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Culture of established induced pluripotent stem cell lines

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Cellular Generation and Phenotyping[1]

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Protocol status: Working
We use this protocol routinely in our group, including for high-throughput culture of iPSC lines, culture of CRISPR/Cas9-edited lines and culture of cell lines adapted from feeder-dependent conditions. These protocols were used when establishing the HipSci cell line bank (http://www.hipsci.org/).

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Keywords: induced pluripotent stem cells, iPSC, feeder free, feeder free iPSCs, pluripotent stem cell lines this protocol, induced pluripotent stem cell line, pluripotent stem cell line, cultured on vitronectin matrix, cell line, crispr screen, vitronectin matrix, cell, streptomycin in the media, streptomycin, culture method,

Abstract

This protocol outlines the method for thawing, passaging and cryopreserving established feeder-free induced pluripotent stem cell lines. In this culture method, cells are kept as aggregates throughout the process. Cells are cultured on vitronectin matrix (full length xeno-free or truncated protein) in E8 or TeSR-E8 media. Cells can be cultured with or without Penicillin-Streptomycin in the media. Cell lines cultured using these protocols are suitable to use for downstream applications including differentiation and CRISPR screens.

Guidelines

We recommend that cells are cultured on vitronectin matrix using E8 or TeSR-E8 media (referred to as culture media in the protocol). Cells can be cultured with or without Penicillin-Streptomycin in the media.

All cell culture should be performed under sterile conditions in a biological safety cabinet.

Materials

MATERIALS

- TeSR™-E8™ Kit for hESC/hiPSC Maintenance 1 Kit **STEMCELL Technologies Inc.** **Catalog #**5990
- Dimethyl sulfoxide (DMSO) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D2650
- Penicillin Streptomycin **Invitrogen - Thermo Fisher** **Catalog #**15140 122
- Gibco™ DPBS no calcium no magnesium **Thermo Fisher Scientific** **Catalog #**14190144
- Falcon™ 15mL Conical Centrifuge Tubes **Fisher Scientific** **Catalog #**14-959-53A
- Essential 8™ Medium **Gibco - Thermo Fisher Scientific** **Catalog #**A1517001
- Vitronectin (VTN-N) Recombinant Human Protein, Truncated **Thermo Fisher** **Catalog #**A14700
- UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher** **Catalog #**15575020
- Falcon 50mL Conical Centrifuge Tubes **Fisher Scientific** **Catalog #**14-432-22
- Knockout serum replacement (KSR) **Gibco - Thermo Fisher Scientific** **Catalog #**10828028
- Nunc 1.8ml Cryotube External Thread Starfoot **Scientific Laboratory Supplies Ltd** **Catalog #**375418K
- Corning® CoolCell® FTS30 Freezing Container for 30 × 1 mL or 2 mL Cryogenic Vials Green **Corning** **Catalog #**432008
- Costar® 6-well Clear TC-treated Multiple Well Plates Individually Wrapped Sterile **Corning** **Catalog #**3516
- Falcon® 6-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate with Lid Individually Wra **Corning** **Catalog #**353046
- Vitronectin XF™ **STEMCELL Technologies Inc.** **Catalog ##**07180
- Y-27632 dihydrochloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**Y0503

Troubleshooting

Safety warnings

Please refer to the manufacturer's documentation and material safety data sheets (MSDS) for the products you are using when following this protocol.

Before start

We recommend that cells are cultured on vitronectin matrix using E8 or TeSR-E8 media (referred to as culture media in the protocol).

All cell culture should be performed under sterile conditions in a biological safety cabinet.

iPSC Thawing

1 **Preparation**
Coat a 6 well plate with vitronectin and incubate according to the manufacturer's instructions.
Prepare complete E8 or TeSR-E8 media according to the manufacturer's instructions.

2 Prepare thawing media by supplementing culture media with rock inhibitor (Y-27632) to a final concentration of 10µM.

3 Add 8ml of thawing media per cryovial being thawed into a sterile 15ml falcon tube(s).

4 Partially thaw the frozen cryovial(s) of iPS cells in a 37 °C water bath until there is a small ice crystal remaining.

5 Add 1ml of thawing media dropwise to each cryovial. Collect the full volume of media and cell suspension and add to the Falcon tube prepared in step 3. iPSCs should be kept as small clumps as much as possible to increase survival efficiency; minimize the amount of pipetting when thawing cells to reduce the number of single cells in the cell suspension.

