gibco
by life technologies

Culturing Human Embryonic Stem Cells (hESCs) on MEF-Conditioned Medium

Publication Part Number MAN0006682 **Revision Date** 22 August 2012

Introduction

Several applications require the maintenance of human embryonic stem cells (hESCs) in the absence of a murine embryonic fibroblast (MEF) feeder layer. This protocol describes the culture of hESCs on matrix-coated plates in the presence of MEF-conditioned media (MEF-CM).

In this system, hESCs are maintained at high density. At confluence, the cells reach 300,000–500,000 cells/cm^2.

Materials Needed

- Dulbecco's Modified Eagle Medium (D-MEM) (Cat. no. 10569-010)
- Fetal Bovine Serum (FBS), ESC-Qualified (Cat. no. 10439-024)
- D-MEM/F-12 (1X) with GlutaMAX™-I (Cat. no. 10565-018)
- KnockOut™ Serum Replacement (KSR) (Cat. no. 10828-028)
- MEM Non-Essential Amino Acids Solution, 10 mM (Cat. no. 11140-050)
- β-mercaptoethanol, 1000X (Cat. no. 21985-023)
- Gibco® Mouse Embryonic Fibroblasts (Irradiated) (Cat. no. S1520-100) or Mitomycin C-treated MEFs
- Basic Fibroblast Growth Factor (bFGF) (Cat. no. PHG0264)
- GlutaMAX™-I (100X) (Cat. no. 35050-079)
- Collagenase Type IV (Cat. no. 17104-019) for enzymatic passaging or StemPro® EZPassage™ Tool (Cat. no. 23181-010) for mechanical passaging
- Cell Scraper (Falcon, Cat. no. 353085)
- Dulbecco's PBS (DPBS) with Calcium and Magnesium (Cat. no. 14040-133)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-144)
- Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix (Cat. no. A1413301 or A1413302) or CELLstart™ CTS™ substrate (Cat. no. A1014201)
- Attachment Factor (Cat. no. S006100)
- Stem Cell Tested 0.22 µm pore size sterile filters (Nalgene, Cat. no. 564-0020 or 156-4020)
- BD Falcon T175 TC-Flasks (Falcon, Cat. no. 353112)
- 37°C water bath
- Appropriate tissue culture plates and supplies

Note: As an alternative to D-MEM/F-12 (1X) with GlutaMAX™-I, KSR, and bFGF, you can use the KnockOut™ ESC/hESC Media Kit (Cat. no. A1412901).

Preparing Media and Materials

10 µg/mL bFGF Solution (1000 µL)

1. To prepare 1 mL of 10 µg/mL bFGF solution, aseptically mix the following components:

bFGF	10 µg
DPBS without Calcium and Magnesium	980 µL
10% KSR	10 µL

2. Aliquot and store at –20°C for up to 6 months.

10 mg/mL Collagenase Type IV Solution

1. Add D-MEM/F-12 to Collagenase Type IV to make a 10 mg/mL solution. Gently vortex to suspend and filter sterilize the solution. This solution can be aliquoted and frozen at –20°C until use.
2. The 10 mg/mL working solution can be used for 2 weeks if properly stored at 2–8°C (store in aliquots to avoid repeated warming).

MEF Medium (for 100 mL of complete medium)

1. To prepare 100 mL of complete MEF medium, aseptically mix the following components:

D-MEM	89 mL
FBS, ESC-Qualified	10 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
β-mercaptoethanol, 1000X	100 µL

2. Complete MEF medium can be stored at 2–8°C for up to 1 week.

Pluripotent Stem Cell (PSC) Culture Medium (for 100 mL complete medium)

1. To prepare 100 mL of complete PSC Culture Medium, aseptically mix the following components:

D-MEM/F-12	79 mL
KSR	20 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
bFGF (10 µg/mL)*	40 µL
β-mercaptoethanol, 1000X	100 µL

*Add bFGF at the time of medium change (final concentration 4 ng/mL).

2. Complete MEF medium can be stored at 2–8°C for up to 4 weeks.

Preparing MEF-Conditioned Medium (MEF-CM)

1. Cover the whole surface of each new culture vessel with Attachment Factor (AF) solution and incubate the vessels for 30 minutes at 37°C or for 1 hour at room temperature. For MEF-CM generation, a T-175 flask is recommended.
2. Using sterile technique in a laminar flow culture hood, completely remove the AF solution from the culture vessel by aspiration just prior to use. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

Note: It is not necessary to wash the culture surface before adding cells or medium.

