TECHNICAL MANUAL

Maintenance of Mouse ES and iPS Cells Using ES-Cult™

STEMCELL™ TECHNOLOGIES

Table of Contents

1.0 Introduction

Mouse embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of day 3.5 blastocysts. Under the appropriate culture conditions, mouse ES cells can be maintained in vitro for extended periods without loss of their capacity to contribute to all cell lineages when reimplanted back into a blastocyst.[1-3] The pluripotency of mouse ES cells, combined with their ease of genetic manipulation and selection, has revolutionized gene function studies in vivo via the generation of transgenic, chimeric, and knockout mice.[4-9]

Mouse ES cells are maintained in an undifferentiated state in vitro by leukemia inhibitory factor (LIF). Upon withdrawal of LIF, cells differentiate into complex structures called embryoid bodies (EBs) that contain cells from each of the three germ layers: endoderm, ectoderm, and mesoderm. These cells can subsequently be induced to differentiate into a variety of cell types[10,11] including pancreatic,[12] neural,[16-24] hematopoietic,[3,28-30] adipocytes,[28] osteoblasts,[31] multipotent endothelial[13-15] and cardiac progenitors.[25-27,32-34]

Mouse ES cell in vitro differentiation provides a unique and powerful system to examine cellular and molecular processes, perform drug screening, and investigate potential tissue engineering and cellular therapy applications.

The discovery that somatic cells can be reprogrammed to become induced pluripotent stem (iPS) cells that closely resemble mouse ES cells using four defined factors[35-38] has increased interest in these cell types considerably.

2.0 Materials and Reagents

2.1 Media and Support Products for the Maintenance of Mouse ES and iPS Cells

PRODUCT	QUANTITY	CATALOG #
ES-Cult™ Mouse ES and iPS Cell Maintenance Kit*	1 Kit	03150
Trypsin-EDTA (0.25%)	500 mL	07901
L-Glutamine	100 mL	07100
DMEM with 4500 mg/L D-glucose	500 mL	36250
LIF, Mouse, Recombinant**	10 µg	02740
Sodium Pyruvate	100 mL	07000
MEM Non-Essential Amino Acid Solution (100X)	100 mL	07600
D-PBS (Without Ca++ and Mg++)	500 mL	37350
0.1% Gelatin in Water	500 mL	07903
Trypan Blue	100 mL	07050
100 mm Treated Tissue Culture Dishes	10/pk 240/case	27125 27127
BMP-4, Human, Recombinant	10 µg	02524
CD-1 Mouse Embryonic Fibroblasts, Day E12.5	1 x 10^6 cells/vial	00321
CD-1 Mouse Embryonic Fibroblasts, Day E14.5	1 x 10^6 cells/vial	00322
Neomycin-Resistant Mouse Embryonic Fibroblasts, Day E13.5	> 3 x 10^6 cells/vial	00323
Hygromycin-Resistant Mouse Embryonic Fibroblasts, Day E13.5	> 3 x 10^6 cells/vial	00324
Puromycin-Resistant Mouse Embryonic Fibroblasts, Day E13.5	> 3 x 10^6 cells/vial	00325
G418	250 mg	03812
Hygromycin B	100 mg	03813
Puromycin	50 mg	73342
60 mm Treated Tissue Culture Dishes	10/pk 400/case	27120 27121
ß-Mercaptoethanol	100 mL	e.g. Sigma M7522
Mitomycin C	1 mg	73272
Monothioglycerol (MTG)	25 mL	e.g. Sigma M6145
Conical tubes (15 mL)	500/case	e.g. Corning 352196
Conical tubes (50 mL)	500/case	e.g. Corning 352070
Serological pipettes (2 mL, 5 mL, 10 mL)	1000/case	e.g. Corning 357507, 357543, 357551

*For a complete list of ES-Cult™ Mouse ES and iPS Cell Maintenance Kit components, refer to section 8.0. The kit does not include fetal bovine serum (FBS).
**mLIF is manufactured by Millipore. mLIF is protected under US Patent nos. 5,187,077, 5,427,925, 5,443,825 and 5,750,654, 6,261,548; European Patent no. 0285 448; and related foreign patents and is not available for resale.

2.2 Antibodies to Characterize Undifferentiated Mouse ES and iPS Cells

PRIMARY ANTIBODIES*					
TARGET ANTIGEN	CLONE	ISOTYPE		QUANTITY	CATALOG #
OCT4 (OCT3)	40	Mouse IgG1		50 µg	60059
SSEA-1	MC-480	Mouse IgM		100 µg	60060
SECONDARY ANTIBODIES*					
TARGETED ANTIGEN	HOST SPECIES	FORMAT	FOR USE WITH	QUANTITY	CATALOG #
Mouse IgG	Goat	FITC	Anti-OCT4 (OCT3)	1.5 mg	60138
Mouse IgM	Goat	FITC	Anti-SSEA-1	1.5 mg	60139

*Optimal working dilutions of primary and secondary antibodies should be determined by the end user.

