Cryopreservation of human pluripotent stem cells in defined conditions

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Introduction

Cryopreservation is a critical step to preserve the integrity of human pluripotent stem cells, however, the recovery after cryopreservation is inefficient with traditional enzymatic methods, such as dispase and collagenase. Due to the technical difficulties of cryopreservation, regular passaging methods are often different from harvest methods used in cryopreservation. Even though individualization with ROCK inhibitor treatment has been successfully used to harvest cells in both passaging and cryopreservation, it is still desirable to have a method that could preserve stem cells without drug treatment. At the same time, serum or other animal products are often used in cryopreservation, which could potentially compromise the future applications of the cells. We developed an enzyme-free method to passage and cryopreserve pluripotent stem cells in chemically defined medium. High recovery efficiency could be achieved in dependent of ROCK inhibitor treatment.

Here we describe how to cryopreserve hESCs and hiPSCs in E8 medium with EDTA dissociation method. For low passage and stock lines, we usually add 10 µM Y27632 to further insure the cryopreservation efficiency.

Protocol

A. Cryopreservation of human ES/iPS cells

1. Maintain cells in E8 medium on matrigel-coated plates, and change medium daily.
2. Split cells with 1:4 ratio 2–3 days before cryo-preservation.
3. When cells reach to ∼70∼80% confluence (2–3 days after passage), prepare label cryo-tubes and 2× cryo-medium (20% DMSO in E8 media) for preservation.
4. Rinse wells to be split with 1 mL/well of warmed EDTA medium to wash away the Mg^{++} and Ca^{++} from the E8 medium.
5. Repeat step 2 to ensure all of the Mg and Ca is removed.
6. Add 1 mL/well EDTA medium, set plate aside and let sit 2–5 minutes (more than 2 minutes will result in very tiny colonies of only 3–5 cells).
7. Remove EDTA/PBS, and swiftly harvest cells with 1 ml E8 medium per 1 well (6-well plate).
8. Drop-wisely add equal volume of 2× cryopreservation media into the EDTA-harvested cell suspension.
9. Aliquote 250–500 ul into each cryotube.

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10. Freeze cells in –80°C in a Cryo container > 2 hours.
11. Preserve cells in liquid nitrogen for long-term storage.
12. ROCK inhibitors can be added into cryopreservation media.

B. Recover human ES/iPS cells in defined conditions.

1. Take cryotube out of liquid nitrogen tank, and put it directly in 37°C water bath.
2. Gently stir water with the tube, and check the disappearance of ice closely.
3. When there is only a small ice particle is floating, transfer tube into a biosafety cabinet.
4. Transfer cells into a 15 ml conical tube
5. Drop-wisely add 10 ml of E8 media, while continuously mix the solution in the tube.
6. Spin cells at 1000 rpm for 5 minutes
7. Remove supernatant, plate cells into 1–3 wells with E8 media.
8. 10 μM ROCK inhibitors can be added into E8 media when plating. To further improve cell survival, plates could be incubated at 5% O_2 for the first 2 days.

Materials

- Cell Materials

Human ESCs
Human iPSCs

- Cell culture media
  - Chemically defined human ESC/iPSC E8 medium: DMEM/F12, 64 mg/L L-Ascorbic acid 2-phosphate magnesium salt, 14 μg/L Sodium Selenite, 10.7 mg/L Holo-transferrin 10.7 mg/L, 100 μg/L basic FGF, 1.8 μg/L TGFβ1, 20 mg/L Insulin. Adjust to pH 7.4 with 340 mOsm osmolality.
  - 2× Cryopreservation medium: 20% DMSO in E8 media. If prefer, 20 μM Y27632 could be added into the medium

- EDTA/PBS (1000 ml)

Ingredient	Amount	Company	Catalog#
PBS	500 ml	Life Technology	14190-250
0.5 M EDTA	0.5 ml	K.D. Biomedical	RGF3130
NaCl	0.9 g	Sigma	5886

- Medium Reagents

Ingredient	**Company**	**Catalog#**
DMEM/F12	Life Technology	11330
L-Ascorbic acid 2-phosphate magnesium salt	Sigma	A8960
Sodium Selenite	Sigma	5886
Sodium Chloride	Sigma	S5886
Holotransferrin	Sigma	T0665
Basic FGF	Peprotech	100-18B
TGFβ1	R&D Systems	240-B/CF
Insulin	Sigma	I9278
Hydrocortisone	Sigma	H0396
Sodium Butyrate	Sigma	B5587

- STUFF

Inverted microscope (i.e., Nikon TE or Olympus IX or Zeiss Promo Vert)
Biosafety cabinet for cell culture
CO_2 incubator with controlling and monitoring system for CO_2, humidity and temperature
Cell culture disposables: Tissue culture dishes, centrifuge tubes, pipettes, pipette tips, cell strainer etc.

Troubleshooting

- Low cell survival
  - Use cells passaged within 2–3 days by EDTA.
  - Keep confluence below 80%.
  - Don't over-treat cells with EDTA, which could generate many single cells.
  - Add ROCK inhibitors as insurance in the process.