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Methods for Culturing Human Embryonic Stem Cells in a Xeno-Free System

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Abstract

Defined pluripotent stem cell culture media is a valuable tool for tracking and analyzing morphological, cell signaling and gene expression changes in human embryonic stem cells. Cultivation of hESC under xeno-free culture settings provides researchers with a consistent and reproducible environment to test experimental hypotheses and move towards translational and clinical research. One of the primary concerns of the xenogeneic culture is the transfer of foreign pathogens or antigens that induce disease or immune response by the patient. These undesirable by-products may come from the use of murine-derived feeder cells, xenogeneic matrices, or from animal-derived components found in the cell culture medium or matrix used to isolate or expand the stem cells. This chapter describes standardized protocols for obtaining consistent and reproducible results for culturing PSC under xeno-free, feeder-free, or feeder-based systems.

Keywords

Xeno-free culture; Pluripotent stem cell; Embryonic fibroblast feeder cells

1 Introduction

Human embryonic stem cells (hESC) were derived and maintained on inactivated murine embryonic fibroblast (MEF) feeder layers (1, 2). Use of MEFs and other components of animal origin for culturing hESC has been a concern owing to the risk of viral infection or pathogen transmission, thus making hESC unsafe for use in regenerative medicine. Defined conditions where all hESC culture components are “xeno-free,” meaning human-derived or synthetic, are required in order to have hESC in a suitable state for therapeutic applications.

Several researchers have been able to successfully derive and propagate hESC lines on a variety of human fibroblast feeder (HFF) layers and also on a recombinant humanized matrix (CELLstart™) for feeder-free cultures. The hESC colonies on human feeders have angular or straight edges as compared to circular colonies of hESC observed on MEF. Cells in the feeder-free xeno-free condition appear to be more flattened on the initial day of culture and colonies get more compact by days 4–5. The hESC derived and propagated in xeno-free conditions remain pluripotent over extended passages, express hESC morphology with high nuclear to cytoplasmic ratio, maintain stable karyotype, and are able to form embryoid bodies (EB) and differentiate into the three germ layers—ectoderm, endoderm,

and mesoderm (3). Apart from the differences in gross colony appearance, all other characteristics of hESC appear to be similar. These protocols will serve to standardize methodologies for cultivating PSC (pluripotent stem cells) under xeno-free conditions (Tables 1, 2, 3).

2 Materials

For xeno-free culturing cells can be cultured on human feeders or a humanized matrix (CELLstart). The protocol will discuss the feeder-based culture followed by feeder-free culture. Unless otherwise specified, all materials are from Life Technologies.

2.1 Reagents

1. CELLstart™ CTS™ defined, humanized substrate for cell culture DPBS CTS™ with calcium chloride and magnesium chloride.
2. ES Cell Qualified FBS.
3. FGF-basic Full Length CTS™ Recombinant Human Protein (100 µg), lyophilized powder.
4. GlutaMAX™-I CTS™.
5. Gentamicin.
6. Human feeders.
7. KnockOut™ CTS™ DMEM (1×), liquid.
8. KnockOut™ SR XenoFree CTS.
9. KnockOut™ SR GF Cocktail CTS™.
10. KnockOut™ DMEME-12 CTS™.
11. 2-Mercaptoethanol (1,000×), liquid.
12. Nonessential amino acids (NEAA).
13. STEMPRO® EZPassage™ Disposable Cell Passaging Tool.
14. TrypLE™ Select CTS™ (1×), liquid, without Phenol Red.

2.2 Equipment

1. Biosafety cabinet.
2. Centrifuge (suitable for 15 mL and 50 mL centrifuge tubes).
3. Colony marker for microscope.
4. Freezer (−20°C).
5. Freezer (−80°C).
6. Gamma irradiator.
7. 37°C, humidified 5% CO₂ incubator.

8. Liquid nitrogen storage tank.
9. Mr. Frosty Nalgene Cryo 1°C Freezing Container.
10. Phase-contrast microscope.
11. Pipet aid.
12. Plastic cryovial rack.
13. 4°C refrigerator.
14. Water bath (37°C).

2.3 Supplies

1. 5 mL, 10 mL, and 25 mL serological pipets.
2. 15 mL and 50 mL centrifuge tubes.
3. 60 mm tissue culture-treated dishes.
4. 1.5 mL cryovials.
5. 60 mm and 100 mm non-tissue culture-treated dishes.