2.3 Products for Differentiation of Mouse ES and iPS Cells

STEMCELL Technologies provides a full range of products and protocols to support the differentiation of mouse ES and iPS cells. Protocols for differentiation are available on our website at www.stemcell.com.

FORMATION OF EMBRYOID BODIES (EBS)	QUANTITY	CATALOG #
AggreWell™400 (8 wells, each with approximately 1,200 microwells per well)	1/pack 5/pack	27845 27945
PRODUCTS FOR DIFFERENTIATION TO HEMATOPOIETIC CELLS	QUANTITY	CATALOG #
ES-Cult™ M3120	40 mL	03120
Iscove's Modified Dulbecco's Medium (IMDM)	500 mL	36150
BIT 9500 Serum Substitute	100 mL	09500
Mouse Recombinant IL-3	100 µg	78042
Human Recombinant IL-6	100 µg	78050
BMP-4, Human, Recombinant	10 µg	02524
Mouse Recombinant SCF	100 µg	78064
Mouse Recombinant GM-CSF	100 µg	78017
EPO, Human, Recombinant	500 U	02625
Blunt-End Needles, 16 Gauge (required for dispensing methylcellulose)	100/pack 2000/pack	28110 28120
3 cc Syringes (required for dispensing methylcellulose)	2000/pack 100/bag	28230 28240
35 mm Culture Dishes	10/pack 500/case	27100 27150

PRODUCTS FOR DIFFERENTIATION TO ENDOTHELIAL CELLS	QUANTITY	CATALOG #
ES-Cult™ M3120	40 mL	03120
Iscove's Modified Dulbecco's Medium (IMDM)	500 mL	36150
ES-Cult™ Endothelial Collagen and Medium Kit	1 kit	05810
Human Recombinant VEGF-165	50 µg	78073
Human Recombinant bFGF	50 µg	78003
Human Recombinant IL-6	100 µg	78050
EPO, Human, Recombinant	500 U	02625
MegaCult-C™ Double Chamber Slides	16/box	04813
Blunt-End Needles, 16 Gauge (required for dispensing methylcellulose)	100/pack 2000/pack	28110 28120
3 cc Syringes (required for dispensing methylcellulose)	2000/pack 100/bag	28230 28240
35 mm Culture Dishes	10/pack 500/case	27100 27150
PRODUCTS FOR DIFFERENTIATION TO NEURAL CELLS	QUANTITY	CATALOG #
ES-Cult™ Basal Medium A	500 mL	05801
ITS Supplement-A	5 mL	07151
ITS Supplement-B	5 mL	07155
N2 Supplement-A	5 mL	07152
N2 Supplement-B	5 mL	07156
NeuroCult™ SM1 Neuronal Supplement	10 mL	05711
Ascorbic Acid	100 mg	07157
Fibronectin	1 mL	07159
Human Recombinant bFGF	50 µg	78003
FGF-8b, Mouse, Recombinant	25 µg	02755
Mouse Recombinant Shh	50 µg	78066
Human Recombinant EGF	500 µg	78006
LIF, Mouse, Recombinant	10 µg	02740
NeuroCult™ Proliferation Supplement (Mouse)	50 mL	05701
NeuroCult™ Basal Medium (Mouse)	450 mL	05700
6-Well Ultra-Low Adherent Plates for Suspension Cultures	6/pack	27145
100 mm Petri Dishes	20/sleeve	27110

2.4 Equipment Required for the Culture of Mouse ES and iPS Cells

- Vertical laminar flow hood certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and > 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with swinging bucket rotor (e.g. Beckman GS-6)

  All protocols described in this Technical Manual can be performed with the brake on.
- Pipette-aid (e.g. Drummond Scientific)
- Hemocytometer
- Inverted microscope with 2X, 4X, and 10X phase objectives
- Isopropanol freezing container (e.g. Nalgene, Fisher; Catalog #1535050)
- -135°C freezer or liquid nitrogen vapor tank

3.0 Preparation of Reagents

The instructions contained in this manual assume mouse ES cell-screened ES-Cult™ products from STEMCELL Technologies are being used. If other reagents are substituted, each should be appropriately tested to ensure it supports the maintenance of mouse ES or iPS cells. All procedures should be carried out using sterile techniques in a certified biological safety cabinet.

Mouse ES or iPS cells can either be maintained on gelatin or on a layer of mitotically inactivated mouse embryonic fibroblasts (MEF). Alternatively, mouse ES or iPS cells can be maintained on a layer of drug-resistant MEF for experiments where drug selection of undifferentiated cells is required. If culturing mouse ES or iPS cell lines on gelatin-coated cultureware, refer to section 3.1. If culturing mouse ES or iPS cell lines requiring MEF feeder layers, refer to section 3.2.

3.1 Gelatin Coating of Tissue Culture-Treated Vessels

In order for certain mouse ES cell lines (e.g. the CCE cell line) or mouse iPS cell lines, as well as feeder layers, to efficiently adhere to the surface of tissue culture-treated vessels, it is necessary to first coat the bottom of the tissue culture-treated vessel with gelatin. Gelatinized tissue culture plates or flasks, therefore, should be used for the first passage of MEF after thawing.

