Date of Issue: Oct 2021

Ver.211010

FCeM® 3D EXPANSION CULTURE

— FCeM® -series Preparation Kit —

FOR RESEARCH USE ONLY

Nissan Chemical
CORPORATION

Table of Contents

I. Description

FCeM® 3D culture medium is a novel medium which enables 3D culture in low viscosity condition as conventional medium. We confirmed the performance with variety of cells including adherent cells.

Tested cell lines (Example)

Cell line	Species	Type
201B7	Human	Induced Pluripotent Stem Cell
253G1	Human	Induced Pluripotent Stem Cell
1383D2	Human	Induced Pluripotent Stem Cell
1383D6	Human	Induced Pluripotent Stem Cell
RPChiPS771	Human	Induced Pluripotent Stem Cell
A375	Human	Malignant Melanoma
A431	Human	Epidermoid Carcinoma
A549	Human	Lung Carcinoma
AGS	Human	Stomach Adenocardinoma
HepG2	Human	Hepato Carcinoma
MIA PaCa-2	Human	Pancreatic Carcinoma
MCF7	Human	Breast Adenocarcinoma
MDA-MB-231	Human	Breast Adenocarcinoma
HeLa	Human	Cervical Cancer
SKOV3	Human	Ovarian Carcinoma
LNCap	Human	Prostate Adenocarcinoma
HCT116	Human	Colon Cancer
MNNG/HOS	Human	Bone Osteosarcoma

This protocol shows iPSC 3D culture method with FCeM® 3D culture medium prepared by FCeM® -series Preparation Kit as an example.

In addition, we developed Sphere Splitting Module and Sphere Collecting Module to realize reproducible culture and ease culture processes. Please get more information by checking our website or contacting us at the contact details described in the end of this document.

Website: https://www.nissanchem.co.jp/eng/products/advance/fcem_3d-ccm.html

II. Materials

- FCeM®-series Preparation Kit (Nissan Chemical, #382-07991 or #385-07981)
- StemFit (Ajinomoto, #AK02N) or mTeSR1 (STEMCELL Technologies, #ST-85850)
- D-MEM/F-12
- D-PBS (-)
- 0.5 mM EDTA in PBS or Dissociation Solution for human ES/iPS cells (Reprocell, #RCHETP002) or CTK solution (0.05 % collagenase IV (Gibco), 0.25 % trypsin (Gibco), and 20 % KSR) or Dispase
- 10 mM Y-27632 in PBS
- TrypLE™ Select Enzyme (1X), no phenol red (Thermo, #12563011) or Trypsin solution
- 10% FBS in D-MEM/F-12

III. Equipment

- iPS cell
- 50 mL Conical Centrifuge Tube
- 15 mL Conical Centrifuge Tube
- 1.5 mL Micro tube
- Serological pipettes and pipettes
- Incubator（37°C, 5% CO_2 condition）
- Centrifuge
- Inverted microscope
- 25 - 37°C water bath
- 4°C refrigerator
- NucleoCounter (chemometec, #NC-200) or Cell counter (BioRad, #TC-20) or Hemocytometer
- Stopwatch
- 60 µm opening pluriStrainer (pluriSelect, #43-50060) or Sphere Collecting Module (Nissan Chemical)
- 50 µm opening CellTrics (Sysmex, #04-004-2327) or Sphere Splitting Module (Nissan Chemical)
- Low attachment 10 cm dish (Corning, #3262)
- Low attachment 6-well plate (ULA, Corning, #3471)
- FCeM® 3D Culture Starter Kit (Nissan Chemical) (optional)

