

Video Article

Cryopreserving and Recovering of Human iPS Cells using Complete KnockOut Serum Replacement Feeder-Free Medium

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Abstract

The discovery in 2006 that human and mouse fibroblasts could be reprogrammed to generate iPS cells¹⁻³ with qualities remarkably similar to embryonic stem cells has created a valuable new source of pluripotent cells for drug discovery, cell therapy, and basic research.

GIBCO media and reagents have been at the forefront of pluripotent stem cell research for years. Knockout DMEM supplemented with Knockout Serum Replacement is the media of choice for embryonic stem cell growth and now iPS cell culture³⁻⁹. This gold standard media system can now be used for feeder-free culture with the addition of Knockout SR Growth Factor Cocktail.

Traditional human ES and iPS cell culture methods require the use of mouse or human fibroblast feeder layers, or feeder-conditioned medium. These culture methods are labor-intensive, hard to scale and it is difficult to maintain hiPS cells undifferentiated due to the undefined conditions. Invitrogen has developed Knockout SR Growth Factor Cocktail to allow you to easily transition your hiPS and hES cell cultures to feeder-free while still maintaining your use of Knockout SR.

Video Link

The video component of this article can be found at <https://www.jove.com/video/2237/>

Protocol

Note: To maintain sterile culture conditions, all of the procedures in this protocol are carried out using sterile laboratory practices and conducted under a laminar flow hood.

Prior to starting, ensure that any media is equilibrated to 37°C and appropriately gassed.

Preparing Geltrex-coated Culture Dishes

Note: see appendix for the use of CELLstart coated culture dishes.

1. Thaw one tube of Geltrex (1 mL) slowly at 2-8°C and dilute 1:100 it in 99 mL of Knockout D-MEM/F-12. Mix the solution gently.
Note: Some iPS cell lines may require a different Geltrex dilution for optimal growth. See appendix for alternative dilutions.
2. Cover the whole surface of each culture dish with the Geltrex solution (1 mL for a 35-mm dish, 1.5 mL for a 60-mm dish).
3. Seal each dish with Parafilm to prevent drying and incubate the dishes for 1 hour at 37°C.
Note: At this point you may store the Geltrex-coated culture dishes at 4°C for up to 1 month. Seal each dish with Parafilm to prevent the Geltrex from drying out.
4. Prior to using, transfer the Geltrex-coated dishes to a laminar flow hood and allow them to equilibrate to room temperature (about 1 hour).

Preparing Complete KnockOut SR Feeder-Free Medium

1. To prepare 1 mL of 10 µg/mL Basic FGF solution, aseptically mix the components listed below. Aliquot the solution and store at -20°C for up to 6 months.

Basic FGF	10 µg
D-PBS	990µL
10% BSA	10 µL

Note: BSA can be substituted with HSA or Knockout SR at the same concentration.

2. To prepare 50 mL of 2 mg/mL Dispase solution, aseptically mix the components listed below. Filter sterilize the solution and store at 4°C for up to 2 weeks.

Dispase	100 mg
D-PBS	50 mL

3. To prepare 100 mL of complete KnockOut SR Feeder-Free (KSR-FF) aseptically combine the components listed in the table below.

Component	Stock Concentration	Final Concentration	Volume
Knockout DMEM/F12 (Cat. no. 12660-012)	-	1X	76.8 mL
GlutaMAX -I (Cat. No. 35050-061)	200 mM	2 mM	1 mL
KnockOut SR (Cat. no. 10828-028)	-	20%	20 mL
KnockOut SR-GFC (Cat. no. A10580-01)	50X	1X	2 mL
bFGF (Cat. no. PHG0024).	10 µg/mL	20 ng/mL	200 µL

You may store the KSR-FF medium at 2-8°C for up to one week.

