ES Cell International Pte Ltd

Methodology Manual
Human Embryonic Stem Cell Culture

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Preparation

Introduction

This manual contains comprehensive methods for the routine culture of ESI's proprietary human embryonic stem cells (hES cells). These methods are in widespread use, have been extensively tested and have been proven to successfully propagate all of ESI's hES cell lines without compromising the karyotype or pluripotency of the cells.

ESI strongly recommends that you employ the methods contained in this manual to generate a supplementary hES cell supply before proceeding with your research. ESI is confident that the detailed protocols contained in this manual will provide you with a solid base from which to launch your hES cell research.

ESI is committed to providing the best possible support for its hES-based products. Our qualified stem cell research scientists are available via telephone or email for technical assistance.

Additionally, ESI conducts a hES cell culture training course at our facility in Singapore. It is highly recommended that cell recipients participate in formal training conducted by qualified ESI stem cell scientists to ensure successful propagation of hES cells.

We at ESI are excited by the huge potential for human ES cell research, and look forward to working with you in the future.

Robert Klupacs
Chief Executive Officer
ESI

"The Complete Embryonic Stem Cell Solution"

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Before shipment

Before receiving your shipment of ESI hES cells, you should **ensure that you have a liquid nitrogen storage container ready** to accept the cells. This container must be able to accommodate either 4.5ml Nunc cryotubes (8.5cm tall by 0.75cm diameter - these are most conveniently stored in 9.5cm high liquid nitrogen freezer boxes) or aluminium cryocanes.

When the shipment arrives

ESI hES cells are shipped in a liquid nitrogen cooled dry shipper. The nitrogen in the shipper is in the vapour phase, so **do not be concerned if there is no liquid in the container upon arrival**.

Inside the dry shipper are aluminium canes holding 5ml vials containing two straws of each cell line requested. You will also receive two 1.5ml vials of Passage 6 mEF cells attached to canes. **The contents of the dry shipper should be transferred to your liquid nitrogen storage container immediately following receipt. Do not allow frozen vials to be exposed to room temperature for more than a few seconds** – the straws containing hES cells will thaw very quickly at room temperature, and refreezing will destroy the cells.

Once the vials are safely stored in your liquid nitrogen container, take the time to read this manual and plan your culture timetable and scale-up procedure.

Before you thaw your first straw of hES cells

Before you begin culturing hES cells in your lab, it is essential that you form a plan for hES cell scale-up and storage. hES cell culture is relatively labour intensive, and hence potentially susceptible to microbial contamination. By generating frozen back-up stock, you can minimize the impact of a contamination or any other lab mishap. **It is recommended that you thaw only one straw of ESI hES cells at a time.**

As with any other serum dependant cell line, some batches of serum may not support hES or mEF growth. ESI recommends that you test batches of serum (over three weeks of hES culture) to ensure they are compatible with hES growth.

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mEF cells

The mEF cells supplied with your shipment can only be split once more to produce mEFs capable of supporting hES growth. Passaging these cells further may result in a reduced ability to support hES cells.

Qualified mEF cells may be purchased from ESI. Please contact us for further information. If you choose not to purchase ESI mEF cells, then it is essential that you produce your own mEF line prior to thawing your first straw of hES cells.

General tips for hES cell culture

ESI hES cells should be kept at 37˚C at all times. Use a warmed platform beside the microscope and a warm stage on the microscope.

Warm all media before it comes into contact with cells. Incubate media in a water bath or your cell culture incubator in 50ml tubes with the top loosened for at least six hours prior to use.

Only passage good quality colonies. The central button or cystic parts of colonies will usually fail to grow or lose pluripotentiality when transferred.

Take care. hES cells are more sensitive to changes in cell culture technique than most mammalian cells.

Pre-thaw checklist

1. Do I have all the equipment required?
2. Do I have all the reagents and media required?
3. Do I have mEF cells ready?
4. Do I have plans to derive new mEF lines, or do I have enough ESI qualified mEFs to support scale-up?

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Preparation

Equipment and reagents

For local distributors, visit the websites listed

Consumables:	Catalogue number:	Website
DMEM Medium	Gibco 11960-044	www.invitrogen.com
L-Glutamine-200mM	Gibco 25030-081	
Penicillin/Streptomycin	Gibco 15070-063	
Non-essential amino acids (NEAA)	Gibco 11140-050	
β-Mercaptoethanol	Gibco 21985-023	
PBS+ (with Ca^{2+} and Mg^{2+})	Gibco 14040-133	
PBS- (without Ca^{2+} or Mg^{2+})	Gibco 14190-144	
Insulin-Transferrin-Selenium (ITS)	Gibco 41400-045	
Trypsin/EDTA (0.25%)	Gibco 25200-056	
HEPES 1M	Gibco 15630-106	
Collagenase IV	Gibco 17104-019	
Mitomycin-C	Sigma M-0503	www.sigmaaldrich.com
Gelatin (Type A from porcine skin)	Sigma G-1890	
Ethylene glycol (EG)	Sigma E-9129	
Dimethylsulfoxide (DMSO)	Sigma D-2650	
Sucrose	Sigma S-7903	
Foetal Bovine Serum (FBS) – Hyclone	Hyclone SH30070-03	www.hyclone.com
Vitrification Straws (Sterile)	LEC Instruments	www.lecinstruments.com
Instrument Sleeves	Leica 11520145	www.leica-ead.com
Centre Well Organ Culture Dishes	Falcon 35-3037	www.bd.com
T-75 Flasks	Falcon 35-3136	
T-175 Flasks	Falcon 35-3112	
15ml Tubes	Falcon 35-2096	
50ml Tubes	Falcon 35-2070	
Cell Scrapers	Falcon 35-3086	
NUNC 4-Well Plates	NUNC 176740	www.nuncbrand.com
1.0ml Cryovials	NUNC 377224	
4.5ml Cryovials	NUNC 379146	
Glass Capillaries 1.0mm Outer diameter	Harvard apparatus GC100T-15	www.harvardbioscience.com
Stericup receiver flasks (500ml)	Millipore SCOOB05RE	www.millipore.com
Steritop 0.22µm filter units (250ml)	Millipore SCGPT02RE	
0.22µm Luer lock filters (syringe filters)	Millipore SLGVR25LS	
Cryo 1˚C Freezing container	Nalgene 5100-0001	www.nalgene.com

Generic equipment:

Class 2 Biohazard Work Station
Dissecting Microscopes
Warm Stages
Incubator (5% CO_2, 37°C)
Aspiration System
Pipetter series covering 1µl to 1000µl
Motorized Pipetters
Centrifuge capable of spinning 15 and 50ml tubes at up to 1000g
-80°C Freezer
-20˚C Freezer
4˚C Refrigerator
37˚C waterbath

Liquid nitrogen storage canister
Liquid nitrogen dewar
Bunsen Burner
Laboratory scales (<0.01g resolution)
Autoclave
Haemocytometer

2.5ml syringes
Aluminium cryocanes
12g and 18g needles
Pasteur pipettes
Liquid Nitrogen supply
Distilled water
Isopropanol

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Preparation

Solutions and media

1% Gelatin Stock Solution:

1. Weigh out 0.4 g of gelatin powder and add to 40ml distilled water in 50ml tube.
2. Mix well.
3. Loosen the lid, place the tubes in a glass beaker covered with foil and autoclave at 121°C for 20 minutes.
4. Store 20ml aliquots at –20˚C

0.1% Gelatin working solution

This solution is used to coat the organ culture dishes that the mitotically inactivated mEF cell layers grow on.

1. Combine 20ml of the 1% gelatin stock solution with 180ml distilled water
2. Store at 4°C. Use or discard within 4 weeks

F-DMEM media

This media is used for the culture of mEF cells.

Component	Volume required
DMEM Media	442.5ml
L-Glutamine-200mM	5ml
FBS	50ml
Penicillin/Streptomycin	2.5ml
Total Volume	**500ml**

1. Combine all the media components.
2. Filter media through a Steritop into a Stericup receiver flask.
3. Store media at 4°C. **Use or discard within 2 weeks.**

hES media

This media is used for the culture of hES cells and pre-equilibration of mEF cells.

Component	Volume required
DMEM Media	457.9ml
NEAA	6ml
Penicillin/Streptomycin	3ml
L-Glutamine-200mM	6ml
FBS	120ml
ITS Supplement	6ml
β-Mercaptoethanol – **Add last**	1.1ml
Total Volume	**600ml**

1. Combine all the media components.
2. Filter media through a Steritop into a Stericup receiver flask.
3. Ensure the flask is kept in the dark (wrap in foil if necessary).
4. Store media at 4°C. **Use or discard within 2 weeks.**

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Preparation

Mitomycin-C stock solution (1mg/ml)

This solution is required to mitotically inactivate the mEF cells prior to seeding hES.

NB: Mitomycin-C is carcinogenic and cytotoxic. Safety glasses and gloves should be used at all times. Mitomycin-C should be discarded according to your local requirements.

1. Add 1ml PBS- to the ampoule containing 2 mg mitomycin-C and dissolve the powder.
2. Remove all of the mitomycin-C solution from the ampoule using an 18G needle and a 2.5ml syringe.
3. Filter the solution through a syringe filter into a 15ml tube.
4. This solution is light sensitive and should be covered in foil.
5. Store at 4°C. Use or discard within 2 weeks.

mEF freezing medium

This cryoprotective medium is used to freeze mEF cells.

1. Add 4.5ml FBS (CSL) and 0.5ml DMSO. Prepare fresh.

0.05% Trypsin/EDTA

Trypsin is used to detach the mEF cells during passages.

1. Dilute 1ml 0.25% Trypsin/EDTA in 4ml PBS-.
2. Store at 4°C. Use or discard within 1 week.

ES-HEPES media

This media is used in both vitrification and thawing.

Component	Volume required
DMEM Media	15.6ml
Foetal Bovine Serum (FBS)	4ml
1M HEPES	0.4ml
Total Volume	**20ml**

1. Combine all the media components.
2. Filter the solution through a syringe filter into a 50ml tube.
3. Store media at 4°C. Use or discard within 1 week.

1 M Sucrose media

This solution is a component of vitrification and thawing solutions.

1. Weigh out 3.42 g of sucrose.
2. Add 6ml DMEM-HEPES media to the sucrose in a 15ml tube.
3. Warm the solution at 37°C to dissolve the sucrose.
4. Make the dissolved solution up to 8ml with DMEM-HEPES media
5. Add 2ml foetal bovine serum (Hyclone)
6. Filter solution through a 0.22μm syringe filter
7. Store the solution at 4°C. Discard any unused solution after one week.

NB: This solution can be stored at -20°C in small aliquots for later use.

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0.2 M Sucrose solution
This is one of the thawing solutions.

Component	Volume required
ES-HEPES Media	4ml
1 M Sucrose Solution	1ml
Total Volume	**5ml**

1. Combine all of the solution components.
2. Store at 4°C. Discard any remaining solution after one week.

0.1 M Sucrose solution
This is one of the thawing solutions.

Component	Volume required
ES-HEPES Media	4.5ml
1 M Sucrose Solution	0.5ml
Total Volume	**5ml**

1. Combine all of the solution components.
2. Store at 4°C. Discard any remaining solution after one week.

10% Vitrification solution

Component	Volume required
ES-HEPES	2ml
Ethylene Glycol	0.25ml
DMSO	0.25ml
Total Volume	**2.5ml**

1. Combine all of the solution components.
2. Store at 4°C. Discard any remaining solution after one week.

20% Vitrification solution

Component	Volume required
ES-HEPES	0.75ml
1 M Sucrose Solution	0.75ml
Ethylene Glycol	0.5ml
DMSO	0.5ml
Total Volume	**2.5ml**

1. Combine all of the solution components.
2. Store at 4°C. Discard any remaining solution after one week.

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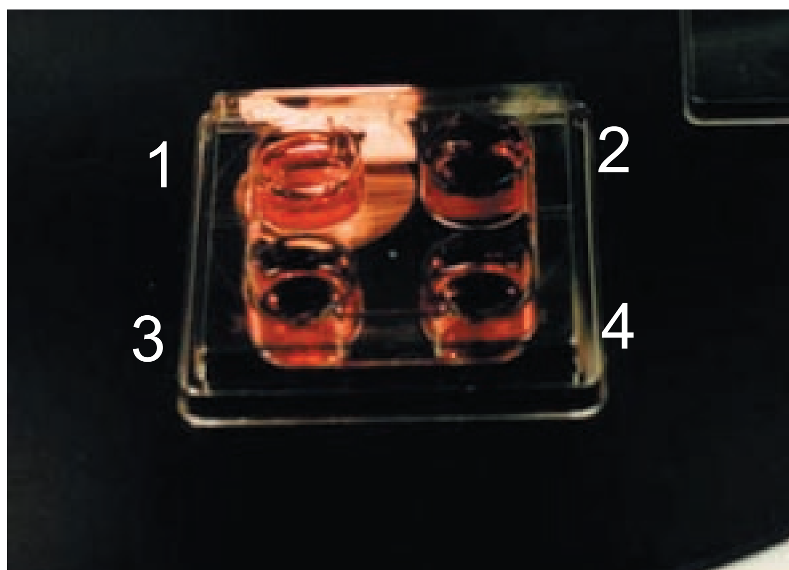
Producing cutting pipettes

A cutting pipette should be pulled from 1.0 mm to approximately 0.25 mm in diameter to produce a sharp cutting edge. If the capillary is pulled too thin or too long it will break when cutting colonies. Capillaries that are too thick tend to tear the colonies. The tip should be broken on a slight angle and have no jagged edges.