IV. 3D culture medium preparation

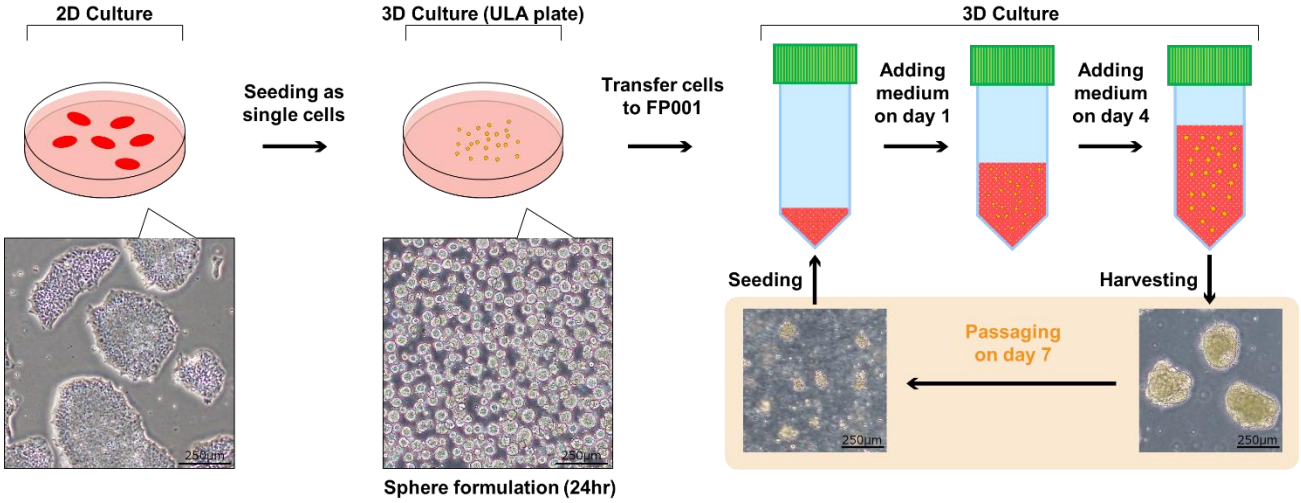
Prepare 3D culture medium with mTeSR1 or StemFit by following the "Instructions for Use" document included in the FCeM® Preparation Kit (Nissan Chemical, #382-07991 or #385-07981).
The recommended FP001 final concentration is 0.016 wt %.

V. Procedure (Single Cell Seeding)

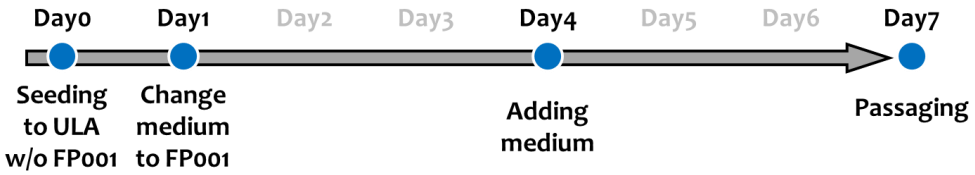
If you passage iPSC by **dissociating into single cells** in your routine 2D culture, please refer to this procedure (e.g. Using iMatrix or Laminin as coating matrix). Otherwise, turn to Procedure VI.

This protocol is based on the suspension culture (3D culture) of 1383D2 human induced pluripotent stem (hiPS) cells in StemFit (Ajinomoto, #AK02N) based 3D culture medium transferred from 2D culture of StemFit medium with iMatrix-511 silk (Matrixome, #892021) coated 6-well plate. If different cell lines or culture media are used, modifications may be required.

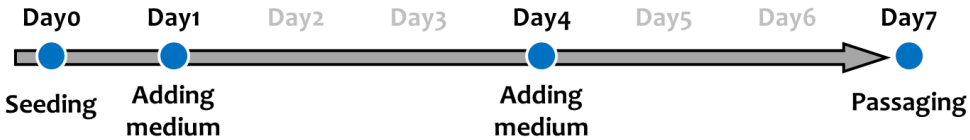
0: Protocol outline



<1st passage from 2D to 3D>



<2nd or subsequent passage from 3D to 3D>



1: Start 3D suspension culture (Day 0)

*Prepare over 2 x 10^6 cells in 2D culture with StemFit and iMatrix-511 silk before starting this process. (e.g. two wells of subconfluent cells in 6-well plate)

*The reagent volume of following instructions is for cells from one well of a 6-well plate.

1. Warm StemFit medium to 25 - 30°C before use.
2. Remove media from the well and wash the cells with 2 mL D-PBS (-).
3. Add 1 mL Dissociation solution (0.5 mM EDTA in PBS) to the well.
4. Incubate at 37°C for 10 minutes.
5. Remove the Dissociation solution and add 1 mL StemFit medium containing 10 µM Y-27632.
6. Pipette until the cells become detached as single cell and transfer the cell suspension to a new 15 mL centrifuge tube.
7. Add 1 mL StemFit medium containing 10 µM Y-27632 to the well and transfer the remaining cells to the 15 mL centrifuge tube.
8. Count cells.
9. Re-suspend 1.8 x 10^6 cells in 9 mL StemFit medium containing 10 µM Y-27632 and seed the cells into a new low attachment 6-well plate at 3 mL/well for 3 wells.
   Note: Do not re-suspend cells with 3D culture medium.
10. Disperse cells uniformly by gently shaking the plate and put the plate into incubator (37°C, 5% CO_2) to get small cell spheres.
    Note: Do not move the plate once put it into incubator.