2.4 Media Preparation for Feeder and Feeder-Free Culture of Pluripotent Stem Cells (PSC)

1. Prepare 500 mL HFF Medium by adding the following: 440 mL KnockOut™ CTST™ DMEM, 50 mL ES Cell Qualified FBS, 5 mL of NEAA, and 5 mL GlutaMAX™-I CTST™.
2. Prepare 500 mL Inactive HFF Medium by combining 440 mL KnockOut™ CTST™ DMEM, 50 mL KnockOut™ SR XenoFree CTST™, 5 mL NEAA, and 5 mL GlutaMAX™-I CTST™.
3. Prepare 100 µg/mL bFGF solution by adding the following: 100 µg of Basic FGF, 990 µL of D-PBS, and 10 µL of 10% BSA.
4. Prepare 100 mL KnockOut SR XenoFree Feeder Medium by adding the following: 83 mL of KnockOut™ DMEM CTST™, 1 mL of GlutaMAX™-I, 15 mL of KnockOut™ SR XenoFree CTST™, 1 mL of NEAA, 20 µL of bFGF CTST™ (see Note 9).
5. Prepare 100 mL KnockOut SR XenoFree Feeder-free Medium by adding the following: 83 mL of KnockOut™ DMEM CTST™, 1 mL of GlutaMAX™-I, 15 mL of KnockOut™ SR XenoFree CTST™, 1 mL KnockOut SR GF Cocktail CTS, 20 µL of bFGF CTST™ (see Note 9).
6. Prepare EB Medium by adding the following: 83 mL of KnockOut™ DMEM, 15 mL of KnockOut™ SR XenoFree, 1 mL of GlutaMAX™, 1 mL of NEAA.
7. Prepare 1:50 CELLstart solution by adding the following: 49 mL of DPBS CTST™ with calcium chloride and magnesium chloride, 1 mL of CELLstart™ CTST™.

⁹Addition of antibiotics to hESC media is optional. Media for hESC culture can be stored at 4°C for about 2 weeks.

8. Prepare hESC KSR XenoFree Freezing Medium by adding the following: 73 mL of KnockOut™ DMEM CTS™, 1 mL of KnockOut™ SR GF Cocktail CTS™, 20 µl of bFGF CTS™, 1 mL of GlutaMAX™-I, and 25 mL of KnockOut™ SR XenoFree CTS™.

3 PSC Culture in Xeno-Free Condition

Workflow diagram of xeno-free culture of pluripotent stem cells (Fig. 1) (see Note 1).

3.1 Recovery of HFF Cells from Liquid Nitrogen

1. Warm HFF Medium before thawing the cells. Place 10 mL HFF Medium in a 15 mL centrifuge tube.
2. Remove HFF vial (e.g., Cat. No. CRL-2429, American Type Culture Collection) from liquid nitrogen and rapidly thaw in a 37°C water bath by gently swirling until just a small frozen chunk remains in the vial.
3. Spray vial with 70% isopropanol to decontaminate it and transfer vial to the biosafety cabinet.
4. Aseptically transfer the entire contents of the vial into the 15 mL centrifuge tube containing 10 mL warm HFF Medium using a 5 mL serological pipet.
5. Rinse the vial with 1-2 mL HFF Medium and add it to the same 15 mL centrifuge tube.
6. Gently pellet cells by centrifuging at $200 \times g$ for 3 min at room temperature.
7. Carefully aspirate the supernatant without disturbing the cell pellet and discard it.
8. Gently “flick” the tube at the base to fully dislodge the cell pellet from the tube bottom.
9. Add the required volume of HFF Medium to the pellet and gently triturate. Transfer the HFF cells to appropriate-sized vessel(s). Incubate in a 37°C incubator with a humidified atmosphere of 5% CO₂.

3.2 Passaging HFF

1. Observe the vessel(s) for confluence; subculture when confluent, but not tightly packed with cells (see Fig. 2). Note: Be sure to scale up and bank cells at low passage number.
2. Rinse cells twice with D-PBS and discard.
3. Add pre-warmed TrypLE Select to HFF cells and place the vessel in a 37°C incubator for 2–3 min. Remove the vessel from the incubator and tap the side with palm to dislodge the HFF cells.

¹All protocols described here must be performed under sterile conditions. For detailed information on sterile techniques, refer to review by Cote (4) and Phelan (5). All cultures should be routinely tested for mycoplasma infection.

