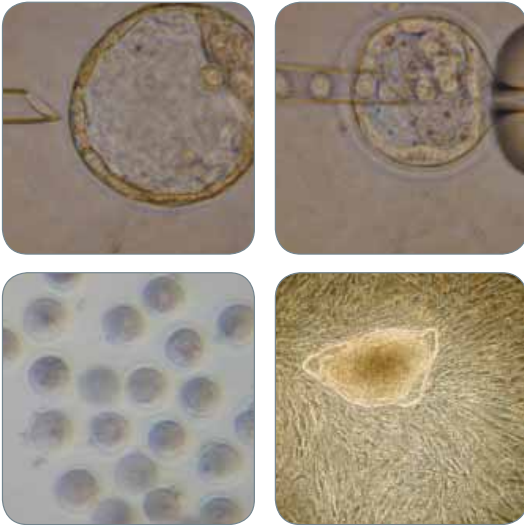


MILLIPORE

Murine Embryonic Stem Cell Culture



INSTRUCTION MANUAL

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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 germ-line tissue. Efficient procedures for the *in vitro* culture and maintenance of pluripotent ES cells have been crucial to the success of gene targeting experiments.

Millipore provides the largest and most comprehensive range of products for your mouse ES cell culture needs.

Highlights of this product range include:

- ESGRO mLIF Medium Supplement for maintaining undifferentiated ES cells
- ESGRO Complete cell culture system, a defined serum-free system for the maintenance and derivation of mouse ES cell lines in the absence of FBS and feeder cells
- RESGRO Culture Medium for the rescue of partially differentiated ES cell lines and improved ES cell derivation
- 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
- Range of ES cell lines derived from different mouse strains
- Mitotically inactivated and antibiotic resistant EmbryoMax® PMEF feeder cell lines
- EmbryoMax ES cell qualified reagents including DMEM, FBS and media supplements
- EmbryoMax media and reagents for mouse embryo culture

Millipore's range of ES cell-qualified reagents provides researchers with convenient and cost effective solutions for the reliable culture of ES cells. These products negate the need for researchers to screen lots of media, reagents and serum, thus delivering significant cost and time savings. Researchers can use the EmbryoMax range of products with confidence, as the same extensive validation processes behind our class-leading ESGRO products are used to qualify these new products for ES cell culture.

The cell culture protocols described in this manual include the *in vitro* culture of murine ES cells using the EmbryoMax range of ES cell qualified products 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 are methods for improving the efficiency of existing ES cell lines (ES cell rescue) and derivation of new 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.
	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
3	Feed ES cells; passage if required	V–VI
4	Feed ES cells; passage if required Prepare Targeting Vector: Linearize ~100 µg of targeting vector (each electroporation requires 1.5–30 µg of linearized vector), and precipitate with EtOH (no need to phenolize).	V–VI
5	Electroporation of ES cells	VII
6–10	Select for ES cell transformants	VIII
9–11	Pick ES cells	IX
12–13	Feed picked ES cells	IX
14	Freeze ES cell clones & retain duplicate wells to grow ES cells for DNA isolation Preparation of Genomic DNA for Southern blot analysis	X–XI X
16–19	Recover clones following analysis using RESGRO Culture Medium	XIII

Procedure for Coating Multiwell Plates with Gelatin Solution

Materials & Reagents required:

- EmbryoMax ES Cell Qualified 0.1% Gelatin Solution (Cat. No. ES-006-B)
- ESGRO Complete Gelatin Solution (Cat. 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 below. Note: Add enough Gelatin Solution to adequately cover the plasticware surface.

Table 3.1: Gelatin solution volume

Plate	Amount / Well
96-well	100 μ L
48-well	300 μ L
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

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.

Plating PMEF Feeder Cells

EmbryoMax Primary Mouse Embryo Fibroblasts (PMEF) feeder cells are supplied as frozen vials containing $5\text{--}6 \times 10^6$ 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:

- 15 mL tubes
- Centrifuge
- EmbryoMax Cryopreserved PMEF Feeder Cells (see Section 19)
- Gelatin coated Tissue Culture Plates or Flasks (see Section 3)
- Incubator, 37 °C/5% CO₂
- PMEF Feeder Cell Medium (see Section 18)
- Pipette
- Water Bath, 37 °C

Procedure:

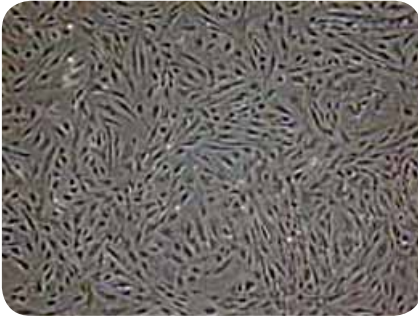
1. Prior to thawing PMEF feeder cells, coat plates/flasks with Gelatin solution (see Section 3)
2. Thaw PMEF vial(s) quickly in a 37 °C water bath and transfer to a 15 mL tube (already containing 10 mL of warm PMEF Feeder Cell Medium). Gently invert the tube to distribute, and centrifuge at 300 xg for 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% CO₂. Use images 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.