3. Plate 9.4×10^6 Mitomycin C-treated or irradiated MEFs in a T-175 flask coated with AF and containing 30 mL of MEF medium
4. The following day, replace the MEF medium with 90 mL of PSC Culture Medium.
5. Collect the PSC Culture Medium, now considered MEF-CM, from the flasks after 24 hours of conditioning for up to seven days in a row.
6. Each day, filter sterilize the collected MEF-CM with a 0.22 µM filter. Filtered MEF-CM can be stored at –20°C until use.
7. At the time of use, thaw the MEF-CM in a 37°C waterbath, and freshly supplement it with additional bFGF (20 ng/mL).

Coating Culture Vessels with Geltrex® LDEV-Free, hESC-Qualified Basement Membrane Matrix

1. Thaw a 5-mL bottle of Geltrex® LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix at 2–8°C overnight.
2. Dilute the thawed Geltrex® solution 1:1 with cold sterile D-MEM/F-12 to prepare 1-mL aliquots in tubes chilled on ice. These aliquots can be frozen at –20°C or used immediately.

Note: Aliquot volumes of 1:1 diluted Geltrex® solution may be adjusted according to your needs.

3. To create working stocks, dilute a Geltrex® aliquot 1:50 with cold D-MEM on ice, for a total dilution of 1:100.

Note: An optimal dilution of the Geltrex® solution may need to be determined for each cell line. Try various dilutions from 1:30 to 1:100.

4. Quickly cover the whole surface of each culture dish with the Geltrex® solution (refer to Table 1).
5. Incubate the dishes in a 37°C, 5% CO_2 incubator for 1 hour.

Note: Dishes can now be used or stored at 2–8°C for up to a week. Do not allow dishes to dry.

6. Aspirate the diluted Geltrex® solution from the culture dish and discard. You do not need to rinse off the Geltrex® solution from the culture dish after removal. Cells can now be passaged directly into MEF-CM onto the Geltrex® matrix-coated culture dish.

Note: CELLstart™ CTS™ substrate may be substituted for Geltrex® hESC-Qualified Matrix (see the Appendix).

Table 1 Volume of Geltrex® hESC-Qualified Matrix Required

Culture Vessel	Surface Area	Volume of Diluted Substrate (mL)
6-well plate	10 cm^2/well	1.5 mL per well
12-well plate	4 cm^2/well	750 µL per well
24-well plate	2 cm^2/well	350 µL per well
35-mm dish	10 cm^2	1.5 mL
60-mm dish	20 cm^2	3.0 mL
100-mm dish	60 cm^2	6.0 mL

Thawing and Plating hESCs

1. Label the Geltrex® matrix-coated dish with the passage number from the vial, the date, and user initials.
2. Remove the vial of hESCs from liquid nitrogen storage using metal forceps.

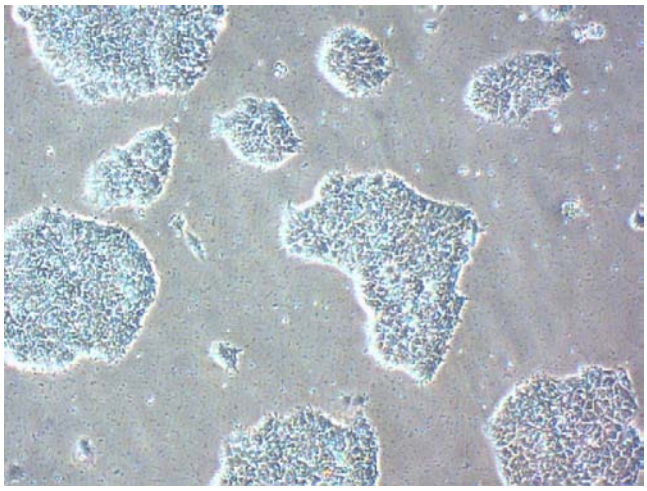
Note: If the vial is exposed to ambient temperatures for more than 15 seconds between removal and thawing, transfer the vial into a container containing a small amount of liquid nitrogen.