6 Centrifuge at 120 rcf for 3 min at room temperature.
120 x g, Room temperature, 00:03:00

7 Aspirate the vitronectin from the labware surface and replace with 1ml of thawing media per well. Do not allow surfaces to dry out.

8 Aspirate the supernatant and gently re-suspend each cell pellet in 1ml of thawing media. Plate the cell suspension into the 1ml of thawing media in the well(s).

9 Agitate the plate gently within a tissue culture incubator set at 37 °C , 5% CO_2 to ensure even distribution of cells across the well.

10 The cells will take 2-24 hours to attach to the surface. Make sure the plate is not disturbed during this time. 24 hours after plating, do a full media change to remove the rock inhibitor.

11 Media change the cells every 24 hours with culture media until they reach 70-80% confluency; at this point they need to be passaged.

iPSC Passaging

12 **Preparation**
Coat a 6 well plate(s) with vitronectin and incubate according to the manufacturer's instructions.

13 Prepare fresh 0.5mM EDTA by diluting UltraPure 0.5M EDTA, pH 8.0 with DPBS(-/-). Store at room temperature and use on day of preparation only.

14 Aspirate the spent medium from cells and wash with 2ml of DPBS(-/-) per well.

15 Aspirate DPBS(-/-) and add 1-2ml of 0.5mM EDTA per well. Rock the dishes to cover the surface of the cells and incubate for 4 min at room temperature.

16 Observe the colonies under a microscope until colonies display shiny ‘halos’ around the edges and holes appear throughout the colonies.

17 Aspirate the EDTA solution and add 2ml of culture medium per well.

18 Collect and dispense medium across the labware surface up to 3 times to detach cells. If colonies are still attached, repeat wash with same volume of fresh media. Avoid creating bubbles. Collect the cells in a falcon tube.

19 Aspirate victronectin from new labware surfaces and replace with 1ml of pre-warmed culture media. Do not allow surfaces to dry out.

20 Add required amount of culture medium to cell suspension depending on desired split ratio. Aim for a split ratio where the cells are passaged approximately every 4-5 days. The split ratio may have to be adjusted depending on cell quality.

21 Plate 1ml of cell suspension into each new well.

22 Agitate the plate gently within a tissue culture incubator set at 37 °C , 5% CO_2 to ensure even distribution of cells across the well.

23 The cells will take 2-24 hours to attach to the surface. Media change the cells every 24 hours. Culture the cells until they reach 70-80% confluency; at this point they can passaged again for further expansion.

iPSC Cryopreservation

24 When colonies are compact and roughly 70% confluent they are ready to be frozen. One confluent well of a 6 well plate should be frozen down into 5 cryovials.

25 Make fresh freezing media using 10% DMSO in KSR (for example 1mL DMSO in 9mL KSR) and store at 4°C until required.

26 Prepare fresh 0.5mM EDTA by diluting UltraPure 0.5M EDTA, pH 8.0 with DPBS(-/-). Store at room temperature and use on day of preparation only.

27 Aspirate the spent medium from the labware and wash with 2ml of DPBS(-/-) per well.

28 Aspirate the DPBS(-/-) and add 2ml of 0.5mM EDTA per well.

29 Rock the dishes to cover the surface of the cells and incubate for 4-8 min at room temperature. Observe the colonies under a microscope until colonies display shiny 'halos' around the edges and holes appear throughout the colonies.

30 Aspirate the EDTA solution and add 2ml of culture medium per well for cell collection.

31 Remove the cells by gently washing the labware surface with the media up to 3 times. If >50% of colonies are still attached, repeat wash with same volume of fresh media. Avoid creating bubbles. Collect the cells into an appropriate Falcon tube.

32 Centrifuge the Falcon tube at 120rcf for 1 min. 120 x g, Room temperature, 00:01:00

33 Aspirate the supernatant and re-suspend pellet in enough of the freezing media (prepared in step 3) to give a total volume of 1mL per cryovial.

34 Dispense 1mL of cell suspension into each cryovial. Place the vials in a CoolCell or similar freezing container and store at -80°C. Note: Once in freezing media, cells should be transferred to the -80°C freezer as quickly as possible to prevent cell death.

35 After 24-48 hours at -80°C, transfer vials from the CoolCell into Liquid Nitrogen storage. The vials can be stored in liquid nitrogen indefinitely.