4. Just before pre-equilibrating the complete medium to temperature and gases, aseptically **add the required volume of 2 mercaptoethanol** (55 mM stock concentration) for a 0.1 mM final concentration. For example, to prepare 100 mL of KSR-FF medium add 182 µL of 55 mM 2-mercaptoethanol (1:550 dilution) Alternatively, the 2-mercaptoethanol may be added to the 1X completed medium and stored at 2-8°C for up to one week.

Preparing Freezing / Cryopreservation Medium

The cryopreservation medium consists of two media; Freezing Medium A and Freezing Medium B. They are added to the cells at different times and must remain separated. They are each 50% of the total volume of Cryopreservation Medium needed. The total volume of Cryopreservation Medium needed is 1 mL for each vial to be frozen. A 90% confluent 60mm dish of hESC can be used to bank 2 vials of hESC in cryopreservation media.

Prepare enough volume of each Freezing Medium with an extra 2-5mL to ensure overage.

Freezing Medium A (50% of final volume):	50% DMEM/F12	50% KSR
Freezing Medium B (50% of final volume):	80% DMEM/F12	20% DMSO

Example:

If you are freezing 20 vials of cells, you will need 20mL of Cryopreservation Medium. Add 4mL for overage for a total of 24mL Cryopreservation Medium. That means you will need 12mL of Freezing Medium A (50% of 24mL) and 12mL of Freezing Medium B (50% of 24mL).

Freezing Medium A (12 mL):	6 mL DMEM/F12	6 mL KSR
Freezing Medium B (12 mL):	9.6 mL DMEM/F12	2.4 mL DMSO

The final composition of the Cryopreservation Medium is 65% DMEM/F12, 25% KSR, and 10% DMSO.

Cryopreserving iPSCs

- Pre-warm the required volume of Dispase in a 37 °C water bath. Refer to Table 1 below for details on the volumes required.
- Pre-equilibrate the required volume of KSR-FF in a 37°C water bath for 15 min. Refer to Table 1 below for details on the volumes required.
- Aspirate the spent medium from the culture vessel using a pipette, and rinse the cells twice with D-PBS.
- Gently add pre-warmed Dispase solution to the culture vessel (e.g., 1 mL of Dispase solution per 60-mm culture dish). Swirl the culture vessel to coat the entire cell surface.
- Incubate the culture vessel at 37°C for 3 minutes.
- Remove the vessel from the incubator, aspirate the Dispase solution, and gently wash the cells with D-PBS.
- Gently scrape the cells off the surface of the culture dish using a cell scraper, and transfer the cells to a sterile 15mL centrifuge tube.
- Rinse the culture dish twice with KSR-FF, gently "spraying off" any cells that have not detached. Pool the rinse medium with the cells in the 15mL tube.
- Centrifuge the tube at 200 x g for 5 minutes at room temperature to pellet the cells.
- Carefully aspirate the supernatant without disturbing the cell pellet and discard it.
- Prepare a sufficient quantity of cryovials. Once the cells are in contact with DMSO, they should be aliquoted and frozen within 2-3 minutes.
- Gently flick the tube, to fully dislodge the cell pellet from the tube bottom and re-suspend the cells in Freezing Medium A. [by gently pipetting up and down using a 5-mL serological pipette]. Following uniform suspension of clumps, add equal volume of Freezing Medium B to it, in a drop wise manner, while gently swirling the cell suspension to mix.
Note: At this point, cells are in contact with DMSO, and work must be performed efficiently. Once cells are in contact with DMSO, they should be aliquoted and frozen within 2-3 minutes.
- Aliquot 1 ml of the cell suspension into each cryovial.
- Quickly place the vials in a Mr. Frosty [to rapidly freeze] and transfer it to -80°C overnight.

15. After overnight storage at -80°C, transfer the cells to a liquid nitrogen tank for long term storage.

iPSC vial thaw and cell recovery

Note: KSR-FF may be replaced with KSR-containing MEF-conditioned medium, or KSR medium for use with feeders. See appendix for instructions for media preparation.