Plating PMEF Feeder Cells *continued*

Table 4.1: Recommended PMEF feeder cell suspension densities

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

Image 4A



PMEF feeder cells at the correct density

Image 4B



PMEF feeder cells at too low density

Image 4C



PMEF feeder cells at too high density

ES Cell Culture using ESGRO Medium Supplement

This manual contains protocols and reagents for the *in vitro* culture of murine ES cells using the EmbryoMax range of ES cell qualified products with ESGRO supplement. Included are methods for ES cell culture with and without the inclusion of a PMEF feeder cell layer. The choice of method is dependent upon the ES cell line used, as certain ES cell lines require the use of feeder cells. When using PMEF cells, the maintenance of undifferentiated ES cells will be improved by the addition of ESGRO supplement to the cell culture medium. For a listing of compatible ES cell lines please refer to Section 20.

Note: *Optimized protocols for the culture of B6-White (Cat. No. SCRO11) and 129S6 (Cat. No. SCRO12) ES cells can be found at www.millipore.com/chemicon.*

5.1 ES Cell Culture without PMEF Feeder Cells

Materials & Reagents required:

- Centrifuge
- EmbryoMax ES Cell Qualified DPBS (Cat. No. BSS-1006-A, Cat. No. BSS-1006-B)
- EmbryoMax ES Cell Qualified FBS (Cat. No. ES-009-B, Cat. No. ES-009-C)
- ES Cell Medium (see Section 18)
- Gelatin coated Tissue Culture Plates (see Section 3)
- Incubator, 37 °C/5% CO₂
- Pipette
- 0.05% Trypsin-0.53 mM EDTA (Cat. No. SM-2002-C)

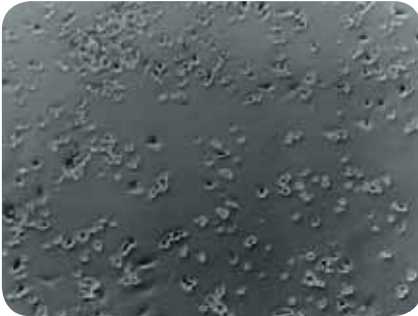
Procedure:

1. Thaw a vial containing 1×10^7 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 $1-1.5 \times 10^6$ cells/25 cm² ($\sim 3 \times 10^6$ cells/100 mm plate). Incubate the plates at 37 °C with 5% CO₂. 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 1:2 ratio.
3. To passage ES cells, prepare two 100 mm gelatinized plates in advance as described (see Section 3). 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).

ES Cell Culture using ESGRO Medium Supplement *continued*

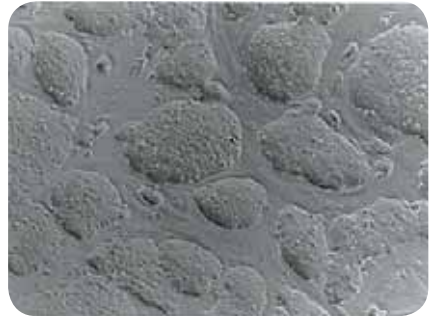
4. Add 5 mL of the cell suspension to each gelatinized plate containing 5 mL of ES Cell Medium. Excess ES cells can be frozen at a concentration of $2\text{--}10 \times 10^6$ cells per vial for future use. Please note that ES cells should always be passaged the day before you intend to electroporate.

Image 5A



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

Image 5B



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

5.2 ES Cell Culture with PMEF Feeder Cells

Materials & Reagents required:

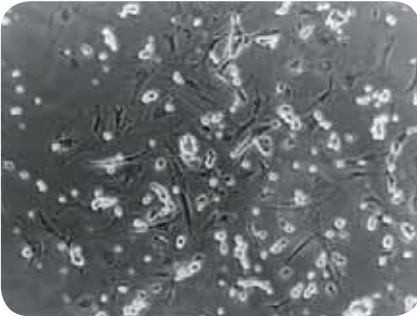
- Centrifuge
- EmbryoMax ES Cell Qualified DPBS (Cat. No. BSS-1006-A, Cat. No. BSS-1006-B)
- EmbryoMax ES Cell Qualified FBS (Cat. No. ES-009-B, Cat. No. ES-009-C)
- ES Cell Medium (see Section 18)
- Incubator, $37\text{ }^{\circ}\text{C}/5\% \text{CO}_2$
- Pipette
- PMEF Feeder cell coated plates (see Section 4)
- 0.05% Trypsin-0.53mM EDTA (Cat. No. SM-2002-C)

Procedure:

1. Thaw a vial containing 1×10^7 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.

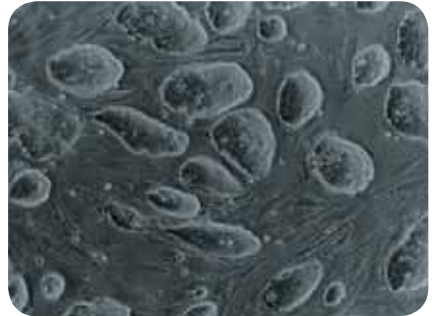
2. Remove the PMEF Feeder Cell Medium from a feeder plate prepared earlier (see Section 4), and seed the ES cells onto the PMEF coated plate at a density of $1-1.5 \times 10^6$ cells/ 25 cm^2 ($\sim 3 \times 10^6$ cells/100 mm plate). Incubate the plates at 37°C with $5\% \text{ CO}_2$. The cells appearance should resemble Image 5C (below).
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 1:2 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 5 mL of the cell suspension to each of the PMEF cell plates containing 5 mL of ES Cell Medium. Excess ES cells can be frozen at a concentration of $2-10 \times 10^6$ cells per vial for future use. Please note that ES cells should always be passaged the day before you intend to electroporate.

