Cell Line: TE04
Lot: 25-Apr-04 p24

Table of Contents

Technion Certificate of Analysis 2

Technion Protocol "Human Embryonic Stem Cells: Laboratory Manual" 4

This material was cultured and frozen using Technion's protocols. WiCell recommends that stem cells should be thawed and established in the conditions in which they were initially frozen prior to transfer to alternate culture platforms. The protocols that were used to produce these cells can be found on the following pages of this document.

If you have any questions or concerns please contact WiCell's technical support staff via our website side at www.wicell.org and we will be happy to assist you.

Thank you,

WiCell

Technion
Faculty of Medicine
Stem Cells Research Center
Human Embryonic Stem Cells laboratory
POB 9649 Haifa 31096, ISRAEL
Tel : 972-4-8295445
Fax: 972-4-8295435
E-mail: mamit@tx.technion.ac.il

הטכניון
הפקולטה לרפואה
מרכז לחקר תאי גזע
מעבדה לחקר תאי גזע עובריים אנושיים
ת.ד. 9649 חיפה 31096
טל: 04-8295445
פקס: 04-8295435

Technion – Isreal Institute of Technology
Human Embryonic Stem Cells
Certificate of Analysis

Human Embryonic Stem Cell Line	I4
Delivered Passages	p15, p22, p23, p24, p25, p26, p28
Batch numbers	12.3.04, 26.4.04, 20.4.04, 30.4.04, 25.4.04

Quality Assurance Testing:
The following tests were performed on each of the batches:

Test	Testing Method	Date	Result	Pass/Fail
Karyotype	G-banding	27.5.04	46, XX	Pass
Mycoplasma	Commercial by PCR reaction	15.5.05	Negative	Pass
Immunostaining	In culture plates	22.4.04	Positive for SSEA4, Oct4, TRA60, TRA81	Pass
Freeze/Thaw survival	Cell viability	3.7.05	Colonies detected	Pass

In addition to the above-stated testing methods, each cell line was tested every six months for the following:

1. Teratoma formation – inclusion of tissues representative of the three embryonic germ layers as demonstrated by histological examination.
2. EB formation – inclusion of cells representative of the three embryonic germ layers as demonstrated by immunostaining for specific antigens and by RT-PCR reactions for representative genes.

Notes:

- The Technion's cell lines are to be used solely for research purposes and not for any clinical use or commercial purposes. Further, the cells may not be transferred to any third party without the Technion’s written consent.
- The lines were not identified as harboring agents known to cause diseases in humans.

- The lines were negative for HIV, Hepatitis A and B, Cytomegalia and Herpes simplex type I & II.
- It is recommended that appropriate safety precautions are used while handling the cells, similarly to those taken with any human specimen.
- The Technion is not liable for any damages or injury resulting from handling, storing or use of the cell lines. The cells should not be used for any therapeutic purposes and may not be used on human subjects.
- The Recipient shall use the Materials in compliance with any and all applicable governmental rules and regulations applicable to the handling or use of Human Embryonic Stem Cell Lines.

Approved by MA, PhD.

Signature:_________________________

Date:_____________________________

Human Embryonic Stem Cells: Laboratory Manual

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

The use of the procedures contained herein is the subject of Patents, Patents applications and other proprietary Intellectual Property owned by the authors and/or by third parties. This document should not be considered an express or implied license under any Intellectual Property rights.

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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

Content:

1. General:

1.1 Required equipment.

1.2 Required materials.

1.3 Gelatin coating of plates

1.4 Preparation of Pasteur pipettes

1.5 Preparation of Freezing solution.

1.6 Preparation of serum free freezing solution.

2. Mouse embryonic fibroblasts (MEF):

2.1 MEF medium.

2.2 Derivation of MEF from pregnant mice.

2.3 MEF splitting.

2.4 MEF freezing.

2.5 MEF thawing.

2.6 Preparation of MEF-covered plates.

3. Human foreskin fibroblasts (HFF):

3.1 HFF medium.

3.2 Derivation of HFF from foreskins.

2.3 HFF splitting.

2.4 HFF freezing.

2.5 HFF thawing.

2.6 Preparation of HFF-covered plates.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

4. Human embryonic stem cells (hES):

4.1 hES cell media:

4.1.1 Normal medium.

4.1.2 Serum- free medium.

4.2 hES cell splitting.