Pre-warm 10mL of KSR-FF medium in a 50 mL tube.

1. Equilibrate the appropriate quantity of Geltrex-coated dishes to room temperature and aspirate any liquid from the dishes just before plating your cells.
2. Carefully remove the hESC (or iPSC) vial from Liquid Nitrogen Storage Tank.
3. Rapidly thaw frozen vial of cells in a 37°C water bath, until just a small frozen chunk remains in the vial.
4. Spray vial with 70% isopropanol to decontaminate it and transfer it to the sterile tissue culture hood.
5. Aseptically transfer the entire contents of the vial into a 15 mL conical tube using a 5 mL pipette.
6. Rinse the vial with 1 mL of KSR-FF and add this to the 15 mL centrifuge tube.
7. Slowly, add 4 mL of KSR-FF drop-wise to cells in the 15mL conical tube. While adding the medium, gently move the tube back and forth to mix the cells. (This reduces osmotic shock to the cells).
8. Centrifuge the 15 mL tube with the cell suspension at 1000rpm (200 x g) for 2 minutes at room temperature.
9. Carefully aspirate and discard the supernatant without disturbing the cell pellet.
10. Re-suspend the cell pellet in pre-warmed KSR-FF (e.g. 4mL/60 mm dish) using a 5 mL pipette and gently pipette the cells up and down until cell pellet is fully dispersed. A viability of greater than 70% is expected, however if the viability is less than 70%, it may take longer for the cells to reach confluence.
11. Using the same pipette, transfer the cell suspension drop-wise to Geltrex-coated culture dish pre-equilibrated to room temperature.
12. Place the culture dish in a 37°C incubator, with a humidified atmosphere of 4 to 6% CO₂ in air. Carefully swirl vessel in a north to south, east to west pattern to evenly distribute the cells.
13. Gently fluid-change the culture dish 24 hrs post-thaw and daily thereafter to remove cell debris and to provide fresh nutrients until the dish is approximately 70-80% confluent. For continued culture and passaging of your iPS cells, refer to our protocol titled "Feeder-Free Culture and Passaging of Human iPS Cells using Complete KnockOut Serum Replacement Feeder-Free Medium"

Expected Results

The cells should be roughly 70-80% confluent prior to cryopreservation. Disperse digestion results in curling up and folding of the colonies along the colony margins. After harvesting the cells they are frozen as small clumps in cryopreservation media. Once the vial is thawed, cells recover and attach to the pre-coated dish as small clumps. They grow rapidly leading to a confluent dish in 5-6 days.

Table 1 - Recommended Volumes

Component	35mm Dish	60mm Dish	100mm Dish
Complete KnockOut SR medium	2 mL	4 mL	10 mL
Geltrex Solution	1 mL	1.5 mL	4-5 mL
Dispase	0.5 mL	1 mL	3-4 mL
D-PBS for rinsing	2 mL	4 mL	10 mL

Please [click here to see the appendix](#).

Disclosures

The authors of this article are employed by Life Technologies that produces reagents and instruments used in this article.

References

1. Takahashi, K. *et al.* Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663-676 (2006).
2. Takahashi, K. *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872 (2007).
3. Yu, J. *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917-1920 (2007).
4. Maherali, N. *et al.* Guidelines and techniques for the generation of induced pluripotent stem cells. *Cell Stem Cell* 3, 595-605 (2008).
5. Li, W. *et al.* Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* 4, 16-19 (2009).
6. Liao, J. *et al.* (2009) Generation of induced pluripotent stem cell lines from adult rat cells. *Cell Stem Cell* 4, 11-15.
7. Dimos, J.T. *et al.* Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* 321, 1218-1221 (2008).
8. Aasen, T. *et al.* Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. *Nat Biotechnol* 26, 1276-1284 (2008).

9. Park, I.H. *et al.* Generation of human-induced pluripotent stem cells. *Nat Protoc* 3, 1180-1186 (2008).