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Serum-free & feeder-free culture protocol

for Human iPS cells using *CELRENA® medium*

CELRENA® medium is a set product of “modified DMEM/F12” medium and supplement “SUBSER-ESrP” designed for iPS cell culture

CD17282

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1. Introduction

It has been strongly desired that completely animal derived-free culture system which do not required serum, and feeder cells to ensuring accurate examination results and biological safety of cultured cells. Additionally, these completely animal derived-free culture system will contribute safety regenerative medicine which will use iPS cells. Modified DMEM/F-12 medium was developed as optimized basal culture medium for self-renewal culture for human iPS cells which especially optimized oxi-redox components for cellular metabolism of human iPS cells. SUBSER-ESrP include proteins and growth factors which required for growth and maintaining pluripotency of human iPS cells. Modified DMEM/F-12 medium and SUBSER-ESrP do not include animal derived factors, and protein components are made from recombinant protein technologies. The combination use of modified DMEM/F-12 medium and SUBSER-ESrP enable completely animal derived-free and feeder cells-free culture for human iPS cells. There are many possibilities in serum-free and feeder-free cell culture of human iPS cells contributes to progression of regenerative medicine.

2. Human iPS cells culture protocol using "CELRENA® medium" (Set medium of modified DMEM/F-12 & SUBSER-ESrP)

2.1 Materials

1. iPS cells, which were pre-cultured on feeder cell with KSR medium for 3 to 4 days after serial passages.
2. CELRENA medium (CSTI, 2008-05), set medium of modified DMEM/F-12 medium (CSTI, 2010-05) and SUBSER-ESrP (CSTI, 2011)
   *The modified DMEM/F12 medium should be stored at 4℃ (do not freeze) and SUBSER-ESrP should be kept at below -30℃ until use.
   SUBSER-ESrP should be stored at 4℃ after thawing and use up as soon as possible, do not repeat freezing and thawing.
3. Dulbecco's Phosphate Buffered Saline (such as CSTI 1102P). We recommend to use CSTI product.
4. Fibronectin (Sigma F1141; 1 mg/ml solution).
5. Dispase (Roche Dispase Ⅱ, 04 942 078) to make Dispase solution.
6. Other required culture equipments
   Tissue culture-treated culture vessel
   Cell scraper
   Plastic tubes for centrifugation or preparation required

2.2 Preparation of culture medium, Dispase solution and Fibronectin coating solution

2.2.1 Preparation of CELRENA medium as iPS cells self-renewal medium

1. Gently mix 1/100 volume of SUBSER-ESrP to modified DMEM/F-12.(CELRENA medium)
   *Recommend to make necessity volume of the medium just before use, see Table 1.
2. Store at 2 to 8˚C until use.

2.2.2 Dispase solution

1. Making Dispase stock solution: Dissolve the Dispase II (Roch, 04942078) with modified DMEM/F-12 medium to 100 unit/mL and sterile by filtration. Aliquot to sufficient volume and freeze them at below -30 ˚C.
2. Aseptically dilute the Dispase stock solution with modified DMEM/F-12 medium until 1 to 1.5 unit/mL prior to use (Dispase solution).
3. Store at 2-8˚C until use.

2.2.3 Coating solution

1. Aseptically dilute Fibronectin solution to 16.7 μ g/mL with PBS(-) in test tube.
2. Store at 2-8℃ until use.

Table 1. Require amount of Fibronectin, Dispase solution and culture medium according to culture surface area of vessels.

Types of culture vessel	Required volume of		
	Fibronectin soln.	Dispase soln.	CELRENA medium
6-well plate	1.4 mL/ well	0.4 mL/well	2 mL/ well
60mm dish	3 mL/dish	1 mL/dish	4 mL/dish
100mm dish	8 mL/dish	3 mL/dish	10 mL/dish

2.3 Transfer of human iPS cells from on feeder culture to feeder-free culture using "CELRENA medium".

2.3.1 Preparation of Fibronectin coating culture vessel

1. Pre-treat the culture vessel with diluted Fibronectin solution according Table 1, and put the dish into CO_2 incubator (37˚C) at least 3 hours or overnight.

