Human Embryonic Stem Cells: Laboratory Manual
(Includes Invitrogen product information)

Michal Amit and Joseph Itskovitz-Eldor.

Department of Obstetrics and Gynecology, Rambam Medical Center and The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology

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Correspondence to Joseph Itskovitz-Eldor M.D., D.Sc., Department of Obstetrics and Gynecology, Rambam Medical Center, P.O.B. 9602, Haifa 31096, Israel.
Tel.: +972-4-854-2536; Fax: +972-4-854-2503;
E-mail: Itskovitz@rambam.health.gov.il.

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1. General:

1.1 Required Equipment:


2. 37 °C Incubators with 5% CO₂.

3. Inverted Phase -Contrast Microscope.

4. Table Centrifuge, preferably with an option for 4 °C.

5. Liquid Nitrogen storage container.

6. Autoclave and oven for sterilization.

7. 500ml autoclaveable glass bottle.

8. Culture dishes  Nunc dishes recommended for hES cell culturing.

9. Pasteur pipettes.

10. Autoclaveable cylinders for Pasteur pipettes.

11. Petri dishes.


13. Sharp Iris scissors.

14. T75 culture flasks.


16. 40μm MESH strainer

17. Glass slides
1.2 Required Materials:

1. **Sterile water**. Invitrogen cat. # 15230

2. Gelatin powder. Recommended type A, from porcine, Sigma G-1890.

3. **Dulbecco’s Modified Eagle’s Medium (DMEM)**. Invitrogen cat #11960.

4. **Fetal Bovine Serum defined (FBSd)**. Invitrogen cat. # 16141


6. **Fetal Calf Serum (FCS)**. Invitrogen cat. # 16010

7. **Penicillin-Streptomycin**. Invitrogen cat. # 15070

8. 70% ethanol.

9. **PBS**. Invitrogen cat. # 20012

10. **Trypsin (TrypLE recommended)** Invitrogen cat. # 12563


12. **L-glutamine**. Invitrogen cat. # 21051

13. **Non essential amino acids**. Invitrogen cat. # 11140

14. **β-Mercaptoethanol**. Invitrogen, cat. # 21985

15. **KO-DMEM**. Invitrogen cat. # 10829.

16. **Serum replacement (SR)**. Invitrogen cat. # 10828.

17. **basic Fibroblasts Growth Factor (bFGF)**. Invitrogen cat. # 13256.

18. **Collagenase type IV**. Invitrogen cat. # 17104

19. **EDTA** 0.5M Invitrogen cat. # 00-5500
1.3 0.1% Gelatin Coating of Plates

All plates should be covered with gelatin before the plating of cells.

1. Rinse an empty 500ml autoclaveable glass bottle with sterile water.
2. Autoclave bottle at 134 °C for 30 minutes.
3. Add 500mg of gelatin powder into cooled bottle (type A, from porcine, Sigma G-1890).
4. Add 500ml of sterile water.
5. Autoclave at 121 °C for one hour. Store at room temperature.
6. Optional: filter the gelatin through 22 µM filter.
7. Cover plating dish according to the following table:

<table>
<thead>
<tr>
<th>Plate/ dish</th>
<th>Volume of gelatin per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 wells</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>6 wells</td>
<td>2 ml</td>
</tr>
<tr>
<td>35 mm</td>
<td>2 ml</td>
</tr>
<tr>
<td>10 c² m</td>
<td>10 ml</td>
</tr>
</tbody>
</table>
8. Leave at room temperature or in incubator for at least two hours.

Note:

It is highly recommended to prepare gelatin-covered- plates 24 hour before use.

Any high quality sterile water may be used.
1.4 Preparation of Pasteur Pipettes

The pasture pipettes are used for the daily medium change.

1. Insert pipettes into autoclaveable cylinders.

2. Place in oven for 4 hours at 180 °C.

**Note:**

We do not recommend the use of autoclave for this procedure as water residue may cause contamination.
1.5 Preparation of Freezing Medium

The following freezing solution is used for hES cells, MEF and HFF.

**Final concentrations:**

- 60% *Dulbecco’s Modified Eagle’s Medium (DMEM)* Invitrogen cat #11960
- 20% Dimethyl sulfoxide (DMSO)
- 20% *Fetal Bovine Serum defined (FBSd)* Invitrogen cat. # 16141

**Preparation:**

1. Pour all materials into a 22- m filter, DMSO last.
2. Filter.
3. Store at 2-8 °C.

**Note:**

The use of different serum reduces the percentage of recovered cells.

