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FCeM® 3D CULTURE STARTER KIT PROTOCOL

ver4.3

Nissan Chemical
CORPORATION

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I. Description

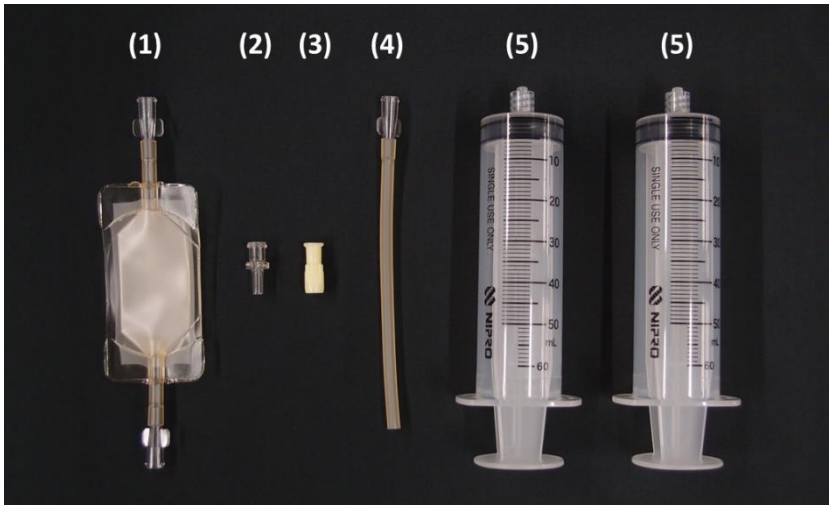
This product is a cell culture kit for ES/iPS cell with FCeM® 3D culture medium. This single use kit makes it possible to collect and passage cell spheres with simple operation. This product is suitable for daily ES/iPS cell culture and mass production study.

【Caution】

- This product ("Product") is designed for research and development use only – Do not use it for other purposes.
- Wear appropriate protective eyewear, clothing, and gloves when handling the Product.
- Nissan Chemical Corporation shall not be liable for any damages as the result of (i) misuse, fault or negligence of or by users or purchasers of the Product, (ii) use of the Product in a manner for which it was not designed, or (iii) improper storage and handling of the Product.
- This protocol is designed for 253G1 human induced pluripotent stem (hiPS) cells grown in mTeSR1 medium with Vitronectin (Thermo, #A14700) coated dish in 2D culture and suspension culture (3D cell culture) of hiPS cells in mTeSR1 based 3D culture medium. If using different cell lines or growth media, the culture schedule and protocol below may need to be modified.

【Components】

1	Sphere collecting module	1 piece, sterile
2	Sphere splitting module	1 piece, sterile
3	Syringe cap	1 piece, sterile
4	Filling nozzle	1 piece, sterile
5	50 mL syringe	2 pieces, sterile



II. Materials

- FCeM®-series Preparation Kit for ES/iPS cells (Nissan Chemical, #382-07991 or #385-07981)
- mTeSR1 (STEMCELL Technologies, #ST-85850)
- D-MEM/F-12
- D-PBS (-)
- 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
  0.25w/v% Trypsin-1mmol/L EDTA·4Na Solution with Phenol Red (FUJIFILM Wako Pure Chemical Corporation, #209-16941)