1. Hold a glass capillary in a Bunsen burner flame until the glass begins to soften (~2 seconds in the blue flame).
2. Remove the capillary from the flame and, holding horizontally, pull at both ends to draw out the heated section.
3. Fold the capillary until the thin section breaks. The thick section of one piece can then be used to break the thin section of the other piece.
4. Carefully wrap the capillaries in foil and autoclave before use.

Thawing plate

Note that there is 1ml of solution in each well.



A hES thawing plate.
1 - 0.2M Sucrose solution (cells are immersed in this solution for 1 minute).
2 - 0.1M Sucrose solution (cells are immersed in this solution for 5 minutes).
3 and 4 - ES-HEPES media (cells are immersed in these solutions for 5 minutes each).

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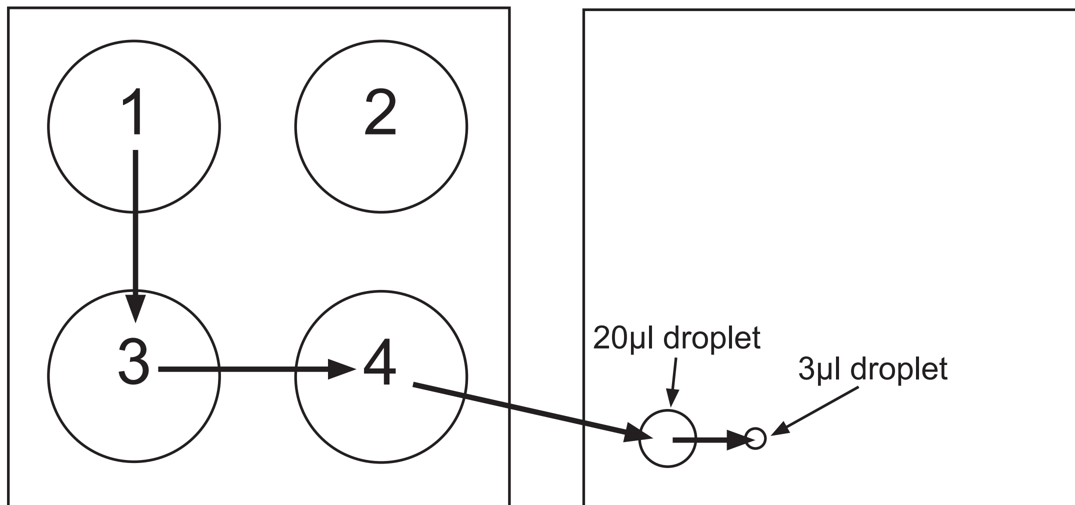
Vitrification plate

Note that there is 1ml of solution in each well.



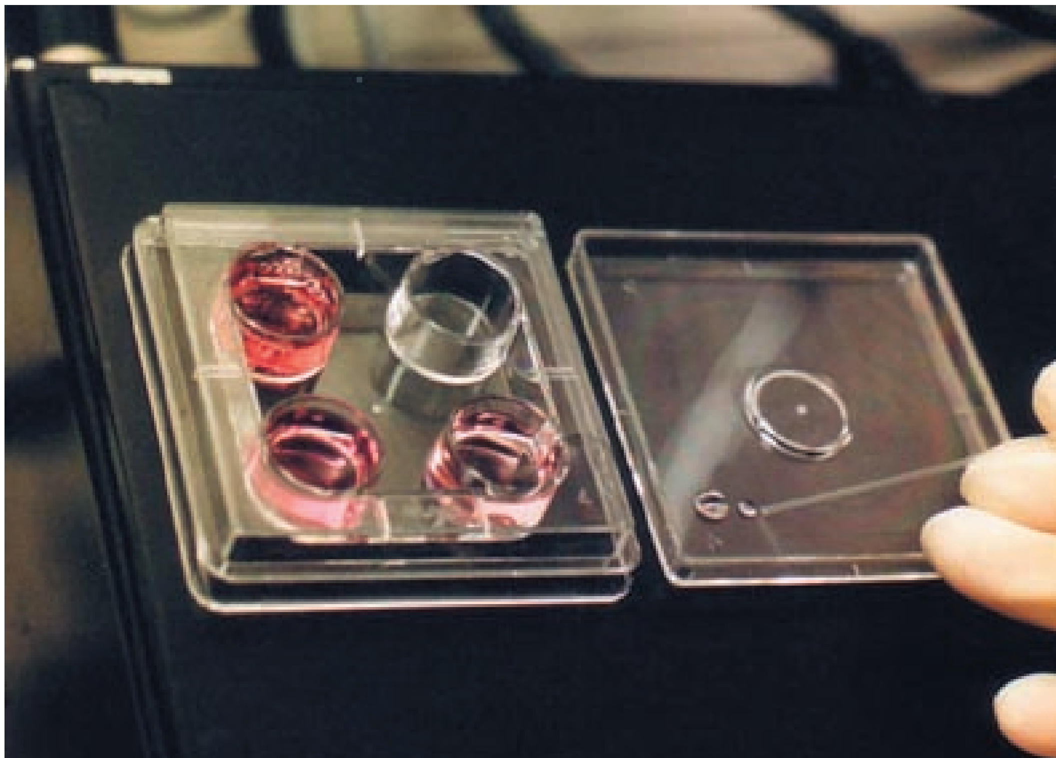


Diagram and photograph of a vitrification plate.
1 - ES-HEPES media.
2 - Empty.
3 - 10% Vitrification Solution (cells are immersed in this solution for 1 minute).
4 - 20% Vitrification Solution (cells are immersed in this solution for 25 seconds).
The 20µl droplet of vitrification solution is prepared before the vitrification procedure begins.
The cells are collected from the 20µl droplet and plated in a 3µl droplet which is then drawn up into the straw by capillary action.