May be used within two weeks of preparation.
2. Mouse Embryonic Fibroblasts (MEF):

2.1 MEF medium:

This medium is used for culture MEF post thawing.

Final concentrations:

90% Dulbecco’s Modified Eagle’s Medium (DMEM) Invitrogen cat #11960.

10% Fetal Calf Serum (FCS) Invitrogen cat. # 16010

Preparation:

1. Pour materials into 22 μm filter unite and filter.

2. Store at 2-8 °C.

Note:

Fetal Bovine Serum defined (FBSd) Invitrogen cat. # 16141 or
newborn calf serum Fetal Calf Serum (FCS) Invitrogen cat. # 16010 is also suitable.

May be used within two weeks of preparation.
2.2 Derivation of MEF from pregnant mice:

Use MEF medium (see 2.1) with the addition of Penicillin-Streptomycin Invitrogen cat. # 15070

Preparation:
1. Use of pregnant ICR mice on the 13-day of conception is recommended. Sacrifice 1 female mouse by brief exposure to CO₂.
2. Wash abdomen with 70% ethanol and dissect the abdominal cavity to expose the uterine horns.
Remove the uterine horns into 10 c² m Petri dish, and wash three times with PBS, Invitrogen cat. # 20012 (See Fig 1 A and B).
3. Using two pairs of watchmakers forceps (Dumont 5, Fine Scientific Tolls recommended product redundant) open each uterine wall and release all embryos carefully without touching the mouse's fur.
4. Wash retrieved embryos three times with PBS, Invitrogen cat. # 20012 see Fig 1 C).
5. Use the same tools, to dissect each embryo from the placenta and membranes, and discard soft tissues as much as possible.
6. Transfer clean embryos into new Petri dish and mince thoroughly using sharp Iris scissors. (see Fig 1 D).
7. Add six ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563 and incubate for at least 10 minutes.
8. Neutralize trypsin using at least 6 ml of MEF culture medium. Transfer the MEF into conical tubes. Use MEF culture medium to wash the plate.

9. Divide evenly into T75 culture flasks. We recommend a ratio of three embryos per flask.

10. Add 20 ml MEF culture medium.

11. Grow the MEF up to three days or until confluent culture. Change medium at least once during culture (do not vacuum the lumps).

12. Freeze the resulting MEF (2.4).

Note:

Other types of mice may be used.

Although pregnant mice at days 12-14 of conception may be used, day 13 of conception is recommended
Fig 1. Preparation of MEF. (A) Mice uterine horns. (B) Released embryos. (C) Resultant mash after thorough mincing of the embryos.
2.3 MEF Splitting:

1. Add 2 ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563 and cover the entire culture-flask surface.

2. Incubate for 6 minutes.

3. Tap side of the flask to loosen the cells. Add 4 ml of culture medium (2.1) to neutralize the trypsin.

4. Remove cell suspension into conical tube and centrifuge for five minutes at 2000 rpm.

5. Remove suspension, re-suspend in desired volume of culture medium (see 2.1) and pipette in order to fracture the pellet.

6. Distribute cell suspension to desired number of culture flasks.

7. Add MEF culture medium to final volume of 10ml.
2.4 MEF Freezing:

1. Remove all lumps possible.
   
   Add 2 ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563

2. and cover the entire culture-flask surface.

3. Incubate for 6 minutes.

4. Tap side of the flask to loosen the cells. Add 4 ml of culture medium (see 2.1) to neutralize the trypsin.

5. Remove cell suspension into conical tube. Let remaining lumps sink and remove cell suspension into clean conical tube.

6. Centrifuge for five minutes at 2000 rpm.

7. Remove suspension, re-suspend in desired volume of culture medium (see 2.1) and pipette in order to fracture the pellet.

8. Drop by drop, add an equivalent volume of freezing medium (see 1.5) and mix gently.

9. Place 1 ml into two-ml cryogenic vials (it is recommended to freeze four vials from one confluent flask).

10. Freeze vials overnight at -70 °C in Nalgene freezing box.

11. Transfer vials into a liquid nitrogen container.

Notes:

Adding the freezing medium drop by drop is crucial for cell recovery.