Image 5C



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

Image 5D



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

Feeder-free and Serum-free ES Cell Culture using ESGRO Complete System

Adapting ES Cell Lines to a Feeder- and Serum-free Environment

The ESGRO Complete cell culture system (see Section 19) is a fully defined, serum- and feeder-free system based on the work of Ying, *et al.* (2003). The cornerstone of this system is the ESGRO Complete Clonal Grade Medium, a defined serum-free medium optimized to grow and maintain undifferentiated mouse embryonic stem cells in the absence of serum and feeder cells. The mouse ES cells should maintain germ line competency.

The following protocol is applicable for adapting both feeder-dependent and feeder-independent mouse ES cells to serum-free cell culture conditions. 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:

- Clonal Grade Medium (Cat. No. SF001-500)
- Basal Medium (Cat. No. SF002-500)
- Accutase™ Solution (Cat. No. SF006)
- Gelatin Solution (Cat. No. SF008)
- DPBS (Cat. No. BSS-1006-C)
- T25 Flasks
- Incubator, 37 °C

Procedure:

1. Grow mouse ES cells to 60% confluence in serum-supplemented medium in a T25 flask with or without feeders.
2. Change medium 24 hours prior to seeding into serum free conditions.
3. Pre-coat T25 flasks with Gelatin Solution.
4. Wash cells once with DPBS. To dissociate cells, add 1 mL Accutase solution. Incubate at 37 °C and allow cells to detach (5–10 minutes). Tap gently and add 5 mL of Basal Medium, mix and spin at 1000 rpm.

Note: It is important not to use standard trypsin. "Balling" of ES cells can occur (see Image 6C).

5. Remove supernatant and resuspend pellet in 5 mL Clonal Grade Medium. Count cells.
6. Plate 1×10^6 cells into a pre-coated T25 flask containing 10 mL pre-warmed Clonal Grade Medium.

7. Observe cell growth over the next 2–3 days. Some residual feeders may remain stuck down, some cell death may be observed and some differentiation may be visible. However, ES cells colonies will continue to grow and may appear to be flatter than those on feeders with a distinct nuclear and cytoplasmic morphology (See Images 6A and 6B).
8. When mES cells are about 60% confluent, perform a 1 in 5 split of the T25 flask into another coated T25 flask containing Clonal Grade Medium.

Note: Do not let ES cells in serum free media become over confluent as they will begin to differentiate.

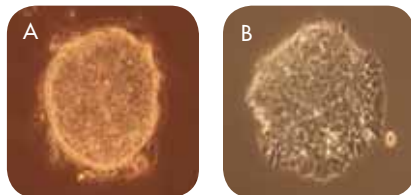
9. To passage the ES cells, remove the media and wash with DPBS buffer. Add 1 mL of Accutase solution per T25 flask.

Note: Do not use standard trypsin.

10. Return to 37 °C incubator and allow cells to detach (5–10 minutes). Tap gently and add 5 mL Basal Medium; mix and spin at 1000 rpm.
11. Remove supernatant. Resuspend pellet in 5 mL Clonal Grade Medium.
12. Cell growth should be observed over the next 2–3 days. The ES cells should be passaged as in steps 9 and 10 an additional 2–3 times. After these passages no or few residual feeders should remain and the remaining ES cells should have only low levels of differentiation (see Image 6D).

Ying, Q., Nichols, J., Chambers, I., Smith, A. (2003). BMP Induction of Id Proteins Suppresses Differentiation and Sustains Embryonic Stem Cell Self-Renewal in Collaboration with STAT3. *Cell* 115:281-292.

Image 6A: Undifferentiated ES cells colonies with a distinct cytoplasmic and nuclear morphology (day 4 of a clonal assay).



(A) Tight round colony

(B) flatter colony

Feeder-free and Serum-free ES Cell Culture using ESGRO Complete System continued

Image 6B: Alkaline phosphatase staining allows for an easy distinction between undifferentiated ES cells (red) and differentiated cells (unstained) on day 5 of the clonal assay.

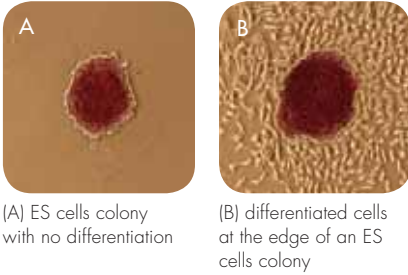


Image 6C: Standard Trypsin use causes the ES cells to lift off the plates in serum free conditions (A) whereas the use of Accutase solution allows for an efficient and gentle way to routinely passage the cells (B).

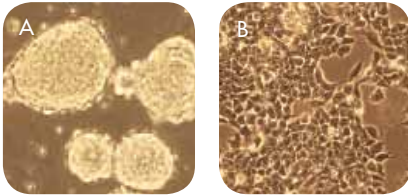
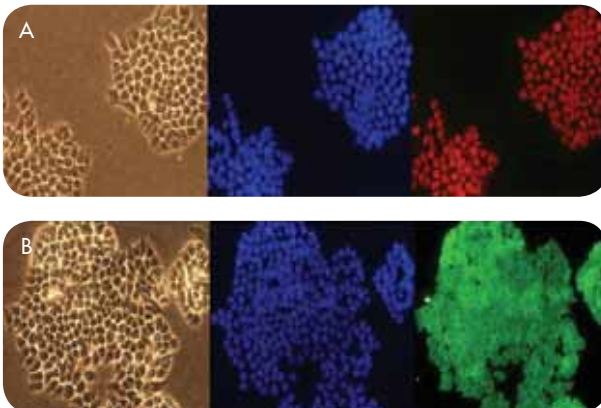


Image 6D: Immunostaining of mouse ES cells grown in Clonal Grade Medium. From left to right for the top panel (A): phase contrast, Hoechst staining and Oct-4 staining. From left to right for the bottom panel (B): phase contrast, Hoechst staining and Nanog staining.