2: Changing medium to 3D culture medium (Day 1)

1. Warm 27 mL 3D culture medium containing 10 µM Y-27632 to 25 - 30°C before use.
2. Transfer cell suspension from 3 wells of 6-well plate to a new 15 mL centrifuge tube.
3. Centrifuge at 100 x g for 3 minutes and remove supernatant carefully.
4. Loosen the cell pellet by tapping tube and add 5 mL 3D culture medium containing 10 µM Y-27632 to the tube.
5. Re-suspend the cell suspension to make it uniform.
6. Transfer the cell suspension to a new low attachment 10 cm dish or 50 mL centrifuge tube and add 22 mL remaining 3D culture medium. Then, re-suspend the cell suspension to make it uniform. When using centrifuge tube as a culture vessel, after closing the centrifuge tube cap, open the cap slightly to keep air flow.
   Note: The cap must not be fully closed.
7. Put the vessel into the incubator (37°C, 5% CO_2).

3: Adding medium (Day 4)

1. Warm 18 mL 3D culture medium containing 10 µM Y-27632 to 25 - 30°C before use.
2. Take the vessel from incubator and add 18 mL 3D culture medium to the vessel.
3. Re-suspend the cell suspension to make it uniform.

4. When using centrifuge tube as a culture vessel, after closing the centrifuge tube cap, open the cap slightly to keep air flow.
   Note: The cap must not be fully closed.
5. Put the vessel into the incubator (37°C, 5% CO_2).

4: Collecting and Passaging (Day 7)

1. Warm StemFit medium, 3D culture medium and D-MEM/F-12 to 37℃.
2. Put 60 µm pluriStrainer on a new 50 mL centrifuge tube.
3. Transfer cell suspension to the strainer and separate cells from 3D medium.
   Note: Be careful not to spill. With tapping the tube, cell suspension easily through strainer.
4. Add 5 mL D-MEM/F-12 to the vessel and transfer the remaining cells to the strainer.
5. Transfer the strainer upside down to a new 50 mL centrifuge tube.
6. Collect cells by adding 10 mL 3D culture medium containing 10 µM Y-27632 to the strainer.
7. Put 50 µm CellTrics Strainer on a new 15 mL centrifuge tube.
8. Make cell suspension flow through the strainer using 1 mL Pipette with 1 mL tip for about 5 seconds while gently press the tip end against the strainer. Repeat this process ten times to complete the passage process.
9. Count cells (operation of **Cell counting**).

Cell counting

A1. After gently pipetting the cell suspension, transfer 1 mL cell suspension to a new 15 mL centrifuge tube.
A2. Add 9 mL D-MEM/F-12 to the 15 mL centrifuge tube and pipette at 10 times.
A3. Centrifuge at 300 x g for 3 minutes and remove supernatant carefully.
A4. Add 250 µL Trypsin solution and suspend the cell pellet.
A5. Incubate for 2 minutes in water bath at 37°C.
A6. Re-suspend the cell pellet firmly 10 times.
A7. Incubate for 2 minutes in water bath at 37°C again.
A8. Re-suspend the cell pellet firmly 10 times to breaking up cell colonies into single cells. Add 250 µL 10% FBS in D-MEM/F-12 and re-suspend the cells to neutralize trypsin.
A9. Count the cells using Cell counter.

10. Adjust seeding density to 2 x 10^5 cells/mL by adding 3D culture medium containing 10 µM Y-27632 and dispense 10 mL cell suspension to a new low attachment 10 cm dish or 50 mL centrifuge tube. When using centrifuge tube as a culture vessel, after closing the centrifuge tube cap, open the cap slightly to keep air flow.
    Note: The cap must not be fully closed.
11. Put the vessel into incubator (37°C, 5% CO2).

5: Adding medium (Day 1 and 4)

1. Warm 20 mL 3D culture medium containing 10 μM Y-27632 to 25 - 30°C before use.
2. Add 20 mL 3D culture medium to the culture vessel.
3. Re-suspend the cell suspension to make it uniform.
4. When using centrifuge tube as a culture vessel, after closing the centrifuge tube cap, open the cap slightly to keep air flow.
   Note: The cap must not be fully closed.
5. Put the vessel into the incubator (37°C, 5% CO_2).

