Murine Embryonic Stem Cell Culture

Procedures & Protocols

Merck Millipore is a division of MERCK

Find the latest stem cell products

www.millipore.com/stemcell

Positively the best place for your antibody research

www.millipore.com/antibodies

- Rely on the combined expertise of Chemicon and Upstate and our 100% Antibody Performance Promise.
- Find antibodies within major and emerging research areas validated against specific applications like Western blot, flow cytometry, HCA, and more.
- Search for antibodies and assays by keyword, gene symbol, Entrez gene number, or UniProt number and refine your search using a number of antibody- and assay-specific filters.
- Quickly link out to associated products across Merck Millipore.

Table of Contents

I.

Introduction

The development of transgenic and gene knockout technology has provided an effective tool for the analysis of gene function. Critical to this has been the ability to isolate and culture murine embryonic stem (ES) cells *in vitro*. Derived from the inner cell mass of early mouse embryos, ES cells contribute to all tissues including germline tissue. Efficient procedures for the *in vitro* culture and maintenance of mouse pluripotent ES cells have been crucial to the success of gene targeting experiments.

Merck Millipore offers a wide range of mouse ES related products providing researchers with convenient and cost effective solutions for the reliable culture of murine ES cells. Merck Millipore is committed to providing the tools needed to advance stem cell research.

Highlights of this product range include:

- ESGRO® mLIF medium supplement for maintaining undifferentiated mouse ES cells.
- ESGRO Complete™ Plus and ESGRO®-2i cell culture media, defined serum-free systems for the maintenance and derivation of mouse ES cell lines in the absence of FBS and feeder cells.
- ES2N serum-free differentiation media for the rapid neuronal differentiation of mouse ES cells.
- RESGRO™ culture medium for the rescue of partially differentiated ES cell lines and improved mouse ES cell derivation.
- STEMCCA™ lentivirus reprogramming kits and reagents for the derivation of induced pluripotent stem cells (IPS) using a single polycistronic lentivirus.
- Active and mitotically inactivated EmbryoMax® PMEF feeder cell lines.
- B6-White™ ES cell line, the first commercially available C57BL/6 $tyrc^{-2J}$ albino line that allows for rapid coat-color determination of successful chimerism in C57BL/6 mice.
- PluriStem® ES cell lines derived from a wide range of inbred strains of mice.
- EmbryoMax® reagents including DMEM, FBS and media supplements for mouse ES culture.

The cell culture protocols described in this instructional manual include the *in vitro* culture of murine ES cells using EmbryoMax products along with ESGRO® mLIF medium supplement, as well as feeder-free and serum-free ES cell culture using the ESGRO Complete™ line of products. Also included in this instructional manual are: methods for serum-free neuronal differentiation of mouse ES cells, iPS cell generation using STEMCCA™ lentivirus kits, cre-recombinase mediated excision of STEMCCA™ genes from iPS cells, derivation and rescue of new and existing ES cell lines using RESGRO™ Culture Medium and ESGRO Complete™ system.

It should be noted that the protocols included in this manual are intended to serve as a guide only, and optimization of culture protocols is encouraged to ensure success.

Experimental Outline for Targeting ES Cells – Step by Step

General Considerations:

Plan out a flow chart from day 1 to day 16–19. Note, depending upon the growth of the ES cells, the days may have to be shifted. Electroporation, screening, picking and preparation of DNA will take 2–3 weeks, including weekends.

Day No.	Application	Section No.
0	Prepare culture plates for plating.	III.
1	Prepare PMEF Feeder cell plates for ES cell expansion & electroporation, if applicable.	IV.
2	Check PMEF Feeder cell plates, and thaw ES cells.	V., XII.
3	Feed ES cells; passage if required.	V., XII.
4	Feed ES cells; passage if required. Prepare Targeting Vector: Linearize ~100 µg of targeting vector (each electroporation requires 15–30 µg of linearized vector), and precipitate with EtOH (no need to phenolize).	V., XII.
5	Electroporation of ES cells.	VI., XIII.
6–10	Select for ES cell transformants.	VII., XIV.
9–11	Pick ES cells.	VIII., XV.
12–13	Feed picked ES cells.	VIII., XV.
14	Freeze ES cell clones & retain duplicate wells to grow ES cells for DNA isolation.	IX., X.
	Prepare Genomic DNA for Southern blot analysis.	IX.
16–19	Recover clones following analysis using RESGRO™ Culture Medium.	XXI.

Procedure for Coating Multiwell Plates with Gelatin Solution

Plate	Amount / Well
96-well	100 µL
48-well	300 µL
24-well	0.5 mL
12-well	1.0 mL
6-well	1.5–2.0 mL
30 mm	1.5–2.0 mL
60 mm	3.0 mL
100 mm	4.0–5.0 mL

Table 3.1: Gelatin solution volume

Materials & Reagents required:

- EmbryoMax® 0.1% Gelatin Solution (Catalogue No. ES-006-B)
- ESGRO Complete™ Gelatin Solution (Catalogue No. SF008)
- Sterile Pipette
- Tissue culture plates

Procedure:

1. Warm 0.1% Gelatin Solution to room temperature prior to use.
2. In a culture hood, under sterile conditions, add Gelatin Solution to each well of the plate as suggested in the table (left). Note: Add enough Gelatin Solution to adequately cover the plasticware surface.
3. Leave the Gelatin Solution in the wells for at least 30 minutes at room temperature, with dish lids on in the laminar flow hood.
4. Remove the Gelatin Solution by aspiration and discard. Immediately add media and cells to the dish. Do not allow the dishes to dry before adding cells.

Mouse ES Cell Culture Using ESGRO® Media Supplement

Plating PMEF Feeder Cells

EmbryoMax® Primary Mouse Embryo Fibroblasts (PMEF) feeder cells are supplied as frozen vials containing 5–6 x 10^6 cells per vial at passage 3 (2–3 population doublings per passage). It is recommended that PMEF feeder cells be plated one day prior to plating ES cells, which guarantees approximately 95% confluence of the PMEF cells. If ES cells are plated earlier than one day after PMEF plating, there may be some small gaps in the feeder layer. Although plating ES cells when gaps are present may not have any detrimental effects on the ES cells, it is not recommended.

Materials & Reagents required:

- EmbryoMax® 0.1% Gelatin Solution (Catalogue No. ES-006-B)
- ESGRO Complete™ Gelatin Solution (Catalogue No. SF008)
- Sterile Pipette
- Tissue culture plates
- PMEF Cells (PMEF-H, PMEF-N, PMEF-CF)

Procedure:

1. Prior to thawing PMEF feeder cells, coat plates/flasks with Gelatin solution (see Section III).
2. Thaw PMEF vial(s) quickly in a 37 °C water bath and transfer to a 15 mL tube (already containing 10 mL of warm PMEF Feeder Cell Medium, Section XXVI). Gently invert the tube to distribute, and centrifuge at 300 xg for 4–5 minutes.
3. Remove supernatant and resuspend the cell pellet in warm PMEF Feeder Cell Medium (see Table 4.1 for volumes).
4. Remove the Gelatin solution from plates/flasks, and aliquot the PMEF feeder cell suspension at the densities recommended in Table 4.1 on the following page.
5. Incubate the PMEF Feeder cells at 37 °C with 5% CO_2. Use Figures 4A, B and C (following page) as a guide for an estimate of correct PMEF density and appearance. Gelatinized plates may be used for 12–14 days.

IV.

Dish Size	Volume (mL)/ flask or well	Growth Area (cm^2)	No. of feeder cells/flask or well
75 cm^2 flask	12	75	3.75×10^6
25 cm^2 flask	6	25	1.25×10^6
100 mm plate	10	56	2.8×10^6
60 mm plate	5	21	1.0×10^6
6-well plate	4	9.5	4.75×10^5
12-well plate	2	4	2.0×10^5
24-well plate	1	2	1.0×10^5
96-well plate	0.1	0.32	1.5×10^4

Table 4.1: Recommended PMEF feeder cell suspension densities

Figure 4A.
PMEF feeder cells at the correct density.

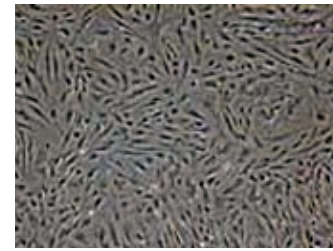


Figure 4B.
PMEF feeder cells at too low density.

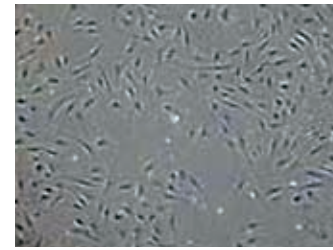


Figure 4C.
PMEF feeder cells at too high density.

ES Cell Culture using ESGRO® Medium Supplement

ESGRO® supplement is a special formulation of mouse LIF protein. Unlike regular LIF, which is sold by weight, each lot of ESGRO® supplement is sold based on its biological activity. The benefits of ESGRO® mLIF medium supplement include consistent inhibition of mouse ES cell differentiation, no batch-to-batch variation and the increased ability to grow mouse ES cells in feeder-free conditions.

V.i ES Cell Culture without PMEF Feeder Cells

Materials & Reagents required:

- Centrifuge
- ESGRO® supplement (Catalogue Nos. ESG1106, ESG1107)
- EmbryoMax® DPBS (Catalogue Nos. BSS-1006-A, BSS-1006-B)
- EmbryoMax® ES Cell Qualified FBS (Catalogue Nos. ES-009-B, ES-009-C)
- ES Cell Medium (see Section XXVI)
- Gelatin coated Tissue Culture Plates (see Section III)
- Incubator, 37 °C/5% CO_2
- Pipette
- 0.05% Trypsin-0.53 mM EDTA (Catalogue No. SM-2002-C)

Procedure:

1. Thaw a vial containing 1x10^7 ES cells into 4 mL of ES Cell Medium (containing ESGRO® supplement at 1000 units/mL) and 4 mL of FBS. Centrifuge (3–5 minutes) and resuspend the cells in 10 mL of ES Cell Medium. Plate the ES cells onto the gelatinized plates at a density of 1–1.5 x 10^6 cells/25 cm^2 (~3 x 10^6 cells/100 mm plate). Incubate the plates at 37 °C with 5% CO_2. The cells appearance should resemble Image 5A (following page).
2. Examine the cells daily to determine if a change of media is required (indicated by a change of media color to yellow). After 2–3 days, ES cell cultures will become crowded with large colonies (see Image 5B). At this point, passage ES cells at a 1:5 ratio.

>> Procedure continued on next page

ES Cell Culture using ESGRO® Medium Supplement (continued)

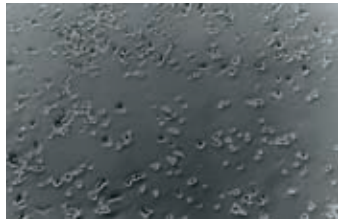


Figure 5A.

ES cells (R1) grown in the absence of PMEFs at the time of plating (10x).

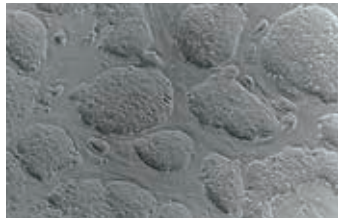


Figure 5B.

ES cells (R1) grown in the absence of feeder cells after 4 days of culture (10x).

3. To passage ES cells, prepare two 100 mm gelatinized plates in advance as described (see Section III). Remove ES Cell Medium, wash plates twice with DPBS, and add 1.2 mL of Trypsin. Incubate plates at 37 °C for 2 minutes, and then add 10 mL of ES cell medium. Pipette vigorously to break up the ES cell aggregates (avoid bubble formation).
4. Add 2 mL of the cell suspension to each gelatinized plate containing 8 mL of ES Cell Medium. Excess ES cells can be frozen at a concentration of 2–10 x 10^6 cells per vial for future use. Please note that ES cells should always be passaged the day before you intend to electroporate.

V.ii ES Cell Culture with PMEF Feeder Cells

Materials & Reagents required:

- Centrifuge
- ESGRO® supplement (Catalogue Nos. ESG1106, ESG1107)
- EmbryoMax® DPBS (Catalogue Nos. BSS-1006-A, BSS-1006-B)
- EmbryoMax® ES Cell Qualified FBS (Catalogue Nos. ES-009-B, ES-009-C)
- ES Cell Medium (see Section XXVI)
- Incubator, 37 °C/5% CO_2
- Pipette
- PMEF Feeder cell coated plates (see Section IV)
- 0.05% Trypsin-0.53mM EDTA (Catalogue No. SM-2002-C)

Procedure:

1. Thaw a vial containing 1x10^7 ES cells into 4 mL of ES Cell Medium (containing ESGRO® supplement at 1000 units/mL) and 4 mL of FBS. Centrifuge (3–5 minutes) and resuspend the cells in 10 mL of ES Cell Medium.
2. Remove the PMEF Feeder Cell Medium from a feeder plate prepared earlier (see Section IV), and seed the ES cells onto the PMEF coated plate at a density of 1–1.5 x 10^6 cells/25 cm^2 (~3 x 10^6 cells/100 mm plate). Incubate the plates at 37 °C with 5% CO_2. The cells appearance should resemble Image 5C.
3. Examine the cells daily to determine if a change of media is required (indicated by a change of media color to yellow). After 2–3 days, the ES cell cultures will become crowded with large colonies (see Image 5D). At this point, passage the ES cells at a 1:5 ratio.
4. To passage ES cells, prepare two 100 mm plates containing PMEF cells as previously described (see Section 4). Remove the ES Cell Medium, wash plates twice with DPBS, and add 1.2 mL of Trypsin. Incubate at 37 °C for 2 minutes. Add 10 mL of ES Cell Medium and pipette vigorously to disperse the ES cell aggregates (avoid bubble formation).
5. Add 2 mL of the cell suspension to each of the PMEF cell plates containing 8 mL of ES Cell Medium. Excess ES cells can be frozen at a concentration of 2–10 x 10^6 cells per vial for future use. Please note that ES cells should always be passaged the day before you intend to electroporate.

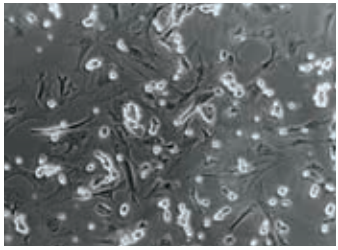


Figure 5C.

ES cells (R1) grown in the presence of PMEFs at the time of plating (10x).

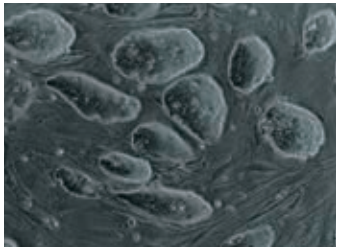


Figure 5D.

ES cells (R1) grown in the presence of feeder cells after 4 days of culture (10x).

VI.