4.3 hES cell freezing.

4.4 hES cell thawing.

4.5 Formation of EBs.

4.6 Formation of teratomas.

5. Feeder Free culture of Human embryonic stem cells (hES):

5.1 Fibronectin coating of plates.

5.2 Culture medium.

5.3 hES cell splitting.

5.4 hES cell freezing.

5.5 hES cell thawing.

5.6 Transfecting hESCs with plasmids.

5.7 Protocol for karyotype preparation.

6. Abbreviations

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

1. General:

1.1 Required Equipment:

1. Class 2 Bio-Safety cabinets.

2. 37 °C Incubators with 5% CO_2.

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.

12. Watchmakers forceps (recomemended Dumont 5, Fine Scientific Tolls).

13. Sharp Iris scissors.

14. T75 culture flasks.

15. Nalgene freezing box. C.N.5100-0001.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

1.2 Required Materials:

1. Sterile water. Recommended Sigma W-3500 or W1503.

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

3. Dulbecco's Modified Eagle's Medium (DMEM). Recommended Invitrogen C.N.41965039.

4. Fetal Bovine Serum defined (FBSd). Recommended Hyclone C.N. SH30070.03

5. Dimethyl sulfoxide (DMSO). Recommended Sigma D-2650.

6. Fetal Calf Serum (FCS), any type. In Israel – FCS Biological Industries C.N. 04-001-1A ES cell tested, do not heat inactivate.

7. Penicillin-Streptomycin (Sigma P-3539).

8. 70% ethanol.

9. PBS.

10. Trypsin EDTA.

11. Mitomycin C. Recommended Sigma C.N. M-4287.

12. L-glutamine.

13. Non essential amino acids.

14. β-Mercaptoethanol.

15. KO-DMEM. Recommended Invitrogen C.N. 10829018.

16. Serum replacement (SR). Recommended Invitrogen C.N. 10828028.

17. basic Fibroblasts Growth Factor (bFGF). Recommended Invitrogen C.N. 13256029.

18. Collagenase type IV. Recommended Invitrogen, C.N 17104019.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.

The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

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:

Plate/ dish	Volume of gelatin per well
4 wells	0.5 ml
6 wells	2 ml
35 mm	2 ml
10 c^2m	10 ml

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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.

The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

1.4 Preparation of Pasteur Pipettes

The pasture pipettes are used or 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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.

The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

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)

20% Dimethyl sulfoxide (DMSO)

20% Fetal Bovine Serum defined (FBSd)

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 one week of preparation.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.

The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

1.6 Preparation of Serum Free Freezing Medium

The following freezing solution is used for hES cells cultured in feeder free conditions.

Final concentrations:

50% Dulbecco's Modified Eagle's Medium (DMEM)

20% Dimethyl sulfoxide (DMSO)

30% Serum replacement (SR, Invitrogen corporation)

Preparation:

1.4. Pour all materials into a 22- m filter, DMSO last.

2.5. Filter.

3.6. Store at 2-8 °C.

Note:

May be used within two weeks of preparation.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

2. Mouse Embryonic Fibroblasts (MEF):

2.1 MEF medium:

This medium is used for culture MEF post thawing.

Final concentrations:

90% DMEM

10% Fetal Calf Serum (FCS)

Preparation:

1. Pour materials into 22 μm filter unite and filter.
2. Store at 2-8 °C.

Note:

FBS or newborn calf serum is also suitable.

May be used within two weeks of preparation.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.