III. Equipment

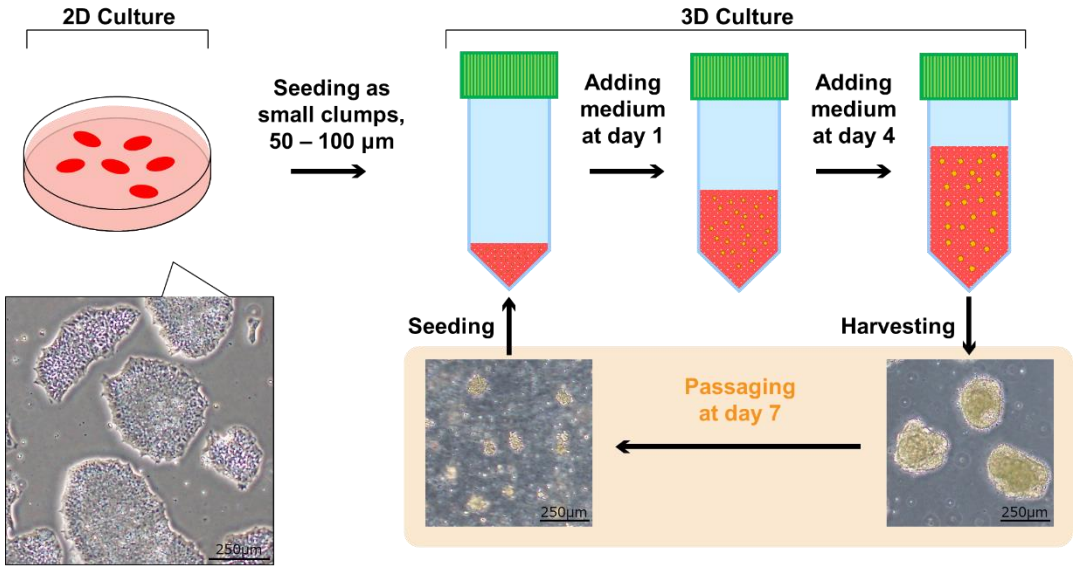
- FCeM® 3D Culture Starter Kit
- 50 mL Conical Centrifuge Tube
- 15 mL Conical Centrifuge Tube
- 1.5 mL Micro tube
- Pipettes and pipette tips
- Serological pipettes
- Incubator（37℃, 5% CO_2 condition）
- Centrifuge
- Inverted microscope
- 25 - 37℃ water bath
- 4℃ refrigerator
- NucleoCounter (chemometec, #NC-200) or Cell counter (BioRad, #TC-20) or Hemocytometer
- Stopwatch

IV. 3D culture medium preparation

The procedure to mix FP001 Solution with mTeSR1 can be found in the "Instructions for Use" document included in the FCeM® Preparation Kit for Stem Cells (Nissan Chemical, #382-07991).
The recommended FP001 final concentration is 0.01 - 0.016 wt %.

V. Procedure

0: Protocol outline



1: Start of suspension sphere culture from conventional adherent culture

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

1. Warm mTeSR1 medium and 3D culture medium to 25 - 30℃ before use.
2. Remove the existing 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℃ 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 over the well until the cells become detached and transfer the cell suspension to a new 15 mL centrifuge tube.
   Note: Not breaking up cell colonies into single cells. Appropriate crump size is from 50 to 100 µm.
7. Add 2 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℃.
A5. Re-suspend the cell pellet firmly at 10 times.
A6. Incubate for 2 minutes in water bath at 37 ℃ again.
A7. Re-suspend the cell pellet firmly at 10 times to breaking up cell colonies into single cells.
A8. Add 250 µL mTeSR1 and re-suspend the cells to neutralize trypsin.
A9. 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. After closing the centrifuge tube cap, open the cap slightly to keep air flow.
13. Put the centrifuge tube into incubator (37℃, 5% CO_2).
    Note: The centrifuge tube cap must not be fully closed.