2.3.2 Transfer to feeder-free culture

1. Choose the well growing iPS cells which was pre-cultured on feeder cells and KSR-medium with a microscopically observation.
2. Make required volume of reconstituted CELRENA medium as shown in Table 1, and warm at 37˚C in the tight closed cap vessel.
3. If the colonies containing the differentiate cells form that shown in Fig 1, should be remove mechanically with aspirator before disperse iPS colonies.
4. Remove the medium containing KSR by aspiration and add recommend volume, see Table 1, of Dispase solution into culture vessel.
5. Incubate the dish in CO_2 incubator (37˚C) for 2 min.
6. Aspirate the Dispase solution and add 5 mL of modified DMEM/F-12 medium. Then, mechanically detach colonies from the culture vessel by using cell scraper and collect the cell lamps into 15 mL centrifuge tube.
7. Add another 5 mL of modified DMEM/F-12 medium to the vessel and collect the remaining cells and transfer to the 15 mL centrifuge tube.
8. Spin down the cellular colonies at 200 rpm (approximately 13 x g) the tube for 1-2 min.
9. Remove the medium by aspiration.

10. Add 10mL of modified DMEM/F-12 medium to the tube and loosen colonies once gently with pipetting.
11. Spin down at 200 rpm (13 x g) for 1-2 min.
12. Remove the medium and add 10 mL of warmed reconstituted CELRENA medium.
13. Split the cells 1:3 or 1:4 and seed the cells in warmed reconstituted CELRENA medium into Fibronectin treated culture vessel. Recommend volume of culture medium show on Table 1.

Fig 1. Two types of differentiate cells appeared on iPS colony

2.3.3 Medium change

*Culture medium should change on the next day of passage.

1. Calculate how much volume need for changing the medium, see Table 1.
2. Newly reconstitute decided volume of CELRENA medium into tight cap plastic tube.
3. Warm the reconstituted CELRENA medium at 37˚C.
4. Remove the medium by aspiration and add pre-warmed CELRENA medium into culture vessel.
5. After that, change to fresh CELRENA medium every 2 days interval.

Attention:

* Do not warm the whole bottle of modified DMEM/F-12 medium and SUBSER-ESrP.

** Do not store CELRENA medium, and prohibit repeat use.

2.3.4 Passage at feeder-free and serum-free culture

1. Make required volume of fresh CELRENA medium as shown in Table 1, in the tight cap vessel and keep at 37˚C.
2. The colonies containing the differentiate cells form that shown in Fig 1, should be remove mechanically with cell scraper before disperse iPS colonies.
3. Remove the medium by aspiration.
4. Add recommend volume, see Table 1, of Dispase solution into culture vessel.
5. Put the vessel into the 5% CO_2 incubator of 37˚C for 1-2min until cell detachment appears around a colony, as shown in Fig 2.
6. Remove the Dispase solution by aspiration, gently tap the dish by finger and add 5 ml of modified DMEM/F-12 medium and gently blow the medium on culture surface with pipetting. And collect detached cells and/or cell-lamps into 15mL tube. (Colonies are so fragile.)
7. Add another 5 mL of modified DMEM/F-12 medium to the vessel and collect the remaining cells and transfer to the 15 mL centrifuge tube.

8. Spin down at 200 rpm (13 x g) for 1-2 min.
9. Remove the medium and add fresh modified DMEM/F-12 medium.
10. Gently pipette just several times until obtain cell of 50-100 iPS cells. (Approximately 100 μm) , as shown in Fig 3.
11. Spin down at 200 rpm (13 x g) for 1-2 min.
12. Remove the medium and add warmed fresh reconstituted CELRENA medium.
13. Split the cells 1:3 or 1:8 and seed the cells/cell-clumps with recommend volume, see table 1, of reconstituted CELRENA medium into culture vessel pre-coated Fibronectin.
14. Put the culture vessel in the 5% CO_2 incubator, and culture for a few days.
15. After for 4 to 5 days culture, passage again as mentioned above.