The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

2.2 Derivation of MEF from pregnant mice:

Use MEF medium (see 2.1) with the addition of Penicillin-Streptomycin (Sigma P-3539).

Preparation:

1. Use of pregnant ICR mice on the 13-day of conception is recommended. Sacrifice 1 female mouse by brief exposure to CO_2.
2. Wash abdomen with 70% ethanol and dissect the abdominal cavity to expose the uterine horns.
3. Remove the uterine horns into 10 c^2m Petri dish, and wash three times with PBS. (See Fig 1 A and B).
4. 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.
5. Wash retrieved embryos three times with PBS. (see Fig 1 C).
6. Use the same tools, to dissect each embryo from the placenta and membranes, and discard soft tissues as much as possible.
7. Transfer clean embryos into new Petri dish and mince thoroughly using sharp Iris scissors. (see Fig 1 D).
8. Add six ml of trypsin/EDTA and incubate for at least 10 minutes.
9. 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.
10. Divide evenly into T75 culture flasks. We recommend a ratio of three embryos per flask.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

11. Add 20 ml MEF culture medium.

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

13. 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

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

Fig 1. Preparation of MEF. (***A***) Mice uterine horns. (***B***) Released embryos.

(***C***) Resultant mash after thorough mincing of the embryos.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

2.3 MEF Splitting:

1. Add 2 ml of trypsin/EDTA 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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

2.4 MEF Freezing:

1. Remove all lumps possible.
2. Add 2 ml of trypsin/EDTA 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 -80 °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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

2.5 MEF thawing:

1. Remove vial from liquid nitrogen and thaw briefly in a 37 °C water bath.
3. When a small pellet of frozen cell remains, clean the vial using 70% ethanol.
4. Pipette the contents of the vial once, and transfer the cells into conical tube.
5. Drop by drop add 2 ml of culture medium (see 2.1).
6. Centrifuge for 5 minutes at 2000 rpm.
7. Re-suspense the pellet in culture medium.
8. 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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

2.6 Preparation of MEF-covered plates

1. Add 8 µg/ml mitimycin C into culture flask and incubate for two hours.

2. Wash four times with PBS.

3. Add 2 ml of trypsin/EDTA and cover the entire culture-flask surface.

4.7. Incubate for 6 minutes.

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

6.9. Remove cell suspension into conical tube.

7.10. Centrifuge for five minutes at 2000 rpm.

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

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

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

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

Note:

MEF number can also be calculate as $3x10^4$ cells per c^2m.

Do not use KO-DMEM containing medium (4.1.2) for plating MEF.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

3. Human Foreskin Fibroblasts (HFF):

3.1 HFF medium:

Final concentrations:

80% DMEM

20% FBSd

2 mM L-glutamine

1% non essential amino acids

0.1% mM β−Mercaptoethanol

Preparation:

1. Pour all materials into 22 μm filter unite and filter.
2. Store at 2-8 °C.

Note:

If desired, human serum (Chemicon) or SR (Invitrogen corporation) may replace FBSd.

May be used within two weeks of preparation.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

3.2 Derivation of HFF from Foreskins:

For HFF derivation we use HFF medium (see 3.1) with the addition of Penicillin-Streptomycin (Sigma P-3539).

Preparation:

1. Place newborn human foreskins in PBS supplemented with Penicillin-Streptomycin.within 48 hours of circumcision .
2. Unfold foreskin and wash three times with PBS.
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.
5. Add six ml of trypsin/EDTA and incubate for at least 30 minutes.
6. Neutralize the trypsin 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.
7. Divide evenly into T25 culture flask at a recommended ratio of two pieces per flask.
8. Add 6 ml HFF culture medium (see 3.1).
9. Grow the HFF until confluent culture. Change medium as needed (do not vacuum the lumps).
10. Freeze the resulting HFF (see 3.4).