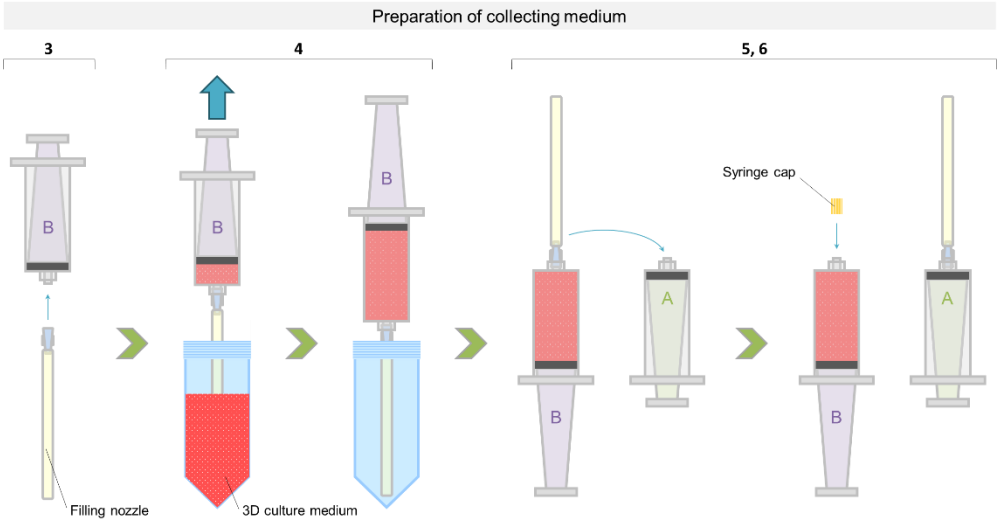
2: Adding medium at day 1 and 4

1. Warm 20 mL 3D culture medium to 25 - 30℃ before use.
2. Adding the medium to cell suspension in 50 mL centrifuge tube.
3. Gently pipette the cell suspension to make it uniform.
4. After closing the centrifuge tube cap, open the cap slightly to keep air flow.
5. Put the centrifuge tube into incubator (37℃, 5% CO_2).
   Note: The centrifuge tube cap must not be fully closed.

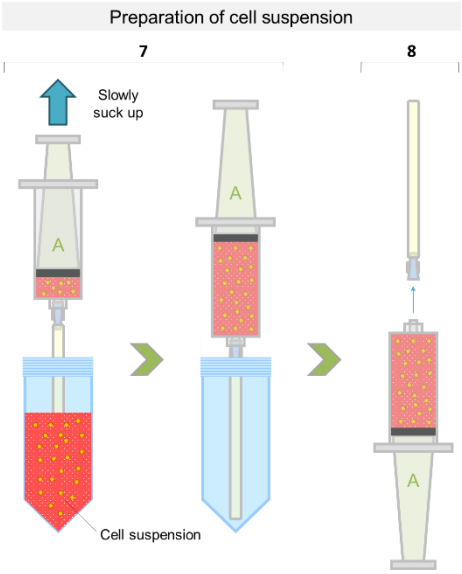
3: Collecting and passaging at day 7

<3-1: Preparation of collecting medium and cell suspension>

1. Warm mTeSR1 medium, 3D culture medium and D-MEM/F-12 to 25 - 30℃ before use.
2. Dispense 50 mL 3D culture medium to a new 50 mL centrifuge tube.