Electroporation of ES Cells

7.1 Protocol for Serum-containing Medium

Materials & Reagents required:

- Electroporator and 0.4 cm cuvette
- EmbryoMax ES Cell Qualified Electroporation Buffer (Cat. No. ES-003-D)
- ES Cell Medium (see Section 18)
- Ice
- Incubator, 37 °C/5% CO₂
- 25–40 µg Linearized construct DNA, ethanol precipitated and dried
- PMEF Feeder cell coated plates (see Section 4)
- 0.05% Trypsin-0.53mM EDTA (Cat. No. SM-2002-C)

Procedure:

1. The evening before the electroporation is to be performed, prepare 4 plates with PMEF cells (see Section 4).
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. 1×10^7 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% CO₂ prior to antibiotic selection.

Electroporation of ES Cells *continued*

7.2 Protocol for Serum-free Medium

Materials & Reagents required:

- EmbryoMax ES Cell Qualified Electroporation Buffer (Cat. No. ES-003-D)
- ESGRO Complete Clonal Grade Medium (Cat. No. SF001-500)
- Accutase Solution (Cat. No. SF006)
- 25–40 µg Linearized construct DNA, ethanol precipitated and dried
- Incubator, 37 °C/5% CO₂
- 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 3). Harvest the ES cells as described previously and determine the cell count. The minimum number of ES cells required for electroporation is 1×10^7 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% CO₂ prior to antibiotic selection.

Selection of ES Cells

8.1 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.

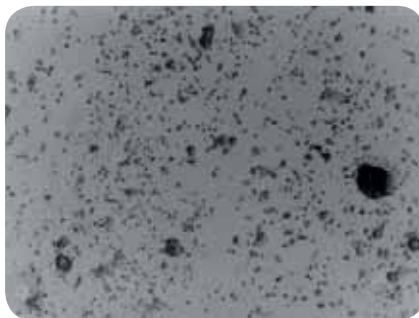
Materials & Reagents required:

- ES Cell Medium supplemented with either Neomycin (G418) or Hygromycin B (see Section 18)
- ES Cell Qualified DPBS (Cat. No. BSS-1006-A, Cat. No. BSS-1006-B)
- Incubator, 37 °C/5% CO₂
- 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 (see Image 8A). Change the culture 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.

Image 8A



Electroporated ES cells (R1) grown in the presence of PMEFs and selected with G418 after 5 days of culture (10x).

Selection of ES Cells *continued*

8.2 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 (Cat. No. SF001-500)
- ESGRO Complete G418 Antibiotic (Cat. No. SF011)
- ESGRO Complete Puromycin Antibiotic (Cat. No. SF012)
- ESGRO Complete Hygromycin B Antibiotic (Cat. No. SF013)
- ES Cell Qualified DPBS (Cat. No. BSS-1006-A, Cat. No. BSS-1006-B)
- Incubator, 37 °C/5% CO₂
- Pipette

Procedure:

1. To select for transformants, add Clonal Grade Medium containing ESGRO Complete 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

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.

9.1 Protocol for Serum-containing Medium

Materials & Reagents required:

- 96-well plate(s)
- ES Cell Medium (see Section 18)
- ES Cell Medium supplemented with Neomycin (150 µg/ml) or Hygromycin B (50 µg/ml)
- Incubator, 37 °C/5% CO₂
- Pipette
- PMEF Feeder cell coated 24-well plates (see Section 4)
- 0.05% Trypsin-0.53mM EDTA (Cat. No. SM-2002-C)
- Microscope

Procedure:

1. The day before picking ES cell colonies, coat an appropriate number of 24-well plates with PMEF Feeder Cells (see Section 4) or gelatin (see Section 3).
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.

Colony Picking *continued*

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 μ L 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 μ L 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 μ L. Incubate at 37 °C and 5% CO₂. Feed every day with medium supplemented with Neomycin (150 μ g/mL) or Hygromycin (50 μ g/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).

9.2 Protocol for Serum-free Medium

Materials & Reagents required:

- ESGRO Complete Clonal Grade Medium (Cat. No. SF001-500)
- ESGRO Complete Clonal Grade Medium supplemented with G418, Hygromycin, or Puromycin (see Section 8)
- Accutase Solution (Cat. No. SF006)
- 0.1% gelatin coated 24-well plates
- 96-well plate(s)
- Pipette
- Incubator, 37 °C/5% CO₂
- 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 3).
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.
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 (see Section 8).
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).

Harvesting and DNA Preparation

Materials & Reagents required:

- 8M Ammonium Acetate Solution
- ES Cell Medium (see Section 18)
- Ice cold 70% Ethanol
- 100% Ethanol
- Lysis Buffer (see Section 18)
- 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 9) 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 (for Southern blot protocol, please see www.chemicon.com/techsupp/southern.asp).