3. Roll the vial between your gloved hands until the outside is free of frost. This should take ~10–15 seconds.
4. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
5. When only an ice crystal remains, remove the vial from the water bath, spray the outside of the vial with 70% ethanol to sterilize, and place it in hood.
6. Pipet the thawed cells gently into a sterile 50-mL conical tube using a 5-mL sterile pipette.
7. Slowly add 10 mL of MEF-CM drop-wise to cells in the 50-mL conical tube. While adding the medium, gently move the tube back and forth to mix the hESCs. This reduces osmotic shock to the cells.
8. Rinse the vial with 1 mL of MEF-CM and add to the 50-mL conical tube with cells.
9. Transfer cell suspension into a 15-mL centrifuge tube. Centrifuge the cells at 200 × *g* for 5 minutes.
10. Aspirate and discard the supernatant.
11. Resuspend the cell pellet in sufficient volume of MEF-CM according to Table 2 by gently pipetting the cells up and down in the tube a few times.
12. Aspirate the excess Geltrex® solution from the prepared dish and slowly add the thawed colonies onto the dish. Move the dish in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface the dish.
13. Place dish gently into the 37°C, 5% CO_2 incubator and incubate the cells overnight.
14. The next day, remove the spent medium with debris using a sterile serological pipet and transfer it into a prepared Geltrex® matrix-coated dish. You can use this dish as a backup in case there is a problem with the main dish.
15. Add fresh MEF-CM to each dish according to the volumes in Table 2. Place both plates gently into a 37°C, 5% CO_2 incubator overnight.
16. Examine cells under the microscope and replace spent medium daily from both plates. If feeding more than one plate, use a different pipette for each well to reduce the risk of contamination. Colonies may not be visible for up to a week.

Table 2 Volume of MEF-CM Required

Culture Vessel	Surface Area	Volume (mL)
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35-mm dish	10 cm^2	2.0 mL
60-mm dish	20 cm^2	4.0 mL
100-mm dish	60 cm^2	10.0 mL

Figure 1 hESCs growing in MEF-CM on a Geltrex® matrix-coated dish



Passaging hESCs

When to Split Cells

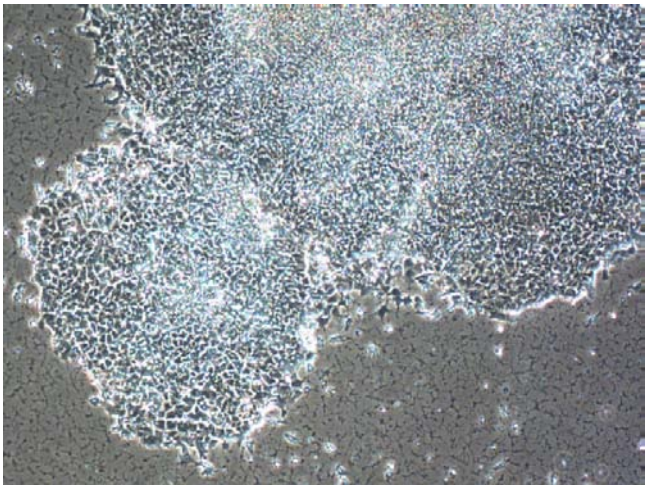
In general, split cells when one of the following occurs:

- hESC colonies are becoming too dense or too large.
- Increased differentiation occurs.

Split Ratio

- The split ratio can vary, though it is generally between 1:2 and 1:4. Occasionally, cells will grow at a different rate and the split ratio will need to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the hESC colonies.
- If the cells look healthy and colonies have enough space, split using the same ratio. If they are overly dense and crowding, increase the ratio. If the cells are sparse, decrease the ratio. Cells will need to be split every 4–10 days based upon appearance.

Figure 2 hESC colonies ready to be passaged. Note the large colony and the close proximity of the colonies to each other.



Enzymatic Passaging Using Collagenase

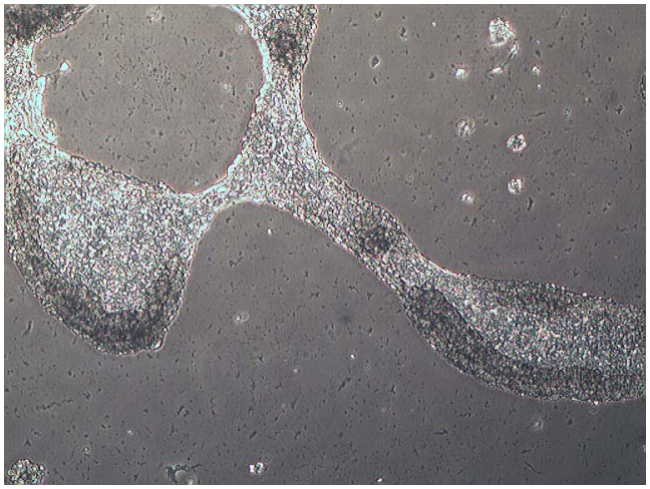
You may passage cells via the enzymatic method as described below, or mechanically as described in the following section.

