Splitting hESC/hiPSC lines on MEF using Accutase

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Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), are known to be vulnerable to apoptosis upon various technical manipulation, such as single cell dissociation, freezing and thawing, etc., which hinder their use for clonal isolation in gene transfer, differentiation and FACS cell sorting.

However, Y-27632, a selective inhibitor of p160-Rho-associated coiled-coil kinase (ROCK) was found to be an effective inhibitor of apoptosis and enhanced survival of hPSCs upon single cell dissociation.

Here we describe how to propagate hPSCs in single cell dissociation using

Accutase, a ready to use cell detachment solution of proteolytic and collagenolytic enzymes and a direct replacement for trypsin solution.

Protocol

- Preparation of MEF feeder plate

1. Coat culture dish with sterile 0.1% gelatin solution for about 5 min at room temperature.
2. Thaw frozen MEF vial at 37°C water bath quickly.
3. Transfer cells into 15 ml conical tube and add fibroblast media (DMEM + 10% FBS).
4. Wash cells via spinning at 1500 rpm, 5 min.
5. Plate MEF at a density of 1.5–2.0 $\times$ 10^4/cm^2.
6. Let MEF settle down at least 8 hours before use.
   (Overnight is best and MEF can be used within 2 days)

- Splitting hPSCs with Accutase

1. Replace media for MEF dish with hES media.
2. Remove differentiated hPSCs colonies under microscope (optional).
3. Aspirate the media of hPSCs and add appropriate amount of Accutase (ex. 2 ml in 60 mm dish).
4. Incubate cells at 37°C incubator for 20 min until cells are in single cell suspension.

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5. Harvest cells into conical tube and add hES media for wash.
   Cell strainer with 40 μm-pore can be used to remove debris and cell clumps (optional).
6. Centrifuge cells for 5 min at 1000 rpm in hESC media to remove any remaining Accutse solution.
7. Resuspend cells with hES media and plate cells onto MEF feeder cells with density of 1.0–2.0 $\times$ 10^4 cells/cm^2.
8. Add Y-27632 with a final concentration of 10 μM.
9. Incubate cells at 37°C incubator for the growth and feed with hES media everyday.

Materials

- Cell Materials

Cell Materials
Mitotically inactivated mouse fetal fibroblasts (PMEF-CF, GlobalStem)
hESCs and hiPSCs

- hES media (1000 ml)

Ingredient	Amount	Company	Catalog#
DMEM/F12	800 ml	Life Technology	11330-032
Knockout serum replacement	200 ml	Life Technology	10829-018
L-Glutamine	5 ml	Life Technology	21051-016
Pen/Strep	5 ml	Life Technology	15070-063
MEM-NEAA	10 ml	Life Technology	11140-050
β–mercaptoethanol	1 ml	Life Technology	21985-023
FGF-2	4 ng/ml	R&D system	233-FB-001MG

- DMEM with 10% FBS (1000 ml)

Ingredient	Amount	Company	Catalog#
DMEM	880 ml	Life Technology	11960-044
FBS	100 ml	Hyclone	16140-071
L-Glutamine	10 ml	Life Technology	21051-016
Pen/Strep	10 ml	Life Technology	15070-063

- Reagents

Name	Company	Catalog#
Ca^2/Mg^2-free DPBS	Life Technology	14190-250
Accutase	Innovative Cell Technology	AT104
Y-27632 dihydrochloride	Tocris	1254
0.1% Gelatin	Millipore	ES-006-B

- STUFF

Inverted microscope (i.e., Nikon TE or Olympus IX)
Biosafety cabinet for cell culture
CO_2 incubator with controlling and monitoring system for CO_2, humidity and temperature
Cell culture centrifuge
Water bath with temperature control
Glass hemocytometer
Cell culture disposables: Tissue culture dishes, centrifuge tubes, pipettes, pipette tips, cell strainer etc.
Gelatin-coated dishes

Troubleshooting

- Cells are still in clump with Accutase treatment
  - Remove clumps with cell strainer
  - Incubate cells enough time
  - Check Accutase
- No colonies attached/survived
  - Wait until single cells form visible colonies for about a couple of days
  - Check Y-27632

Reference

Watanabe, K., Ueno, M., Kamiya, D., Nishiyama, A., Matsumura, M., Wataya, T., Takahashi, J.B., Nishikawa, S., Nishikawa, S., Muguruma, K., and Sasai, Y. (2007). A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnology *25*, 681–686.