding PBS instead of StemFit and detaching and collecting cells by pipetting also showed no difference in cell survival and proliferation rates and expression of undifferentiated markers (OCT3/4).

## Cryopreservation of iPS cells

### ■ Materials

☐ Cells: 6-well plate seeded with cells

Cell status before cryopreservation



(Scale bar; 200 μm)

☐ Reagents: StemFit AK03N (StemFit medium) (Ajinomoto)
PBS (Nakalai Tesque, 14249-24)
0.5×TrypLE Select solution
Trypan blue solution (Sigma-Aldrich, T8154)
STEM-CELLBANKER (Zenogen Pharma, CB045/047)

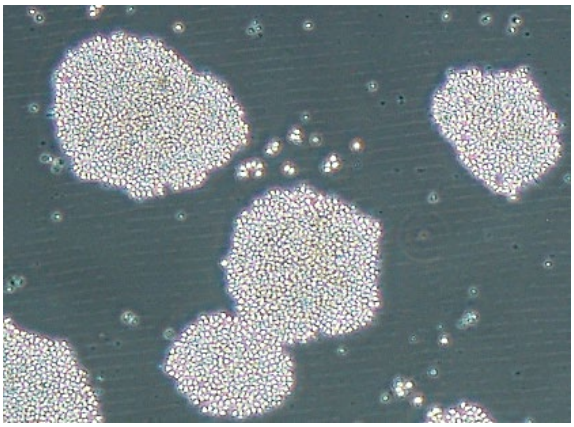
☐ Equipment: BICELL (Japan Freezer, BICELL)

☐ Plasticware: 15/50 mL conical tubes
Plastic Pipettes
Cell scraper
Cryovials (1.0 mL)

### ■ Procedure

1) Check the condition of the cells using a microscope (cell viability, presence of iPS cell-like colonies) and take photographs if necessary.
2) Remove medium and add 1 mL of PBS. Wash all cells by shaking the plate, then remove PBS.

3) Add 1 mL of 0.5×TrypLE Select solution and shake the plate to spread the solution throughout.
4) Return 6-well plate to the incubator and leave for up to 10 minutes.
5) Examine the cells using a microscope to see whether cell-cell adhesions areloosened for almost all cells and whether individual cells can be recognized.



6) After removing 0.5 x TrypLE Select solution, gently add 2 mL of PBS and wash the entire cell by shaking the plate. Then remove PBS.
7) Add 1 mL of new StemFit medium and scrape the cells with a cell scraper* (If multiple clones or strains are to be passed together, scrape cells in all wells with cell scrapers before proceeding).
8) Gently pipet the cells to break them apart (10 times) and collect them in a new tube.
9) Transfer the cell suspension to a 50 mL tube (Combine all well suspensions in one tube).
10) Mix the cell suspension by pipetting (6 times) and roughly measure total liquid volume with scales on the pipette surface.
11) Remove 10 µL of cell suspension, and add 10 µL of Trypan-blue solution, mix and count the cells.
12) Calculate the number of cryovials to freeze based on total number of viable cells (the number of cryovialsx 2.4 x $10^5$ cells).
13) Calculate the number of viable cells required from the total number of cryovials (the number of cryovials x 2.4 x $10^5$ cells)
14) Prepare new conical tubes and aliquot cell suspension with required amount of viable cells.
15) Perform centrifugation (160 x g, 23°C, 5 min). Prepare the required number of new cryotubes.
16) After centrifugation, remove the supernatant.

17) Resuspend the pellet by gentle tapping, add STEM-CELLBANKER to a concentration of 1.2 x $10^6$ cells/mL and suspend by pipetting (8 times). Dispense 200 µL of the cell suspension into each cryovials.
18) After aliquoting, the cryovials are placed in pre-cooled bicelles and frozen at -80°C.
19) Transfer to liquid nitrogen tank within a few days for storage.

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Note: In our internal validation, the method of adding PBS instead of StemFit and detaching and collecting cells by pipetting also showed no difference in cell survival and proliferation rates and expression of undifferentiated markers (OCT3/4).

# Additional information

## Preparation of StemFit(AK03N) medium

☐ Reagents: StemFit AK03N A solution 400 mL (stored at 4°C)
StemFit AK03N B solution 100 mL (stored at -30°C)
StemFit AK03N C solution 2 mL (stored at -30°C)

Dissolve liquids B and C at 4°C or room temperature (at least 8 hours, o/n).

☐ Plasticware: Disposable pipettes

### Procedure

1) Mix liquid B by pipetting and add to a bottle of liquid A.
2) Add 2 mL of liquid C to the bottle of liquid A.
3) Close the lid of liquid A tightly and mix well.
4) Dispense the mixture into 50 mL tubes or bottles.
5) Store at 4°C or -80°C.

## Preparation of 0.5×TrypLE Select solution (0.5 mM EDTA/PBS final concentration 0.75 mM)

☐ Reagents: Cell Therapy Systems TrypLE Select CTS (Thermo Fisher Scientific, A12859)
0.5 mmol/L-EDTA/PBS solution (Nacalai Tesque, 13567-84)
☐ Plasticware: Disposable pipettes
☐ Equipment: 250 mL filter system (0.2 μm filter)

### Procedure

1) Dispense 90 mL of TrypLE Select into a 250 mL storage bottle.
2) Add 90 mL of 0.5 mM EDTA/PBS solution to the storage bottle in 1) and mix by pipetting (about 5 times).
3) Close the bottle lid and mix well.

*The expiration date should be 6 months at room temperature (if the expiration date of the original reagent is less than 6 months, follow the expiration date of the original reagent).

## Reagent amount and seeded cell number according to culture scale.

**Table 1. Amount of laminin (iMatrix-511MG; 0.5 mg/mL) and medium used in the coating**

| Scale | Cultivation area | laminin quantity (iMatrix-511) | PBS volume | Amount of StemFit+Y medium |
|---|---|---|---|---|
| 6-well plate*. | 9.2 $cm^2$ | 9.2 µL | 1.5 mL | 1.5 mL |
| 90 mm Dish | 57 $cm^2$ | 57 µL | 9.3 mL | 9 mL |

*per well

**Table 2: Reagent amounts during cell passaging.**

| Scale | 0.5× TrypLE Select amount | Amount of PBS added (After TrypLE treatment) | StemFit Addition Amount (During cell detachment) |
|---|---|---|---|
| 6-well plate*. | 1 mL | 2 mL | 1 mL |
| 90 mm Dish | 3 mL | 10 mL | 5 mL |

*per well

**Table 3. number of cells seeded during cell passages.**

| Scale | Number of cells seeded |
|---|---|
| 6-well plate*. | 1.3 x $10^4$ cells |
| 90 mm Dish | 8×$10^4$ cells |

*per well

Created by: Kyoto University iPS Cell Research Foundation
Cell Preparation Facility FiT

## References:

1. A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells
M. Nakagawa et al, *Scientific Reports*, 4:3594 (2014) DOI: 10.1038/srep03594

2. An Efficient Non-viral Method to Generate Integration-Free Human iPS Cells from Cord Blood and Peripheral Blood Cells
K. Okita et al, *Stem Cells*, 31(3):458-66 (2013) DOI: 10.1002/stem.1293