1. Dispense sufficient gelatin into a tissue culture-treated plate or flask so that it completely covers the bottom.

   Note: Suggested volumes are 3 mL per T-25 cm^2 flask or 60 mm tissue culture-treated dish; 7 - 8 mL per 100 mm tissue culture-treated dish or T-75 cm^2 flask. To coat other sizes of tissue cultureware, scale the volume of gelatin by the surface area of the vessel to be coated.

2. Incubate for at least 20 minutes at room temperature (15 - 25°C).

3. Aspirate all of the gelatin solution and allow the remainder to evaporate by leaving the container in the hood with the lid or cap open until no traces of the liquid remain. Replace the lid once the surface is dry.

   Note: If not used immediately, the gelatinized dishes or flasks can be stored at room temperature (15 - 25°C) for one day or at 2 - 8°C for at least 2 weeks.

3.2 Plating and Passaging Mouse Embryonic Fibroblasts (MEF)

MEF (Catalog #00321/00322) and drug-resistant MEF (Catalog #00323/00324/00325) are available from STEMCELL Technologies; these can be expanded and used up to passage 5. Drug-resistant MEF can be used for experiments where drug selection of undifferentiated cells is required.

Note: High serum concentrations and gelatin increase the plating efficiency of MEF from the frozen state. Gelatinized tissue culture dishes or flasks should be used for the first passage of MEF after thawing.

1. Prepare a 50 mL tube with 20 mL of medium consisting of DMEM with 4500 mg/L D-glucose containing 30% FBS and 10^{-4} M ß-mercaptoethanol.

2. Quickly thaw one vial of frozen MEF by gently shaking in a 37°C water bath. Remove the vial from the water bath and wipe with 70% ethanol to sterilize the outside of the vial.

3. Add cells *dropwise* to the 50 mL tube of medium containing 30% FBS (prepared in step 1) with gentle agitation. Using a sterile 5 mL or 10 mL pipette, mix by gently pipetting up and down 2 - 3 times.

4. Transfer the appropriate amount of medium containing the cells to a gelatinized, tissue culture-treated plate or flask.
5. Incubate the plate(s) or flask(s) at 37°C and 5% CO_2 with 95% humidity overnight.
6. The next day, prepare 20 mL of medium consisting of DMEM with 4500 mg/L D-glucose containing 10% FBS and 10^{-4} M ß-Mercaptoethanol. Replace the culture medium with this fresh medium and allow the cells to grow to confluence, usually 2 - 4 days.

   Note: Once MEF have become confluent, they can be passaged up to 5 times.
7. To passage cells, aspirate medium and rinse cultures once with D-PBS (Without Ca++ and Mg++).
8. Add sufficient Trypsin-EDTA (0.25%) at room temperature (15 - 25°C) to cover the cells.

   Note: Use approximately 2 mL for a 60 mm dish or T-25 cm^2 flask; 4 mL for a 100 mm dish or T-75 cm^2 flask.
9. Incubate for 3 - 5 minutes at 37°C or just until the cells begin to lift off the plate.
10. After the incubation period, use a serological pipette to transfer the cells to a 15 mL tube containing approximately 2 - 4 mL of a solution of DMEM with 4500 mg/L D-glucose containing 10% FBS. Then immediately centrifuge the cells at 300 x *g* for approximately 8 minutes to pellet the cell suspension.

    Note: The serum is present in this wash step to inactivate residual trypsin activity.
11. Aspirate the medium and resuspend the cell pellet in approximately 2 mL of DMEM with 4500 mg/L D-glucose containing 10% FBS. Pipette up and down against the bottom of the tube 4 - 6 times to ensure that the cell pellet is disrupted to a single-cell suspension.
12. Re-plate MEF in an appropriate amount of DMEM with 4500 mg/L D-glucose containing 10% FBS. It is recommended to re-plate at a ratio of 1:5. However, this ratio may vary depending on the preparation and passage number of the MEF.

3.3 Inactivation of MEF

MEF used for feeder layers must be mitotically inactivated before use. This is often conveniently done while the cells are still attached to the tissue culture plate. Alternatively, if cells are to be maintained and/or expanded further, harvest the fibroblasts with Trypsin-EDTA (0.25%) and re-plate a portion at a 1:5 dilution. The remainder of the cells can then be irradiated in a tube.

1. Mitotically inactivate the MEF using either irradiation or treatment with mitomycin C.

   a) Inactivation by irradiation:

   Expose the MEF either on plate(s) or in tube(s) to 6,000 - 10,000 rads (60 - 100 Gy) from a gamma or x-ray source. Large batches of MEF may be inactivated and frozen at recommended densities (see step 2 below) so that they may be thawed and used directly at a later date.

   b) Inactivation by mitomycin C treatment:

   Mitomycin C is light sensitive and cytotoxic. Please refer to the SDS for hazard information. Stock solutions should be divided into 10 mL aliquots and stored at -20°C in the dark.

i. Once the MEF are confluent, replace the growth medium with a solution of DMEM with 4500 mg/L D-glucose containing 5% FBS and 10 µg/mL mitomycin C and return the MEF to the 37°C incubator with 5% CO_2 and 95% humidity for 3 hours.