We collect all resultant flasks from the same mice and mark them with a batch number, due to variations between different batches.
2.5 MEF thawing:

1. Remove vial from liquid nitrogen and thaw briefly in a 37 °C water bath.
2. When a small pellet of frozen cell remains, clean the vial using 70% ethanol.
3. Pipette the contents of the vial once, and transfer the cells into conical tube.
4. Drop by drop add 2 ml of culture medium (see 2.1).
5. Centrifuge for 5 minutes at 2000 rpm.
6. Re-suspend the pellet in culture medium.
7. Remove cells suspension into culture flasks and add 10 ml of culture medium.

Notes:

Adding the medium drop by drop is crucial for cell recovery.
It is recommended to thaw one vial into a T75 culture flask. If the batch of MEF is sluggish you may thaw two vials per flask.
Do not thaw more than four vials at once.
2.6 Preparation of MEF-covered plates

1. Add 8 ng/ml mitomycin C into culture flask and incubate for two hours.

2. Wash four times with PBS, Invitrogen cat. # 20012

3. Add 2 ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563 recommended and cover the entire culture-flask surface.

4. Incubate for 6 minutes.

5. Tap side of the flask to loosen the cells. Add 4 ml of culture medium (see 4.1) to neutralize the trypsin.

6. Remove cell suspension into conical tube.

7. Centrifuge for five minutes at 2000 rpm.

8. Remove suspension, re-suspend in 10 ml of culture medium (see 4.1) and pipette in order to fracture the pellet.

9. Count cells and re-suspend in desired medium volume (see 4.1).

10. Add cell suspension into culture dishes. We recommend on $4 \times 10^5$ cells per well in six-well plates.

11. Let set for at least two hours before plating hES cells.

Note:

MEF number can also be calculate as $3 \times 10^4$ cells per $cm^2$.

Do not use KO-DMEM containing medium (4.1.2) for plating MEF.
3. Human Foreskin Fibroblasts (HFF):

3.1 HFF medium:

Final concentrations:

80% Dulbecco’s Modified Eagle’s Medium (DMEM) Invitrogen cat #11960.

20% Fetal Bovine Serum defined (FBSd). Invitrogen cat. # 16141

2 mM L-glutamine. Invitrogen cat. # 21051

1% Non essential amino acids. Invitrogen cat. # 11140

0.1% mM β-Mercaptoethanol Invitrogen, cat. # 21985

Preparation:

1. Pour all materials into 22 μm filter unite and filter.

2. Store at 2-8 °C.

Note:

If desired, human serum or SR may replace FBSd.

May be used within two weeks of preparation.
3.2 Derivation of HFF from Foreskins:

For HFF derivation we use HFF medium (see 3.1) with the addition of Penicillin-Streptomycin. Invitrogen cat. # 15070

Preparation:

Place newborn human foreskins in PBS. Invitrogen cat. # 20012

1. supplemented with Penicillin-Streptomycin. Invitrogen cat. # 15070 within 48 hours of circumcision.

2. Unfold foreskin and wash three times with PBS. Invitrogen cat. # 20012

3. Cut into small pieces using sharp Iris scissors (approximately eight pieces per foreskin).

4. Transfer clean pieces into a new Petri dish and mince thoroughly using sharp Iris scissors.

Add six ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563 and incubate for at least 30 minutes.

5. Neutralize the Trypsin (TrypLE recommended) Invitrogen cat. # 12563 using at least 6 ml of HFF culture medium (see 3.1). Transfer the HFF into conical tubes. Use HFF culture medium to wash the plate.

6. Divide evenly into T25 culture flask at a recommended ratio of two pieces per flask.

7. Add 6 ml HFF culture medium (see 3.1).

8. Grow the HFF until confluent culture. Change medium as needed (do not vacuum the lumps).

9. Freeze the resulting HFF (see 3.4).
Note:

Culture flasks may be covered with gelatin (see 1.3).
3.3 HFF Splitting:

1. Add 2 ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563 and cover the entire culture-flask surface.

2. Incubate for 6 minutes.

3. Tap side of the flask to loosen the cells. Add 4 ml of culture medium (see 3.1) to neutralize the trypsin.

4. Remove cell suspension into conical tube and centrifuge for five minutes at 1500 rpm.

5. Remove suspension, re-suspend in culture medium (3.1) and pipette in order to fracture the pellet.

6. Distribute cell suspension to desired number of culture flasks.

7. Add 6 ml of HFF culture medium (3.1).
3.4 HFF Freezing:

1. Remove all lumps as much as possible.

2. Add 2 ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563 and cover the entire culture-flask surface.

4. Incubate for 6 minutes.

5. Tap side of the flask to loosen the cells. Add 4 ml of culture medium (3.1) to neutralize the trypsin.

6. Remove cell suspension into conical tube. Let remaining lumps sink and remove cell suspension into clean conical tube.