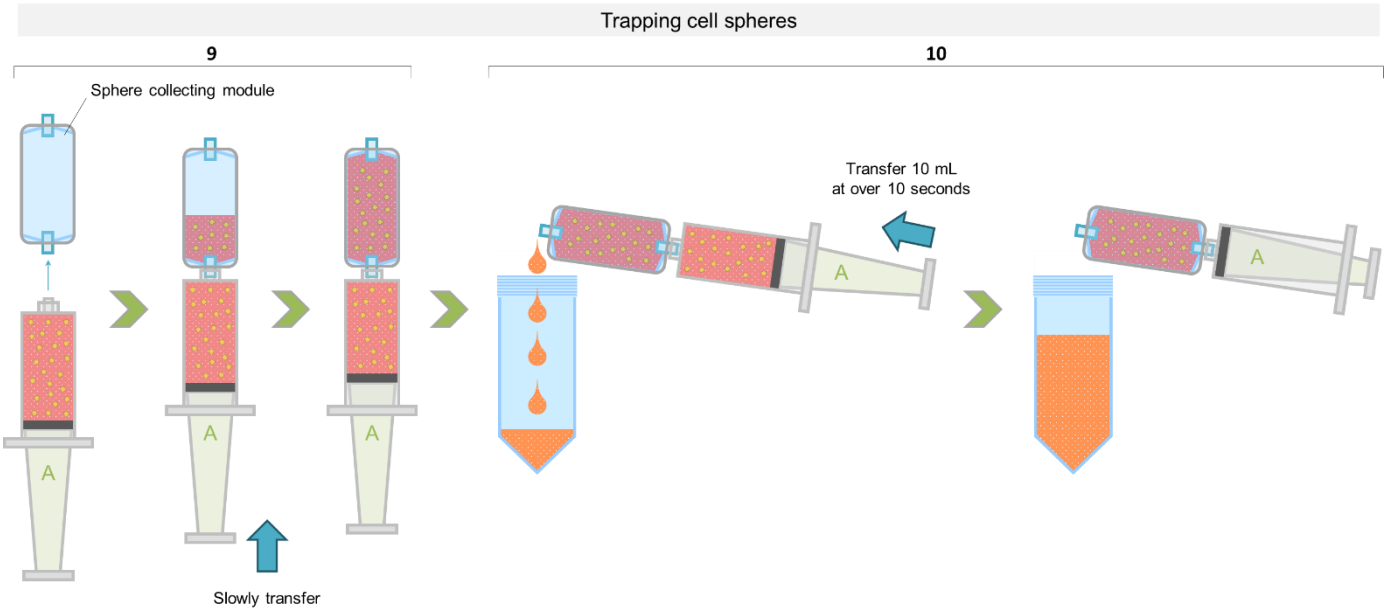


3. Unseal two syringes and put one syringe "syringe A" on centrifuge tube rack to keep not touched the tip around. Putting filling nozzle to another syringe "syringe B" tip.
4. Suck up the 3D culture medium (prepared step: 2) into the syringe B with filling nozzle. Direct the syringe tip up and push air out of the syringe completely.
5. Remove the filling nozzle from the syringe B and put the nozzle to syringe A tip. Put the syringe B on centrifuge tube rack to keep not touched the tip around.
6. Put syringe cap to syringe B tip and put the syringe B on centrifuge tube rack.