Electroporation of ES Cells - Protocol for Serum-containing Medium

Materials & Reagents required:

- Electroporator and 0.4 cm cuvette
- EmbryoMax® Electroporation Buffer (Catalogue No. ES-003-D)
- ES Cell Medium (see Section XXVI)
- Ice
- Incubator, 37 °C/5% CO_2
- 25–40 µg Linearized construct DNA, ethanol precipitated and dried
- PMEF Feeder cell coated plates (see Section IV)
- 0.05% Trypsin-0.53mM EDTA (Catalogue No. SM-2002-C)

Procedure:

1. The evening before the electroporation is to be performed, prepare 4 plates with PMEF cells (see Section IV).
2. The morning that the electroporation is to be performed feed the ES cells fresh ES Cell Medium.
3. Later that afternoon, harvest the ES cells as described previously, and determine the cell count. $1x10^7$ ES cells is the minimum number of ES cells required for electroporation. If there is excess, freeze the cells down as previously described.
4. Centrifuge the cells required for electroporation at 300 xg for 10 minutes, then aspirate the medium.
5. Resuspend the ES cell pellet in 600 µL of Electroporation Buffer.
6. 25–40 µg of knockout construct DNA (purified) should already be linearized, ethanol precipitated and dried as a pellet. In a sterile hood, dissolve the DNA pellet in 30 µL of Electroporation Buffer, and then add the solution to the ES cells. Mix well and leave for 5 minutes at room temperature.
7. Place the ES cells in a 0.4 cm electroporation cuvette. Electroporate the suspension at 500 µFD, 0.24 kV. The time constant produced should be between 6.9 and 7.9 milliseconds (optimal 7.2). Following electroporation, place the cuvette on ice for 10 minutes.
8. Transfer the electroporated ES cells to 40 mL of ES Cell Medium and mix gently using a Pasteur pipette.
9. Plate the ES cell suspension (10 mL per feeder plate, total of 4 plates). Ensure that the PMEF Feeder Cell Medium is removed prior to the addition of cells.
10. Incubate for approximately 36 hours at 37 °C and 5% CO_2 prior to antibiotic selection.

Selection of ES Cells - Protocol for Serum-containing Medium

Prior to selection, it is recommended that a kill curve should be determined for each ES cell line in order to determine the exact drug concentration to be used. The following selection regimes can be used as a guide:

- **Neomycin (G418) in 129SVEV and 129SVJ cells:** 350 µg/mL for two days, then 150 µg/mL for the rest of the selection.

  Total 5–7 days selection

- **Neomycin (G418) in C57BL6/J cells:** 275 µg/mL for one day, 200 µg/mL for the second day, then 150 µg/mL for the rest of the selection.

  Total 6–8 days selection

- **Hygromycin B in 129SVEV and 129SVJ cells:** 100 µg/mL for two days, then 75 µg/mL for one day, then 50 µg/mL for the rest of the selection.

  Total 7–10 days selection

Materials & Reagents required:

- ES Cell Medium supplemented with either Neomycin (G418) (Catalogue No. 345810) or Hygromycin B (Catalogue No. 400050)
- EmbryoMax® DPBS (Catalogue Nos. BSS-1006-A BSS-1006-B)
- Incubator, 37 °C/5% CO_2
- Pipette

Procedure:

1. To select for transformants, add ES cell medium containing either Neomycin (G418) or Hygromycin B.
2. After 48 hours, cell death should be apparent. Change theculture medium daily if there is excessive debris evident or if the medium is discoloring, otherwise every other day is sufficient. If the debris is adhering to the living cells, wash the cells gently with sterile DPBS before changing the medium, taking care not to dislodge the feeder cell layer. ES cells should be cultured for approximately 10 days following electroporation.

VIII.

Colony Picking - Protocol for Serum-containing Medium

Depending upon the cell line and the media used, ES cell colonies are generally ready for picking 5–10 days after electroporation. The most suitable colonies to select are those that appear rounded or oval in shape, with a phase contrast bright edge and often a dark necrotic center. Differentiated colonies are flat and often surrounded by fibroblast like cells that form cobblestone-like structures. These cells should be avoided when selecting cells. ES cells can be picked onto either gelatinized plates or a PMEF feeder cell layer depending upon the ES cell line used. If gelatinized plates are preferred, please disregard the use of feeder cells as described in the procedure below.

Materials & Reagents required:

- 96-well plate(s)
- ES Cell Medium (see Section XXVI)
- ES Cell Medium supplemented with Neomycin (150 µg/mL) or Hygromycin B (50 µg/mL)
- Incubator, 37 °C/5% CO_2
- Pipette
- PMEF Feeder cell coated 24-well plates (see Section IV)
- 0.05% Trypsin-0.53mM EDTA (Catalogue No. SM-2002-C)
- Microscope

>> Procedure continued on next page

Procedure:

1. The day before picking ES cell colonies, coat an appropriate number of 24-well plates with PMEF Feeder Cells (see Section IV) or gelatin (see Section III).
2. Prior to selecting ES cell colonies, ensure that you are wearing gloves, a gown and face mask. All surfaces including the microscope, bench, tip boxes and pipette should be wiped with ethanol prior to use.
3. Inspect the ES cell cultures at 4x magnification. Colonies selected for picking should be spaced well enough apart to ensure no contamination from surrounding colonies. When a desired colony is found, circle the colony with a pipette tip to loosen the surrounding fibroblast layer. With the pipette set to a volume of 15 µL, scrape the colony with the pipette tip to dislodge the colony, then aspirate (the colony is often visible inside the tip). Transfer the picked colony to an empty well in a 96-well plate.
4. Continue picking and transferring ES cell colonies to fresh wells using a new tip each time, until a suitable number is picked. Clones are often picked in batches of 48 cells to prevent fatigue.
5. Add a single drop of Trypsin to each well and incubate at 37 °C for 2 minutes. During this period, replace the PMEF Feeder Cell Media in the 24-well plates with 500 µL of ES cell medium.
6. Disperse each ES cell colony in the 96-well plate by using a pipette to break up each colony, avoiding excessive foaming. Transfer the suspension to the 24-well plate (including foam) containing 500 µL of ES cell medium, using a fresh tip for each well.
7. When all the colonies are transferred, mix each well using a clean pipette tip set to 400 µL. Incubate at 37 °C and 5% CO_2. Feed every day with medium supplemented with Neomycin (150 µg/mL) or Hygromycin (50 µg/mL) antibiotics.
8. New colonies should be evident within a few days. If the colonies are too close in proximity to each other, disperse them using a 1 mL pipette tip to break up the colonies and spread the cells (Trypsin is not required as colonies break up very easily). Each well should be evenly covered with colonies before harvesting.
9. Continue changing the ES cell medium every day until a good coverage of colonies in each well is achieved (typically 7–10 days).

IX.

Harvesting and DNA Preparation

Materials & Reagents required:

- 8M Ammonium Acetate Solution
- ES Cell Medium (see Section XXVI)
- Ice cold 70% Ethanol
- 100% Ethanol
- Lysis Buffer (see Section XXVI)
- Microcentrifuge tubes
- Phenol/Chloroform/Isoamyl Alcohol (24:24:1)
- Pipette
- TE buffer

Procedure:

1. Label an appropriate number of microcentrifuge tubes to identify each well (eg. 1.7A1 – electroporation 1, plate 7, well A1).
2. Resuspend the ES cell cultures (from Section VIII) using a pipette set to 400 µL. Transfer 400 µL of the resultant cell suspension (total volume 500 µL) to each microcentrifuge tube.
3. After all wells have been harvested, add 500 µL of ES cell medium to each well containing ~100 µL of cell suspension, and return to incubator for 3–5 days. Change the cell media as required.
4. Collect the ES cells that have been transferred to the microcentrifuge tubes by centrifugation (30 seconds). Aspirate and discard the media.
5. Add 300 µL of fresh Lysis Buffer to each tube (there is no need to resuspend cells). Incubate at 37 °C overnight (not in a water bath).
6. The next day, add 37.5 µL of 8M Ammonium Acetate to each tube, then 350 µL of Phenol/Chloroform/Isoamyl Alcohol (it is recommended to do this in a fume hood). Mix by inversion approximately 5 times. DO NOT VORTEX! Centrifuge for 5 minutes.
7. Remove the upper aqueous layer, leaving any interface behind. Transfer to 750 µL (3 volumes) of 100% Ethanol and mix well. Often a precipitate is immediately visible.
8. Facilitate DNA precipitation by incubating each tube at –20 °C for 1 hour. Following this period, collect the DNA pellet by centrifugation for 10 minutes. Wash the pellet with 300 µL ice-cold 70% Ethanol and repeat the centrifugation for 5 minutes. Remove the liquid carefully, taking care not to disturb the pellet. Air-dry the pellet.
9. Redissolve the DNA pellet in 100 µL of TE (less if pellet is very small). Allow the pellet to completely dissolve for 2 hours at 65 °C then overnight at 4 °C.
10. Prior to restriction enzyme digestion, heat the DNA solution to 65 °C for 10 minutes. If pipetting is very difficult, pipette the solution straight from the 65 °C block. Depending upon the size of the original DNA pellet, between 10–30 µL of DNA should be used for Southern blot analysis.

Freezing Plates

Materials & Reagents required:

- Dry Ice
- EmbryoMax® Freezing Medium, 2x (Catalogue No. ES-002-D)
- ES Cell Medium (see Section XXVI)
- Parafilm® Film
- Pipette
- 0.05% Trypsin-0.53mM EDTA (Catalogue No. SM-2002-C)

Procedure:

1. When the ES cell colonies have regrown (see Section 10, step 3), wash with DPBS and add 35 µL of Trypsin. Incubate for 10 minutes at 37 °C, and then add 65 µL of ES Cell Medium.
2. Disperse the cells with a pipette and transfer into a replica plate containing 65 µL of cold EmbryoMax® Freezing Medium (2x).
3. Wrap each plate with Parafilm film and place on dry ice for 20 minutes. Transfer to –80 °C to freeze. Plates can keep for a number of months.
4. To thaw, add 150 µL of ES Cell Medium plus selection agent to each well. Thaw plate quickly by placing in a 37 °C incubator.
5. Transfer the thawed plates to a hood and manually pipette each well. Transfer the contents to a fresh 24-well plate with PMEF feeders (if required).
6. The next day, change the media to remove DMSO. Incubate the plate for up to 2 weeks to allow colonies to establish.

XI.

Karyotyping ES Cells

Materials & Reagents required:

- Depex Mounting Medium
- ES Cell Medium (see Section XXVI)
- Fixative (MeOH:Glacial Acetic Acid, 3:1)
- Hypotonic KCl Solution
- Leishman's Stain (see Section XXVI)
- Microscope Slides
- Pipette
- 0.05% Trypsin-0.53mM EDTA (Catalogue No. SM-2002-C)
- Xylene

Procedure:

1. This method is recommended for use with actively growing cultures of ES cells (i.e. 1–2 day cultures).
2. One day prior to karyotyping, passage a 70% confluent ES cell plate at a 1:2 ratio.
3. At least 3 hours prior to karyotyping, transfer the ES cells into fresh medium.
4. Trypsinize the ES cells and transfer the cell suspension to a conical tube. Centrifuge the cells at 300 xg for 5 minutes, then aspirate the medium. Avoid allowing the pellets to dry out.
5. Resuspend each cell pellet in 8 mL of hypotonic KCl solution, gently flicking the tube to avoid clumping and ensure an even suspension.
6. Incubate the tube at 37 °C for 10 minutes (this may vary for each type of cell line used).
7. Add 2 mL of freshly made fixative (MeOH:Glacial Acetic Acid, 3:1) and mix by gentle inversion.

>> Procedure continued on next page

8. Centrifuge cells (300 xg, 5 minutes) and aspirate the supernatant.
9. Using a Pasteur pipette, carefully add 2 mL of fixative solution drop wise, with gentle mixing to avoid clumping. Add an additional 6 mL of fixative and mix by gentle inversion of the tube.
10. Centrifuge cells (300 xg, 5 minutes) and aspirate supernatant.
11. Repeat steps 8 & 9 three times.
12. Resuspend the pellet in 1 mL of fixative (this volume may need to be adjusted slightly according to pellet size).
13. To make cell spreads, first humidify the surface of a dried cold slide by exhaling on the slide surface while holding the slide at a 45° angle. Using a Pasteur pipette, carefully dispense one drop of the suspended cells onto the top surface of the slide and allow to air dry.

Staining:

1. Stain slides with freshly made Leishman's stain for 8 minutes.
2. Rinse in running water for 1 minute and air dry.
3. Clear slides in 2 changes of xylene and mount cover slip using Depex mounting medium.

Notes:

- Colcemid is not used in this method, as the mitotic index of actively growing ES cells is generally high enough to obtain an ideal chromosome spread.
- High quality slides are recommended. Slides should be soaked in 100% ethanol overnight and dried with lint-free tissue before use. As it is important to have slides cold before use, slides can be stored in the refrigerator or freezer in an ethanol bath prior to making cell spreads.
- Most labs use 0.56% KCl and some labs use 0.2% KCl + 0.2% Na Citrate as an alternative. This depends entirely on the cell types being analyzed. The time in KCl is crucial — too short and the chromosomes will be too tightly packed; too long and they will not remain in their appropriate group.

Notes

Mouse ES Cell Culture using ESGRO Complete™ PLUS and ESGRO®-2i Media

XII.i ESGRO Complete™ PLUS System

Adapting ES Cell Lines to a Feeder- and Serum-free Environment using ESGRO Complete™ PLUS Medium

ESGRO Complete™ PLUS Clonal Grade Medium is a defined serum-free medium provided with a selective GSK3β inhibitor to enhance viability of mouse embryonic stem (ES) cells and increased maintenance of pluripotency in the absence of serum and feeder cells. This medium is intended for mouse ES cell expansion and growth at clonal density in serum-free conditions. The mouse ES cells should maintain germline competency.

The following protocol is applicable for adapting both feeder-dependent and feeder-independent mouse ES cells to serum-free cell culture conditions using ESGRO Complete™ PLUS system. It is important to pre-warm all reagents to 37 °C prior to use and avoid using glassware, as ES cells in serum-free media are sensitive to any residual detergent. The use of disposable plasticware in any manipulations is strongly recommended.

Materials & Reagents required:

- ESGRO Complete™ PLUS Clonal Grade Medium (Catalogue No. SF001-500P)

  Which Contains:
  ESGRO Complete™ Clonal Grade Medium: (Catalogue No. SF001-B) One (1) 500 mL bottle containing clonal grade medium.

  GSK3β Inhibitor Supplement: (Catalogue No. SF012-250) One (1) 250 µL vial containing selective GSK3β inhibitor.
- ESGRO Complete™ Basal Medium (Catalogue No. SCR002-500)
- ESGRO Complete™ Gelatin (Catalogue No. SF008)
- ESGRO Complete™ Accutase™ Solution (Catalogue No. SF006)
- D-PBS (Catalogue No. BSS-1006-B)

Procedure:

The first split is performed using standard trypsin. All subsequent passaging should be performed in Accutase™ solution. Feeder cells naturally deplete within 3-4 passages, at which time the cells are considered adapted.