4. Gently triturate the cells gently and transfer them to a sterile 15 mL centrifuge tube. Rinse flask twice with HFF Medium and pool with cells in the tube.
5. Gently pellet cells by centrifuging at room temperature for 5 min at $200 \times g$.
6. Aseptically, aspirate the supernatant without disturbing the cell pellet and discard it.
7. Gently “flick” the bottom of the tube to fully dislodge the cell pellet from the tube bottom.
8. Add the required volume of pre-warmed HFF Medium to the pellet and gently triturate.
9. Subculture active feeders between 1:4 and 1:8 in HFF Medium for routine maintenance.

3.3 Irradiating and Cryopreserving Human Fibroblast Feeder Layers

1. Remove the confluent vessel(s) from the incubator.
2. Aspirate the spent medium from the vessel(s).
3. Add pre-warmed TrypLE solution (2 mL for a 60 mm dish) and transfer to a 37°C incubator.
4. After 1 min, remove the vessels and observe under the microscope.
5. When cells begin to detach, transfer dish to biosafety cabinet.
6. Gently aspirate the TrypLE solution.
7. Add pre-warmed HFF Medium to the vessels and wash off the cells. Collect the cell suspension in a 50 mL centrifuge tube.
8. Centrifuge the tube at $200 \times g$ for 5 min at room temperature.
9. Gently aspirate the supernatant and resuspend the cell pellet in a small volume of pre-warmed HFF Medium, obtaining a single cell suspension.
10. Determine the viable cell count by trypan blue exclusion method using a hemocytometer.
11. To inactivate HFF, expose to gamma irradiation at a dose of 30 Gy.
12. Plate inactive HFF at $7 \times 10^4/\text{cm}^2$ in the desired cell culture vessel(s) that have been pre-coated with CELLstart™ CTS (see below) (see Note 7).

3.4 CELLstart CTS Coating of Culture Vessels for Feeder and Feeder-Free Culture of PSC

1. Dilute CELLstart™ CTS™ at 1:50 in D-PBS CTS™ with calcium chloride and magnesium chloride. Pipet gently to mix. Do not vortex.

⁷Plate HFF at recommended seeding density to maintain pluripotent hESC.

2. Add diluted CELLstart™ CTS™ to coat the entire surface of the culture vessel (e.g., 0.078 mL/cm²). Swirl or gently tap the vessel to distribute the diluted CELLstart™ CTS™ evenly.
3. Place culture vessels containing diluted CELLstart™ CTS in a 37°C incubator with a humidified atmosphere of 5% CO₂, for 1–2 h.
4. After incubation, remove coated vessels from the incubator. For immediate use, place vessels at room temperature. For use the next day, carefully wrap vessels containing diluted CELLstart™ CTS™ with Parafilm®, and store at 2–8°C.
5. Immediately before use, remove all CELLstart™ CTS diluent from the vessel. It is not necessary to rinse vessels following removal of CELLstart™ CTS™.
6. If growing cells in a feeder-free system proceed with plating hESCs on the CELLstart™ CTS™-coated vessel at the desired density.

3.5 Thawing and Plating Inactive HFFs

1. Remove the cryovial containing inactivated HFFs from the liquid nitrogen storage tank.
2. Thaw vial of HFF cells in a 37°C water bath, until just a small frozen chunk remains in the vial.
3. Spray vial with 70% isopropanol to decontaminate it and transfer vial to the biosafety cabinet.
4. Aseptically transfer the entire contents of the vial into a 15 mL centrifuge tube containing 10 mL warm Inactive HFF Medium (containing KSR XenoFree) using a 5 mL serological pipet.
5. Rinse the vial with 1–2 mL Inactive HFF Medium and add it to the same 15 mL centrifuge tube.
6. Gently pellet HFF by centrifuging for 5 min at 200 × *g* at room temperature.
7. Carefully aspirate the supernatant without disturbing the cell pellet and discard it.
8. Gently “flick” the tube at the base to fully dislodge the cell pellet from the tube bottom.
9. Resuspend the cell pellet with warm Inactive HFF Medium, and determine the viable cell count by trypan blue exclusion method using a hemocytometer.
10. Plate inactive HFF at 7×10^4 cells/cm² in the desired CELLstart-coated vessels. Place the vessel in a 37°C incubator with a humidified atmosphere of 5% CO₂ (see Note 7).
11. Swirl vessel in a north to south, east to west pattern to evenly distribute feeder cells.
12. Use the inactive HFF culture vessels within 3–4 days after plating.