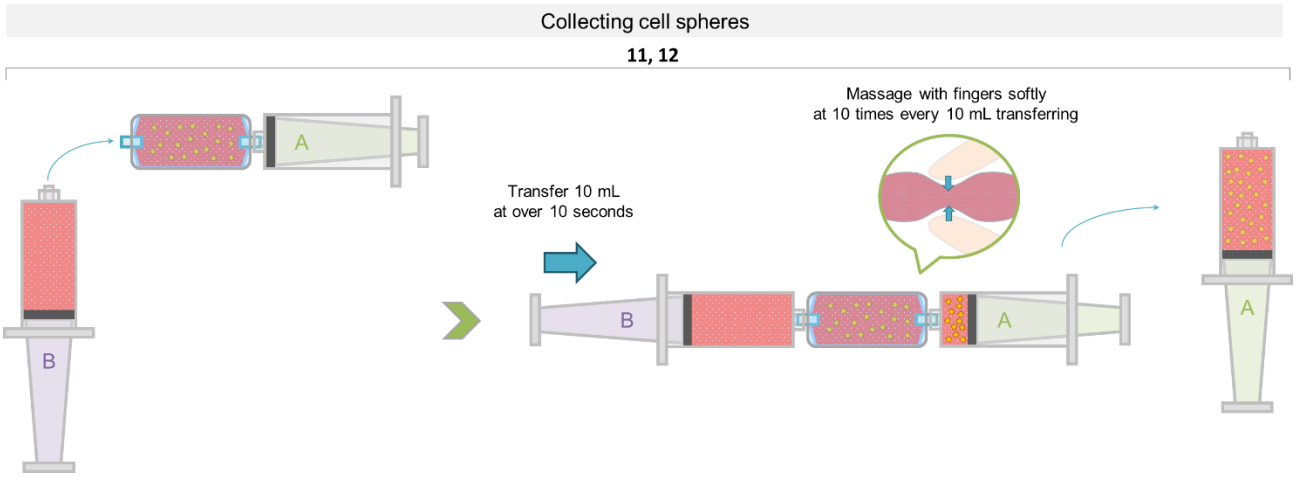


7. Slowly suck up cell suspension with the syringe A.
8. Remove the filling nozzle from syringe A.

<3-2: Trapping and collecting cell spheres>

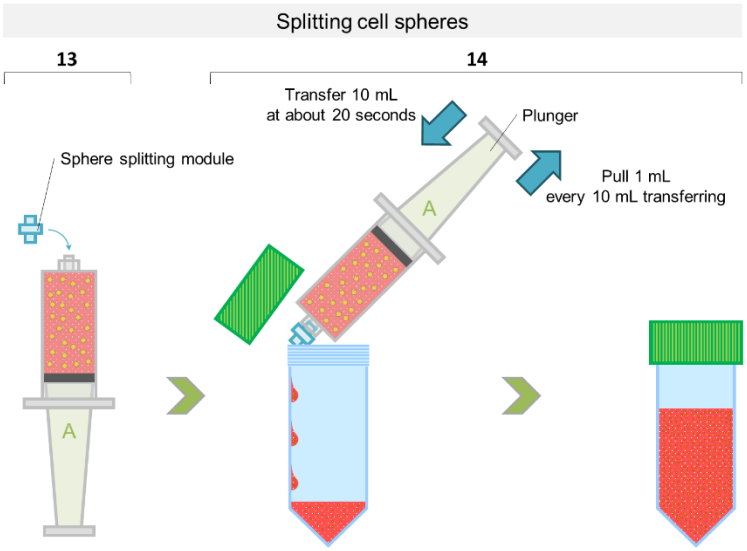


9. Connect the syringe A to the sphere collecting module firmly.
   Note: Sphere collection module is symmetrical structure.
   Direct the sphere collecting module up and slowly transfer cell suspension to the module to fill the module with cell suspension.
10. Transfer cell suspension to sphere collecting module slowly; 10 mL cell suspension is transferred at over 10 seconds. (e.g. if you collect 50 mL cell suspension, it takes about 1 minute to transfer it to sphere collecting module.) Receive waste with 50 mL centrifuge tube.



11. Connect the syringe A to the sphere collecting module with air released.
    Transfer 3D culture medium to sphere collecting module slowly; 10 mL 3D culture medium is transferred at over 10 seconds, and massage sphere collecting module with fingers softly at 10 times every 10 mL transferring.
12. After all 3D culture medium transferred, pull the syringe B plunger to collect residual liquid in the sphere collecting module.
13. Remove the sphere collecting module from syringe B.

<3-3: Splitting cell spheres>



14. Put sphere splitting module to syringe B tip filled with cell suspension firmly.
15. Prepare a new 50 mL centrifuge tube and place tip of sphere splitting module to inner side of the 50 mL centrifuge tube lightly.
    Transfer cell suspension 10 mL at about 20 seconds with pulling plunger 1 mL every 10 mL transferring.
16. Count cells (operation of **Cell counting**).

Cell counting

A1. After gently pipetting the cell suspension, transfer 3 mL cell suspension to a new 15 mL centrifuge tube.
A2. Add 12 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℃.
A6. Re-suspend the cell pellet firmly at 10 times.
A7. Incubate for 2 minutes in water bath at 37℃ again.
A8. Re-suspend the cell pellet firmly at 10 times to breaking up cell colonies into single cells. Add 250 µL mTeSR1 and re-suspend the cells to neutralize trypsin.
A9. Count the cells using Cell counter.

17. Adjust seeding density to 2 - 3 x 10^5 cells/mL by adding 3D culture medium and dispense the cell suspension to a new 50 mL centrifuge tube.
18. Add Y-27632 to the cell suspension at 10 µM final concentration and gently pipette the cell suspension to make it uniform.
19. After closing the centrifuge tube cap, open the cap slightly to keep air flow.
20. Put the centrifuge tube into incubator (37℃, 5% CO2).
    Note: The centrifuge tube cap must not be fully closed.

VI. Appendix

Troubleshooting

<About Starter Kit>

Problem	Possible reason	Solution
Cell recovery rate with sphere collecting module is not good.	Cell spheres are too small. *Sphere collecting module separates cell spheres and 3D culture medium by sphere size.	Culture cell spheres until the diameter size become about 200 μm.
	Time to transfer cell spheres to sphere collecting module is too short.	Transfer cell suspension to sphere collecting module more slowly; 10 mL cell suspension is transferred at over 20 seconds.
	Cell spheres remain in sphere collecting module.	Transfer 3D culture medium to sphere collecting module more slowly; 10 mL cell suspension is transferred at over 20 seconds.
		Massage sphere collecting module with fingers softly at more 10 times every 10 mL transferring.
		Transfer fluids to sphere collecting module without containing air.
Splitting efficiency with sphere splitting module is not good.	Splitting velocity is too high.	Transfer cell suspension 10 mL at over 20 seconds.
	Cells are stuck in sphere splitting module.	Transfer cell suspension with pulling plunger 1 mL every 5 mL transferring.
	Cell density is too high.	Dilute cell suspension at under 3 x 10^5 cells/mL.

<About 3D cell culture>

Problem	Solution
After 3D culture, cell spheres don't precipitate by centrifugation.	Dilute cell suspension over 5 times by D-MEM/F-12.
	Re-suspend cell suspension more 10 times after dilution.
Cell spheres gathered up side in the centrifuge tube.	Gently mix the tube by pipetting.
	Warm 3D culture medium at 37℃ 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.

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