Note: Use 7 - 10 mL of solution per 100 mm plate or 20 - 25 mL of solution per T-75 cm^2 tissue culture-treated flask.

ii. Remove the solution and wash the MEF at least 2 times with DMEM with 4500 mg/L D-glucose if the plate is to be used directly, or with D-PBS (Without Ca++ and Mg++) if the cells are to be trypsinized and re-plated.

2. If dividing a large plate of inactivated MEF between several smaller plates, or if MEF were inactivated in tubes, re-plate inactivated MEF in ES-Cult™ Maintenance Medium (section 3.4) such that a uniform feeder monolayer of cells is formed.

   Recommended cell densities for plating inactivated MEF feeder layers are as follows:

   - 100 mm dish: 1.7 - 2 x 10^6 cells
   - 60 mm dish: 1 x 10^6 cells
   - 24-well plate: 1 x 10^5 cells per well
   - 96-well plate: 3 - 5 x 10^4 cells per well

   Note: Once MEF have been inactivated, gelatinized dishes are no longer required – instead, use high-quality tissue culture-treated plates. Inactivated feeder layers should be used for mouse ES or iPS cell culture within 10 days.

3.4 Preparation of ES-Cult™ Maintenance Medium

Unless large numbers of mouse ES or iPS cultures are to be maintained, it is highly recommended that medium be prepared in 50 mL aliquots, which can then be stored at 2 - 8°C for up to 1 week. If desired, add 100 U/mL penicillin and 10 µg/mL streptomycin.

Note: Aliquots stored at 2 - 8°C for longer than 1 week should not be used due to the instability of the MTG and mLIF.

ES-CULT™ MAINTENANCE MEDIUM			
COMPONENT	CATALOG #	VOLUME ADDED FOR 50 ML	FINAL CONCENTRATION
Fetal Bovine Serum (FBS; ES cell qualified)	---	7.5 mL	15 %
*Sodium Pyruvate	07000	0.5 mL	1 mM
L-Glutamine	07100	0.5 mL	2 mM
MEM Non-Essential Amino Acid Solution (100X)	07600	0.5 mL	0.1 mM
LIF, Mouse, Recombinant (10 µg/mL)	02740	50 µL	10 ng/mL
†MTG (1:100 dilution in DMEM)	Sigma M6145	43 µL	100 µM
*DMEM with 4500 mg/L D-glucose	36250	to final volume of 50 mL	-

*It is not necessary to add sodium pyruvate if DMEM with 4500 mg/L D-glucose (Catalog #36250) is used, as it already contains this supplement. If other sources of DMEM are used, check the formulation to determine if addition of sodium pyruvate is required.
†MTG working solution is prepared by diluting MTG (Sigma #M6145) 1 in100 in DMEM with 4500 mg/L D-glucose.

Only the highest quality reagents should be used in preparing the ES-Cult™ Maintenance Medium. With the exception of monothioglycerol (MTG) and fetal bovine serum (FBS), all components essential for the preparation of ES-Cult™ Maintenance Medium are available as pre-screened ES-Cult™ products from STEMCELL Technologies. If non-ES-Cult™ products are substituted, it is essential that they are pre-tested to ensure their ability to maintain mouse ES or iPS cells in the undifferentiated state. Ensure that the FBS used is ES cell qualified.

4.0 Maintenance Culture for Mouse ES and iPS Cells

4.1 Thawing and Plating of mouse ES and iPS Cells

1. Ensure that sufficient numbers of gelatinized plates or dishes of MEF are available (refer to sections 3.1 or 3.2 and 3.3).
2. Warm the ES-Cult™ Maintenance Medium prepared in section 3.4 to room temperature (15 - 25°C) or 37°C.
3. To a 15 mL tube, add 9 mL DMEM with 4500 mg/L D-glucose containing 10% FBS.
4. Remove the cryovial containing frozen mouse ES or iPS cells from the freezer and quickly thaw by gently shaking the vial in a 37°C water bath. Remove the vial from the water bath and wipe the vial with 70% ethanol.
5. Add the cells slowly and *dropwise* into the tube prepared in step 3 and centrifuge at 300 x *g* for 8 minutes.
6. Aspirate the supernatant and resuspend the cells in approximately 1 mL of ES-Cult™ Maintenance Medium.
7. Pipette gently up and down to ensure that the cell pellet is disrupted to a single-cell suspension.

   Note: Transferring clumps of cells to the culture dish will promote differentiation. Ensure a single cell suspension is obtained before transferring the cells.
8. Dilute a sample of resuspended cells 1:1 in Trypan Blue, mix gently, and allow the resulting solution to sit for 5 - 15 minutes. Count viable, unstained cells using a hemocytometer.