Note:

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

Culture flasks may be covered with gelatin (see 1.3). If needed, Kanamycin may also be added to the culture medium.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

3.3 HFF Splitting:

1. Add 2 ml of trypsin/EDTA 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).

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

3.4 HFF Freezing:

1. Remove all lumps as much as possible.
2. Add 2 ml of trypsin EDTA 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 -80 °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.

.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

3.6 Preparation of HFF-Covered Plates

1. Add 8 μg/ml mitimycin C into culture flask and incubate for two hours.
2. Wash four times with PBS.
3. Add 2 ml of trypsin EDTA 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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

4. Human Embryonic Stem Cells (hES):

4.1 hES cell media:

4.1.1 Normal medium:

Final concentrations:

80% DMEM or Knockout DMEM (ko-DMEM, Invitrogen).

20% FBSd (Hyclone C.N SH30070.03)

1% non-essential amino acid

1 mM L-glutamine

0.1 mM β-mercaptoethanol

Preparation:

1. Pour all materials into 22 μM filter unit, and filter.
2. Store at 4°C.

Note:

We tested several FBS; none were able to support hES cell as the HyClone FBSd.
Usable within two weeks.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

4.1.2 Serum free medium:

Final concentrations:

85% Ko-DMEM

15 % Serum replacement (SR, Invitrogen)

1% non-essential amino acid

1 mM L-glutamine

0.1 mM β-mercaptoethanol

4 ng/ml basic Fibroblasts Growth Factor (bFGF, Invitrogen)

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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

4.2 hES cell Splitting:

Splitting medium:

1 mg / ml collagenase (Invitrogen, type IV C.N 17104019)

DMEM (Gibco BRL, C.N. 41965-039)

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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