1. Grow Mouse ES cells to 60% confluence in serum-supplemented medium in a T25 flask with or without feeders.
2. Precoat T25 flasks or 6 well plates with Gelatin Solution (Catalogue No. SF008).
3. Wash cells once with D-PBS (Catalogue No. BSS-1006-B). To dissociate cells, add 0.05% trypsin (Catalogue No. SM-2002-C), and incubate at 37 °C for 5-10 minutes.
4. Pipet up and down to ensure a single cell suspension and transfer cells to a 15 mL tube with 10 mL of prewarmed serum-containing medium.
5. Spin cells down at 1000 rpm, discard medium, and resuspend in ESGRO Complete™ PLUS medium.
6. Count cells and plate at a density of: 1-5x10^5 cells/ 6 well plate or 2.5-12x10^5 cells/ T25 flask in a volume of 3 mL or 10 mL ESGRO Complete™ PLUS medium, respectively.
7. Next day it may be necessary to passage the cells again: Wash cells once with D-PBS (Catalogue No. BSS-1006-B). To dissociate cells, add 1.5 mL Accutase™ solution (Catalogue No. SF006) per T25 flask, or 0.5 mL per 6 well plate. Incubate at RT and allow cells to detach (2-5 minutes).

   IMPORTANT NOTE: Do not use standard trypsin from this point onward. Standard trypsin affects the attachment properties of the cells, which form clusters of free floating cells. When passaging cells in Accutase™ solution, incubate no more than 2-5 minutes at RT or the least amount of time. Resuspend cells to a single cell suspension, as otherwise the cells tend to clump together to form floating colonies. Compared to regular trypsin, some cells in Accutase™ solution may require more rigorous resuspension.
8. Tap flask to remove all colonies. It may be necessary to pipette up and down to obtain a single cell solution. To wash the cells, prepare a 15 mL tube with 5 mL of Basal Medium (Catalogue No. SF002-500), and add the single cell suspension, mix and spin at 1000 rpm.
9. Repeat the wash: Remove supernatant, add again 5 mL of Basal Medium, and resuspend cells.

>> Procedure continued on next page

10. Count the cells and and spin at 1000 rpm. Remove supernatant and resuspend pellet in ESGRO Complete™ PLUS medium.
11. Plate 1-5x10^5 cells /6 well plate or 2.5-12x10^5 cells / T25 flask containing 3 mL or 10 mL pre-warmed ESGRO Complete™ PLUS medium, respectively.
12. Observe cell growth over the next 1-3 days. Some residual feeders remain, and initially some cell death may be observed. However, mES cell colonies will continue to grow and will appear bright with a similar morphology as in serum containing medium. When mES cells are 60-90% confluent, cells may be passaged again.
13. Repeat step 7-11 once or twice more for a total of 3-4 passages, until all feeder cells are depleted. Depending on the growth vigor of the cells a 1:5 split routine can be adopted every 2-3 days.

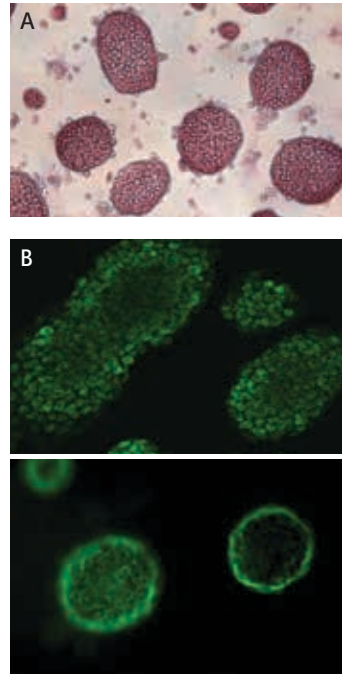


Figure 12A.

To confirm pluripotency of ES cells after many passages in ESGRO Complete™ clonal grade medium supplemented with GSK3β inhibitor, cells were stained for alkaline phosphatase (A) and immunostained for Oct-4 (B) and SSEA-1 (Figure 2B).

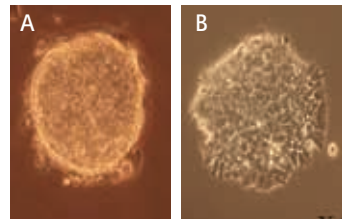
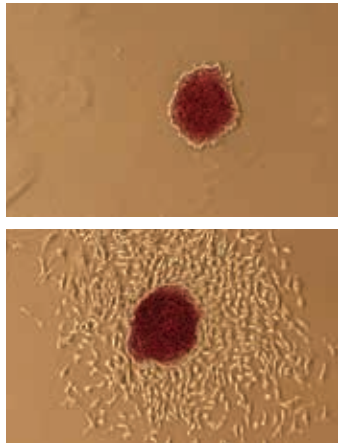


Figure 12B.

(A) Tight round colony (B) flatter colony. Undifferentiated ES cells colonies with a distinct cytoplasmic and nuclear morphology (day 4 of a clonal assay).



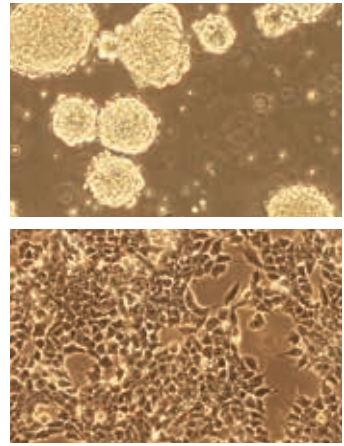


Figure 12C.

(Top) ES cells colony with no differentiation (Bottom) differentiated cells at the edge of an ES cells colony. Alkaline phosphatase staining allows for an easy distinction between undifferentiated ES cells (red) and differentiated cells (unstained) on day 5 of the clonal assay.

Figure 12D.

Standard Trypsin use causes the ES cells to lift off the plates in serum free conditions (Top) whereas the use of Accutase™ solution allows for an efficient and gentle way to routinely passage the cells (Bottom).

XII.ii ESGRO®-2i System

Adapting ES Cell Lines to a Feeder- and Serum-free Environment using ESGRO®-2i Medium

ESGRO®-2i Medium is a defined, LIF containing medium provided with a selective GSK3β inhibitor and Mek1/2 inhibitor for the culture of mouse embryonic stem (ES) and induced pluripotent stem (iPS) cells and increased maintenance of pluripotency. This medium is intended for mouse pluripotent stem cell expansion and growth at clonal density in serum-free conditions.

The following protocol is applicable for adapting both feeder-dependent and feeder-independent mouse ES cells to serum-free cell culture conditions using the ESGRO®-2i system. It is important to pre-warm all reagents to 37 °C prior to use and avoid using glassware, as ES cells in serum-free media are sensitive to any residual detergent. The use of disposable plasticware in any manipulations is strongly recommended.

Materials & Reagents required:

- ESGRO®-2i Medium (Catalogue No. SF016-100)

  Which contains:
  ESGRO®-2i Basal Medium with LIF: One (1) 100 mL bottle containing basal medium supplemented with LIF, pH 7.1-7.3.

  GSK3 Inhibitor Supplement: One (1) 50 µL vial containing selective GSK3β inhibitor supplement.

  Mek1/2 Inhibitor Supplement: One (1) 10 µL vial containing selective Mek 1/2 inhibitor supplement.

- ESGRO Complete™ Basal Medium (Catalogue No. SCR002-500)
- ESGRO Complete™ Gelatin (Catalogue No. SF008)
- ESGRO Complete™ Accutase™ solution (Catalogue No. SF006)
- EmbryoMax® D-PBS (Catalogue No. BSS-1006-B)

Procedure:

NOTE: If ES cells were cultured in serum-containing medium, the first split can be done in your regular trypsin, and then all subsequent passaging is performed in Accutase™ solution. Feeder cells naturally deplete within 3-4 passages, at which time the cells are considered adapted.

1. Grow mES cells to 60% confluence in serum-supplemented medium in a 6-well plate with or without feeders.
2. Pre-coat T25 flasks or 6-well plates with Gelatin Solution (Catalogue No. SF008).
3. Wash cells once with D-PBS (Catalogue No. BSS-1006-B). To dissociate cells, add 0.05% trypsin and incubate at 37 °C for 5-10 minutes.

>> Procedure continued on next page

4. Pipette up and down to ensure a single cell suspension and transfer cells to a 15 mL tube with 10 mL of pre-warmed serum-containing medium.
5. Spin cells down at 1000rpm, discard supernatant, and resuspend in ESGRO®-2i medium.
6. Count cells and plate at a density of: 0.5-5 x10^5 cells per 6-well in a volume of 4 mL ESGRO®-2i medium. **NOTE:** Depending on the density of the cell culture, it may be necessary to passage the cells the next day.
7. Completely remove medium (no need to wash cells with PBS). To dissociate cells, add 0.5-1 mL Accutase™ solution (Catalogue No. SF006) per 6-well. Incubate at RT and allow cells to detach (2-5 minutes). Do not let cells become confluent.

   IMPORTANT NOTE: Do not use standard trypsin from this point onward. Standard trypsin affects the attachment properties of the cells, which form clusters of free floating cells.
8. Pipette up and down to obtain a single cell solution. To wash the cells, prepare a 15 mL tube with 5 mL of Basal Medium (Catalogue No. SF002-500), or DMEM/F12, and add the single cell suspension, mix and spin at 1000 rpm.
9. Repeat the wash: Remove supernatant, add again 5 mL of Basal Medium or DMEM/F12, and resuspend cells.
10. Count the cells and spin at 1000 rpm. Remove supernatant and resuspend pellet in ESGRO®-2i medium.
11. Passage cells at a ratio of 1:2–1:5 and add in 4 mL pre-warmed ESGRO®-2i Medium per 6-well plate.
12. Observe cell growth over the next 1-3 days. Some residual feeders remain, and initially some cell death may be observed. However, mES cell colonies will continue to grow and will appear bright with a similar morphology as in serum-containing medium. When mES cells are 60-90% confluent, cells may be passaged again.
13. Repeat step 7-11 once or twice more for a total of 1-2 passages, until all feeder cells are depleted. Depending on the growth vigour of the cells, a 1:5 split routine can be adopted every 2-3 days.

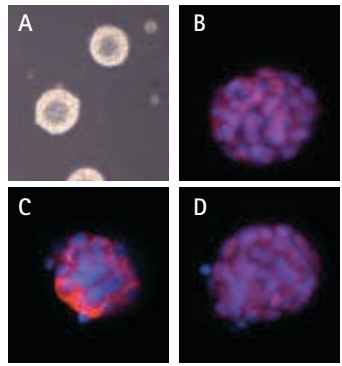


Figure 12F.

Prolonged pluripotent culture in ESGRO®-2i. 129SvEv mESCs were cultured for 10 or more passages. Pluripotency was assessed A) morphologically using bright field microscopy, B) with anti-Oct4 antibody staining, C) with anti-SSEA-1 antibody staining, and D) with anti-Sox2 antibody staining.

Electroporation of ES Cells - Protocol for Serum-free Medium

Materials & Reagents required:

- EmbryoMax® Electroporation Buffer (Catalogue No. ES-003-D)
- ESGRO Complete™ Clonal Grade Medium (Catalogue No. SF001-500P)
- Accutase™ Solution (Catalogue No. SF006)
- 25–40 µg Linearized construct DNA, ethanol precipitated and dried
- Incubator, 37 °C/5% CO_2
- Electroporator and 0.4 cm cuvette

Procedure:

1. The morning that the electroporation is to be performed feed the ES cells with fresh Clonal Grade Medium.
2. Later that afternoon, prepare 4 plates with 0.1% Gelatin solution (see Section III). Harvest the ES cells as described previously and determine the cell count. The minimum number of ES cells required for electroporation is $1x10^7$ cells. If there are excess cells, freeze down as previously described.
3. Centrifuge the cells required for electroporation at 300 xg for 3 minutes, then aspirate the medium.
4. Resuspend the ES cell pellet in 600 µL of Electroporation Buffer.
5. 25–40 µg of knockout construct DNA (purified) should already be linearized, ethanol precipitated and dried as a pellet. In a sterile hood, dissolve the DNA pellet in 30 µL of Electroporation Buffer, and then add the solution to the ES cells. Mix well and leave for 5 minutes at room temperature.
6. Place the ES cells in a 0.4 cm electroporation cuvette. Electroporate the suspension at 3 µFD, 0.8 kV (BioRad Gene Pulser®). The time constant produced should be between 6.9 and 7.9 milliseconds (optimal 7.2). Following electroporation, leave the cuvette at room temperature for 10 minutes.
7. Transfer the electroporated ES cells to 40 mL of Clonal Grade Medium and mix gently using a Pasteur pipette.
8. Plate the ES cell suspension (10 mL per plate, total of 4 plates). Ensure that the 0.1% Gelatin solution is removed prior to the addition of cells.
9. Incubate for approximately 36 hours at 37 °C and 5% CO_2 prior to antibiotic selection.

Selection of ES Cells - Protocol for Serum-free Medium

Please note that in serum-free medium conditions, a lower concentration of selection antibiotic is recommended.

Materials & Reagents required:

- ESGRO Complete™ Clonal Grade Medium (Catalogue No. SF001-500P)
- G418 Antibiotic
- Puromycin Antibiotic
- Hygromycin B Antibiotic
- EmbryoMax® DPBS (Catalogue Nos. BSS-1006-A, BSS-1006-B)
- Incubator, 37 °C/5% CO_2
- Pipette

Procedure:

1. To select for transformants, add Clonal Grade Medium containing G418, Hygromycin B, or Puromycin selection antibiotics.
2. After 48 hours, cell death should be apparent. Change the culture media daily if there is excessive debris evident or if the media is discoloring, otherwise every second day is sufficient. If the debris is adhering to the living cells, wash the cells gently with sterile DPBS before changing the media.
3. ES cells should be cultured for approximately 5 to 10 days following electroporation.

Colony Picking - Protocol for Serum-free Medium

Materials & Reagents required:

- ESGRO Complete™ Clonal Grade Medium (Catalogue No. SF001-500P)
- ESGRO Complete™ Clonal Grade Medium supplemented with G418, Hygromycin, or Puromycin
- Accutase™ Solution (Catalogue No. SF006)
- 0.1% gelatin coated 24-well plates
- 96-well plate(s)
- Pipette
- Incubator, 37 °C / 5% CO_2
- Microscope

Procedure:

1. The day of picking ES cell colonies, coat an appropriate number of 24-well plates with 0.1% Gelatin solution (see Section III).
2. Prior to selecting ES cell colonies, ensure that you are wearing gloves, a gown and face mask. All surfaces including the microscope, bench, tip boxes and pipette should be wiped with ethanol prior to use.
3. Inspect the ES cell cultures at 4x magnification. Colonies selected for picking should be spaced well enough apart to ensure no contamination from surrounding colonies. When a desired colony is found, and with the pipette set to a volume of 15 µL, scrape the colony with the pipette tip to dislodge the colony, then aspirate (the colony is often visible inside the tip). Transfer the picked colony to an empty well in a 96-well plate.
4. Continue picking and transferring ES cell colonies to fresh wells using a new tip each time, until a suitable number is picked. Clones are often picked in batches of 48 cells to prevent fatigue.