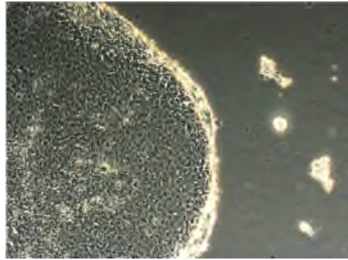

Fig 2. Partially detached colony after Dispase treatment

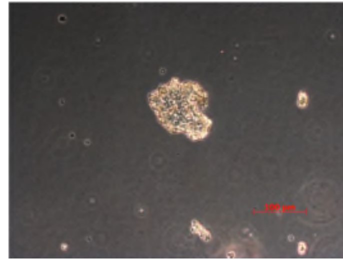

Fig 3. Most suitable size for seeding

3. Single-cell culture of human iPS cells using r-TE and s-TI

3.1 Materials

1. iPS cells, which were pre-cultured on feeder cells and KSR medium for 2 to 3 passages.
2. CELRENA medium (CSTI, 2008-05), set of modified DMEM/F-12 medium (CSTI, 2010-05) and SUBSER-ESrP (CSTI, 2001)
   *The modified DMEM/F12 medium should be stored at 4℃ (do not freezing) and SUBSER-ESrP should be kept at below -30℃ untile use.
   SUBSER-ESrP should be stored at 4℃ after thawing and use up as soon as possible, do not repeat freezing and thawing.
3. Dulbecco's Phosphate Bufferd Saline (such as CSTI 1102P)
4. Sterilized 4mM EDTA/PBS(-) solution
5. r-TE (CSTI, 1210), recombinant trypsin solution
6. s-TI(CSTI, 1220), synthetic trypsin inhibitor solution
7. Corning® Synthemax® Ⅱ (Corning, 3535XX1; 10mg) to make coating solution.
8. Sterilized distilled water
9. Y-27632 (wako, 257-00511)
10. Other required culture equipments
    Tissue culture-treated culture vessel
    Cell scraper
    Plastic tubes for centrifugation or reagent preparation required

3.2 Preparation of Synthemax® Ⅱ coating solution.

3.2.1. Coating solution

1. Before preparation, sterilized distilled water by autoclave or filtration.
2. Add 10 ml of sterilized distilled water to Synthemax Ⅱ vial containing 10 mg of Synthemax Ⅱ powder.
3. Mix gently until the powder dissolves completely, the solution is used as concentrated stock for coating solution.
4. Before coating, dilute 1mg/ml stock solution to 0.025mg/ml solution by distilled water. Dilution factor is 1:40.
5. 1mg/ml stock solution should be stored at 4℃.

Table 2. Require volume of coating solution, r-TE solution and s-TI solution for the kind of culture ware.

Types of culture vessel	Required volume of		
	Synthemax-2 soln. 4mM EDTA/PBS	r-TE soln.	s-TI soln.
6-well plate	2 mL/ well	0.5 mL/well	0.5 mL/well
60mm dish	4 mL/dish	1 mL/dish	1 mL/dish
100mm dish	8 mL/dish	3 mL/dish	3 mL/dish

3.3 Transfer of human iPS cells from on feeder culture to single-cell culture using “CELRENA medium”

3.3.1 Preparation of SynthemaxⅡ coating culture ware

1. Pre-treat the culture dish with diluted SynthemaxⅡ solution according Table 2, and put the dish at room temperature for 2 hours or overnight.

3.3.2 Transfer to single-cell culture

1. Make required volume of reconstituted CELRENA medium as shown in table 1, and warm at 37℃.
2. Remove the medium containing KSR by aspiration and add recommend volume, see table 2, of 4mM EDTA/PBS(-) solution into culture vessel.
3. Aspirate the EDTA/PBS(-) solution and Add required volume, see table 2, of r-TE.
4. Incubate the dish in CO_2 incubator (at 37℃) for 2 to 4 minutes.
5. Add required volume, see table 2, of s-TI.
6. Add 5ml of modified DMEM/F-12 medium and detach the cells by blowing medium or cell scraper.
7. Collect the cell suspension to centrifugation tube.
8. Add another 5 mL of modified DMEM/F-12 medium to the dish and collect the remaining cells and transfer to the 15 mL centrifuge tube.
9. Centrifuge at 1000 rpm for 3 minutes at 4℃.
10. Remove supernatant and add appropriate volume of modified DMEM/F-12 medium.
11. Pipette several time gently and count cell number.
12. Seed 5,000 to 10,000 cells/cm^2 of cells with CELRENA medium to synthemax-2 coated culture vessel and add 10μM Y-27632 to culture medium.
13. Put the vessel in 5% CO_2 incubator.
14. After for 5 to 7 days culture, iPS cells are suitable for passage. Passage again as mentioned above.

3.3.3 Medium change

1. On the second day from seeding, change culture medium to fresh CELRENA medium. Y-27632 is not added to fresh CELRENA medium.Y-27632 is added only at the time of seeding.
2. On and after the first medium change, culture medium should be changed everyday.

Attention:

*Single-cell culture is tend to differentiate than colony-culture.

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