Freezing Plates

Materials & Reagents required:

- Dry Ice
- EmbryoMax ES Cell Qualified Freezing Medium, 2x (Cat. No. ES-002-D)
- ES Cell Medium (see Section 18)
- Parafilm® Film
- Pipette
- 0.05% Trypsin-0.53mM EDTA (Cat. No. SM-2002-C)

Procedure:

1. When the ES cell colonies have regrown (see Section 10, step 3), wash with DPBS and add 35 μ L of Trypsin. Incubate for 10 minutes at 37 °C, and then add 65 μ L of ES Cell Medium.
2. Disperse the cells with a pipette and transfer into a replica plate containing 65 μ L of cold EmbryoMax ES Cell Qualified 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 μ L 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.

Karyotyping ES Cells

Materials & Reagents required:

- Depex Mounting Medium
- ES Cell Medium (see Section 18)
- Fixative (MeOH:Glacial Acetic Acid, 3:1)
- Hypotonic KCl Solution
- Leishman's Stain (see Section 18)
- Microscope Slides
- Pipette
- 0.05% Trypsin-0.53mM EDTA (Cat. No. SM-2002-C)
- Xylene

Procedure:

This method is recommended for use with actively growing cultures of ES cells (i.e. 1–2 day cultures).

1. One day prior to karyotyping, passage a 70% confluent ES cell plate at a 1:2 ratio.
2. At least 3 hours prior to karyotyping, transfer the ES cells into fresh medium.
3. 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.
4. Resuspend each cell pellet in 8 mL of hypotonic KCl solution, gently flicking the tube to avoid clumping and ensure an even suspension.
5. Incubate the tube at 37 °C for 10 minutes (this may vary for each type of cell line used).
6. Add 2 mL of freshly made fixative (MeOH:Glacial Acetic Acid, 3:1) and mix by gentle inversion.
7. Centrifuge cells (300 xg, 5 minutes) and aspirate the supernatant.
8. 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.
9. Centrifuge cells (300 xg, 5 minutes) and aspirate supernatant.
10. Repeat steps 8 & 9 three times.
11. Resuspend the pellet in 1 mL of fixative (this volume may need to be adjusted slightly according to pellet size).
12. 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.

Recovery of Recombinant Clones using RESGRO Culture Media

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 (Cat. No. SCM001, Cat. No. SCM002)
- EmbryoMax ES Cell Qualified L-Glutamine Solution (Cat. No. TMS-002-C)
- Incubator, 37 °C/5% CO₂
- PMEF Feeder cell coated culture plates (see Section 4)
- Pipette
- 70 mm fiberglass prefilter
- Cellulose acetate, PVDF or PES filters

Table 13.1:

Other well sizes and suggested volumes of medium:

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

Procedure:

1. Prepare RESGRO Culture Medium for use by adding 10 mL of ES Cell Qualified 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. Filter the solution using only cellulose acetate, PVDF, or PES filters together with the 70 mm fiberglass prefilter.
3. 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.
4. 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.
5. Gently pipette the content of the vial up and down several times (removal of the cryoprotective medium is not necessary).
6. Dispense the cells into the wells of the 6-well plate containing RESGRO Culture Medium.
7. Gently disperse the cells by shaking to ensure a homogenous distribution.
8. Disperse cells again after 35 minutes and 1 hour by gently shaking.
9. 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.
10. 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.

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/chemicon for additional data and information.

Materials & Reagents required:

- RESGRO Culture Medium (Cat. No. SCM001, Cat. No. SCM002)
- EmbryoMax ES Cell Qualified L-Glutamine Solution (Cat. No. TMS-002-C)
- Cellulose acetate, PVDF or PES filters
- 70 mm fiberglass prefilter
- Centrifuge
- ES Cell Medium (see Section 18)
- Incubator, 37 °C/5% CO₂
- Pipette
- PMEF Feeder cell coated culture plates (see Section 4)
- 0.05% Trypsin-0.53mM EDTA (Cat. No. SM-2002-C)
- Water Bath, 37 °C

Procedure:

1. Prepare RESGRO Culture Medium for use by adding 10 mL of ES Cell Qualified L-Glutamine Solution (200mM) to 500 mL of RESGRO Culture Medium.
2. Filter the solution using only cellulose acetate, PVDF, or PES filters together with the 70 mm fiberglass prefilter.
3. Culture the ES cells in RESGRO Culture Medium for 2 passages on a monolayer of PMEF feeder cells.
4. After 2 passages, replate 1/3–1/5 of the cell suspension on the same size plate without PMEF feeder cells.
5. 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.

Rescue of ES Cell Lines using RESGRO Culture Medium *continued*

6. Collect the supernatant (which will contain the undifferentiated cells) and discard the dish containing the differentiated cells.
7. Centrifuge the supernatant and remove the medium.
8. Add 0.5 mL of Trypsin-EDTA to the cell pellet.
9. Pipette up and down with a 1 mL pipette (do not use pipette tip of smaller volume).
10. Place the cell suspension in a water bath at 37 °C for 1.5 minutes.
11. Pipette up and down, 10 times (with a 200 µL pipette tip or a 1 mL pipette).
12. Add 9.5 mL of RESGRO Culture Medium.
13. Centrifuge and remove the supernatant.
14. 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, replat ES cells at a lower density the following day.

Table 14.1:
Improved efficiency of Murine ES cell lines using RESGRO Culture Medium

ES Cell Line	Medium* & Method used	Number of Embryos Transferred	Number of Pups Born	Number 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 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%					

*Traditional medium: basal medium supplemented with ESGRO

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.