>> Procedure continued on next page

5. Add a single drop of Accutase™ solution to each well and incubate at 37 °C for 2 minutes. During this period, replace the 0.1% Gelatin solution in the 24-well plates with 500 µL of Clonal Grade Medium.
6. Disperse each ES cell colony in the 96-well plate by using a pipette to break up each colony, avoiding excessive foaming. Transfer the suspension to the 24-well plate (including foam) containing 500 µL of Clonal Grade Medium, using a fresh tip for each well.
7. When all the colonies are transferred, mix each well using a clean pipette tip set to 400 µL. Incubate at 37 °C and 5% CO_2. Feed every day with Clonal Grade Medium supplemented with G418, Hygromycin, or Puromycin antibiotics.
8. New colonies should be evident within a few days. If the colonies are too close in proximity to each other, disperse them using a 1 mL pipette tip to break up the colonies and spread the cells (Accutase™ solution is not required as colonies break up very easily). Each well should be evenly covered with colonies before harvesting.
9. Continue changing the Clonal Grade Medium every day until a good coverage of colonies in each well is achieved (typically 7–10 days).

XVI.-XVIII. Harvesting and DNA Preparation, Freezing Plates and Karyotyping for Serum-Free Media

Refer to Protocols in Section IX-XI (pages 14-17).

Mouse ES Differentiation using ES2N Medium and Embryoid Body Formation

Serum Free Monolayer Neural Differentiation using ES2N Medium

ES2N Complete medium is a defined serum-free formulation that has been optimized for the differentiation of mouse embryonic stem (ES) and induced pluripotent stem (iPS) cells into neurons. When used in combination with ESGRO Complete™ Plus medium, mouse ES and iPS cells readily differentiate into neurons in a monolayer assay within 9-12 days on gelatin coated culture dishes.

Materials & Reagents required:

- ES2N Complete Medium Kit (Catalogue No. SCM082)

  Which Contains:
  ES2N Basal Medium: (Catalogue No. SCM083) One (1) 245 mL bottle containing standard base medium.

  Neuro27 Medium Supplement: (Catalogue No. SCM013-S) One (1) 5 mL vial containing neural supplement.

  Neuro2 Medium Supplement: (Catalogue No. SCM012-S) One (1) 1.25 mL vial containing N2-like supplement.
- ESGRO Complete™ Plus Clonal Medium (Catalogue Nos. SF001-500P, SF001-100P)
- ESGRO Complete™ Basal Medium (Catalogue Nos. SF002-500, SF002-100)
- ESGRO Complete™ Gelatin (Catalogue No. SF008)
- Accutase™ Solution (Catalogue No. SF006)
- EmbryoMax® D-PBS (Catalogue No. BSS-1006-B)
- Basic FGF; FGF-2 (Catalogue No. GF003)
- EGF (Catalogue No. GF144)

Procedures:

The following protocol is for the direct differentiation into neurons from both feeder-dependent and feeder-independent mouse ES and iPS cells.

Serum-free and feeder-free adapted mouse ES and iPS cells

1. 3-4 days prior to the experiment, thaw and culture in ESGRO Complete™ Plus medium.
2. Passage cells by adding the least amount of Accutase™ to cover the cells, gently tap the culture dish.

>> Procedure continued on next page

3. Transfer the single cell solution into a vial with 5-10 mL ESGRO Complete™ Basal medium. Spin down for 5 minutes at 1000 rpm and remove supernatant. At this point a very small amount of cells (10 µL) can be removed for a cell count.
4. Repeat the wash in step 3.
5. Plate cells in an appropriate sized culture vessel coated with 0.1% Gelatin. As a rule it is best to passage cells once or twice in the ESGRO Complete™ Plus medium starting with the neuronal differentiation assay.
6. Start with step 1 of the Neuronal Differentiation Assay with a confluent ES cell culture.

Serum and feeder dependent mouse ES and iPS cells

1. Wash cells with PBS.
2. Add the least amount of trypsin to cover the cells, tap the culture dish, and transfer the single cell suspension in a vial with 5-10 mL serum-containing medium.
3. Spin at 1000 rpm for 5 minutes, discard supernatant.
4. Repeat wash using 5-10 mL ESGRO Complete™ Basal Medium. Remove a small amount of resuspended cells for a cell count and spin 5 minutes at 1000 rpm. Discard supernatant.
5. Resuspend cells in ESGRO Complete™ Plus medium and plate cells on 0.1% Gelatin coated dishes.
6. Culture at 37 °C with 5% CO_2 until confluent. Passage cells until feeder cells are depleted. Generally, cells are feeder free within 2-3 passages.

On the day of the neuronal differentiation experiment:

1. Coat 6-well dishes with 0.1% gelatin 1- 4 hours at room temperature. NOTE: Use fresh Gelatin. Do not coat for less than one hour or more than 4-6 hours.
2. Passage serum-free and feeder-free mouse ES and iPS cells with Accutase™, wash twice and count cells with a hemocytometer.
3. Plate 1-3 x 10^4 cells/cm^2 (1-3x 10^5 cells per 6-well plate). NOTE: Plating the correct density is crucial. Each cell line needs optimization.
4. Remove gelatin and add correct number of cells in 30 – 200 µL volume of ES2N Complete medium.
5. Add 3 - 4 mL of ES2N Complete medium to each well and place at 37 °C incubator with 5% CO_2.
6. Change medium every 1-2 days, gently aspirate undifferentiated cells. Depending on the cell line there can be cell death around day 3-5 associated with the assay.

>> Procedure continued on next page

The assay is completed on day 9-12, with up to 80-90% of neurons generated. Neurons may be kept in medium for an additional 4-7 days for analysis. During the assay, neural stem cells (NSCs) and precursor cells form rosettes on day 4-6. At this point NSCs can be isolated by replating cells in ES2N Complete mediuim containing bFGF and EGF and following the protocol for neural stem cell maintenance below.

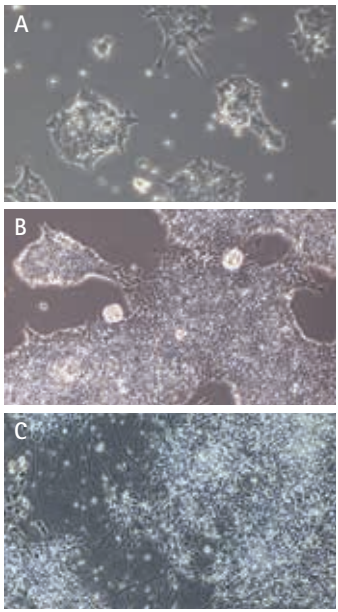


Figure 19A.

Timeline and morphology of 129S6 cells in the ES2N Neuronal differentiation assay. (a) Day 2 after plating. (b) Day 4 after plating, cells acquire neuronal precursor morphology. (c) Day 9 after plating, mature neurons become visible.

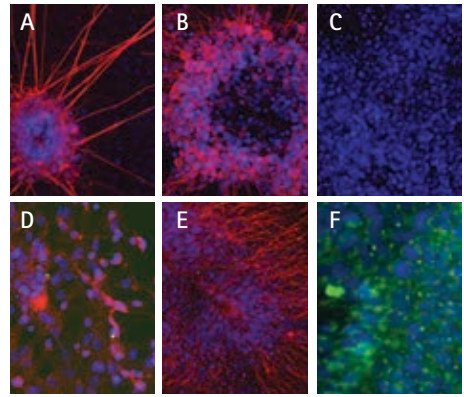


Figure 19B.

Immunocytochemistry analysis of mature neurons at day 12 differentiated from 129S6 mESCs. (a) Mature neurons stained with tubulin antibody. (b) Mature neurons stained with MAP-2 antibody. (c) Antibody staining of the Oct 4 pluripotency marker shows no Oct4 expression, only the nuclear DAPI staining is visible. (d) Antibody staining with the astrocyte marker GFAP. (e) Antibody staining with the neural stem cell marker nestin and (f) Antibody staining with oligodendrocyte marker O1. All stainings are overlaid with blue DAPI nuclear staining.

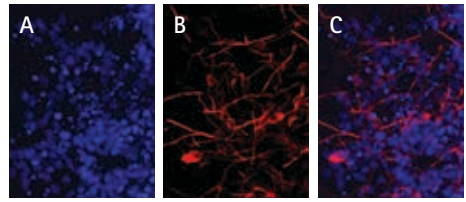


Figure 19C.

iPS cells were differentiated into neurons in ES2N complete medium.
(A) DAPI nuclear staining.
(B) Tubulin antibody staining.
(C) Overlay of tubulin and DAPI staining.

Protocol for isolation and maintenance of Neural Stem Cells (NSCs):

1. Aseptically add bFGF and EGF (both at 20ug/mL final concentration) to the ES2N Complete medium.
2. Coat 6-well dishes with matrix, if required. Note: the strongest adherence of NSCs occurs on fibronectin coated wells, followed by laminin/ polyornithine, and lastly gelatin.
3. Briefly wash cells with PBS. Add just enough Accutase™ solution to cover adherent cells and incubate at RT for 2-4 minutes.
4. Tap on the culture vessel and pipet up and down to obtain a single cell suspension. Collect cells in Accutase™ solution and transfer into a vial containing 5mL prewarmed ES2N Basal Medium.
5. Centrifuge for 5 minutes at 1000 rpm and resuspend in ES2N Basal Medium. This is your first wash.
6. Count cells with the hemocytometer. Spin cells down for 5 minutes at 1000rpm, resuspend in ES2N Basal Medium. Spin down again for 5 minutes at 1000rpm, this is your second wash.
7. Discard supernatant and resuspend cells in ES2N complete Medium containing bFGF and EGF, and transfer to a new culture well.
8. Plate 1-3 x 10^4 cells/cm^2 (1-3 x 10^5 cells/6-well).
9. Add medium to a final 3 - 4 mL of ES2N complete medium containing EGF and bFGF to each 6-well and place at 37 °C incubator with 5% CO_2.
10. Change medium every day.

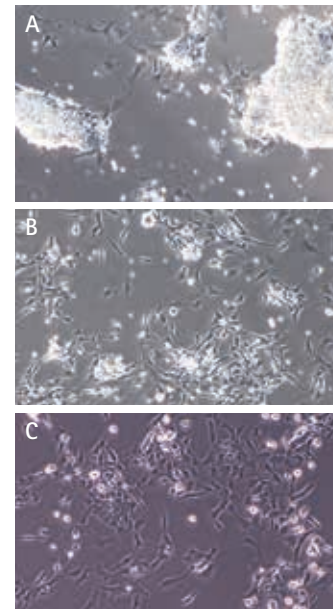


Figure 19D.

Isolation of neural precursor cells (NPC) from 129SvEv mESCs. (a) Neuronal rosettes at day 5 after plating in ES2N Complete medium before passaging and single cell re-plating. (b) Passage 2 after NPC isolation in ES2N Complete medium supplemented with bFGF and EGF. (c) Passage 3 after NPC isolation in ES2N Complete medium supplemented with bFGF and EGF.

Serum Containing Neural Differentiation via Embryoid Body Formation

The Mouse Embryonic Stem Cell Neurogenesis Kit (Catalogue No. SCR101) provides a system designed for the neural differentiation of mouse ES cells using the 3-dimensional culturing technique known as embryoid body formation. The kit contains all the reagents necessary to fully differentiate mouse ES cells into βIII-tubulin positive neurons *in vitro* following a modified 4-/4+ differentiation protocol (Bain, et al., 1995).

Materials & Reagents required:

- Mouse Embryonic Stem Cell Neurogenesis Kit (Catalogue No. SCR101)

  Which contains:
  Embryoid Body (EB) Formation Medium: (Catalogue No. SCM018) Five (5) 100 mL bottles.

  Accutase™ (Catalogue No. SCR005): One (1) 100 mL bottle.

  Neural Inducer A Solution: One (1) 200 µL vial of 500 µM retinoic acid.

  Mouse Laminin, 200UG: One (1) vial of 200 µg mouse laminin.

  Poly-L-ornithine Solution: One (1) vial of 2 mg poly-L-ornithine (10 mg/mL).

- Petri Dishes (BD Catalogue No. 351008)
- Phosphate-Buffered Saline (1X PBS) (Catalogue No. BSS-1005-B)
- Mouse anti-SSEA-1 (Catalogue No. MAB4301-50UG) One (1) vial of 50 µg IgM monoclonal.
- Mouse anti-βIII tubulin (Catalogue No. MAB1637-50UL) One (1) vial of 50 µL IgG monoclonal.

Procedures:

Removal of Feeders from Mouse ES Cell Culture

If mouse embryonic stem cells are grown on a mouse embryonic fibroblast feeder (MEF) layer, it is important that the feeder layer is removed before mouse ES cells are differentiated. The following protocol is recommended.

1. Carefully remove the medium used to culture mouse ES cells from the 10 cm tissue culture plate and wash the plate twice with 1X PBS.
2. Apply 5 mL Accutase™ solution and incubate in a 37 °C incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of the cells (both ES and MEFs) by gently tapping the side of the plate with the palm of your hand.
4. Apply 5 mL Complete ES Cell Media w/ 15% Serum and LIF (Catalogue No. ES-101-B) (pre-warmed to 37 °C) to the plate and use this medium to collect the cells onto a 15 mL conical tube.
5. Centrifuge the tube at 300 xg for 2-3 minutes to pellet the cells.
6. Discard the supernatant.
7. Apply 10 mL Complete ES Cell Media w/ 15% Serum and LIF (prewarmed to 37 °C) to the conical tube and resuspend the cells thoroughly.

   IMPORTANT: Do not vortex.
8. Transfer the cell suspension to a fresh sterile 10 cm plate that has been pre-coated with 0.1% gelatin solution.
9. Incubate in 37 °C incubator for 30 minutes.
10. After 30 minutes, remove the medium and any non-attached cells and transfer this cell suspension onto another 0.1% gelatin coated 10 cm plate.

    NOTE: Mouse embryonic fibroblasts (MEFs) adhere more readily to the tissue-culture plate than undifferentiated mouse ES cells and thus a significant number of MEFs can be removed by this differential adhesion step.
11. Repeat step 9 and 10 two to three more times.
12. Repeat the whole procedure (steps 1 through 11) for at least three passages to ensure that any residual MEFs are removed from the mouse ES cell culture.

Formation of Embryoid Bodies (4- Stage)

1. Carefully remove the medium used to culture feeder-free mouse ES cells from the 10 cm tissue culture plate and wash the plate twice with 1X PBS.
2. Apply 5 mL Accutase™ solution and incubate in a 37 °C incubator for 3-5 minutes.
3. Inspect the plate and ensure the complete detachment of the cells (both ES and feeders) by gently tapping the side of the plate with the palm of your hand.
4. Apply 5 mL Embryoid Body (EB) Formation medium (pre-warmed to 37 °C) to the plate and use this medium to collect the detached cells and transfer the cell suspension to a 15 mL conical tube.
5. Centrifuge the tube at 300 xg for 2-3 minutes to pellet cells.
6. Discard the supernatant.
7. Apply 2 mL EB Formation Medium to the conical tube and resuspend the cells thoroughly.