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Preparation

Example production schedule

Day	mEFs	hES
Thursday		
Friday	Thaw mEF cells	
Weekend		
Monday	Prepare mitotically inactivated mEF plates (1 per straw hES)	
Tuesday	Change media (hES media onto mEFs)	
Wednesday		Thaw hES colony pieces
Thursday		Colony pieces attach
Friday	Passage mEF cells	Change media
Weekend		Change media
Monday	Prepare mitotically inactivated mEF plates (6 per plate of hES)	Change media
Tuesday	Change media (hES media onto mEFs)	Change media
Wednesday		Cut and transfer hES colonies

Thawing Schedule (yellow: first Thursday–Wednesday)

Maintenance Schedule (green: second Thursday–Wednesday)

Please take the time to adapt this production schedule to your own needs. Take into account FBS batch testing and generation of new mEF lines.

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Mouse embryonic fibroblast preparation

The procedure for deriving new mEF lines and passaging them for use is described on page 22.

New (unqualified) mEF cell lines **need to be tested for at least three hES passages to ensure that they can support hES growth**. Since only small numbers of mEF plates are required initially, excess mitomycin-C treated mEFs should be frozen down in small quantities for each week of use.

A healthy mEF cell feeder layer should be even and confluent to support the growth of transferred hES colonies. The mEF cells should only be used at low passage numbers as their ability to support hES cell growth generally declines from Passage 7 onwards.

Thawing passage 6 mEF cells

Prepare: Pre-warmed F-DMEM media

NB - The cryoprotective medium contains DMSO, which is cytotoxic. It is essential to work quickly to minimize exposure of thawed cells to DMSO.

1. Remove the mEF vial from liquid nitrogen and thaw immediately in a 37˚C water bath.
2. Add the cell suspension slowly into 10ml of warm F-DMEM media in a 15ml tube and mix. Rinse the cryovial with 1ml fresh of F-DMEM media.
3. Centrifuge the cell suspension at 2000 rpm for 2 minutes.
4. Aspirate the supernatant and resuspend the pelletted cells in 3ml F-DMEM media. Add this to a T-75 flask containing 20ml F-DMEM media (the flask should now contain 23ml).
5. Incubate at 37˚C in 5% CO_2 overnight.

Splitting mEF cells

Prepare: Pre-warmed PBS-
Pre-warmed F-DMEM media
Pre-warmed 0.05% Trypsin/EDTA

1. Examine the mEFs under a phase contrast microscope. Cells should be ~90% confluent. If they are too sparse, then leave them in the incubator for a further 12-24 hours.
2. Wash the flask containing mEF cells three times with 20ml PBS-.
3. Add 1.5ml 0.05% Trypsin/EDTA to the flask. Ensure the entire cell surface is covered.
4. Incubate the cells for 1-2 minutes only. Tap flasks to detach cells - some cells will remain.
5. Add 7ml of F-DMEM media to the flask and pipette gently up and down to disaggregate the cells. Transfer the cell suspension to a 15ml tube.
6. Wash flasks with 3ml F-DMEM and add this to the cell suspension in the 15ml tube. Check the flasks under the microscope to ensure trypsinisation has been effective (it is normal for up to 10% of cells to remain).

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7. Centrifuge the cell suspension at 2000 rpm for 2 minutes.

8. Aspirate the supernatant and resuspend cells in 6ml of F-DMEM media. Use a haemocytometer to count the mEF cells and determine total cell number

9. Seed the cells into T-75 flasks at a density of 1.7 - 2.0 x 10^4 cells/cm^2 in a total volume of 20ml of F-DMEM media. You should get about three T-75 flasks from the original flask.

10. Incubate the flasks in a 37°C, 5% CO_2 incubator for two days by which time they should be >90% confluent.

Preparation of mitotically inactivated mEFs

Prepare:

- Pre-warmed F-DMEM media
- Pre-warmed hES media
- Pre-warmed Distilled Water
- 0.1% gelatin solution
- Pre-warmed PBS-
- Pre-warmed Trypsin/EDTA
- Mitomycin-C Solution
- T-75 flask containing passage 7 mEFs

1. Determine the number of mEF plates you will require and label the plates appropriately. You do not necessarily need to use all the passage 7 mEFs immediately - excess mitomycin C treated mEFs may be frozen.

2. Coat each plate with 1ml of 0.1% gelatin for at least one hour before the addition of mEF cells. Add 5ml distilled water to the outside reservoir of each organ culture plate at the same time.

3. Aspirate media from T-75 flask containing mEF cells. Mix 100 μL mitomycin-C in 20ml of F-DMEM (i.e. 10μg/ml) and add to T-75 flask. Swirl flask gently and then place in 5% CO_2 incubator.

4. Leave mitomycin-C solution in flask for a minimum of 2.5 hours and a maximum of 3 hours.

5. Wash each flask once with 20ml F-DMEM media. Gently swirl the media.

6. Wash each flask twice with 20ml PBS-. Gently swirl the media.

7. Add 1.5ml 0.05% Trypsin/EDTA to each flask and leave on the cells for 1-2 minutes. Tap flask gently to remove cells.

8. Add 7ml of F-DMEM media to each flask and pipette gently up and down to further disaggregate the cells. Use the cell suspension to wash down the sides of the flask several times. Transfer the cell suspension to a 15ml tube.

9. Centrifuge the cells at 2000 rpm for 2 minutes.

10. Aspirate the supernatant from the cell pellet and resuspend the cells in 4ml **hES media**. Pipette the cells up and down gently to ensure they are disaggregated.

11. Combine the cells from all flasks. Count the mEF cells using a haemocytometer and determine total cell number.

12. Dilute the cells to a concentration of 1.75 x 10^5 cells per ml in hES media.

13. Remove gelatin solution and add 1ml of the cell suspension to each organ culture plate. Each

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flask should yield approximately 3 x 10^6 to 4 x 10^6 cells, which is enough for approximately 15-20 organ culture dishes (mEFs are seeded at 6 x 10^4 cells/cm^2).

13. Transfer the plates to the 5% CO_2 incubator with great care. Swirling the dish concentrates the cells in the centre resulting in an uneven feeder layer which may not adequately support hES growth.