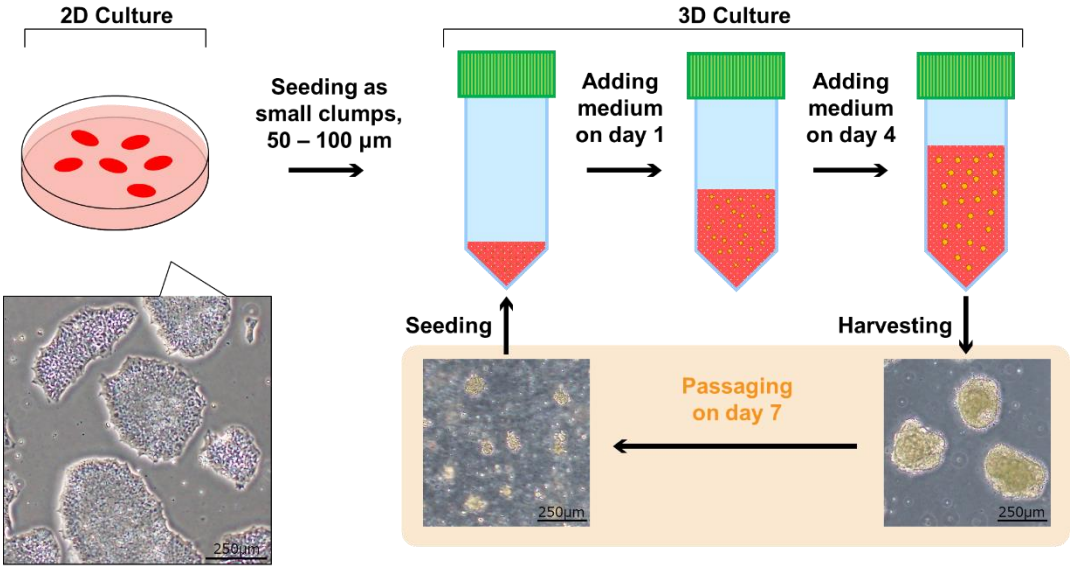
* Repeat step 4 ”Collecting and Passaging (Day 7)” and step 5 ”Adding medium (Day 1 and 4)”.

VI. Procedure (Clump Seeding)

If you passage iPSC by **dissociating into small clumps** in your routine 2D culture, please refer to this procedure of the protocol (e.g. Using Vitronectin or Matrigel as coating matrix).

This protocol is based on the suspension culture (3D culture) of 253G1 human induced pluripotent stem (hiPS) cells in mTeSR1 (STEMCELL Technologies, #ST-85850) based 3D culture medium transferred from 2D culture of mTeSR1 medium with Vitronectin (Thermo, #A14700) coated 6-well plate. If different cell lines or culture media are used, modifications may be required.

0: Protocol outline



1: Start 3D suspension culture (Day 0)

*Prepare over 2 x 10^6 cells in 2D culture with mTeSR1 and Vitronectin before starting this process. (e.g. two wells of subconfluent cells in 6-well plate)

*The regent volume of following instructions is for cells from one well of a 6-well plate.

1. Warm mTeSR1 medium, 3D culture medium, PBS and D-MEM/F-12 to 25 - 30°C before use.
2. Remove media from the cells and wash the cells with 2 mL D-PBS (-).
3. Add 1 mL Dissociation solution (e.g. Dissociation Solution for human ES/iPS cells or CTK solution or Dispase).
4. Incubate at 37°C in an incubator for 3 - 5 minutes until the cells begin to slough off the well.
5. Remove the Dissociation solution and add 1 mL mTeSR1 medium.
6. Gently pipette until the cells become detached and transfer the cell suspension to a new 15 mL centrifuge tube.
   Note: Don't break up cell colonies into single cells. Appropriate crump size is from 50 to 100 µm.
7. Add 1 mL mTeSR1 medium to the well and transfer the remaining cells to the 15 mL centrifuge tube.
8. Count cells (operation of **Cell counting**).

Cell counting

A1. Transfer 500 µL cell suspension into 1.5 mL Micro tube.
A2. Centrifuge at 100 x g for 3 minutes and remove supernatant.
A3. Add 250 µL Trypsin solution and suspend the cell pellet.
A4. Incubate for 2 minutes in water bath at 37°C.
A5. Re-suspend the cell pellet firmly 10 times.
A6. Incubate for 2 minutes in water bath at 37 °C again.
A7. Re-suspend the cell pellet firmly 10 times to breaking up cell colonies into single cells. Add 250 µL 10% FBS D-MEM/F-12 and re-suspend the cells to neutralize trypsin.
A8. Count the cells using Cell counter.