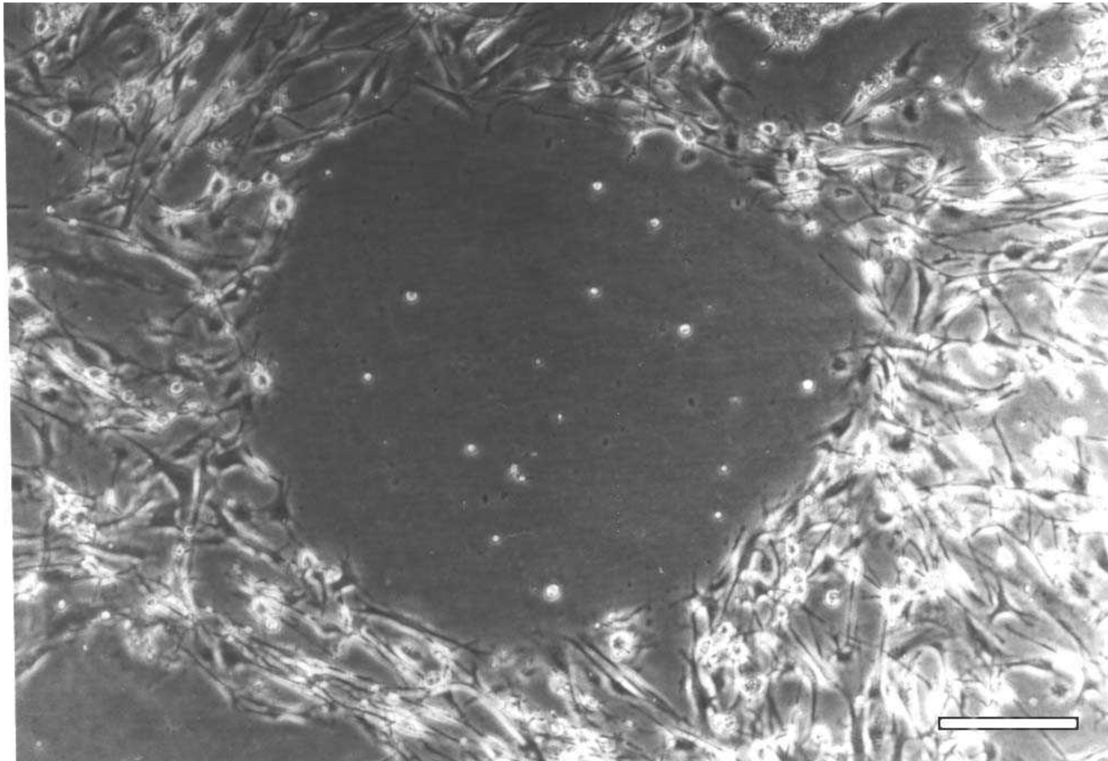

Fig 2: MEF after collagenase treatment. Note the remaining circular area after the detachment of the hES cell colony from the MEF feeder layer. Bar 25 μM.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

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 -80° 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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

4.4 hES Cell Thawing:

1. Remove vial from liquid nitrogen.

2. Gently swirl vial in 37^{0}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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

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 using cell-scraper.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

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 fiveminutes 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.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5. Feeder Free Culture of Human Embryonic Stem Cells (hES):

5.1 50µgr/10 cm^2 Fibronectin Coating of Plates

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

6.1. Dilute fibronectin to desirable concentration. Recommended; 1 mg of fibronectin (Sigma human foreskins fibroblast cellular fibronectin F6277; Sigma human plasma fibronectin F2006; Chemicon human plasma fibronectin FC010-10) to 10 ml of sterile water (Sigma W1503).

2. Optional: filter the fibronectin through 22 µM filter**.**

Can be store up to two weeks at 4 °C.

7.3.Cover plating dish according to the following table:

Plate/ dish	Volume of fibronectin per well
4 wells	0.3 ml
6 wells	0.5 ml
35 mm	0.5 ml

8.4.Leave at room temperature or in incubator for at least one hour.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5. If water were used to dissolve the fibronectin there is no need to collect the fibronectin residues before plating the cells. It is recommended to plate the cells directly on the fibronectin residues.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5.2 hES cell media:

Final concentrations:

85% Ko-DMEM

15% Serum replacement (SR, Invitrogen)

1% non-essential amino acid

2 mM L-glutamine

0.10.2 mM β-mercaptoethanol

4 ng/ml human recombinant basic Fibroblasts Growth Factor (bFGF, Invitrogen corporation)

0.12 ng/ml human recombinant Transforming Growth Factor β1 ($TGF_{\beta 1}$, R&D Systems)

1000 unites/ml human recombinant Leukemia Inhibitory Factor (LIF, R&D Systems)

Preparation:

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

2. Store at 4°C .

Notes:

May be used within two weeks of preparation.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5.3 hES cell Splitting:

Splitting medium:

1 mg / ml collagenase (Invitrogen, type IV C.N 17104019)

DMEM (Gibco BRL, C.N. 41965-039)

Splitting protocol:

1.2.Remove medium from well. Add 0.5 ml splitting medium, and incubate for at least 1 hour. Most colonies will flout.

2. Add 1 ml of culture medium (see 5.2) and gently collect cells with 5-ml pipette. Differentiated cells and "human feeders" formed at the edge of the colonies will remain on the plate.

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 (5.2) and plate directly on fibronectin-covered plate.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.

The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5.4 hES Cell Freezing:

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

2. Add 1 ml culture medium (5.2) 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 (5.2).

5. Drop by drop add an equivalent volume of freezing medium (see 1.6) and mix gently.

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

7. Freeze overnight at –80° C (we use freezing boxes from Nalgene).

8. Transfer to liquid nitrogen on the following day.

Notes:

Adding the freezing medium (1.6) drop by drop is crucial for cell recovery.

Do not fracture the cells into small clumps. Freezing boxes may be left at 80° C for up to three days.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.

The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5.5 hES Cell Thawing:

1. Remove vial from liquid nitrogen.

2. Gently swirl vial in 37^{0}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 (5.2).

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 (5.2).