   IMPORTANT: Do not vortex.
8. Count the number of cells using a hemacytometer.
9. Aliquot 2-3 x 10^6 cells in 10 mL EB Formation Medium and place in a sterile 10 cm bacterial Petri dish or ultra low attachment Petri dish.
10. Incubate the cells in 37 °C, 9-10% CO_2 incubator for two days. After two days, there should be numerous floating embryoid bodies (EBs) formed.
11. On the second day, transfer the floating EBs and the entire medium in the 10 cm Petri dish to a 50 mL conical tube.
12. Wash the 10 cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50 mL conical tube.
13. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube.
14. Using a 10 mL or larger pipette, carefully remove the supernatant.

    NOTE: We do not recommend aspirating with a vacuum.
15. Resuspend the EBs in 10 mL EB Formation Medium (pre-warmed to 37 °C).
16. Transfer the entire cell suspension to a sterile 10 cm Petri dish. Incubate the EBs in 37 °C, 9-10% CO_2 incubator for an additional two days.

Induction of Neuronal Differentiation (4+ Stage)

1. After a total of 4 days in suspension culture, collect the EBs to a 50 mL conical tube.
2. Wash the 10 cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50 mL conical tube.
3. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube. Set aside.
4. Prepare the Neural Induction medium by adding 10 µL Neural Inducer A Solution to 10 mL EB Formation Medium.
5. To the tube containing the EBs, carefully remove and discard the supernatant with a 10 mL or larger pipette.

   NOTE: We do not recommend aspirating the supernatant with a vacuum.
6. Resuspend the EBs in 10 mL Neural Induction Medium (from step 4) and transfer the suspension to a fresh Petri dish.
7. Incubate the dish of cells in 37 °C, 9-10% CO_2 incubator for two days.
8. After two days, repeat steps 1 through 7 for a total of 4 days in the Neural Induction Medium.

Expansion of Neuronal Cells from Embryoid Bodies (8- Stage)

1. From step 8 of section titled "Induction of Neuronal Differentiation," gently swirl the 10 cm Petri dish containing the neural induced EBs counterclockwise for several circular rotations.

   NOTE: Use caution to avoid sloshing the media over the side of the plate as this may increase the risk of cell contamination. The swirling process helps localize all of the EBs to the center of the 10 cm Petri dish.
2. Immediately place the 10 cm Petri dish under a microscope and count the number of EBs that have localized to the center of the 10 cm Petri dish. This is your total number of EBs in the culture. Typical yields are 50 to 100 EBs per 10 cm Petri dish.
3. Using a 10 mL pipette, collect and transfer the EB suspension to a 50 mL conical tube.
4. Wash the 10 cm Petri dish twice with 10 mL EB Formation Medium and transfer each wash to the same 50 mL conical tube.
5. Leave the conical tube at room temperature for 15 minutes to allow the EBs to settle to the bottom of the tube.
6. Using a 10 mL or larger pipette, remove and discard the supernatant.

>> Procedure continued on next page

7. Resuspend the EBs in 2 mL EB Formation Medium (pre-warmed to 37 °C).
8. Plate approximately 10 to 20 EBs to each well of a poly-L-ornithine and laminin coated 8-well chamber slide.
9. Exchange the medium in each well with 0.5 mL fresh EB Formation Medium every two days for a total of 8 days. Neuronal like cells and their processes should extend and migrate from the attached EBs in as little as four days.

   NOTE: Use extreme care when exchanging the medium as the cells are loosely adherent. Always leave behind a small volume of medium to ensure that the cells do not dry out.

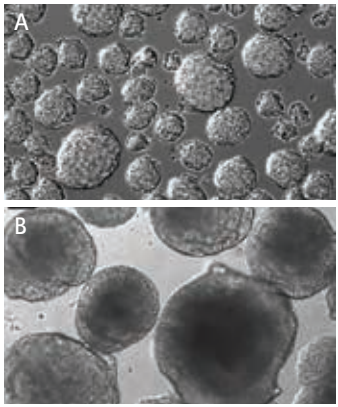


Figure 20A.

Embryoid bodies (EB) formed after the culture of dissociated mouse ES cells (Catalogue No. SCR012) in EB formation medium for 2 days (A). Morphology of EBs after treatment with retinoic acid for three days (B). 10X magnification.

Staining Protocol (for 8-well chamber slides)

1. Carefully aspirate the medium and wash the wells three times with 1X PBS (5 minutes per wash).

   NOTE: Use extreme care when exchanging media as the cells are loosely adherent. Always leave behind a small volume of medium to ensure that the cells do not dry out.
2. Fix the cells by incubation in 4% paraformaldehyde for 30-40 minutes at room temperature.
3. Carefully aspirate the fixative and rinse three times (5-10 minutes each) with 1X PBS. Aspirate after each rinse.
4. Wash the cells with 0.5 mL Non-Permeable Blocking Solution (5% normal donkey serum in 1X PBS) three times (5 minutes each). Aspirate after each wash.
5. Apply 0.25 mL fresh Blocking Solution for at least 2 hours at room temperature or overnight at 4 °C.

   IMPORTANT: Do not shake the cells.

   NOTE: For optimal results, use the Blocking Solution (e.g. 5% normal donkey serum, 0.3% Triton X-100 in 1X PBS) with the antibody directed against the neuronal cell marker, βIII-tubulin. Use the Non-Permeable Blocking Solution (5% normal donkey serum in 1X PBS) with the antibody directed against the mouse ES cell marker, SSEA-1.

>> Procedure continued on next page

6. Dilute the primary antibodies included in this kit to working concentrations in the appropriate blocking solutions. For optimal results, the following antibody dilutions are recommended for immunocytochemistry (see images): Mouse anti-SSEA-1, IgM monoclonal: 1/500 dilution of 1.7 mg/mL, final 3.4 µg/mL Mouse anti-βIII-tubulin, IgG monoclonal: 1/500 to 1/1000 dilution of monoclonal antibody.
7. In a separate control well, depending upon the specific antibody used, add equivalent concentrations of mouse IgG or mouse IgM to 0.25 mL of the appropriate blocking solution.
8. Incubate the cells in primary antibody overnight at 4°C.

   IMPORTANT: Do not shake.
9. The next day, wash the cells three times with 1X PBS (5-10 minutes each wash) and three times with the appropriate blocking solution.
10. At the completion of the last wash, leave the cells in the appropriate blocking solution for at least 30 minutes.
11. Dilute secondary antibodies in the appropriate blocking solution just before use. The following secondary antibody can be used, donkey anti-mouse IgG Cy3 conjugated (Catalogue No. AP192C) and donkey anti-mouse IgM Cy3 conjugated (Jacksons Laboratories) at a 1:500 dilution.
12. Overlay the cells with the secondary antibody blocking solution for 2 hours at room temperature in the dark.
13. Wash 3-5 times (5-10 minutes each) with 1X PBS.
14. Counterstain the cell nuclei with DAPI / 1X PBS solution.
15. Mount a glass coverslip over the chamber slides using antifading mounting solution (e.g. DABCO/PVA)
16. Visualize the cell staining with a fluorescent microscope.

    NOTE: Be sure to use the correct filter to visualize fluorescent-labeled cells.

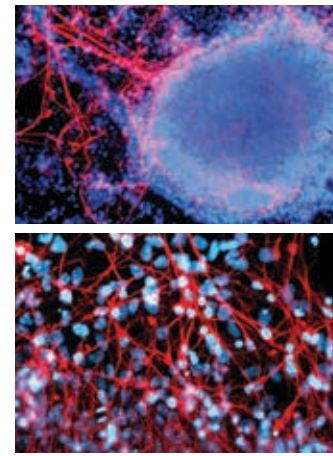


Figure 20B.

Differentiated neurons emanating from the EB demonstrate complex networks and high levels of branching (top). Differentiation of mouse ES cells to βIII-tubulin positive neurons (40X magnification) (bottom). Red: neurons labeled with βIII-tubulin antibody. Blue: cell nuclei labeled with DAPI.

Mouse ES Culture using RESGRO™ Medium

Recovery of Recombinant Clones Using RESGRO™ Culture Medium

RESGRO™ Culture Medium allows for the culture of ES cells on gelatinized culture dishes. Even in the absence of a PMEF feeder cell layer, ES cells maintain their undifferentiated character and their germline transmission capability for at least 5 passages when cultured with RESGRO™ Culture Medium. After trypsinization, pure ES cell suspensions without fibroblast cells can be obtained. Fibroblast cells will no longer interfere during blastocyst injections, diploid aggregations, tetraploid aggregations and electroporations.

Materials & Reagents required:

- RESGRO™ Culture Medium (Catalogue Nos. SCM001, SCM002)
- EmbryoMax® L-Glutamine Solution (Catalogue No. TMS-002-C)
- Incubator, 37 °C/5% CO_2
- PMEF Feeder cell coated culture plates (see Section IV)
- Pipette
- Cellulose acetate, PVDF or PES filters. For more information on sterile filtration products, visit: www.millipore.com

Procedure:

1. Prepare RESGRO™ Culture Medium for use by adding 10 mL of L-Glutamine Solution (200 mM) to 500 mL of RESGRO™ Culture Medium. If required, filter the solution using only cellulose acetate, PVDF or PES filters.
2. Dispense 3 mL of RESGRO™ Culture Medium into each well of a 6-well plate covered with PMEF feeder cells and place in a 37 °C incubator for 1 hour.
3. Thaw a frozen vial of ES cells in a water bath at 37 °C. Remove the vial just before the last trace of ice has melted.
4. Gently pipette the content of the vial up and down several times (removal of the cryoprotective medium is not necessary).
5. Dispense the cells into the wells of the 6-well plate containing RESGRO™ Culture Medium.
6. Gently disperse the cells by shaking to ensure a homogenous distribution.
7. Disperse cells again after 35 minutes and 1 hour by gently shaking.
8. Following attachment of the ES cells to the feeders (2–3 hours), gently remove the RESGRO™ Culture Medium and replace it with 4 mL of fresh medium.
9. Replace the RESGRO™ Culture Medium daily with fresh medium. Ensure that ES cell colonies do not come into contact with each other by passaging cells every 2–3 days.

Wells	Amount / Well
96	200 µL
48	500 µL
24	1.0 mL
12	1.5 mL
6	4.0 mL
100 mm plate	10.0 mL

Table 21.1: Other well sizes and suggested volumes of medium

Rescue of ES Cell Lines using RESGRO™ Culture Medium

RESGRO™ Culture Medium has the capacity to rescue traditional ES cell lines that have started drifting and either generate low percentage chimeras or have lost germline transmission capability. Differentiated ES cells not visible with traditional ES cell culture medium, will become visible with RESGRO™ medium. After 2 passages, a clear difference is seen between differentiated and undifferentiated ES cells. At that moment, it is recommended to perform a subcloning to select the undifferentiated cells.

The selection procedure should be repeated if some differentiation is still present after one subcloning procedure.

Please refer to **www.millipore.com** for additional data and information.

Materials & Reagents required:

- RESGRO™ Culture Medium (Catalogue Nos. SCM001, SCM002)
- EmbryoMax® L-Glutamine Solution (Catalogue No. TMS-002-C)
- Cellulose acetate, PVDF or PES filters
- Centrifuge
- ES Cell Medium (see Section XXVI)
- Incubator, 37 °C/5% CO_2
- Pipette
- PMEF Feeder cell coated culture plates (see Section IV)
- 0.05% Trypsin-0.53mM EDTA (Catalogue No. SM-2002-C)
- Water Bath, 37 °C

Procedure:

1. Prepare RESGRO™ Culture Medium for use by adding 10 mL of L-Glutamine Solution (200mM) to 500 mL of RESGRO™ Culture Medium.
2. Culture the ES cells in RESGRO™ Culture Medium for 2 passages on a monolayer of PMEF feeder cells.
3. After 2 passages, replate 1/3–1/5 of the cell suspension on the same size plate without PMEF feeder cells.
4. After 2 days, a clear difference will be observed between 3-dimensional (undifferentiated) and flat growing (differentiated) colonies. By tapping the dish, the undifferentiated colonies will detach.
5. Collect the supernatant (which will contain the undifferentiated cells) and discard the dish containing the differentiated cells.
6. Centrifuge the supernatant and remove the medium.
7. Add 0.5 mL of Trypsin-EDTA to the cell pellet.
8. Pipette up and down with a 1 mL pipette (do not use pipette tip of smaller volume).
9. Place the cell suspension in a water bath at 37 °C for 1.5 minutes.
10. Pipette up and down, 10 times (with a 200 µL pipette tip or a 1 mL pipette).
11. Add 9.5 mL of RESGRO™ Culture Medium.
12. Centrifuge and remove the supernatant.
13. Add an appropriate volume of RESGRO™ Culture Medium, which will depend upon the final volume that you prefer to plate the cells. For 6-well plates, it is recommended that the cells be suspended in 4 mL of RESGRO™ Culture Medium. Plate 1/3–1/6 of the ES cells on wells containing mitotically inactivated PMEF feeder cells. Alternatively, ES cells can be cultured in ES Cell Medium containing ESGRO® supplement.

    NOTE: Avoid contact between the colonies. If the ES cells have been plated at too high a density, replate ES cells at a lower density the following day.

ES Cell Line	Medium & Method used	No. of Embryos Transferred	No. of Pups Born	No. of Chimeras Born	Percentage Chimerism
R1#19 Knockout clone	Traditional medium Blastocyte injection	56	7	1	1 x 10%
R1#19 Knockout clone	RESGRO™ medium Blastocyte injection	64	27	20	3 x 5%
					3 x 10%
					1 x 20%
					2 x 30%
					4 x 40%
					2 x 50%
					2 x 60%
					2 x 70%
					1 x 80%
129SvEv Wild-type clone	Traditional medium Diploid aggregation	40	28	4	1 x 2%
					1 x 5%
					1 x 10%
					1 x 50%
129SvEv Wild-type clone	RESGRO™ medium Diploid aggregation	106	25	25	11 died
					1 x 10%
					1 x 90%
					12 x 100%
C57B1/6 Knockout clone	Traditional medium Blastocyte injection	50	8	0	0
C57B1/6 Knockout clone	RESGRO™ medium Blastocyte injection	96	38	19	2 died
					1 x 2%
					3 x 5%
					4 x 10%
					1 x 20%
					2 x 30%
					1 x 60%
					3 x 70%
					2 x 80%

Table 22.1: Improved efficiency of Murine ES cell lines using RESGRO™ Culture Medium

ES Cell Line Derivation using RESGRO™ Culture Medium

The efficiency of ES cell derivation is greatly strain dependent. To date, very few murine ES cell lines are available from inbred strains other than 129 strains, and those derived have generally been obtained with low success rates. Furthermore, ES cells derived from strains other than 129 are in general more difficult to propagate *in vitro*. Especially at high passage number and after genetic manipulation, these cell lines generate chimeras less efficiently and contribute less frequently to the germline.

RESGRO™ Culture Medium enables the efficient derivation and maintenance of ES cell lines from several inbred mouse strains, including certain strains that were previously considered to be non-permissive for ES cell derivation. A recent study demonstrated that RESGRO™ medium allowed the derivation of ES cell lines from inbred strains other than 129 (including FVB, a strain previously considered to be non-permissive for ES cell derivation and C57Bl/6N, BALB/c, 129/SvEv and DBA/2N mouse strains).