14. After 24 hours, aspirate the media and replace it with 1ml **hES media**.

Freezing excess mitotically inactivated mEF cells

Prepare: Freshly prepared mEF freezing solution
1.5ml cryovials - label these with the date and mEF details prior to freezing
Excess mitomycin C treated mEF cells

1. Determine the total number of excess cells you want to freeze. Cells should be frozen in 0.5ml aliquots containing 8.75 x 10^5 cells. At this rate, when thawed, each vial will seed 5 organ culture dishes.

2. Centrifuge the cell suspension at 2000 rpm for 2 minutes.

3. Aspirate the supernatant from the cell pellet and resuspend the cells in the mEF freezing solution. Mix well.

4. Dispense 0.5ml of the cell suspension in 1.5ml cryovials.

5. Place the cryovials in a Nalgene Cryo 1˚C freezing container and store at -80°C for 24 hours.

6. Transfer the cryovials to liquid nitrogen.

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Thawing hES cells

Prepare: Pre-warmed thawing plate
Dewar containing liquid nitrogen
mEF plates

1. Collect the cryovial containing freezing straws in a container filled with liquid nitrogen.
2. Remove a straw from the cryovial using forceps.
3. Working quickly, hold the straw between the thumb and middle finger and submerge the narrow end in the first well of the thawing plate (containing 0.2M Sucrose) at a slight angle.
4. As soon as the liquid column melts (almost immediatly) place a finger on the top of the straw. As the gases in the straw expand, they force the liquid from the straw. If any liquid remains inside the straw, it may be expelled using a 200μl pipetter by placing the tip in the other end of the straw and pushing the plunger.
5. After one minute, transfer the colony pieces to the next well containing 0.1 M Sucrose Solution.
6. After 5 minutes transfer the colony pieces to the next well containing ES-HEPES Media.
7. After 5 minutes transfer the colony pieces to the next well containing ES-HEPES Media.
8. After 5 minutes, transfer the pieces to prepared mEF cell plates.

Step 4 - By placing a finger over the end of the straw, gases within the straw warm and expand, forcing the liquid from the straw.

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hES colony transfer

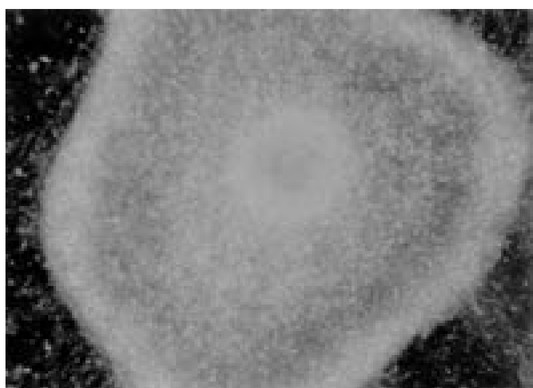
Colony transfer is one of the most difficult aspects of hES cell culture. Professional training at ESIs Singapore facility is recommended

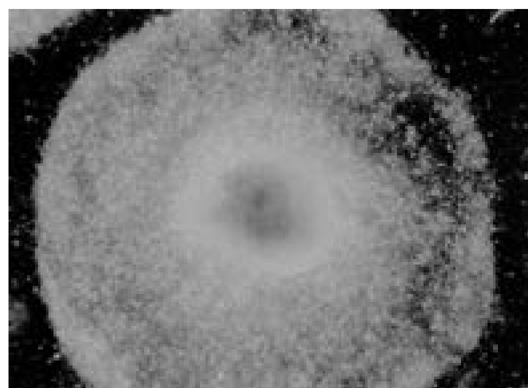
Colonies are transferred on day 7 when they should be 2-3 mm in diameter. Only colonies with good morphology should be transferred (transferring poor colonies will result in a loss of pluripotency). Large colonies are generally cut into 9 pieces, 8 of which are transferred (the central 'button' should not be transferred). Smaller colonies should be cut into fewer pieces. Care should be taken to exclude mEFs.

Rule of thumb: Each large colony contains approximately 1-1.2 x 10^5 cells. Each colony should be cut into pieces containing approximately 1-1.4x10^4 cells per piece.

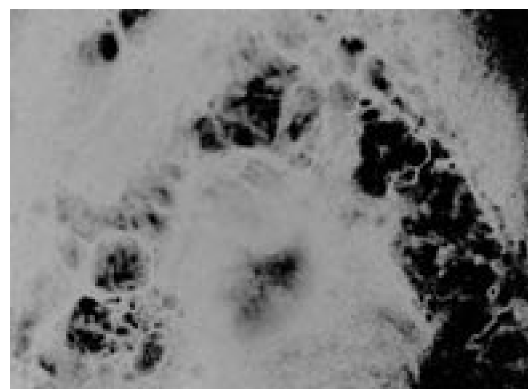
hES colony rating scale

Rating	Description
Excellent	Large colonies with even morphology and well defined edges. The cells should be homogenous and should appear as if they are sitting flat on the feeders (ie. not cystic). Excellent colonies should yield 7-8 transferrable pieces.
Good	Colonies that are smaller or not as thick as excellent colonies but still contain predominantly transferable material. Good colonies should yield 4-6 transferrable pieces
Fair	Colony has grown out from central button but is small or mainly cystic. Poor colonies are generally not used for transfers but may yield 1-3 transferrable pieces
Poor	Colonies that either have not grown at all or are exhibiting a cystic or filamentous, well differentiated morphology. NB. These colonies should not be used for transfer


An example of an excellent colony


An example of a good colony

An example of a fair colony


An example of a poor colony

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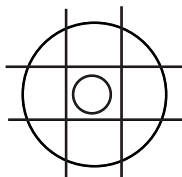
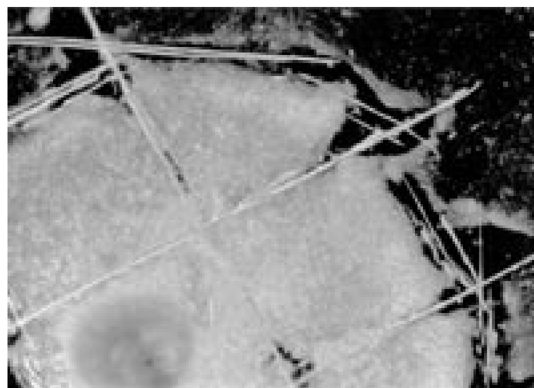
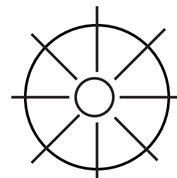
Cutting hES colonies

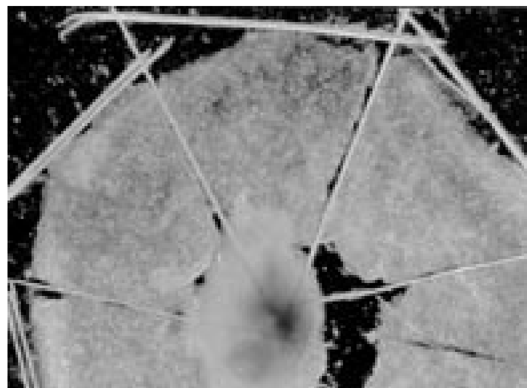
The technique you choose for cutting hES colonies depends entirely upon personal preference. The two methods in use at ESI are grid cutting and wheel-like cutting. Both methods involve cutting a colony into pieces of approximately equal size.