Table 15.1:

Efficiency of ES Cell Derivation and Germline Competence with RESGRO Culture Medium

Mouse Strain	Blastocysts Cultured (n)	Established ES Cell Lines (n)	(%)	No. Germline Competent ES Cell Lines/ No. ES Cell Lines Cultured
C57Bl/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

Materials & Reagents required:

- RESGRO Culture Medium (Cat. No. SCM001, Cat. No. SCM002)
- EmbryoMax ES Cell Qualified L-Glutamine Solution (Cat. No. TMS-002-C)
- 96-well plates coated with PMEF Feeder cells (see Section 4)
- Cellulose acetate, PVDF or PES filters
- 70 mm fiberglass prefilter
- Incubator, 37 °C/5% CO₂
- 0.25% Trypsin-1 mM EDTA (Cat. 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 4).
2. Prepare RESGRO Culture Medium for use by adding 10 mL of ES Cell Qualified L-Glutamine Solution (200 mM) to 500 mL of RESGRO Culture Medium.
3. Filter the solution using only cellulose acetate, PVDF, or PES filters together with the 70 mm fiberglass prefilter.
4. 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).
5. After attachment of the blastocysts, replace the medium completely on a daily basis with RESGRO Culture Medium.
6. 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-1 mM EDTA on a 96-well plate covered with a monolayer of PMEF feeder cells.
7. Culture the ES cells until 70–80% confluent, then replate on larger culture dishes.
8. Passage ES cells every 2–4 days on freshly prepared feeder layers, and replace with fresh RESGRO Culture Medium daily.

Derivation of ES cells from Delayed Blastocysts using the ESGRO Complete Derivation Kit

The ESGRO Complete Derivation Kit (see Section 19) is intended for the derivation of mouse ES cells from delayed blastocysts in serum-free conditions. In this system, the derivation of ES cells involves subjecting the embryos to delayed implantation or diapause, allowing the embryos to attach to the substrate for a few days, and then disrupting the cell contacts by disaggregation and replating.

Materials & Reagents required:

- **ESGRO Complete Derivation Kit (Cat. No. SF003):**
 - Clonal Grade Medium (Cat. No. SF001-100): One 100 mL bottle
 - Basal Medium (Cat. No. SF002-100): One 100 mL bottle
 - DBIM: Delayed Blastocyst Incubation Medium (Cat. No. SF010): One 100 mL bottle
 - Trypsin Solution (Cat. No. SF007-20): One 20 mL bottle
 - Gelatin Solution (Cat. No. SF008-100): One 100 mL bottle
 - Complete Freezing Medium (Cat. No. SF005): One 50 mL bottle
- **Injection solution 1:** Tamoxifen (Sigma catalog no. T5648).
To prepare stock solution (10 mg/mL), dissolve 100 mg in 10 mL ethanol. Store at 4 °C for up to one month. To prepare working solution, dilute 100 µL of stock solution in 9.9 mL propylene glycol (100 µg/mL). Store at 4 °C for up to one month. For injections use 100 µL per animal i.p.
- **Injection solution 2:** Depo-Provera® solution (Medroxyprogesterone 17-acetate) (Sigma M1629). Prepare a stock 150 mg/mL aqueous suspension using sterile distilled water. Dilute with sterile phosphate buffered saline to a concentration of 30 mg/mL. Store at 4 °C for up to one year. For injections use 100 µL per animal subcutaneously.
- **Injection needles** need to be made using a micropipette puller:
 - Transfer needles A: For the handling of Blastocysts and outgrowths
 - Transfer needles B: Thin needles for disaggregating outgrowths and colonies
 - Transfer needles C: Extra thin for disaggregating outgrowths and colonies
- Embryo handling pipette
- 30 mL universal tube (Sterilin)
- 4-well, 6-well, 12-well, and 96-well tissue culture plates
- Center well organ culture dishes (Falcon 35-3037)
- 60 mm tissue culture dish
- 25 cm² tissue culture flasks
- Tissue culture flow hood
- Incubator, 37 °C/7% CO₂

- Sterile phosphate buffered saline
- Stereo-microscope
- Inverted microscope

Procedure:

1. Set up matings and monitor for plugged females the following morning. The most common strain of mice used is 129/Sv or 129/Ola, but ES cell lines can be derived from other strains such as C57BL/6.
2. Exactly 2 days after the plugs are detected inject the plugged females with 100 μL of Injection solution 1 as an IP injection, and 100 μL of Injection solution 2 subcutaneously. Make sure Solutions 1 and 2 are made up as described on previous page. Failure to do this could be lethal to the animals.
3. After 4 to 5 days flush the delayed embryos from the uterus with Basal Medium into a 6 cm^2 tissue culture dish using standard techniques. Place the blastocysts into non-coated center well organ culture dish (do not add the gelatin to the plasticware at this stage). Using transfer needle A, transfer the embryos into a gelatin coated well dish containing DBIM pre-incubated in the tissue culture incubator. Incubate embryos at 37 $^{\circ}\text{C}$ in a 7% CO_2 tissue culture incubator in this medium for a further 4–5 days (without media change). During this time the morphology of the embryo will change. The embryo may float freely or lightly attach. It is normal not to see any form of cell outgrowth at this stage in serum-free conditions.
4. Set up 4-well dishes in the flow hood by coating with Gelatin Solution for 30 minutes. Remove the buffer and allow to dry for 15 minutes. To these add Clonal Grade Medium and pre-incubate in the tissue culture incubator.
5. Place 50 μL drops of Trypsin solution onto a non-coated 6-well tissue culture dish. Transfer each delayed blastocyst into these drops one at a time. Leave the embryo for 2–3 minutes only. Watch for morphology change by microscopy. Individual cell structures will become visible in the embryo and, although some dissociation of cells will occur during this time, the embryo should remain largely intact.