9. Transfer 2 – 3 x 10^6 cells to a new 50 mL centrifuge tube.
10. Centrifuge at 100 x g for 3 minutes and remove supernatant.
11. Tapping the 50 mL centrifuge tube to disperse cell pellet and re-suspend the cell pellet with 10 mL 3D culture medium containing 10 µM Y-27632.
12. Put 50 µm CellTrics Strainer on a new 15 mL centrifuge tube.
13. Make cell suspension flow through the strainer using 1 mL Pipette with 1 mL tip for about 5 seconds while gently press the tip end against the strainer.
14. Transfer cell suspension to a new low attachment 10 cm dish or 50 mL centrifuge tube. When using centrifuge tube as a culture vessel, after closing the centrifuge tube cap, open the cap slightly to keep air flow.
    Note: The cap must not be fully closed.
15. Put the centrifuge tube into incubator (37°C, 5% CO_2).

2: Adding medium (Day 1 and 4)

1. Warm 20 mL 3D culture medium to 25 - 30°C before use.
2. Add 20 mL 3D culture medium to the culture vessel.
3. Re-suspend the cell suspension to make it uniform.
4. When using centrifuge tube as a culture vessel, after closing the centrifuge tube cap, open the cap slightly to keep air flow.
   Note: The cap must not be fully closed.
5. Put the vessel into the incubator (37°C, 5% CO_2).

3: Collecting and Passaging (Day 7)

1. Warm mTeSR1 medium, 3D culture medium and D-MEM/F-12 to 37℃.
2. Put 60 µm pluriStrainer on a new 50 mL centrifuge tube.
3. Transfer cell suspension to the strainer and separate cells from 3D medium.
   Note: Be careful not to spill. With tapping the tube, cell suspension easily through strainer.
4. Add 5 mL D-MEM/F-12 to the vessel and transfer the remaining cells to the strainer.
5. Transfer the strainer upside down to a new 50 mL centrifuge tube.
6. Collect cells by adding 10 mL 3D culture medium containing 10 µM Y-27632 to the strainer.
7. Put 50 µm CellTrics Strainer on a new 15 mL centrifuge tube.
8. Make cell suspension flow through the strainer using 1 mL Pipette with 1 mL tip for about 5 seconds while gently press the tip end against the strainer. Repeat this process ten times to complete the passage process.
9. Count cells (operation of **Cell counting**).

Cell counting

A1. After gently pipetting the cell suspension, transfer 1 mL cell suspension to a new 15 mL centrifuge tube.
A2. Add 9 mL D-MEM/F-12 to the 15 mL centrifuge tube and pipette at 10 times.
A3. Centrifuge at 300 x g for 3 minutes and remove supernatant carefully.
A4. Add 250 µL Trypsin solution and suspend the cell pellet.
A5. Incubate for 2 minutes in water bath at 37°C.
A6. Re-suspend the cell pellet firmly 10 times.
A7. Incubate for 2 minutes in water bath at 37°C again.
A8. Re-suspend the cell pellet firmly 10 times to breaking up cell colonies into single cells. Add 250 µL 10% FBS in D-MEM/F-12 and re-suspend the cells to neutralize trypsin.
A9. Count the cells using Cell counter.

10. Adjust seeding density to 2 x 10^5 cells/mL by adding 3D culture medium containing 10 µM Y-27632 and dispense the 10 mL cell suspension to a new low attachment 10 cm dish or 50 mL centrifuge tube. When using centrifuge tube as a culture vessel, after closing the centrifuge tube cap, open the cap slightly to keep air flow.
    Note: The cap must not be fully closed.
11. Put the vessel into incubator (37°C, 5% CO2).

* Repeat step 2 ”Adding medium (Day 1 and 4)” and step 3 ”Collecting and Passaging (Day 7)”.

VI. Appendix

Troubleshooting

Problem	Solution
After 3D culture, cell spheres don't precipitate by centrifugation.	Dilute cell suspension over 5 times by D-MEM/F-12.
	Pipette the cell suspension (up and down) at 10 times after the DMEM/F12 dilution.
Cell spheres gather upside in the centrifuge tube.	Gently mix the tube by pipetting.
	Warm 3D culture medium at 37°C before use.
Cells do not growth in 3D condition.	The cells may not be used to the new 3D environment. Please contact us.

If you have any questions or encounter any problems or need help with any aspect related to these instructions, please contact us.

Nissan Chemical
CORPORATION

LIFE SCIENCE MATERIALS DEVELOPMENT DEPT.

2-5-1 NIHONBASHI, CHUO-KU, TOKYO 103-6119, JAPAN

TEL: +81-3-4463-8370　FAX:+81-3-4463-8371

E-mail: fcem@nissanchem.co.jp