The following protocol is based upon that used by Schoonjans L. et al. (Stem Cells 21:90-97. 2003). Please refer to this reference for comprehensive details on the application of RESGRO™ Culture Medium for ES cell derivation.

Mouse Strain	Blastocysts Cultured	Established ES Cell Lines		No. Germline Competent ES Cell Lines / No. ES Cell Lines Cultured
	(n)	(n)	(%)	
C57B1/6N	35	18	51	10/11
FVB/N	20	8	40	6/9
BALB/c	34	15	44	7/7
129SvEv	10	6	60	4/4
DBA-2/N	34	13	38	3/3

Table 23.1: Efficiency of ES Cell Derivation and Germline Competence with RESGRO™ Culture Medium

Materials & Reagents required:

- RESGRO™ Culture Medium (Catalogue Nos. SCM001, SCM002)
- EmbryoMax® L-Glutamine Solution (Catalogue No. TMS-002-C)
- 96-well plates coated with PMEF Feeder cells (see Section IV)
- Cellulose acetate, PVDF or PES filters
- Incubator, 37 °C/5% CO_2
- 0.25% Trypsin-1mM EDTA (Catalogue No. SM-2003-C)

Procedure:

1. Collect 3.5 to 4.5 day old blastocyst stage mouse embryos and plate on a 96-well dish covered with a freshly prepared monolayer of PMEF feeder cells (see Section IV).
2. Prepare RESGRO™ Culture Medium for use by adding 10 mL of L-Glutamine Solution (200 mM) to 500 mL of RESGRO™ Culture Medium.
3. During the first 2 days remove only 75% of the medium and replace gently with fresh RESGRO™ Culture Medium (in order to avoid detachment of the blastocysts).
4. After attachment of the blastocysts, replace the medium completely on a daily basis with RESGRO™ Culture Medium.
5. After 5–6 days in culture, remove the inner cell mass (ICM) outgrowth from the trophoectoderm. Replate the cells following trypsinization with 0.25% Trypsin-1mM EDTA on a 96-well plate covered with a monolayer of PMEF feeder cells.
6. Culture the ES cells until 70–80% confluent, then replate on larger culture dishes.
7. Passage ES cells every 2–4 days on freshly prepared feeder layers, and replace with fresh RESGRO™ Culture Medium daily.

Notes

Cellular Reprogramming using STEMCCA™

Reprogramming using STEMCCA Lentiviral Technology

Induced pluripotent stem (iPS) cells can be generated from somatic cells by the ectopic expression of the four Yamanaka transcription factors, Oct4, Klf4, Sox2, and c-Myc (OKSM). Merck Millipore's STEMCCA™ polycistronic (OKSM) lentivirus reprogramming kits contain high titer Cre-excisable and non-Cre-excisable polycistronic (OKSM) lentivirus and Polybrene® transfection reagents that have been validated for the generation of mouse and human iPS cells from mouse embryonic fibroblasts (MEFs) and human foreskin fibroblasts (HFFs), respectively.

Materials & Reagents required:

- EF1α-STEMCCA (OKSM) Lentivirus: (Catalogue Nos. SCR510, SCR511, SCR530, SCR531, SCR544, SCR545, SCR512, SCR513, SCR548) One (1) vial containing 15 µL of high titer lentivirus. For exact titer refer to the label on the front of manual.
- Polybrene® 10 mg/mL: (Catalogue No. TR-1003-G) One (1) vial containing 50 µL of 10mg/mL stock of Polybrene® transfection reagent.
- 6-well plates, culture flasks, dishes (TC grade)
- PMEF Feeder Cell Culture Medium (see formulations Section XXVI)
- PMEF cells, not mitomycin-C treated (Catalogue No. PMEF-CFL)
- EmbryoMax® 0.1% Gelatin Solution (Catalogue No. ES-006-B)
- EmbryoMax® Complete ES Cell Media w/15% FBS and mLIF (Catalogue No. ES-101-B)
- Trypsin-EDTA Solution (0.25% Trypsin & 1 mM EDTA) (Catalogue No. SM-2003-C)
- Accutase™ Cell Dissociation Solution (Catalogue No. SCR005)
- Recombinant Human FGF-2 (Catalogue No. GF003)
- PMEF cells, growth-arrested, mitomycin-C treated (Catalogue No. PMEF-CF)
- Adenovirus expressing Cre recombinase and GFP (Vector Biolabs Catalogue No. 1710)
- DNeasy® Blood and Tissue Kit (QIAGEN Catalogue No. 69504).

Procedure:

Part 1: Reprogramming Mouse Embryonic Fibroblasts

IMPORTANT NOTE: The following protocol has been optimized using early passage primary mouse embryo fibroblasts (MEFs) and can be used as a guide to further optimize reprogramming of other somatic cells derived from rodents.

DAY 0

1. Coat a sterile 6-well plate with 0.1% gelatin solution (Catalogue No. ES-006-B). Use 2 mL volume per well. Incubate for at least 30 minutes before using. Aspirate the gelatin solution just before seeding the MEFs or target rodent cells.
2. Make up 50 mL PMEF Feeder Cell Culture Medium (see formulations Section XXVI). Sterile filter with 0.22 µm filter.
3. Seed 1 x 10^5 actively proliferating p3 mouse embryonic fibroblasts (Catalogue No. PMEF-CFL) in 3 mL PMEF Feeder Cell Culture Medium into each well of a 0.1% gelatin coated 6-well plate. Incubate overnight in a 37 °C, 5% CO_2 incubator. It is recommended to use early passage MEFs.

DAY 1

4. Replace the media with 3 mL fresh PMEF Feeder Cell Culture Medium per well. Add 1.5 µL Polybrene® transfection reagent (Catalogue No. TR-1003-50UL) to each well that is to be infected with the virus. Final Polybrene® concentration should be 5 µg/mL. Set the plate aside in 37 °C, 5% CO_2 incubator until ready to add the virus.
5. Using the equation provided below, determine the volume of virus required to achieve a multiplicity of infection (MOI) of at least 20. Please make note of the titer as the viral titer may vary slightly from lot to lot. An MOI of 20 will typically yield 3-7 mouse iPS cell colonies from p3 MEFs. Using an MOI < 20 is not recommended as the results are variable and may yield very few to no colonies.

Virus volume (µL) required = (Number MEFs)(Desired MOI)(1000 µL)/ (Virus Titer (IFU/mL)

EXAMPLE: If the number of cells in the well at the time of transduction is 1 x 10^5, the viral titer is 3 x 10^8 IFU/mL, and a desired MOI is 20, then the volume of virus required for 1 well of a 6 well plate is:

$$\frac{(1\times10^5)(20)(1000\ \mu L)}{(3\times10^8\ IFU/mL)} = 6.6\ \mu L$$

NOTE: Use the actual viral titer located on the label on the front of the manual in the equation above to determine the actual volume of virus to add.

6. Thaw 1 vial of EF1α-STEMCCA™-LoxP (OKSM) Lentivirus at room temperature and quickly place the vial on ice after it is thawed. Quickly centrifuge the vial to spin down the contents. Keep the virus on ice and proceed immediately to the next step.
7. Add the required volume of thawed virus directly to the wells containing the attached MEFs (from Step 4). Gently rock the plate from side to side to thoroughly mix the virus onto the MEFs. Incubate overnight in a 37 °C, 5% CO_2 incubator.

DAY 2

8. Exchange PMEF Feeder Cell Culture Medium with 3.0 mL Complete ES Cell Media with 15% FBS and LIF (Catalogue No. ES-101-B).

DAY 4 – DAY 13

9. Exchange with 3 mL fresh Complete ES Cell Media with 15% FBS and LIF (Catalogue No. ES-101-B) every other day for a total of 10 – 13 days. Mouse iPS cell colonies start to emerge around day 7–10.
10. Mouse iPS cell colonies can be selected and clonally expanded (typically around Day 12 – 14) when they reach an approximate size where the colony fits into the frame of a 10X magnification view.

Part 2: Clonal Expansion of Mouse iPS Colonies

At approximately Day 14 or when the mouse iPS colonies are of sufficient size (see step 10), they can be picked for clonal expansion, freezing, and subsequent excision with Adeno-Cre recombinase.

DAY BEFORE PASSAGING

11. One day prior to passaging the iPS colonies, prepare a fresh 6-well plate with inactivated MEFs to support the expansion of the mouse iPS cells as follows.
    a. Coat each well of a fresh sterile 6-well plate with 2 mL of 0.1% gelatin solution (Catalogue No. ES-006-B). Incubate for 30 minutes at 37 °C. Set aside until ready to receive inactivated MEFs.
    b. Aspirate the 0.1% gelatin coating solution from each well before seeding the inactivated MEFs. Thaw inactivated MEFs (Catalogue No. PMEF-CF). Count the number of thawed MEFs and seed 4 x 10^5 cells per well of a 6-well dish. Use PMEF Feeder Cell Culture Medium to culture the cells. Total volume per well should be 3 mL. Incubate overnight in a 37 °C, 5% CO_2 incubator.

DAY OF PASSAGING

12. On the day of passaging add 100 mL of 0.25% Trypsin-EDTA (prewarmed to 37 °C) to a 15 mL conical tube for each mouse iPS colony to be picked. For example, for 10 iPS colonies, prepare 10 separate conical tubes containing 0.25% trypsin-EDTA. Set aside.
13. Under a dissecting microscope in a laminar flow hood, scrape and pipette up one iPS colony and deposit the pieces into the 15 mL conical tube containing 0.25% trypsin-EDTA. Repeat this step for each iPS colony to be clonally expanded, being careful to keep each iPS colony in separate conical tubes.
14. Incubate the 15 mL conical tubes at room temperature for 5-10 minutes to allow the trypsin to dissociate the cell colonies.
15. Add 5 mL fresh Complete ES Cell Media with 15% FBS and LIF to each 15 mL conical tube containing the dissociated cell colonies.
16. Using a 5 or 10 mL pipette, slowly pipette up and down to break apart any remaining cell clumps.
17. Discard the media from the plate of inactivated MEFs (from Step 11) and add the dissociated mouse iPS colony from each 15 mL conical tube (approximately 5 mL volume) into separate wells of the 6-well dish containing inactivated MEFs. Incubate the 6-well dish in a 37 °C, 5% CO_2 incubator.

    NOTE: It is important to avoid cross-colony contamination between mouse iPS clones. Therefore, each well of the 6-well dish should only contain dissociated cells from one mouse iPS colony.
18. Replace with fresh Complete ES Cell Media with 15% FBS and LIF the following day and every other day thereafter for two weeks or until the mouse iPS colonies are 80% confluent.
19. When mouse iPS clones are 80% confluent, they can be further expanded and frozen. Freezing back vials of each mouse iPS clone before proceeding with the Adeno-Cre recombinase excision is strongly recommended.
20. After freezing back multiple vials of cells per clone, each mouse iPS clone can be grown in a T25 flask to 80% confluency for subsequent excision analysis.

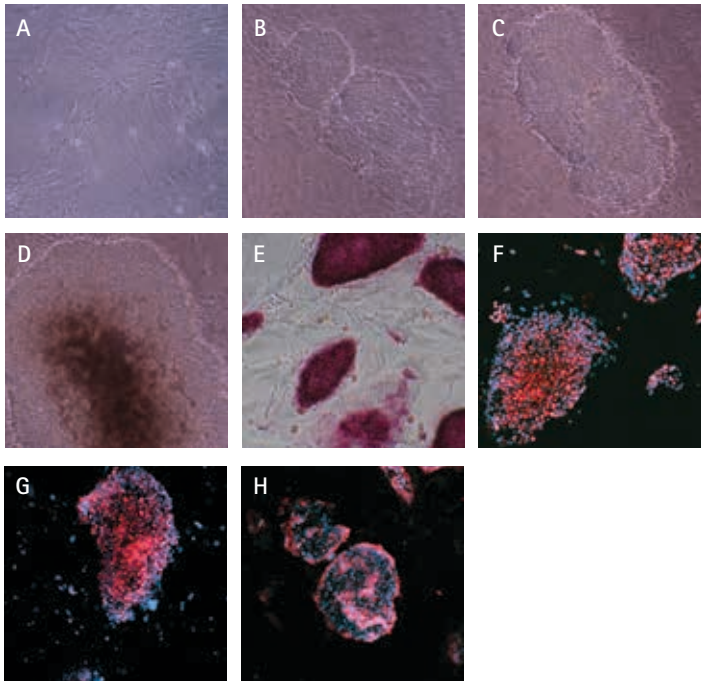


Figure 24A.

Mouse iPS cells derived from MEFs (passage 3) infected with the STEMCCA™ constitutive polycistronic (OKSM) lentivirus have cell morphology and staining characteristics of mouse ES cells. Phase contrast images of uninfected (A) and a single mouse iPS cell colony monitored over the course of five (B), seven (C), and ten (D) days after infection. Mouse iPS colonies that filled the frame of a 10X magnification view were deemed ready to be passaged (C). Overgrown colonies developed necrotic cells at the center (D). Passage 3 mouse iPS cells exhibited high alkaline phosphatase activity (E, SCR004) and expressed high levels of Oct4 (F), Sox2 (G), and SSEA-1 (H). Cell nuclei were counterstained with DAPI (blue).

Part 3: Excision of Ef1α-STEMCCA™-LoxP Viral Transgenes

IMPORTANT NOTE: A full demonstration of excision requires a lengthy protocol of cloning and subcloning iPS colonies and will require an average of 30-50 days to complete.

ONE DAY PRIOR TO EXCISION ANALYSIS:

21. Prepare a 10 cm plate with inactivated MEFs to support the expansion of the mouse iPS clones. Refer to step 11 for exact protocol. Please note that for 10 cm plates, inactivated MEFs should be plated at 1 x 10^6 cells.

DAY OF EXCISION ANALYSIS:

22. To each T25 flask containing approximately 80% confluent mouse iPS clones (from step 20), add 3-5 mL of 0.25% trypsin-EDTA and incubate in a 37 °C incubator for 3 minutes.
23. Inspect the flask and ensure the complete detachment of cells by gently tapping the side with the palm of your hand. Confirm under the microscope that the cells are in single-cell suspension.

    NOTE: It is critical to obtain a single cell suspension of each mouse iPS colony before initiating the adenoviral Cre recombinase infection.
24. Apply 10 mL Complete ES Cell Media with 15% FBS and LIF (pre-warmed to 37 °C) to each flask. Pipette up and down several times to ensure single cell suspension and collect the cell suspension to a 15 mL conical tube.
25. Centrifuge for 5 minutes at 300 xg to pellet the cells. Remove the supernatant and resuspend with 10 mL fresh Complete ES Cell Media with 15% FBS and LIF (pre-warmed to 37 °C). Pipette up and down several times to ensure single cell suspension.
26. MEF depletion: Transfer the singly dissociated cell suspension to a fresh gelatin coated T75 flask. Incubate in a 37 °C, 5% CO_2 incubator for 45 minutes to allow any MEFs to adhere to the flask.
27. After 45 minutes, collect the floating mouse iPS cells into 15 mL conical tube.