1. Label a new Geltrex® matrix-coated dish with the cell line name, the new passage number, the date, the split ratio, and user initials. Return the dish to the incubator.
2. Under a dissecting microscope, remove differentiated colonies from the dish to be passaged.
3. Aspirate the spent medium from the dish with a Pasteur pipette, and rinse the dish once with Dulbecco's PBS (DPBS) without Calcium and Magnesium
4. Add Collagenase Type IV (10 mg/mL) solution to the dish containing hESCs. Adjust the volume of Collagenase Type IV for various dish sizes (e.g., 35-mm dishes require 1 mL of Collagenase IV).
5. Incubate the dish(es) for 5–7 minutes in a 37°C, 5% CO_2 incubator. Note that the incubation times may vary among different batches of collagenase; therefore, examination of the colonies is needed to determine the appropriate incubation time.

Note: As an alternative to Collagenase Type IV, you may use Dispase at a concentration of 2 mg/mL and incubate the dish(es) for 2–3 minutes in a 37° C, 5% CO_2 incubator.

6. Stop the incubation when the edges of the colonies are starting to pull away from the plate (see Figure 3).

Figure 3 PSC colony pulling away from the Geltrex® matrix layer after treatment with Collagenase Type IV



7. Aspirate the Collagenase Type IV Solution with a Pasteur pipette. Remove the collagenase carefully without disturbing the attached cell layer.
8. Add MEF-CM to each dish. Use a 5-mL pipette to gently blow the cells off the surface of the dish while pipetting up and down. Make sure to pipet gently to minimize the formation of bubbles.
9. After the hESCs have been removed from the surface of the well, pool the contents of the wells into a 15-mL conical tube.
10. Using a 5-mL pipette, add MEF-CM to the dish to wash and collect any residual cells. Pipet up the medium and cells, and then add the collected cells to the 15-mL tube.
11. Pipet cells up and down gently a few times in the 15-mL tube to further break up cell colonies. Pipet carefully to reduce foaming.

Note: Avoid making a single cell suspension.

12. Centrifuge at 200 × *g* for 5 minutes, and then aspirate the supernatant from the hESC pellet.
13. Resuspend the pellet with an appropriate amount of MEF-CM (refer to Table 2). This is dependent on the split ratio and the number of dishes used.

14. Mix the cell suspension well with a 10-mL pipette. Be careful not to break up the colonies too much or cause bubbles in the media.
15. Add appropriate volume of cell suspension to each dish. Return the dish to the incubator.
16. Move the dish(es) in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the dishes.
17. Incubate cells overnight to allow colonies to attach. Replace spent medium daily.

Note: While cells are attaching, be careful when opening and closing the incubator doors to avoid disturbing the even distribution of cells on the surface of the wells.

Mechanical Passaging Using the StemPro® EZPassage™ Disposable Cell Passaging Tool

1. Replace the medium in the dish containing the cells with fresh MEF-CM.
2. Under a laminar flow hood, open the package containing the EZPassage™ tool and remove the tool.
3. Hold the culture vessel in one hand and pull (roll) the EZPassage™ tool across the entire dish in one direction. Apply gentle but firm pressure so that the entire roller blade touches the dish and maintains uniform pressure during the rolling action.
4. Keep rolling the EZPassage™ tool parallel to the first pass until the entire dish has been covered.
5. Rotate the culture dish 90°, and then repeat rolling the cell layer as described above.
6. When you are finished, discard the EZPassage™ tool and do not reuse. Use a cell scraper to lift cell clusters off the plate, if necessary.
7. Using a serological pipette, rinse the dish with MEF-CM so that the cut colonies are suspended in the medium.
8. Transfer the medium containing the colonies to a 15-mL sterile tube.
9. Seed the cell colonies on dishes coated with Geltrex® matrix at an appropriate density.
10. Place the plates into a 37°C, 5% CO_2 incubator. Shake the plates gently to evenly spread out cells.