   Note: For more information on cell counting, please refer to the Product Information Sheet for Trypan Blue (Document #29603), available at www.stemcell.com or contact us to request a copy.
9. Resuspend the cells to an appropriate volume and place in culture vessel: 5 mL for a 60 mm dish or T-25 cm^2 flask; 10 mL for a 100 mm dish or T-75 cm^2 flask. Incubate at 37°C with 5% CO_2 and 95% humidity.
10. The next day, aspirate the medium and replace with fresh ES-Cult™ Maintenance Medium.

    Note: Tiny clusters of adherent cells should be visible on the culture dish - these are the beginnings of healthy mouse ES or iPS cell colonies.

4.2 Passage and Maintenance of Mouse ES and iPS Cells

The passage of mouse ES cells is done before the cells are confluent and prior to the growth medium becoming acidic or yellow in color, indicating nutrients have been depleted. The density of the mouse ES cell colonies should not exceed 50 to 70% of the surface of the culture vessel. In part, the frequency of passage is dependent upon the growth rate of the cells, but most cell lines require passage every second day. It is critical that the cells are passaged regularly since overgrowth of the cultures promotes the differentiation of mouse ES and iPS cells.

1. Aspirate the medium from the culture vessel.
2. Rinse cultures once with D-PBS (Without Ca++ and Mg++).
3. Add sufficient room temperature (15 - 25°C) Trypsin-EDTA (0.25%) to cover the cells. This would be approximately 2 mL for a 60 mm dish or T-25 cm^2 flask, or 4 mL for a 100 mm dish or T-75 cm^2 flask.
4. For most mouse ES or iPS cell lines on gelatinized dishes, incubate at 37°C for 3 - 5 minutes or just until the cells begin to lift off the plate. For mouse ES or iPS cells maintained on MEF feeder layers, incubate at room temperature (15 - 25°C) for 2 - 3 minutes or just until the cells begin to lift off the plate.
5. After the appropriate incubation period, use a serological pipette and pipette up and down to dislodge any remaining cells on the plate. Then transfer the cells to a 15 mL tube containing approximately 2 - 4 mL of a solution of DMEM with 4500 mg/L D-glucose containing 10% FBS. Immediately centrifuge the cells at 300 x *g* for approximately 8 minutes to pellet the cell suspension.

   Note: The serum is present in this wash step to inactivate residual trypsin activity.
6. Aspirate the media and resuspend the cell pellet in approximately 2 mL of ES-Cult™ Maintenance Medium. Pipette up and down against the bottom of the tube 4 - 6 times to ensure that the cell pellet is disrupted to a single-cell suspension.

   Note: It is absolutely essential to ensure a single-cell suspension is achieved, as transfer of cell clumps will promote differentiation.
7. In order to perform a 1:10 cell split, transfer 0.2 mL of cells onto a freshly prepared gelatinized plate or flask, or onto a freshly prepared plate or flask of MEF feeders, containing the appropriate volume of ES-Cult™ Maintenance Medium (section 3.4).

   Note: Mouse ES or iPS cells do not grow well if they are plated at too low a density. On the other hand, if the density is too high, differentiation is promoted. It is best to aim for a situation where adherent cells cover approximately 40 - 50% of the surface area of the culture vessel at 24 hours after plating.

   Absolute numbers of cells transferred to the various sizes of culture dishes are dependent upon the mouse ES or iPS cell line used; approximately 5 x 10^5 cells per T-75 cm^2 flask or 100 mm dish and 1 - 3 x 10^5 cells per T-25 cm^2 flask or 60 mm dish are good starting points. When working with a new cell line, it is recommended to set up multiple concentrations.
8. Continue to passage as required to maintain the cells in an undifferentiated state, usually every other day.

   Note: Keep careful record of the number of times your mouse ES or iPS cells have been passaged. Prolonged culture, even under optimal conditions, usually results in a reduction in differentiation ability in vitro.

4.3 Cryopreservation of Mouse ES and iPS Cells

It is advisable to freeze a large number of mouse ES or iPS cells that have been passaged as few times as possible in order to ensure an adequate stock of low-passage number cells for your experiments.

1. Label an appropriate number of cryovials, making sure to include the freezing date and the name and passage number of the mouse ES or iPS cell line. Pre-chill the cryovials to -70°C in a styrofoam rack.

   Note: Chilling the vials in this way will allow them to remain cold throughout subsequent manipulations at room temperature (15 - 25°C). Alternatively, the cryovials may be pre-chilled and kept on ice.

2. Prepare an adequate volume of ice-cold ES-Cult™ Cryopreservation Solution as follows:

   Pre-chill the following two components to 2 - 8°C, combine, and place on ice:

   - FBS (50%)
   - ES-Cult™ Maintenance Medium (40%) (section 3.4)

   Slowly add dropwise:

   - DMSO (10%)

   Note: Do not pre-chill the DMSO as this will cause it to crystallize.