    NOTE: It is critical to remove as many MEFs as possible before initiating the adenoviral Cre recombinase infection to ensure that the adenovirus is not diluted by the MEFs. If necessary, repeat the MEF depletion (steps 26-27).
28. Centrifuge at 300 xg for 10-15 minutes to pellet the cells.
29. Remove supernatant and resuspend the cell pellet in a small volume (1 mL) of Complete ES Cell Media with 15% FBS and LIF. Pipette up

and down, being careful not to introduce bubbles, to ensure that cells are singly dissociated. If cells are not singly dissociated, use a fire-polished Pasteur pipette to pipette to a single cell suspension.

30. Count the number of cells using a hemocytometer.
31. Aliquot 1 x 10^5 singly dissociated cells to a microcentrifuge tube.
32. Thaw the Adeno-Cre recombinase (Vector Biolabs Catalogue No. 1710) on ice until ready to use.
33. Using the equation provided below, determine the volume of virus required to achieve a multiplicity of infection (MOI) of 3000.

Virus volume (mL) required = (Number iPS cells from step 31) (Desired MOI)(1000ul)/(Virus Titer (IFU/mL)

EXAMPLE: If the number of cells being transduced is 1 x 10^5, the viral titer is 1 x 10^{10} IFU/mL, and the desired MOI is 3000, then the volume of virus required is:

(1 x 10^5 cells)(3000 MOI)(1000 µL)/ (1 x 10^{10}IFU/mL)

= 30 uL virus required

34. Add the calculated volume of Adeno-Cre recombinase (from step 33) to the microcentrifuge tube containing the singly dissociated cells (from step 31).
35. Bring the final volume in the microcentrifuge tube to 250 mL by adding Complete ES Cell Media with 15% FBS and LIF.
36. Incubate the microcentrifuge tube in a 37 °C, 5% CO_2 incubator for 6 hours. During this incubation time, pipette the cell mixture up and down every two hours to ensure that the cells remain in a single cell suspension.
37. After the 6 hour incubation, add the entire cell suspension (approximately 250 mL) to the 10 cm plate containing inactivated MEFs from step 21.
38. Inspect the plate under a microscope to ensure that the iPS cells are in single cell suspensions after plating onto MEF feeder layer.
39. Replace media the following day and every other day thereafter with fresh Complete ES Cell Media with 15% FBS and LIF until colonies start to appear. Colonies will become apparent after 2-3 weeks.
40. After 2-3 weeks, manually pick as many individual colonies as possible from the 10 cm plate. Select smaller colonies that are spaced apart as these colonies have more likely arisen from single cells. Follow the protocol outlined in Section 2, steps 11 – 19 to expand individual colonies from the 10 cm plate to separate wells of 6-well plate for subsequent expansion of clones for freezing and genomic DNA isolation for PCR analysis of excision. It is important to avoid cross

colony contamination as this will affect the PCR excision analysis.

NOTE: Freezing back multiple vials of each mouse iPS clone that has undergone the Cre recombinase excision reaction before proceeding with PCR analysis is strongly recommended.

Part 4: PCR Analysis of Genomic DNA

41. Isolate genomic DNA from mouse iPS colonies that have undergone the excision process (from step 40). As a control, also isolate genomic DNA from mouse iPS colonies that have not undergone the excision process (from step 19). Using a commercial kit, follow the manufacturer's instructions regarding the isolation of genomic DNA. For example, the DNeasy® Blood and Tissue Kit from QIAGEN (Catalogue No. 69504) may be used to obtain genomic DNA from a confluent T25 flask of each mouse iPS clone (typical cell yield = 5×10^5 – 1×10^6 cells).

42. Use the following primers (not provided) to set up PCR reactions for the analysis of the excision reaction in the iPS clones.

    NOTE: The WPRE primer set is specific to the viral genome while the GAPDH primer set is used to normalize for cDNA template between PCR reactions.

    Marker Primer Sequence Product Size

    Forward 5' - ACG AGC ACA AGC WPRE TCA CCT CT - 3'

    Reverse 5' - TCA GCA AAC ACA GTG CAC ACC - 3'

    350 bp

    GAPDH Forward 5' - CCT TCA TTG ACC TCA ACT AC - 3'

    Reverse 5' - GGA AGG CCA TGC CAG TGA GC -3'

    500 bp

43. For each iPS clone, prepare the following PCR reaction mix for each primer set (WPRE and GAPDH) in separate, clean RNAse-free PCR tubes.

    Component Per Reaction

    - 10X PCR Reaction Buffer 5.0 mL*
    - 10 mM dNTPs 1.0 mL
    - 50 mM $MgCl_2$ 1.5 mL*
    - 10 mM primer mix (WPRE or GAPDH) 1.0 mL
    - Taq Platinum DNA Polymerase 0.4 mL*
    - 200 ng genomic DNA template x mL
    - Sterile distilled water to final 50 mL

    NOTE: The volumes of the 10X PCR reaction buffer, $MgCl_2$ and DNA polymerase should be based upon the instructions provided with the DNA polymerase enzyme.

44. Thoroughly mix the reaction mixture by pipetting up and down.

45. Briefly centrifuge PCR tubes.
46. Transfer the reactions to a PCR machine and perform PCR using the following optimized cycling parameters:
    a. Initial denaturation at 94 °C for 2 minutes.
    b. 30 cycles of:
    95°C 30 seconds
    65°C 45 seconds
    72°C 45 seconds
    c. Final extension at 72 °C for 10 minutes.
47. After the PCR reactions have terminated, the samples can be stored at 4 °C. For long term storage, samples should be stored at -20 °C.
48. Analysis of Results: Following the PCR reaction, prepare a 1.5% agarose gel containing ethidium bromide and directly load 10 to 25 mL of each PCR reaction mix containing an appropriate amount of 5X loading dye to each well. Include a molecular weight marker with bands between 100 bp to 1000 bp in an adjacent well. The gel can be analyzed when the red dye has migrated to 2/3 distance from the loading point.

    The GAPDH band is used to normalize for gel loading. Ideally a band corresponding to 500 bp (GAPDH) should be present at comparable intensity in all the PCR samples.

    Presence of a 350 DNA band, corresponding to the WPRE transcript indicates that the viral transgene has not been excised in the particular mouse iPS clone.

    Absence of a 350 DNA band indicates that the viral STEMCCA™ transgene has been successfully excised in the particular mouse iPS clone.

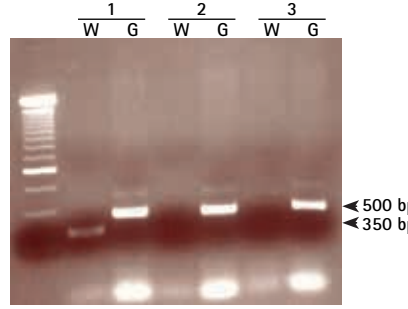


Figure 24B.

PCR analysis of mouse iPS clones that have undergone Cre recombinase excision. Presence of a 350 bp PCR product, corresponding to the WPRE transcript specific to the STEMCCA™ lentiviral genome indicates that the viral transgene has not been excised in the particular mouse iPS clones (G, clone 1). The absence of a 350 bp PCR product indicates that the viral STEMCCA™ transgene has been successfully excised (G, clones 2, 3). PCR reactions were normalized using GAPDH. A 500 bp PCR product corresponding to GAPDH is present at comparable intensity in all PCR samples. W = WPRE; G = GAPDH.

Notes

Troubleshooting Information

The two most common problems encountered when generating knockout mice are (1) ES cell differentiation, and (2) the inability to generate chimeras once a targeted ES cell clone has been established.

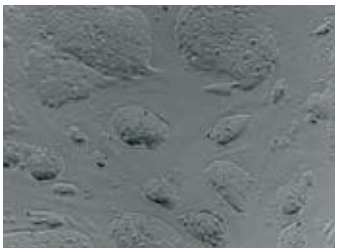


Figure 25A.
Optimal confluence of ES cells (10x).

In Table 25.1 are a number of common causes of ES cell differentiation and recommendations on how to help prevent differentiation from occurring. The extent of ES cell differentiation can be determined by examining the morphology of the ES cell colonies, or more thoroughly assessed using Merck Millipore's ES Cell Characterization Kit or Alkaline Phosphatase Detection Kit (see Section XXVII). These kits contain monoclonal antibodies to ES cell markers and reagents for Alkaline Phosphatase detection that permit a discrimination of pluripotent and differentiated ES cells.

Also included in Table 25.2 are a number of reasons that often cause the slow growth of ES cells. Table 25.3 contains possible causes for the lack of generating chimeras.

Cause of Slow ES Cell Growth	Recommendations
Incorrect concentration of mLIF	ESGRO® mLIF medium supplement should be used at the recommended concentration of 1000 units/mL in ES cell media.
Expiry date of ESGRO® Media	Always check the date of each batch prior to use. Medium should be less than 4 weeks old as glutamine by-products could be toxic to ES cells. ES cells should be fed with fresh media every 2–3 days or when media discolors.
Serum	New batches of Fetal Bovine Serum should be tested for the effect of inducing differentiation. ES cell medium usually requires a serum concentration between 10% and 20%. We recommend the use of EmbryoMax ES cell qualified serums.
Lack of passaging	ES cells should be passaged every 2–3 days as frequent passaging removes differentiated cells. Only undifferentiated ES cells will survive frequent passaging. Refer to Images 25A–25G for illustrations of ES cell confluency and differentiated ES cells.
Disinfectants	Disinfectants such as Roccal® or Lysol® preparations should be avoided in the Tissue Culture Room and incubator where ES cells are cultured. Some disinfectants have been suspected to cause differentiation by use in water baths and aerosols created by spray wiping. The use of 70% ethanol to clean tissue culture surfaces is recommended.
Feeder cells	If differentiation cannot be controlled using ESGRO® mLIF medium supplement with gelatinized plates, it may be necessary to culture ES cells on a feeder layer. Please refer to Section 4 for the recommended number of growth arrested fibroblasts. ESGRO® supplement should be added at a concentration of 1000 units/mL to assist in maintaining undifferentiated ES Cells.
Incubator settings	Ensure that the incubator readings are correct at 37 °C and 5% CO_2.
Rate of growth of ES cells	Slow growing ES cells will be most likely to undergo differentiation. Increase serum concentration if cells are not growing quickly enough.
Gelatin	It is preferable to use cell culture grade gelatin at all times (even if using feeder layers) as gelatin minimizes surface differences on the tissue culture plates. EmbryoMax® ES cell qualified 0.1% gelatin solution is recommended.
Low level of contamination	Regular use of Pen/Strep in media can often mask a low level contamination with agents such as mycoplasma. It is recommended to routinely test your ES cell lines for mycoplasma contamination on a periodic basis.

Table 25.1: Common causes of ES cell differentiation and recommendations.

Cause of Slow ES Cell Growth	Recommendations
Insufficient serum	Ideally a concentration of between 10% and 20% serum is used; however, this will vary according to each ES cell strain. It is recommended to routinely test new batches of serum to determine the optimum concentration required for fast growth without inducing excessive differentiation. We recommend the use of EmbryoMax® ES cell qualified serum.
Low number of ES cells	ES cells grow optimally when slightly crowded. Plate a higher density from frozen stocks or passage cells to increase individual colony numbers. Refer to the images below for illustrations of ES cell confluency.

Table 25.2: Causes of slow growth of ES cells and recommendations.

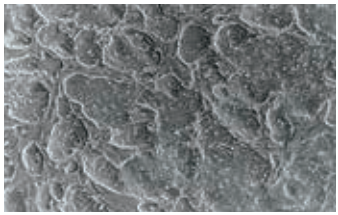


Figure 25B.
Over confluent ES cells (10x).

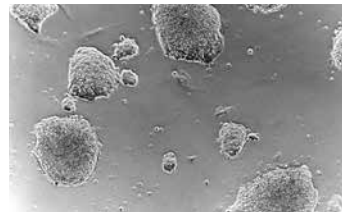


Figure 25D.
ES cells plated at low density (10x). Cells require another passage to increase individual colony numbers.

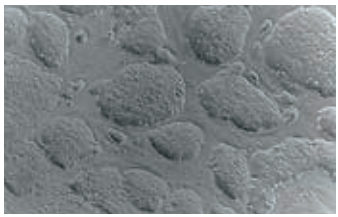


Figure 25C.
ES Cells at a density ready for passage (10x).

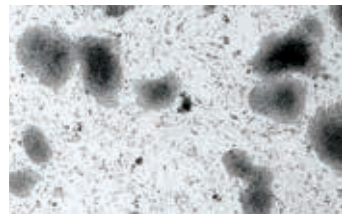


Figure 25E.
ES cells plated at too low density with fibroblasts (10x). Cells require another passage to increase individual colony numbers. Note the darkened areas in the center of each colony where ES cells are dying.

Cause of Lack of Chimeras	Recommendations
Time taken to culture the ES cells	Once the targeted ES cell clone is identified, minimize the time that the ES cells remain in culture. It is preferable to passage ES cell lines no more than 1–2 times prior to microinjection and/or aggregation.
Karyotype the ES cells	The routine karyotype analysis of ES cells is recommended, particularly when the ES cell lines are reaching a high passage number.
Undetected differentiation	Some degree of differentiation may go undetected by routine microscopic examination, and therefore regular passage of the ES cells is necessary. For a detailed assessment of ES cell differentiation, Merck Millipore's ES Cell Characterization Kit or Alkaline Phosphatase Detection Kit are recommended (see Section XXVII). Please refer to Section XXII of this manual for information on the use of RESGRO™ Culture Medium for the removal of differentiated ES cells within a cell line.
ES cell passage number	In general, the best chimeras are generated from low passage number ES cells. If cell lines have been cultured for a high number of passages, it is recommended to use a lower passage frozen stock.
Number of ES cells microinjected	Most often ES cell lines are microinjected at approximately 8–12 cells/blastocyst.
Time taken prior to microinjection	It is recommended that ES cells be microinjected as soon as possible after removing them from the tissue culture plates, and not left on ice or at room temperature for extended periods of time.
Compatibility of ES cells and mouse strain	Prior to any microinjection or aggregation procedures, please check the combination of embryo strains and ES cells, as some strains have been reported to be incompatible.

Table 25.3: Causes for the lack of chimeras.

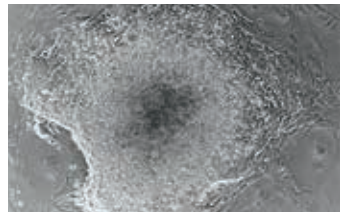


Figure 25F.

Highly Differentiated ES cells (25x). Note the loss of discreet ES cell colony border, the formation of cobblestone-like cells and ES cells differentiating into fibroblasts that extend outwards from the colony.

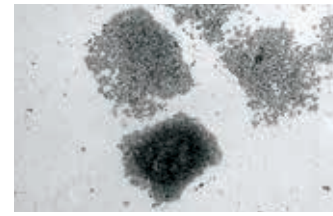


Figure 25G.