Figure 5 PSC colony after being cut with the StemPro® EZPassage™ Disposable Cell Passaging Tool

APPENDIX

A. In the PSC Culture Medium, DMEM/F12 containing GlutaMAX™-I (Cat. no 10565-018) can be substituted with the following alternatives**:**

i. KnockOut™ DMEM/F-12 (Cat. no. 12660-012)

To prepare 100 mL of complete PSC Culture Medium using KnockOut™ DMEM/F-12, aseptically combine the components listed in the table below.

Component	Stock Concentration	Final Concentration	Volume
KnockOut™ DMEM/F-12 (Cat. no. 12660-012)	—	1X	78 mL
KnockOut™ SR (Cat. no. 10828-028)	—	20%	20 mL
GlutaMAX™-I (Cat. no. 35050-061)	200 mM	2 mM	1 mL
MEM Non-Essential Amino Acids Solution (Cat. no. 11140-050)	10 mM	0.1 mM	1 mL
bFGF (Cat. no. PHG0024)	10 µg/mL	4 ng/mL	40 µL
β-mercaptoethanol (Cat. no. 21985-023)	1000X	1X	100 µL

ii. KnockOut™ DMEM (Cat. no. 10829-018)

To prepare 100 mL of complete PSC Culture Medium using KnockOut™ DMEM, aseptically combine the components listed in the table below.

Component	Stock Concentration	Final Concentration	Volume
KnockOut™ DMEM (Cat. no. 10829-018)	—	1X	78 mL
KnockOut™ SR (Cat. no. 10828-028)	—	20%	20 mL
GlutaMAX™ -I (Cat. no. 35050-061)	200 mM	2 mM	1 mL
MEM Non-Essential Amino Acids Solution (Cat. no. 11140-050)	10 mM	0.1mM	1 mL
bFGF (Cat. no. PHG0264)	10 µg/mL	4 ng/mL	40 µL
β-mercaptoethanol (Cat. no. 21985-023)	1000X	1X	100 µL

B. Alternative bFGF pack sizes

Product Name	Cat. no.	Product Size
FGF-basic (AA 1-155) Recombinant Human	PHG0264	10 µg
FGF-basic (AA 1-155) Recombinant Human	PHG0266	25 µg
FGF-basic (AA 1-155) Recombinant Human	PHG0261	100 µg
FGF-basic (AA 1-155) Recombinant Human	PHG0263	1 mg

C. Dissociation Enzymes/Tools for Harvesting hESC

Dissociation Enzyme /Tools	Application	Suggested concentration
StemPro® EZPassage™ tool (Cat. no. 23181-010)	Manual passaging	Sterile, disposable tool
StemPro® Accutase® (Cat. no. A11105-01)	Monolayer of cells post passage, Dissociation into single cells	1X ready to use (1–2 minutes incubation at 37°C)
Dispase (Cat. no. 17105-041)	Colony-like morphology post passage	2 mg/mL (2–3 minutes incubation at 37°C)
TrypLE™ Express (Cat. no. 12604-021)	Dissociation to single cells	1X ready to use

D. Coating Culture Dishes with CELLstart™ CTS™ Substrate

CELLstart™ CTS™ is a xeno-free substrate that supports the growth of hESCs in feeder-free medium.

1. Dilute CELLstart™ CTS™ at 1:50 in D-PBS with Calcium and Magnesium. Pipet gently to mix. Do not vortex.
2. Add enough diluted CELLstart™ CTS™ to coat the entire surface of the culture vessel (refer to Table 3). Swirl or gently tap the vessel to distribute the diluted CELLstart™ CTS™ evenly.
3. Incubate the coated vessels in a 37°C, 5% CO_2 incubator for 1–2 hours.
4. After incubation, the coated vessels are ready for immediate use or can be stored at 2–8°C for up to a week.
5. Just before use, pipet the CELLstart™ CTS™ solution from the culture vessel. It is not necessary to rinse the vessel following removal of CELLstart™ CTS™.
6. Proceed with plating hESCs on the CELLstart™ CTS™-coated vessel at the desired density.

Table 3 Volume of CELLstart™ CTS™ Substrate Required

Culture Vessel	Surface Area	Volume of Diluted Substrate (mL)
6-well plate	10 cm^2/well	750 µL per well
12-well plate	4 cm^2/well	250 µL per well
24-well plate	2 cm^2/well	160 µL per well
35-mm dish	10 cm^2	750 µL
60-mm dish	20 cm^2	3.0 mL
100-mm dish	60 cm^2	5.0 mL