8.8. Place cell suspension on one well of 6- well plate, or on a 4- well plate, previously covered with fibronectin (5.1).

Note:

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

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5.6 Transfecting hESCs with plasmids:

hESCs preparation:

24-48 before the transfection prepare the cells as follows:

1. Incubate the cells for 5 minutes with trypsin-EDTA.
2. Neutralize the trypsin with serum containing medium and centrifuge for 5 minute at 1200rpm.
3. Break the cells using 1000 µl tip into small clumps, and plate the cells on previously prepared culture plate covered with feeders carrying the appropriate resistance. The recommended seeding concentration should be twice the normal (in our hand 4 million cells per 10 cm^2).

The cells are ready to use when small colonies of 3-10 cells can be recognized.

The transfection using FuGENE 6:

1. To 95 µl of OptiMEM medium (Gibco, c.n. 31985-047) add, drop by drop, 5 µl of FuGENE reagent (Roche, c.n.11815 091001). Leave at room temp for 5 minutes.

2. Add, drop by drop, the reagent solution (prepared at 1) to a tube containing 3 µl of the plasmid (recopmended concentration of 1 µgr/µl). Leave at room temperature for 15 minutes.

3. Transfer the cells to OptiMEM at a minimum volume to avoid the well from drying (for a well at 6-well plate 200µl). Drop by drop, add the FuGENE solution (from 2). Incubate for four hours.

4. Add to the well culture medium (do NOT collect the transfection solution). It is recommended to use serum free medium, such as ko-DMEM and ko-serum replacement.

5. 24 hours post the transfection remove medium from the well and add fresh culture medium.

6. 48 hours post the transfection start the selection using appropriate antibiotic.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

5.7 Protocol for Karyotype Preparation

The values are calculated for one of six-well plates (10 cm^2). About 2 million ESCs which will result in at least 20 metaphases. Use dividing cells on the third or fourth day post passaging.

1. Dilute Colcemid solution with salt solution in ratio $1_{(colcemid)}:2_{(Salt\ solution)}$. (Karyomax Colcemid solution, Gibco BRL, C.N.15210-040; salt solution see below). 30µl:60µl is recommended.
2. Change the medium of the examined well with 2 ml fresh ES cell medium. Add 25µl colcemid solution (Step 1).
3. Incubate for 30 minutes.
4. Remove medium and collect the cells with trypsin.
5. Centrifuge 5 minutes at 1,200 rpm.
6. Collect medium and add 2 ml of Hypotonic Solution pre-warmed at 37°C.
7. Incubate 30 minutes at 37°C.
8. Add, drop by drop, 1 ml of fixer (see below) on the Hypotonic Solution.
9. Incubate 5 minutes at room temperature.
10. Centrifuge 5 minutes at 1,200 rpm.
11. Aspirate all liquid and add 2 ml of fixer.
12. Repeat stages 8-11 twice.
13. On the third repeat, leave the fixer and keep at -20 ^{o}C. Can be stored for a few months.

Fixer
1:3 (Acetic acid : Methanol) = 16ml : 48ml
Must be kept at -20 ^{o}C throughout the procedure. Use a fresh fixer for each preparation.

Hypotonic Solution
KCl 0.56g/100ml H_20 + Na citrate 0.5g/100ml H_20
Final volume 200 ml of solution.

Salt solution
Na citrate 1gr/ H_20 200 ml, and Kcl 1.12 gr / H_20 200 ml. Mix 100 ml of each.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.

6. 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.

Leukemia Inhibitory Factor – LIF.

MEF – Mouse Embryonic Fibroblasts.

SR - Serum Replacement.

$TGF_{\beta 1}$ - Transforming Growth Factor β1.

Copyright © 2002 Technion – Israeli Institute of Technology. All Rights reserved.
The information is copyrighted in Israel and worldwide by international treaty.
Revised; October 2002.