Grid cutting





Wheel-like cutting

hES colony transfer procedure

Prepare: Pre-warmed hES Media
Cutting pipettes and instrument sleeve

1. Place 1ml hES media into each of two organ culture plates. These plate will be used as rinse plates and should be stored in the incubator while not in use. Swap between the two plates every 5 minutes to prevent the media from becoming too alkaline.
2. Using a cutting pipette held in an instrument sleeve, cut the undifferentiated parts of each colony (see “Cutting hES Colonies”).
3. Use a 20μl micropipette tip to nudge colony pieces from the plate. Be very gentle and avoid tearing the colony.
4. Aspirate the colony pieces into a 20μl pipette and rinse in the rinse plate.
5. Transfer 7 to 9 colony pieces to each mEF plate. Place the colony pieces evenly around the plate to prevent colonies from growing together. Move plate very carefully to the incubator to prevent colony pieces moving before they attach (approx 24-48 hours).
6. Replace the media on the hES plates daily with fresh hES media, beginning 48 hours after transfer.

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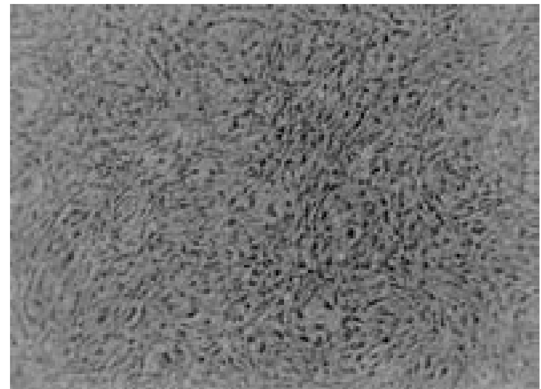
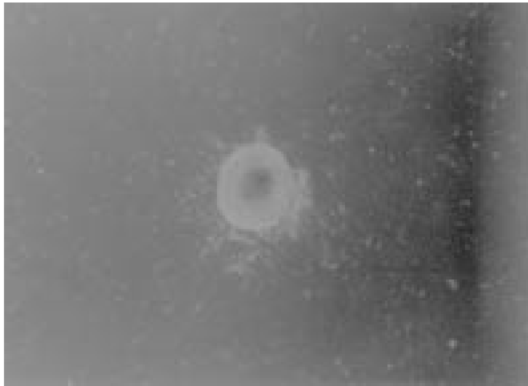
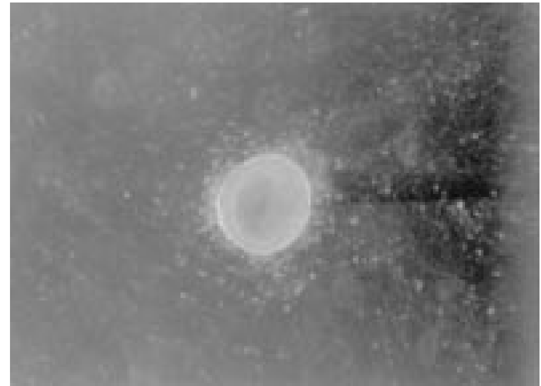
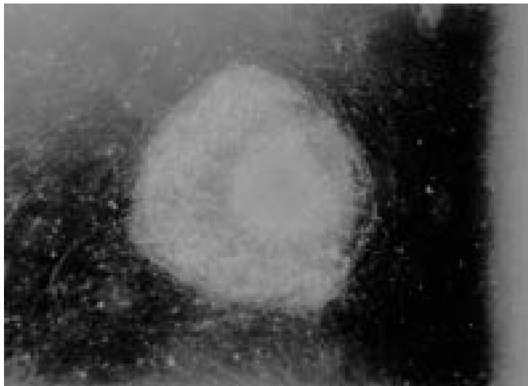
Colony morphology after transfer

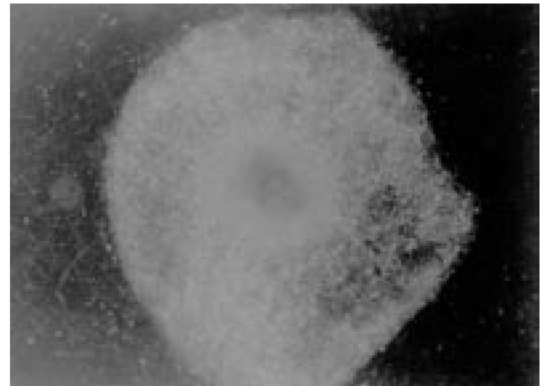
In the early stages of learning to culture hES cells, it is helpful to look at the colonies every day to get an understanding of how they grow. The following photo series shows good colony morphology at each day.