3. To harvest mouse ES or iPS cells, aspirate the medium from the culture vessel.
4. Rinse cultures once with approximately 4 - 8 mL of D-PBS (Without Ca++ and Mg++).
5. Add sufficient Trypsin-EDTA (0.25%) at room temperature (15 - 25°C) to cover the cells. This would be approximately 2 mL for a 60 mm dish or T-25 cm^2 flask; 4 mL for a 100 mm dish or T-75 cm^2 flask.
6. For most mouse ES or iPS cell lines on gelatinized dishes, incubate for 3 - 5 minutes at 37°C or just until the cells begin to lift off the plate. For mouse ES or iPS cells maintained on MEF feeder layers, incubate for 2 - 3 minutes at room temperature (15 - 25°C) or just until the cells begin to lift off the plate.
7. After the appropriate incubation period, use a serological pipette to transfer the cells to a 15 mL tube containing approximately 2 - 4 mL of DMEM with 4500 mg/L D-glucose containing 10% FBS. Immediately centrifuge the cells at 300 x *g* for approximately 8 minutes to pellet the cell suspension.

   Note: The serum is present in this wash step to inactivate residual trypsin activity.

8. Aspirate the medium and briefly place the cell pellet on ice for 1 - 2 minutes while removing the prepared cryovials (step 1) from the freezer.
9. Retrieve the cryovials prepared in step 1 from the freezer and remove the lids once in the tissue culture hood. Keep the cryovials in the styrofoam rack (or on ice if not pre-chilled to -70°C) to minimize warming.
10. Resuspend the cell pellet in ice-cold ES-Cult™ Cryopreservation Solution (see step 2) at a concentration of between 5 x 10^5 - 1 x 10^6 cells/mL if the cells were maintained on gelatin. Mouse ES or iPS cells maintained on MEF may be frozen at a lower concentration, as feeder cells offer some protection during cryopreservation.
11. Transfer 1 mL of the resuspended cell solution to each pre-chilled, labeled cryovial within the styrofoam rack and replace the lid.

    Note: It is important to work quickly to minimize the time the cells are in contact with the ES-Cult™ Cryopreservation Solution before being placed in a -70°C freezer.

12. Place the styrofoam rack containing the vials of cells into a large styrofoam box and place in a -70°C freezer.

    Note: The combination of the styrofoam rack within the styrofoam box ensures that freezing occurs slowly and reduces cell mortality. Alternatively, place the cryovials into an isopropanol freezing container (e.g. Nalgene, Fisher Catalog #1535050).

13. After 24 hours, the cryovials can be transferred to liquid nitrogen or a -135°C freezer for long-term storage.

    Note: Be sure to carefully record the passage number of the frozen mouse ES or iPS cell stocks.

5.0 Helpful Hints

The following are a list of common problems associated with the maintenance of mouse ES or iPS cells. Possible causes for these difficulties are given to help ensure success with culturing mouse ES and iPS cell lines.

1. Cell death
   - Lack of MTG in the maintenance media, or MTG not freshly added to the maintenance media
   - Passaging the cells at too low a density
   - Toxicity of one of the reagents

     Note: All ES-Cult™ products have been pre-screened and found to exhibit no observable toxicity to a commonly used mouse ES or iPS cell line.

2. Mouse ES or iPS cell colonies lift off the culture plate
   - Petri dishes used rather than treated tissue cultureware
   - Gelatin solution prepared incorrectly

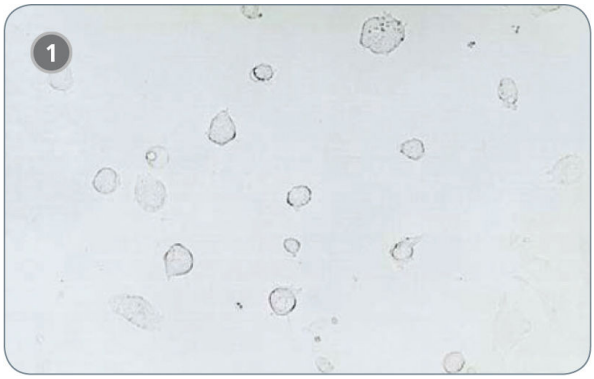
     Note: We recommend using 0.1% Gelatin in Water (Catalog #07903).
   - Excessive differentiation
   - FBS used was not pre-screened

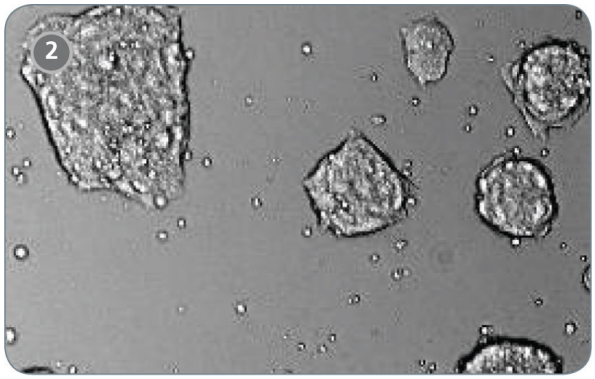
3. Excessive differentiation
   - Failure to obtain a single-cell suspension when passaging mouse ES or iPS cells
   - Insufficient or inactive mouse LIF
   - Plating too many cells in the culture vessel