Note: Do not use standard trypsin. *The use of ESGRO Complete Trypsin solution is required.*

6. Remove the embryo from the Trypsin solution with transfer needle B. Be careful to transfer the embryo in the smallest quantity of trypsin possible. To do this pre-fill the needle with a small amount of Clonal Grade Medium and then in the smallest possible volume of Trypsin take up the embryo into the needle using the embryo handling pipette. Next place in the 4-well plates made previously and using the same needle vigorously pipette up and down until the embryo disassociates to almost a single cell suspension. In some cases the smaller bore needle C is required instead of needle B.

Note: Do not use standard trypsin.

Derivation of ES cells from Delayed Blastocysts the using ESGRO Complete Derivation Kit *continued*

7. Incubate the 4-well dishes at 37 °C in 7% CO₂ for five or more days without media change until colonies are formed.
8. Prepare the 96-well plate by coating in Gelatin solution for 30 minutes, then removing the buffer and allowing to dry for 15 minutes. Add Clonal Grade Medium and pre-incubate in the tissue culture incubator.
9. Prepare an uncoated 6 cm² tissue culture dish with drops of Trypsin solution as done previously. From the 4-well dish remove the colonies by using transfer needle A and place them in the trypsin drops for 2–3 minutes only. Watch for morphology change by microscopy, again the colony will largely be intact. Now move the colony using needle B (pre-filled with Clonal Grade Medium and taking only the smallest possible amount of the trypsin solution). Needle C may be required if the colonies are small. Expel the colonies into wells on the 96-well plate and disaggregate the colony by gently pipetting up and down. Incubate until the colonies are sufficiently large for further passage. Regularly monitor growth of the colonies by microscopy.

Note: Do not use standard trypsin.

10. When ready to passage colonies prepare 4-well plates by coating in the Gelatin solution for 15 minutes, removing the buffer and allowing to dry for 25 minutes. Remove the media from the cells and add 100 µL Trypsin solution and incubate for 2 minutes in the tissue culture incubator. Disaggregate by pipetting gently using a Gilson or similar hand pipette. Remove contents of the well and add to a universal tube containing 5 mL of Basal Medium to neutralize the trypsin. Spin the universal tube at 1500 rpm in a bench top centrifuge. Carefully remove the supernatant and resuspend the cell pellet in a small volume of Clonal Grade Medium and transfer this to the prepared gelatin coated 4-well dish.

Note: Do not use standard trypsin.

11. Continue to expand the colonies into larger coated wells and dishes. For instance, from 4-well plate to a 12-well plate to a small tissue culture flask and then a medium size flask.
12. When cell culture is established seed some cells for freezing into small coated tissue culture flask. Cells to be frozen should be in late log phase growth. It is recommended that the ESGRO Complete Freezing Medium is used for this procedure.

Image 16A: ES cell colonies form 5 days after disaggregation of outgrowths (A). Undifferentiated ES cells have a distinct nuclear morphology that is easy to identify (B).

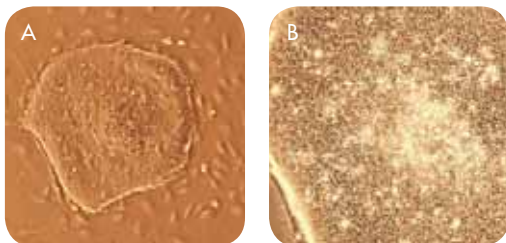
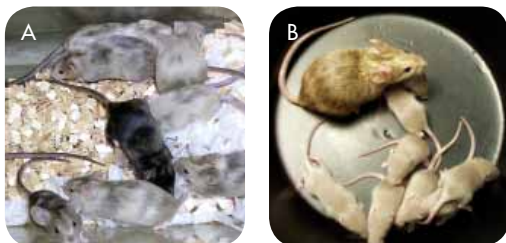


Image 16B: Chimeras produced after microinjection of ES cells derived in serum-free conditions (A). Confirmation of germline transmission was seen as gray pups born to outbred mice (B).



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.

Listed below in Table 17.1 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 Millipore's ES Cell Characterization Kit or Alkaline Phosphatase Detection Kit (see Section 19). 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 17.2 are a number of reasons that often cause the slow growth of ES cells. Table 17.3 contains possible causes for the lack of generating chimeras.

Table 17.1: Common causes of ES cell differentiation and recommendations

Cause of Differentiation	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 17A–17G (pages 35 & 36) 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.

Table 17.1 continued: Common causes of ES cell differentiation and recommendations

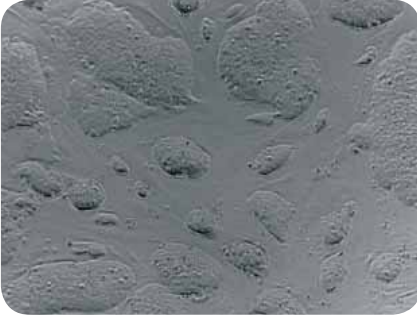
Cause of Differentiation	Recommendations
Feeder cells	If differentiation cannot be controlled using ESGRO mLIF medium supplement with gelatinized plates, it may be necessary to culture ES cells on a feeder layer. Please refer to Section 4 for the recommended number of growth arrested fibroblasts. ESGRO 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 17.2: Causes of slow growth of ES cells and recommendations

Cause of Slow 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 on the following page for illustrations of ES cell confluency.