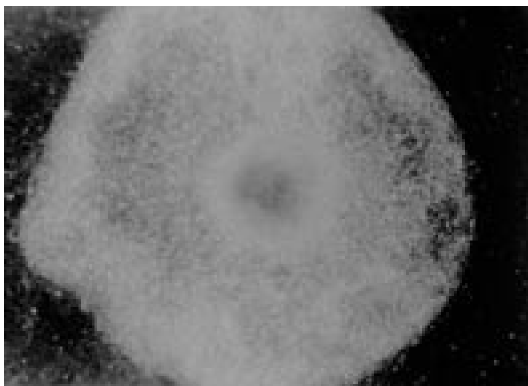

mEF feeder layer

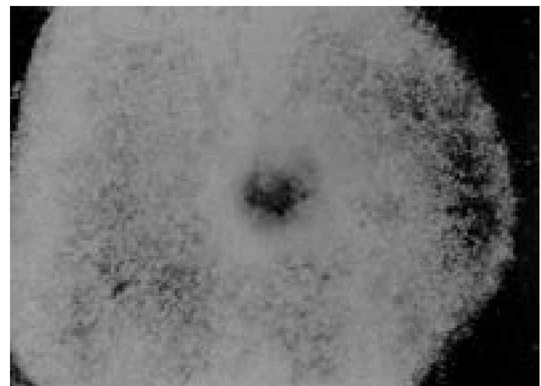

hES colony 1 day post transfer


hES colony day 2


hES colony day 4


hES colony day 5


hES colony day 6


hES colony day 7

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Enzymatic ‘bulk culture’ of hES cells

This method has been adapted from methods published by Thomson et al (JA Thomson, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall, JM Jones, *Science* 282:1145-7). It differs from the manual hES colony transfer methods described on page 18 in that hES colonies are dissociated using the enzyme collagenase IV rather than being physically dissected into pieces.

Researchers may choose to use this method when large quantities of hES cells are required as cultures may be scaled-up to large culture flasks.

Split ratio and frequency
ESI suggests that you observe cultures daily and make your own assessment of when cells should be passaged. When establishing the method in your own lab, we suggest you begin by passaging 1:6 every 7 days. At ESI, we generally passage between 1:4 and 1:8 every 7 days.

Prepare:
Live hES cultures
Freshly prepared culture vessels coated with mitomycin C treated mEFs (see page 14). The size and number of culture vessels depends upon the split ratio selected.
Pre-warmed PBS+
Pre-warmed hES media
Collagenase IV solution in PBS+ (200U/ml)
Cell scraper(s)

1. Aspirate media from hES culture and rinse 3 times in PBS+
2. Add Collagenase IV solution (0.5ml per organ culture dish, 1ml per 25cm^2 flask, 3ml per 75cm^2 flask).
3. Incubate at 37˚C for 8 minutes
4. If passaging cells from organ culture dishes, use a 1ml pipette to gently scrape cell clumps from dish. If passaging from flasks, use a cell scraper gently.
5. Transfer cells to a 12ml tube.
6. Rinse the dish with 2 volumes of hES media.
7. Using a motorized pipette, partially disaggregate the hES pieces. Aim to break the cells up so that most of the large pieces are approximately 1/10th to 1/20th of the size of a mature colony (ie aim to break the cells into 5000-10000 cell clumps).
8. Spin the cells down at 600g for 2 minutes. Remove the supernatant and resuspend the cell pellet in 10ml hES media.
9. Again, spin the cells down at 600g for 2 minutes. Remove the supernatant and resuspend cells in hES media.
10. Seed cell clumps onto mEF coated culture flasks as appropriate.

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Vitrification

Prepare: Pre-warmed vitrification plate
5ml labelled cryovials & cryocanes.
Vitrification straws
Pre-warmed hES media
Liquid nitrogen in Dewar

Transferring colony pieces between wells of the vitrification plate (steps 4,6 and 7)

The colony pieces are drawn up by capillary action (step 10)

1. Punch one hole through the side and one through the lid of each cryovial. This is best done using an 18g needle heated in a Bunsen flame.
2. Place the cryovials attached to cryocanes in the liquid nitrogen to cool.
3. Follow the colony transfer procedure up to step 4. In other words, cut colony pieces and place them in a rinsing plate. NB. ESI recommends cutting slightly larger than normal hES pieces to allow for cell death associated with the freezing process.
4. Using a 20µl pipettor, transfer the colony pieces to the first well (the holding well, containing ES-HEPES) of the vitrification plate.
5. Make a 20 µL drop of 20% Vitrification Solution on the inside of the 4-Well plate lid.
6. Transfer 6 to 9 colony pieces from the first to the second well (containing 10% vitrification solution). **Leave for 1 minute.**
7. After 1 minute, transfer the colony pieces from the second to the third well (containing 20% vitrification solution). **Leave for 25 seconds.** The colony pieces may swirl around – use a pipette tip to keep them away from the sides of the well.
8. After 25 seconds, transfer the colony pieces in the smallest possible volume to the 20 µL drop.
9. Using a second pipettor, pick up the colonies from the 20 µL drop in 3 µL and make a small, high droplet on the lid.
10. Immediately touch the narrow end of the vitrification straw to the side of the droplet at about a 30° angle to the plane of the dish. The droplet should be drawn up by capillary action to make a 1 mm media column in the straw. Occasionally some pieces are not drawn up. It is better to leave these behind than risk damaging all the tissue by taking too long.
11. Holding the straw at a 45° angle, plunge it into liquid nitrogen.
12. Transfer the straw into the cryovial in the liquid nitrogen.

NB: It should take 2-3 minutes to go from step 6 to step 12. Taking longer than 3 minutes may compromise the post-thaw viability of hES cells.

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Deriving new mEF lines

This method is a modified version of a method first described in: Robertson E.J. (1987), Embryo-derived stem cell lines. In Teratocarcinoma and Embryonic Stem Cells: A Practical Approach. ed E.J. Robertson, Oxford, UK: IRL Press.

Recommended mouse strains

ESI uses mEFs derived from day 13.5 *post-coitum* 129sv inbred mouse embryos. mEFs from some other inbred mice may also support hES colony growth.

It is important to note that mEF feeder cells are not immortal, and hence each mEF preparation has a finite capacity to support hES growth.

NB: The following procedures are a guide only & apply to a healthy cell line that grows well. If your line is not as robust you may need to reduce the passage ratios or increase culture time.

Deriving primary (P0) mEF lines

Prepare: Dissection scissors
Forceps
Pre-warmed PBS-
Pre-warmed Trypsin-EDTA (0.25%)
Pre-warmed F-DMEM Media
7-8 embryos (13.5 days from conception) freshly dissected in sterile PBS-

1. Remove placenta and all membranes from each foetus.
2. Remove the head and internal viscera (brightly coloured abdominal and thoracic contents) from each foetus.
3. Wash the foetuses twice in 10 cm petri dishes containing 15ml PBS-. Transfer foetuses to the lid of a 10 cm petri dish.
4. Mince the foetal material finely in 2ml of PBS- using dissection scissors before transferring to a 50ml tube.
5. Add 2ml Trypsin/EDTA (0.25%) per foetus to 50ml tube (use some trypsin solution to wash lid of petri dish) and pipette cell mixture up and down to disaggregate cells.
6. Incubate at 37°C for 5 minutes with agitation.
7. Check the cell suspension to ensure it has dispersed. If not, pipette cell suspension up and down and leave for a further 2 minutes.
8. Neutralise trypsin with 35ml F-DMEM Media. Mix well using a pipette.
9. Allow large pieces of cellular debris to settle out for 2 minutes.
10. Transfer supernatant to T-175 flask and add F-DMEM to make up to 55ml total volume.
11. Incubate flask for 6 hours at 37°C in a 5% CO_2 incubator.
12. Replace media with 55ml F-DMEM and incubate for 24 hours at 37˚C in a 5% CO_2 incubator until confluent (~24 hours).