   Excessive differentiation is characterized by any of the following:
   - The presence of large numbers of flattened colonies in which the individual cells are visible
   - Mouse ES or iPS cell colonies lifting off the culture dish
   - The presence of many round floating cells
   - The presence of round mouse ES or iPS cell colonies with a clearly defined external membrane surrounding the colony

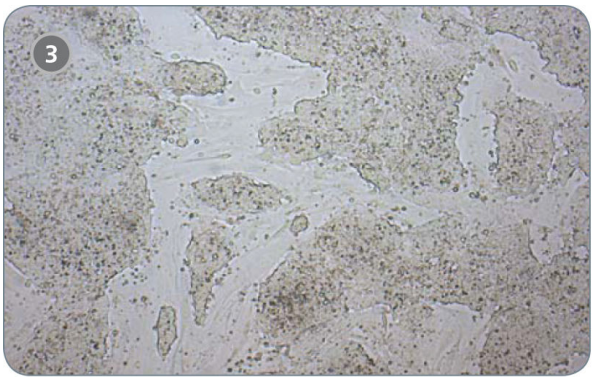
6.0 APPENDIX 1: The Morphology of Undifferentiated Mouse ES Cells

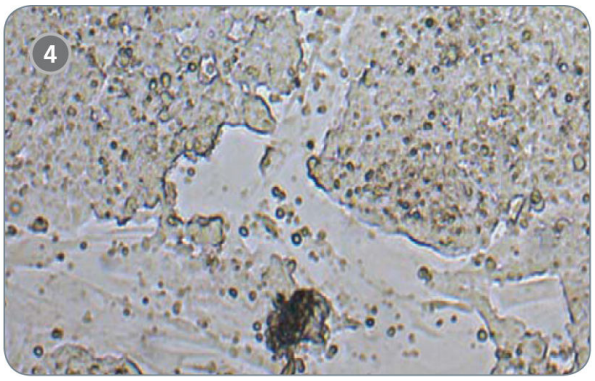
Undifferentiated mouse ES cells have a large nucleus, minimal cytoplasm, and one or more prominent dark nucleoli. It should be difficult to identify individual cells within the mouse ES cell colony, as there are non-distinct cytoplasmic membranes between the cells. Colonies appear amorphous without a distinct or common shape. Signs of differentiation include the ability to distinguish individual cells within the mouse ES cell colony by the defined cytoplasmic membrane for the cells. The colony may appear to spread and cells appear flattened. Cells may lift off the dish.



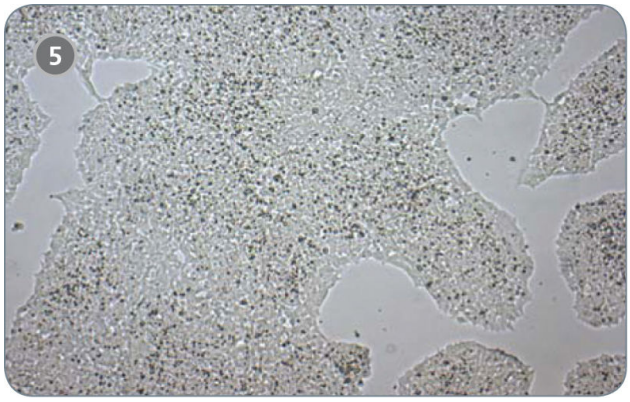


Undifferentiated mouse ES cell colonies. Colonies are dense with distinct, tight borders, and individual cells are not visible. Colonies are not touching one another.
1) Low magnification; 2) High magnification.





Differentiated mouse ES cell colonies on MEF. Colonies are merging, with a loss of border integrity.
3) Low magnification; 4) High magnification.



Differentiated mouse ES cell colonies on gelatin. Colonies are merging, with a loss of border integrity. 5) Low magnification.

7.0 APPENDIX 2: Mouse ES and iPS Cell Pre-Differentiation Culture

For in vitro differentiation to be successful, it is critical to use mouse ES or iPS cells of a low passage number that have been carefully maintained. Generally, it is ideal to use cells which have been in culture for 10 days or less, although this is not always possible. Cells cultured for longer periods may be used, but the efficiency of in vitro differentiation will be reduced.

The presence of MEF can also decrease the efficiency of mouse ES or iPS cells differentiation in vitro. There are several methods for the removal of MEF feeder cells from mouse ES and iPS cell culture prior to differentiation. The three most common methods are described in sections 7.1- 7.3.

For detailed information on differentiation protocols to hematopoietic, neural or endothelial cells, please refer to the Technical Manual: In vitro Hematopoietic Differentiation of Mouse ES and iPS Cells (Document #28415) or the Product Information Sheet for ES-Cult™ Endothelial Collagen and Medium Kit (Document #29584) available on our website at www.stemcell.com or contact us to request a copy.

