Serum-free & feeder-free culture protocol for Human iPS cells using CELRENA® medium

Ver. 1.1

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

2014, Apr

Cell Science & Technology Institute, Inc.

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

Introduction

It has been strongly desired that completely animal derived-free culture system which do not required serum or serum replacement, 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 medical techniques.

1. Materials

1. iPS cells, which were pre-cultured on feeder cell and 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 cultureware
   Cell scraper
   Plastic tubes for centrifugation or preparation required

2. Preparation of culture medium, Dispase solution and Fibronectin coating solution

2-1. Reconstitution 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. Dispase solution

1. Make 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).
4. Store at 2-8˚C until use.

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, Despase solution and culture medium according to the kind of culture 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

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

3-1. Preparation of Fibronectin coating culture ware

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

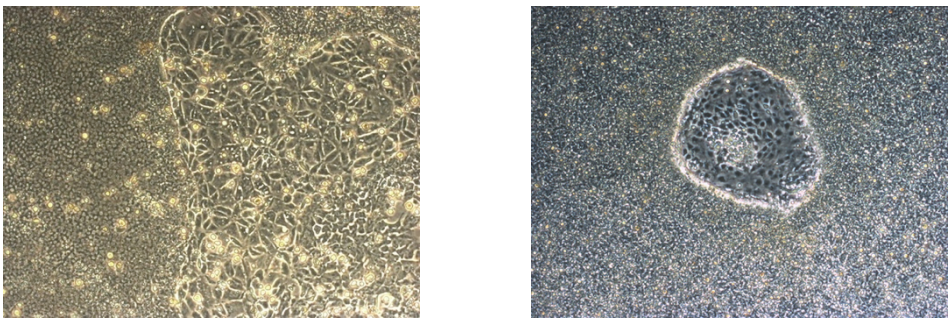
3-2. Transfer to feeder-less culture

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 cap vissel.
3. The colonies containing the differentiate cells form that shown in Fig 1, should be remove mechanically with cell scraper 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 dish 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.
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-12medium to the tube and loosen pipette 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 immature iPS colony



3-4. Medium change

*Culture medium should change 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.

3-5 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 of Dispase solution into culture vessel, see Table 1.
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 new 5 mL of modified DMEM/F-12 medium.
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-lumps of 50-100 iPS cells. (Approximately 100 μ m) , 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-lamps 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

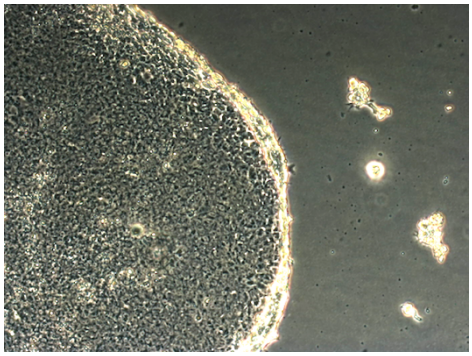
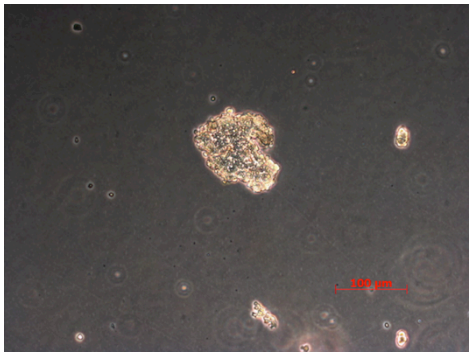


Fig 3. Most suitable size for seeding



* This protocol is restricted to the research purpose only.

Cell Science & Technology Institute Inc. (CSTI)
1-16-16 Seika-en, Aoba-ku Sendai City, 982-0262, Japan

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