7. Centrifuge for five minutes at 1500 rpm.

8. Add culture medium (3.1) and pipette up and down in order to brake to cells pellet.

9. Drop by drop, add an equivalent volume of freezing medium (1.5) and mix gently.

10. Place 1 ml into two-ml cryogenic vials (we place 1-2 vials per one confluent flask).

11. Freeze vials overnight at -70 °C in Nalgene freezing box.

12. Transfer vials into liquid nitrogen container.

Note:
Adding the freezing medium drop by drop is crucial for cell recovery.
3.5 HFF Thawing:

1. Remove vial from liquid nitrogen and quickly thaw it in 37 °C water bath.
2. When a small pellet of frozen cell remains, clean the vial using 70% ethanol.
3. Pipette the contents of the vial up and down once, and transfer the cells into conical tube.
4. Drop by drop add 2 ml of culture medium (see 3.1).
5. Centrifuge for five minutes at 1500 rpm.
6. Re-suspend the pellet in culture medium (3.1).
7. Remove cells suspension into culture flask and add 6 ml of culture medium (see 3.1).

Notes:

Adding the medium drop by drop is crucial for cell recovery.

Thaw one vial into one T25 culture flask.

Do not thaw more than four vials at once.
3.6 Preparation of HFF-Covered Plates

1. Add 8 ng/ml mitomycin C into culture flask and incubate for two hours.

2. Wash four times with PBS Invitrogen cat. # 20012
   Add 2 ml of Trypsin (TrypLE recommended) Invitrogen cat. # 12563 and cover the entire culture-flask surface.

4. Incubate for 6 minutes.

5. Tap side of the flask to loosen cells. Add 4 ml of culture medium (see 4.1) to neutralize the trypsin.

6. Remove cell suspension into conical tube.

7. Centrifuge for five minutes at 1500 rpm.

8. Add 10 ml of culture medium (see 4.1) and pipette up and down in order to break to cell pellets.

9. Count cells and re-suspend in desired medium volume (4.1).

10. Add cell suspension into culture dishes. We recommend 4x10^5 cells per well in six-well plates.

11. Let set for at least five hours before plating hES cells.

Notes:
HFF number can be calculate as 3x10^4 cells per c^2m.
If possible, set plate overnight before plating hES cells.
4. Human Embryonic Stem Cells (hES):

4.1 hES cell media:

4.1.1 Normal medium:

Final concentrations:

80% **Dulbecco’s Modified Eagle’s Medium (DMEM)**. Invitrogen cat #11960 or Knockout DMEM **KO-DMEM**. Invitrogen cat. # 10829.

20% **Fetal Bovine Serum defined (FBSd)**. Invitrogen cat. # 16141

1% **Non essential amino acids**. Invitrogen cat. # 11140

mM **L-glutamine** Invitrogen cat. # 21051

0.1 mM **β-Mercaptoethanol** Invitrogen, cat. # 21985

Preparation:

1. Pour all materials into 22 μM filter unit, and filter.

2. Store at 4°C.
4.1.2 Serum free medium:

Final concentrations:

80% KO-DMEM Invitrogen cat. # 10829.

20% Serum replacement (SR) Invitrogen cat. # 10828.

1% Non essential amino acids. Invitrogen cat. # 11140

0 mM L-glutamine. Invitrogen cat. # 21051

0.1 mM β-Mercaptoethanol. Invitrogen, cat. # 21985

4 ng/ml basic Fibroblasts Growth Factor (bFGF). Invitrogen cat. # 13256

Preparation:

1. Pour all materials into 22 μM filter unit, and filter.

2. Store at 4°C.

Notes:

Do not use this medium for MEF-covered plate preparation. Prepare MEF-covered plate using normal culture medium (see 4.1.1) and change the medium before plating hES cells.