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Passaging mEF cell cultures

Prepare: Pre-warmed PBS-
Pre-warmed Trypsin-EDTA (0.05%)
Pre-warmed F-DMEM Media

1. Ensure the mEFs are confluent in the flask.
2. Wash flask three times with PBS- (20ml for a T75 flask, 7ml for a T25 flask).
3. Add Trypsin-EDTA (0.05%; 1.2ml for a T75 flask, 0.8ml for a T25 flask) for 1-2 minutes. Tap flask to remove cells.
4. Add 10ml F-DMEM Media to flask and mix.
5. Transfer suspension to a 15ml tube and centrifuge at 500g for 2 minutes.
6. Aspirate supernatant from pellet and resuspend cells in 3ml F-DMEM.
7. Perform a cell number and viability count.
8. Seed in flasks at 2 x 10^4 cells per cm^2 and note passage number
9. Incubate flasks at 37°C in a 5% CO_2 incubator until confluent (~48h).

Cells should be derived and passaged according to these procedures until passage 6. Passage 6 mEFs are then used to generate mitotically inactive mEF plates as described on page 14-15. ESI recommends that you freeze aliquots of mEFs down at each passage and store in liquid nitrogen for future use.

Freezing mEF cells

Prepare: Pre-warmed PBS-
Pre-warmed Trypsin-EDTA (0.05%)
Pre-warmed F-DMEM Media
mEF freezing solution
Labelled cryovials
Haemocytometer
Nalgene Cryo 1˚C freezing container

1. Ensure the mEFs are confluent in the flask.
2. Wash each flask three times with 20ml PBS-.
3. Add Trypsin-EDTA (0.05%) for 1-2 minutes. Tap flask to remove cells.
4. Add 5-10ml F-DMEM media to each flask and mix. Transfer the cell suspension to a tube.
5. Perform a cell number and viability count on a haemocytometer.
6. Centrifuge cells at 500g for 2 minutes.
7. Aspirate supernatant from cell pellet. Resuspend cells at 3 x 10^6 cells per ml in freezing solution.

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8. Dispense 0.5ml of cell suspension into each cryovial.

9. Place the cryovials into a Nalgene Cryo 1˚C freezing container and store at -80°C for 24 hours.

10. Move the cryovials into LN_2 storage.

Thawing mEF cells

Prepare: Pre-warmed F-DMEM Media 37˚C water bath

1. Transport cryovial of Passage 2 mEFs on ice from liquid nitrogen storage.
2. Thaw at 37°C in water-bath, leaving an ice sliver.
3. Immediately transfer the suspension from the cryovial to 10ml F-DMEM Media in a tube.
4. Centrifuge cells at 2000 rpm for 2 minutes.
5. Aspirate supernatant from pelleted cells and resuspend cells in 3ml F-DMEM Media.
6. Perform a cell and viability count.
7. Seed the cells in flasks at 2×10^4 cells per cm^2
8. Incubate flask until confluence (around 48 hours).

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Troubleshooting

Problem	Possible cause	Solution
hES cell colonies do not grow.	Poor quality FBS Poor quality mEFs mEFs too sparse	A high grade FBS with low endotoxin levels is essential. Always batch test new serum. Not all mEF lines support the growth of undifferentiated hES colonies. Compare your mEF line/s with the mEFs supplied by ESI to ensure that they are suitable. Increase the seeding density of mitotically inactivated mEFs by 10%
hES cell colonies grow upward rather than spreading out.	Poor support from mEF layer.	See points above, but also ensure that the mEFs are not over-trypsinised. It is better to leave a few mEF cells attached to the flask than to leave the trypsin on too long. Seeding density of mEFs. Different mEF lines may need to be seeded at higher density to ensure a healthy confluent plate.
hES colonies showing a lot of differentiation.	Poor quality media Too many colonies per plate	Check the media composition and ensure that you are using all of the recommended components. Ensure that additives are aliquoted out to avoid multiple freeze-thaw cycles. Ensure that media is not used after the use-by date. Ensure that the culture media is changed daily (except for the first day post-seed). Ensure that you are only plating 6-9 colonies per plate. More colonies per plate will deplete the nutrients in the media quickly and put nutritional stress on the colonies.
Small hES colonies.	Cutting small colony pieces.	If you continuously cut small colony pieces, the resulting colonies will become smaller and smaller. If the colonies are small, cut much larger than normal (try twice the size to start) to ensure a similar cell number as a larger piece.
MITC mEF plates not confluent.	Under-seeding plates.	Increase the seeding rate for plates. If you have used a different mouse strain, the seeding rate may need to be higher (eg. 1.85 x 10^5 cells/cm^2). Alternatively, some lines that proliferate well may be seeded slightly lower (eg. 1.7 x 10^5/cm^2).
Patchy or thin mEF layer on flasks or on MITC plates.	mEF freezing procedure not optimal or mEFs passaged too long.	If the mEFs have been frozen down in poor FBS or it has taken a long time to freeze them down, then it takes longer for the cells to recover. Ensure that flasks are confluent before passaging the mEF cells and that freezing is optimal. Make up fresh batches of freezing solution with fresh components each time. Passage 6 is the optimal passage to use the mEFs at. mEFs passaged further lose the ability to support hES colony growth.
mEF cells not confluent in flasks at expected time points.	mEF cell require longer periods to grow up to confluence.	Some mEF lines do not grow as quickly or split as well. For these lines you will need to determine how long it requires them to reach confluence and then split at this point. You may need to seed the line at a higher concentration per flask to ensure that the cells reach confluence. Ensure that you split the cells just as they reach confluence. Splitting them too early means low cell yields. Splitting them after they have become very confluent means that they enter the G0 phase of cell growth and will take longer to start growing at the next passage

Please feel free to contact ESI if you have any other problems or questions.

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