Highly Differentiated ES cells — plated at too low density (10x). Note that these cells are beyond rescue by passaging.

Formulations

PMEF Feeder Cell Culture Medium

Description	% (v/v)	Catalogue No.
EmbryoMax® DMEM, 500 mL	N/A	SLM-220-B
EmbryoMax® ES Cell Qualified Fetal Bovine Serum	10%	ES-009-B
EmbryoMax® Penicillin-Streptomycin	1%	TMS-AB2-C
EmbryoMax® L-Glutamine Solution (100x)	1%	TMS-002-C

Serum Containing Mouse ES Cell Culture Medium

Description	% (v/v)	Catalogue No.
EmbryoMax® DMEM	N/A	SLM-220-B
EmbryoMax® ES Cell Qualified Fetal Bovine Serum	15–20%	ES-009-B
EmbryoMax® Nucleosides (100x)	1%	ES-008-D
EmbryoMax® Penicillin-Streptomycin (100x)	1%	TMS-AB2-C
EmbryoMax® Non-Essential Amino Acids (100x)	1%	TMS-001-C
EmbryoMax® L-Glutamine Solution (100x)	1%	TMS-002-C
EmbryoMax® 2-Mercaptoethanol (100x)	1%	ES-007-E
ESGRO® mLIF Medium Supplement	1000 units/mL	ESG1106

NOTE: For selection add 150–350 µg/mL of Neomycin G418 (Sigma, G-9516) or 75–100 µg/mL of Hygromycin B (Sigma, H-3274).

Lysis Buffer

Description	Qty
1M Tris pH 8.5	2 mL
0.5M EDTA	0.2 mL
20% SDS	0.2 mL
5M NaCl	0.8 mL
Proteinase K (20 mg/mL)	100 µL
RNaseA (10 mg/mL)	100 µL
Nanopure water	16.8 mL

Leishman's Stain

Make to 0.2% w/v solution in methanol.

Ordering Information

Mouse Embryonic Stem Cell Lines

Description	Qty	Catalogue No.
PluriStem™ B6-White Murine ES cell line	5 x 10^6 cells	SCR011
PluriStem™ 129S6 Murine ES cell line	5 x 10^6 cells	SCR012
EmbryoMax® 129/SVEV (S6) Murine ES cell line	5 x 10^6 cells	CMTI-1
EmbryoMax® C57/BL6 Murine ES cell line	5 x 10^6 cells	CMTI-2
ESGRO Complete™ Adapted C57/ BL6 Mouse ES Cell Line	5 x 10^6 cells	SF-CMTI-2
PluriStem® C57BL/6N Murine ES Cells	5 x 10^6 cells	SCC050
PluriStem® BALB/C Murine ES Cells	5 x 10^6 cells	SCC052
PluriStem® FVB/N Murine ES Cells	5 x 10^6 cells	SCC053
PluriStem® DBA/2N Murine ES Cells	5 x 10^6 cells	SCC054
PluriStem® C3H/HeN Murine ES Cells	5 x 10^6 cells	SCC055
PluriStem® NZW Murine ES Cells	5 x 10^6 cells	SCC013
MilliTrace™ Constituitive GFP Reporter Mouse Embryonic Stem Cell Kit	1 x 10^6 cells	SCR082
MilliTrace™ Nanog GFP Reporter Mouse Embryonic Stem Cell Kit	1 x 10^6 cells	SCR089

Primary Mouse Embryo Fibroblasts

Description	Qty	Catalogue No.
EmbryoMax® Primary Mouse Embryo Fibroblasts,Hygro Resistant,Strain C57/BL6	2.5 x 10^7 cells	PMEF-H
EmbryoMax® Primary Mouse Embryo Fibroblasts,Hygro Resistant, Not Mytomycin C treated, Strain C57/BL6	2.5 x 10^7 cells	PMEF-HL
EmbryoMax® Primary Mouse Embryo Fibroblasts, Neo Resistant, Strain FVB	2.5 x 10^7 cells	PMEF-N
EmbryoMax® Primary Mouse Embryo Fibroblasts, Neo Resistant, Not Mytomycin C treated, Strain FVB	2.5 x 10^7 cells	PMEF-NL
EmbryoMax® Primary Mouse Embryo Fibroblasts, Strain CF1, Mytomycin C treated	2.5 x 10^7 cells	PMEF-CF
EmbryoMax® Primary Mouse Embryo Fibroblasts, Not Mytomycin C Treated, Strain CF1	2.5 x 10^7 cells	PMEF-CFL

ESGRO Complete™ PLUS and ESGRO®-2i Cell Culture System

Description	Qty	Catalogue No.
ESGRO Complete™ PLUS Clonal Grade Medium	100 mL	SF001-100P
ESGRO Complete™ PLUS Clonal Grade Medium	500 mL	SF001-500P
ESGRO Complete™ Basal Medium	100 mL	SF002-100
ESGRO Complete™ Basal Medium	500 mL	SF002-500
ESGRO®-2i Medium	100 mL	SF016-100
ESGRO®-2i Medium	200 mL	SF016-200
ESGRO Complete™ Switch Kit	1 kit	SF004
ESGRO Complete™ Freezing Medium	50 mL	SF005
Accutase™ Solution for ESGRO Complete™ Medium	100 mL	SF006
ESGRO Complete™ Gelatin	500 mL	SF008
ESGRO Complete™ Enzyme Free Dissociation Solution	100 mL	SF009

Kits for ES Cell Differentiation

Description	Qty	Catalogue No.
ES2N Basal Medium	245 mL	SCM083
ES2N Complete Medium Kit	250 mL	SCM082
Mouse Embryonic Stem Cell Adipogenesis Kit	10rxn/kit	SCR100
Mouse Embryonic Stem Cell Neurogenesis Kit	10rxn/kit	SCR101
Embryoid Body (EB) Formation Medium	100 mL	SCM018

Kits for Detection of ES Cell Differentiation

Description	Qty	Catalogue No.
ES Cell Characterization Kit	1 kit	SCR001
Alkaline Phosphatase Detection Kit	1 kit	SCR004

Culture Media & Supplements

Description	Qty	Catalogue No.
ESGRO® mLIF Medium Supplement	1 x 10^6 units	ESG1106
ESGRO® mLIF Medium Supplement	1 x 10^7 units	ESG1107
Leukemia Inhibitory Factor, Recombinant Mouse	5 µg	LIF2005
Leukemia Inhibitory Factor, Recombinant Mouse	10 µg	LIF2010
Complete ES Cell Media with 15% FBS Serum and LIF	500 mL	ES-101-B
RESGRO™ Culture Medium	250 mL	SCM001
RESGRO™ Culture Medium	500 mL	SCM002
EmbryoMax® ES Cell Qualified FBS	500 mL	ES-009-B
EmbryoMax® DMEM, Low-bicarb, w/o glutamine & sodium pyruvate	500 mL	SLM-220-B
EmbryoMax® DMEM, Low-bicarb, w/o sodium pyruvate	500 mL	SLM-120-B
EmbryoMax® DMEM, w/o glutamine & sodium pyruvate	500 mL	SLM-021-B
EmbryoMax® Cell Culture, Freezing Medium (2x)	50 mL	ES-002-D
EmbryoMax® Cell Culture, Freezing Medium (2x)	10 x 10 mL	ES-002-10F
EmbryoMax® Cell Culture, Freezing Medium (2x)	5 x 10 mL	ES-002-5F

EmbryoMax® Culture Reagents

Description	Qty	Catalogue No.
EmbryoMax® 0.1% Gelatin in Sterile Water	500 mL	ES-006-B
EmbryoMax® Electroporation Buffer	50 mL	ES-003-D
EmbryoMax® Nucleosides (100x)	50 mL	ES-008-D
EmbryoMax® MEM Non-Essential Amino Acids (100x)	100 mL	TMS-001-C
EmbryoMax® 2-Mercaptoethanol (100x)	20 mL	ES-007-E
EmbryoMax® Filtered Light Mineral Oil	100 mL	ES-005-C
EmbryoMax® L-Glutamine Solution (100x)	100 mL	TMS-002-C
EmbryoMax® Penicillin-Streptomycin Solution	100 mL	TMS-AB2-C
EmbryoMax® DPBS (no Mg^{2+} or Ca^{2+})	1 L	BSS-1006-A
EmbryoMax® DPBS (no Mg^{2+} or Ca^{2+})	500 mL	BSS-1006-B
EmbryoMax® DPBS	1 L	BSS-1005-A
EmbryoMax® DPBS	500 mL	BSS-1005-B
EmbryoMax® 1M HEPES Buffer Solution	100 mL	TMS-003-C
EmbryoMax® Ultra Pure H_2O	1 L	TMS-006-A
EmbryoMax® Ultra Pure H_2O	500 mL	TMS-006-B
EmbryoMax® Ultra Pure H_2O	100 mL	TMS-006-C

EmbryoMax® Mouse Embryo Culture Reagents

Description	Qty	Catalogue No.
EmbryoMax® M2 Medium (1x), Phenol Red	50 mL	MR-015-D
EmbryoMax® M2 Medium (1x), Phenol Red & hyaluronidase	10 mL	MR-051-F
EmbryoMax® M2 Medium (1x), Powdered Media Kit	1 x 50 mL	MR-015P-D
EmbryoMax® M2 Medium (1x), Powdered Media Kit	5 x 50 mL	MR-015P-5D
EmbryoMax® M2 Medium (1x), Powdered Media Kit	5 x 10 mL	MR-015P-5F
EmbryoMax® Modified M16 Medium (1x), w/o Phenol Red	50 mL	MR-010-D
EmbryoMax® Modified M16 Medium (1x), Phenol Red	50 mL	MR-016-D
EmbryoMax® Modified M16 Medium (1x), Powdered Media Kit	1 x 50 mL	MR-010P-D
EmbryoMax® Modified M16 Medium (1x), Powdered Media Kit	5 x 50 mL	MR-010P-5D
EmbryoMax® Modified M16 Medium (1x), Powdered Media Kit	5 x 10 mL	MR-010P-5F
KSOM w/ 1/2 Amino Acids (1x), Glucose & Phenol Red	50 mL	MR-121-D
KSOM w/ 1/2 Amino Acids (1x), Glucose	50 mL	MR-106-D
KSOM w/ 1/2 Amino Acids (1x), Glucose w/o BSA	50 mL	MR-107-D
KSOM Powdered Media Kit	5 x 10 mL	MR-020P-5F

Cell Dissociation Reagents

Description	Qty	Catalogue No.
Accutase™ Solution	100 mL	SCR005
Accumax™ Solution	100 mL	SCR006
Enzyme Free Cell Dissociation Solution, Hank's Based	500 mL	S-004-B
Enzyme Free Cell Dissociation Solution, Hank's Based	100 mL	S-004-C
Enzyme Free Cell Dissociation Solution, PBS Based	500 mL	S-014-B
Enzyme Free Cell Dissociation Solution, PBS Based	100 mL	S-014-C
Trypsin 0.25% (1x)	100 mL	SM-2001-C
Trypsin 0.05%, EDTA (1x)	100 mL	SM-2002-C
Trypsin 0.25%, EDTA (1x)	100 mL	SM-2003-C
Low Trypsin 0.25%, High EDTA	100 mL	SM-2004-C
Low Trypsin 0.25%, High EDTA, w/ Phenol Red	100 mL	SM-2005-C

STEMCCA™ Reprogramming Kits

Description	Qty	Catalogue No.
Human STEMCCA™ Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	2 x 15 µL Lentivirus	SCR544
Human STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	2 x 15 µL Lentivirus	SCR545
Human STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKS/L-Myc) Lentivirus Reprogramming Kit	1 x 15 µL Lentivirus	SCR548
Mouse STEMCCA™ Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	1 x 15 µL Lentivirus	SCR510
Mouse STEMCCA™ Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	3 x 15 µL Lentivirus	SCR530
Mouse STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	1 x 15 µL Lentivirus	SCR511
Mouse STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	3 x 15 µL Lentivirus	SCR531
Mouse STEMCCA™ Dox-Inducible Polycistronic (OKSM) Lentivirus Reprogramming Kit	2 x 15 µL Lentivirus	SCR512
Mouse STEMCCA™ Cre-Excisable Dox-Inducible Polycistronic (OKSM) Lentivirus Reprogramming Kit	2 x 15 µL Lentivirus	SCR513
Mouse STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKS) Lentivirus Reprogramming Kit	2 x 15 µL Lentivirus	SCR518
Mouse iPS Cell Boost Supplement	1 Kit	SCM087
Human iPS Cell Boost Supplement	1 Kit	SCM088
Human iPS Cell Boost Supplement II	1 Kit	SCM094
iPS Selection Kit	1 Kit	SCR502

ESGRO® Medium Supplement Compatible ES Cell Lines

Cell Line	Reference
E14TG2a	Templeton N. et al. (1997). Gene Therapy 4: 700.
R1	Kywa S. et al. (1997). Exp. Anim. 46(4): 311.
D3	Tian L. et al. (1997). Biol. Reprod. 57:561.
J1	Doi Y. et al. (1998). J. Virology 72(2): 1586.
CCE (129/Sv)	Camenisch G. et al. (1996). Nucleic Acids Res. 24(19): 3707.
CGR8	Mehlan P. et al. (1997). J. Biol. Chem 272(50): 31657.
T/T GM6.15	Wilson V. & Beddington R. (1997). Dev. Biol. 192: 45.
AB1	Kyuwa S. (1997). Exp. Anim. 46(4): 311.
HD5	Williams L. et al. (1988). Nature 336: 684.
CBL63	Williams L. et al. (1988). Nature 336: 684.
GK129/2	Norris D. et al. (1994). Cell 77: 41.
PGK12.1	Norris D. et al. (1994). Cell 77: 41.

Table 28.1: Cell lines compatible with ESGRO® mLIF Medium Supplement alone.

Cell Line	Reference
H200	Gagneten et al. (1997). Nucleic Acids Res. 25(16): 3326.
CJ7	Rosti V. et al. (1997). J. Clin. Invest. 100(5): 1028.
AB1	Rucker E. et al. (1997). Mol. Rep. Dev. 48: 324.
MRL	Goulet J. et al. (1997). J. Immunol. 159(9): 4376.

Table 28.2: Cell lines compatible with ESGRO® mLIF Medium Supplement and feeder cell layer.

To Place an Order or Receive Technical Assistance

In Europe, please call Customer Service:

France: 0825 045 645
Germany: 01805 045 645
Italy: 848 845 645
Spain: 901 516 645 Option 1
Switzerland: 0848 645 645
United Kingdom: 0870 900 4645

For other countries across Europe, please call: +44 (0) 115 943 0840

Or visit: **www.merckmillipore.com/offices**

For Technical Service visit: **www.merckmillipore.com/techservice**

Get Connected!

Join Merck Millipore Bioscience on your favorite social media outlet for the latest updates, news, products, innovations, and contests!

facebook.com/MerckMilliporeBioscience

twitter.com/Merck4Bio

MERCK MILLIPORE

www.merckmillipore.com

Lit. No. PB4090ENEU 05/12 LS-SBU-12-06032