May be used within two weeks of preparation.
4.2 hES cell Splitting:

Splitting medium:

1 mg / ml Collagenase type IV. Invitrogen cat. # 17104

Dulbecco’s Modified Eagle’s Medium (DMEM). Invitrogen cat #11960.

Splitting protocol:

1. Remove medium from well. Add 0.5 ml splitting medium, and incubate for at least 30 minutes.

2. Add 1 ml of culture medium (see 4.1) and gently scrape cells with 5-ml pipette. The MEF feeder layer will remain on the plate (see fig 2).

3. Collect cell suspension and put into conical tube.

4. Centrifuge 3 minutes at 800 rpm at a recommended temperature of 4 °C.

5. Re-suspend cells in media (4.1) and plate on feeder-covered plate.

Notes:

For effective separation of hES cell from the feeder, longer collagenation is recommended.

hES cells may be incubated in collagenase for up to three hours.
Fig 2: MEF after collagenase treatment. Note the remaining circular area after the detachment of the hES cell colony from the MEF feeder layer. Bar 25 μM.
4.3 hES Cell Freezing:

1. Add splitting medium (see 4.2) and incubate for at least 30 minutes.

2. Add 1 ml culture medium and gently scrape the cells using 5-ml pipette and transfer into conical tube.

3. Centrifuge 3 minutes at 800 rpm at a recommended temperature of 4 °C.

4. Re-suspend cells in culture medium.

5. Drop by drop add an equivalent volume of freezing medium (see 1.5) and mix gently.

6. Put 0.5 ml into 1-ml cryogenic vial.

7. Freeze overnight at –70°C (we use freezing box from Nalgene).

8. Transfer to liquid nitrogen on the following day.

Notes:

Adding the freezing medium (1.5) drop by drop is crucial for cell recovery.

Do not fracture the cells into small clumps.
4.4 hES Cell Thawing:

1. Remove vial from liquid nitrogen.

2. Gently swirl vial in 37\(^\circ\)C water bath.

3. When a small pellet of frozen cell remains, wash vial in 70% ethanol.

4. Pipette content of vial up and down once to mix.

5. Place contents of vial into conical tube and add, drop by drop, 2 ml of culture medium.

6. Centrifuge 3 minutes at 800 rpm at a recommended temperature of 4 °C.

7. Remove supernatant and re-suspend cells in 3 ml medium.

8. Place cell suspension on one well of 6- well plate, or on a 4- well plate.

Note:

Adding the medium drop by drop is crucial for cell recovery.
4.5 Formation of Embryoid Bodies (EBs):

1. Remove medium from well. Add 0.5 ml splitting medium (see 4.2), and incubate for at least 30 minutes.

2. Add 1 ml of culture medium (see 4.1) and gently scrape cells with 5-ml pipette.

3. Collect cell suspension and place into conical tube.

4. Centrifuge 3 minutes at 800 rpm at a recommended temperature of 4 °C.

5. Re-suspend cells in media (4.1) using Gillson 1000 μM tip and plate on 58 mm Petri dish.

6. Add 6 ml of medium.

Note:

If EBs attach to the dish, scrape them off gently.
4.6 Formation of Teratomas:

1. Scrap hES cells drawn from three confluent wells (out of a six well plate) using policeman rubber scraper.
2. Centrifuge cell for five minutes at 1200 rpm.
3. Leave as little medium as possible.
4. Inject cells into the rear leg muscle of 4-week-old male SCID-beige mice, using 18 or 21 g needle.
5. Ten weeks after injection resulting teratomas may be examined.

Notes:
Higher amounts of cells will ensure teratoma formation but will reduce the mice capability to carry them.
Approximately six weeks after injection, touching can identify teratoma formation.
Injection into one leg of each mouse is advisable in order to avoid its suffering.
hES cells may be collected using collagenase splitting.
5. Abbreviations:

BFGF - Basic Fibroblasts Growth Factor
DMEM - Dulbecco’s Modified Eagle’s Medium
DMSO - Dimethyl sulfoxide
EB – Embryoid Bodies.
FBSd - Fetal Bovine Serum defined
FCS - Fetal Calf Serum
hES – Human Embryonic Stem cells.
HFF – Human Foreskin Fibroblasts.
Ko-DMEM - Knockout DMEM.
MEF – Mouse Embryonic Fibroblasts.
SR - Serum Replacement.