3.6 Recovery of Cryopreserved hESCs on Feeder and Feeder-Free System

1. Rapidly thaw frozen vial of cells in a 37°C water bath, until a small frozen piece remains in the vial.
2. Decontaminate vial with 70% isopropyl alcohol.
3. Aseptically transfer the entire contents of the vial into a 15 mL centrifuge tube (see Note 3).
4. Gently add 3 mL pre-warmed KnockOut SR XenoFree Feeder or Feeder-free Medium dropwise to the centrifuge tube containing thawed hESC (see Note 4). Rinse the vial with 1–2 mL additional medium and add to the same centrifuge tube (see Note 8).
5. Gently pellet cells by centrifuging at 200 ×g at room temperature for 2 min.
6. Carefully aspirate the supernatant without disturbing the cell pellet and discard.
7. Gently “flick” the tube to fully dislodge the cell pellet from the tube bottom.
8. Carefully add the desired volume of pre-equilibrated KnockOut SR XenoFree Feeder or Feeder-free Medium to the hESC pellet. Do *not* triturate cells.
9. Gently invert the centrifuge tube containing hESCs to mix cells. Using a pipet, transfer the cells to inactive HFF culture vessel for feeder-based system or CELLstart-coated only culture vessel for feeder-free system (see Subheadings 3.7.14–3.7.15) (see Note 6).
10. Place vessel in a 37°C incubator with a humidified atmosphere of 5% CO₂. Carefully swirl vessel in a north to south, east to west pattern to evenly distribute hESC.
11. Fluid change vessel 24 h post-thaw and daily thereafter, until approximately 70–80% confluent (see Fig. 3) (see Notes 2, ⁵).

3.7 Passaging hESC on Feeder and Feeder-Free Culture

1. Observe hESC under the microscope and confirm that the cells are ready to be subcultured (when 70–80% confluent).
2. Physically remove any differentiated hESC colonies prior to passaging the culture. A 20 G 1½” needle attached to a syringe works well for removing differentiated hESCs.

³Use a 5 mL serological pipet when thawing cells from liquid nitrogen and for passaging and plating hESC.

⁴Always pre-warm hESC medium before use.

⁸Prepare a frozen stock of cells in control medium prior to adaptation to a new culture condition.

⁶Plate thawed hESC at a high seeding density; not all cells will be viable, attach, and expand.

²hESC cultures must be fluid-changed daily for optimal growth without differentiation.

⁵Timing of passage is critical. For best results, hESC should be nearing confluence (70–80%) at the time of passage. If passaged too light or overgrown, hESC will differentiate.

3. Pre-warm the required volumes of TrypLE™ Select CTS™ to 37°C, and pre-equilibrate the required volume of appropriate KnockOut SR XenoFree Medium to temperature and gases before use. Minimize dwell time.
4. Remove spent medium from culture vessel using a pipet, and discard.
5. Rinse hESCs twice with DPBS CTS™ without calcium chloride, magnesium chloride (1×), liquid.
6. Gently add pre-warmed TrypLE™ Select CTS™ to the culture vessel (e.g., 1 mL/60 mm dish). Swirl vessel to coat the entire cell surface.
7. Place culture vessel in a 37°C incubator for 2–3 min.
8. Remove vessel from the incubator. Gently tap the sides of the dish to dislodge cells.
9. Transfer cells to a sterile 15 mL centrifuge tube.
10. Rinse dish twice with pre-warmed wash medium, gently “spraying off” any cells that have not detached, and pool with cells in tube. Do not triturate!
11. Pellet hESCs by centrifugation at 200 ×g for 2 min at room temperature.
12. Carefully aspirate the supernatant without disturbing the cell pellet and discard it.
13. Gently “flick” the bottom of tube to fully dislodge the cell pellet from the tube bottom.
14. Gently resuspend hESCs in pre-equilibrated complete medium using a 2 mL or 5 mL serological pipet. Do not triturate!
15. Transfer hESCs to a fresh inactive HFF-plated culture vessel or CELLstart-coated culture vessel at the desired cell ratio or seeding density. A 1:2 split is recommended during adaptation, or 8×10^4 cells/cm². For routine maintenance, cells can be split at 1:4–1:8, or 4×10^4 cells/cm² using appropriate KnockOut™ SR XenoFree CTS™ Medium. Adjust densities as needed to suit your particular hESC line (see Note 5).
16. Place vessel in a 37°C incubator, with a humidified atmosphere of 5% CO₂. Carefully swirl vessel in a north to south, east to west pattern to evenly distribute hESC.
17. Gently fluid-change culture the next day to remove cell debris and to provide fresh nutrients, and daily thereafter.
18. Observe hESCs daily and passage by the above protocol whenever required (approximately every 3–5 days).

Note: Cultures can also be passaged manually by using Stempro EZ passaging tool to cut the colonies in small clumps, followed by collecting the colonies by using a cell scraper; resume with step 10.