7.1 Removal of MEF Using Gelatin-Coated Plates

For differentiation to neural, pancreatic-islet like cells and endothelial cells, the most common method to remove MEF is to plate the cells on a gelatin-coated plate at the last passage prior to beginning differentiation. Please refer to sections 3.1 and 4.2 for guidance on Gelatin Coating of Tissue Culture Vessels and Passage and Maintenance of Mouse ES and iPS Cells, respectively. Cells can then be grown in ES-Cult™ Maintenance Medium (section 3.4) containing mLIF for 24 - 48 hours. In the presence of mLIF, the few remaining mitotically inactivated MEF that are transferred when the mouse ES or iPS cells are passaged will not survive.

7.2 Pre-Plating to Remove MEF Prior to Pre-Differentiation

Pre-plating is another option to remove MEF prior to pre-differentiation of mouse ES or iPS cells. Replating the cells on a tissue culture surface directly allows the MEF to stick down, but the mouse ES or iPS cells will float or loosely adhere to the dish.

1. Passage mouse ES or iPS cells as described in section 4.2, steps 1 - 5.
2. Aspirate the medium and resuspend the cell pellet in an appropriate amount of pre-differentiation medium or ES-Cult™ Maintenance Medium without mLIF (section 3.4). Pipette gently up and down to ensure that the cell pellet is disrupted to a single-cell suspension.
3. Plate cells onto a tissue culture dish or flask and incubate at 37°C and 5% CO_2 with 95% humidity for 1 hour.
4. Harvest the non-adherent or loosely adherent mouse ES or iPS cells by drawing up the supernatant.
5. Count viable cells using Trypan Blue. Dilute a sample of resuspended cells 1:1 in Trypan Blue, mix gently, and allow the resulting solution to sit for 5 - 15 minutes. Count viable, unstained cells using a hemocytometer. Most of the MEF will be removed.

   Note: For more information on cell counting, please refer to the Product Information Sheet for Trypan Blue (Document #29603) available at www.stemcell.com or contact us to request a copy.

7.3 Preparation of MEF Extracellular Matrix (ECM) for Specific Applications

The presence of MEF decreases the ability of mouse ES or iPS cells to differentiate in vitro. Therefore, one passage on gelatinized dishes prior to differentiation is preferable. This is sometimes problematic, however, due to the reduced plating efficiency of MEF-maintained mouse ES or iPS cells on gelatin only. One way to reduce this problem is to passage the mouse ES or iPS cells on the ECM prepared from MEF. This procedure can be used with very sensitive mouse ES or iPS cells that do not respond well to being cultured on gelatin alone and require a substrate on which to grow. Instructions for preparing a MEF ECM are outlined below.

1. Plate MEF onto gelatin-coated culture dishes as described in section 3.2 at recommended cell densities.

   Note: Use of gelatin-coated dishes is critical for proper adherence of the MEF ECM.

2. Once MEF are confluent, remove the medium by aspiration and rinse the adherent cells with D-PBS (Without Ca++ and Mg++) at room temperature (15 - 25°C).

   Note: MEF should just have reached confluence, but should be not overgrown. This is very critical since overgrowth prevents adherence of the extracellular matrix.

3. Cover the plate with a solution of 0.5% Triton™ X-100 (e.g. Sigma #T9284) in D-PBS (Without Ca++ and Mg++).

   Note: Suggested volumes are approximately 4 mL per 100 mm dish, or 1.5 mL per 60 mm dish.

4. Incubate at room temperature (15 - 25°C) for up to 5 minutes.

   Note: It should be possible to see lysis of the cells and the presence of a matrix remaining on the gelatinized dish.

5. Carefully aspirate the Triton™ X-100 solution and rinse the plate 3 times with D-PBS (Without Ca++ and Mg++).

   Note: Considerable caution should be taken during these rinse steps so as to completely remove all the detergent without disrupting the adhering ECM. ECM should be used immediately following preparation.

6. Prepare mouse ES or iPS cells in ES-Cult™ Maintenance Medium as described in section 3.4 and continue to maintain the culture (see section 4.2).

8.0 ES-Cult™ Mouse ES and iPS Cell Maintenance Kit

The ES-Cult™ Mouse ES and iPS Cell Maintenance Kit* (Catalog #03150) includes:

COMPONENT	SIZE	QUANTITY
Trypsin-EDTA (0.25%)	500 mL	1
L-Glutamine	100 mL	1
DMEM with 4500 mg/L D-glucose	500 mL	1
LIF, Mouse, Recombinant**	10 µg	1
Sodium Pyruvate	100 mL	1
MEM Non-Essential Amino Acid Solution (100X)	100 mL	1
D-PBS (Without Ca++ and Mg++)	500 mL	1
0.1% Gelatin in Water	500 mL	1
Trypan Blue	100 mL	1
100 mm Treated Tissue Culture Dishes	10/pk	2

*Kit does not include fetal bovine serum (FBS).

**mLIF is manufactured by Millipore. mLIF is protected under US Patent nos. 5,187,077, 5,427,925, 5,443,825 and 5,750,654, 6,261,548; European Patent no. 0285 448; and related foreign patents and is not available for resale.

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