3.8 Cryopreservation of hESC Using KnockOut SR XenoFree

1. Follow the protocol for passaging hESC with appropriate KnockOut SR XenoFree Medium mentioned in Subheading 3.7.
2. At step 14 in Subheading 3.7, gently resuspend the cell pellet with a 50/50 mix of appropriate KnockOut SR XenoFree Medium and hESC KSR XenoFree Freezing Medium (to yield 10% DMSO) using a 5 mL pipet and triturate.
3. Dispense 1 mL of the cell suspension per cryovial using a 5 mL pipet, mixing thoroughly between dispenses (see Note 3).
4. For best results, vials should be cryopreserved using a controlled rate freezing device (e.g., CryoMed® Freezer or Mr. Frosty Nalgene Cryo 1°C Freezing Container), following manufacturer's instructions.
5. The next day, transfer vials to permanent liquid nitrogen storage. Expect some cell death at recovery, and freeze hESC at a higher density than would normally be passaged to facilitate recovery from liquid nitrogen storage. For example, if cells are routinely passaged at 1:5, a 1:3 or 1:4 densities are recommended for freezing hESC (see Note 6).

3.9 EB Formation

1. Culture hESC in KnockOut SR XenoFree Medium as desired, for a minimum of three passages.
2. When the culture is 70–80% confluent, dissociate hESC enzymatically as previously described, or manually using the Stempro EZ passaging tool and cell scraper (see Subheading 3.7).
3. Dilute the cell clumps at 1:4 in EB Medium and transfer to non-tissue culture-treated 60 mm dishes (4 mL/60 mm dish). Note: No pre-coating of dishes is necessary. Transfer 60 mm dishes to a 37°C incubator, with a humidified atmosphere of 5% CO₂.
4. On day 2, observe EBs (they will be floating/in suspension culture). Transfer the entire EB suspension from the non-tissue culture-treated dish to a 15 mL centrifuge tube (use a separate tube for every 60 mm dish). Let the EBs settle to the bottom of the tube.
5. When most of the EBs have settled out, aspirate and discard the supernatant, leaving the EBs behind.
6. Gently, resuspend EBs with 4 mL fresh EB Medium and return to the non-tissue culture-treated dishes. Transfer to a 37°C incubator, with a humidified atmosphere of 5% CO₂.
7. On day 4, repeat the fluid change procedure (steps 4 and 5 above), but this time, resuspend EBs in 10 mL EB Medium.
8. Transfer 5 mL EBs to each of two non-tissue culture-treated 100 mm dishes. Add 5 mL EB Medium to each 100 mm dish and return dishes to 37°C incubators.

9. Continue fluid-changing the 100 mm EB dishes every 2–3 days, as described above. Some of the EBs will attach to the dishes and expand. Continue fluid-changing the attached EBs.
10. Let the cells culture for 21 days (or desired time course).
11. Fix and stain EB outgrowths to examine by immunocytochemistry or lyse and harvest them (e.g., with TRIzol reagent) to examine gene expression by RT-PCR, microarray, etc. (Fig. 4).

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Fig. 1.
The workflow diagram of culturing hESC on human feeders and in feeder-free culture with CELLstart™ CTS matrix

Fig. 2.
Morphology image of human feeders cultured on CELLstart™ CTS-coated dishes

Fig. 3. Morphology image of hESC cultured on HFF in KSR XenoFree Medium (**a**) and stained with alkaline phosphatase (**b**). Morphology image of feeder-free hESC cultured in KSR XenoFree Medium on CELLstart matrix (**c**) and ICC (**d**) for cells expressing pluripotent marker Oct-4

Fig. 4. EB formation image shows floating embryoid bodies at day 4 **(a)** and attached differentiating EB at day 21 **(b)**

Table 1
Initial HFF seeding information (passage 0)

Flask size	HFF seeding density	Initial volume of pre-warmed HFF medium (mL)
T-75	5.25×10^6	15
T-160	1.12×10^7	30
T-225	1.575×10^7	50

Table 2
Trypsin and TrypLE express volumes for dissociation

Flask size	Suggested volume (mL)
T-75	1.5
T-160	3
T-225	5

Table 3

Establishing passage 1 HFF T-flasks

Initial vessel	Passage 1 vessels	Passage 2 vessels
T-75	(2) T-160 flasks	(8) T-160 flasks
T-160	(4) T-160 flasks or (3) T-225 flasks	(16) T-160 flasks or (12) T-225 flasks
T-225	(4) T-225 flasks	(16) T-225 flasks