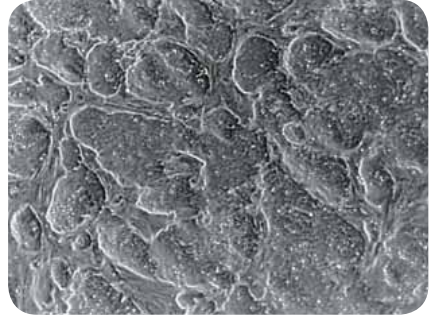
Troubleshooting Information *continued*

Image 17A



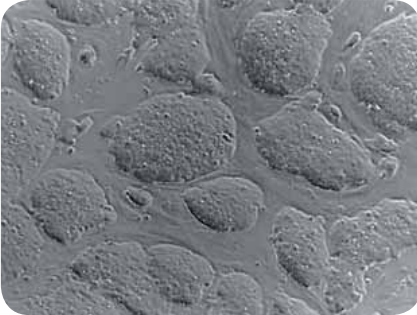
Optimal confluence of ES cells (10x).

Image 17B



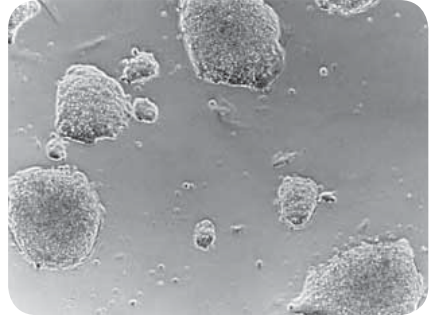
Over confluent ES cells (10x).

Image 17C



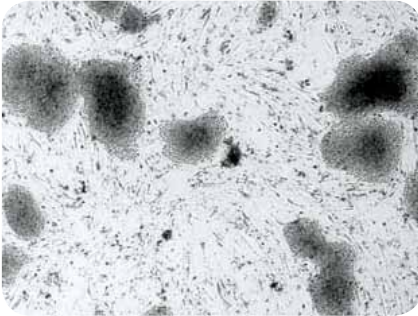
ES Cells at a density ready for passage (10x).

Image 17D



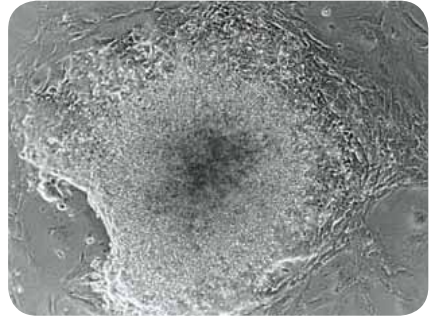
ES cells plated at low density (10x). Cells require another passage to increase individual colony numbers.

Image 17E



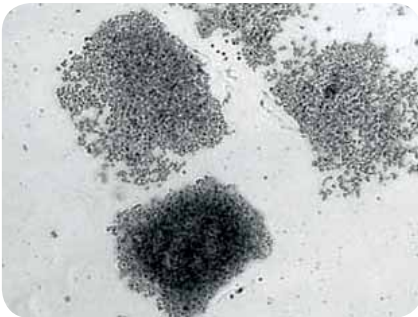
ES cells plated at too low density with fibroblasts (10x). Cells require another passage to increase individual colony numbers. Note the darkened areas in the center of each colony where ES cells are dying.

Image 17F



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. It is possible to attempt to rescue this colony by passaging several times, however it is not usually recommended.

Image 17G



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

Troubleshooting Information continued**Table 17.3:** Causes for the lack of chimeras

Cause for 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, Millipore's ES Cell Characterization Kit or Alkaline Phosphatase Detection Kit are recommended (see Section 19). Please refer to Section 14 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.

Formulations

PMEF Feeder Cell Culture Medium

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

Serum-containing ES Cell Medium

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

Note: For selection add 150–350 $\mu\text{g}/\text{mL}$ of Neomycin G418 (Sigma, G-9516) or 75–100 $\mu\text{g}/\text{mL}$ of Hygromycin B (Sigma, H-3274)

Lysis Buffer

Component	Quantity
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	Quantity	Cat. No.
PluriStem B6-White Murine ES cell line	5 x 10 ⁶ cells	SCRO11
PluriStem 129S6 Murine ES cell line	5 x 10 ⁶ cells	SCRO12
PluriStem 129/SVEV (S6) Murine ES cell line	5 x 10 ⁶ cells	CMTI-1
PluriStem C57/BL6 Murine ES cell line	5 x 10 ⁶ cells	CMTI-2
PluriStem DBA-1 Murine ES cell line	5 x 10 ⁶ cells	CMTI-3

Primary Mouse Embryo Fibroblasts

Description	Quantity	Cat. No.
EmbryoMax PMEF, Hygro Resistant, Strain C57/BL6	2.5 x 10 ⁷ cells	PMEF-H
EmbryoMax PMEF, Neo Resistant, Strain FVB	2.5 x 10 ⁷ cells	PMEF-N
EmbryoMax PMEF, Hygro Resistant, Not Mitomycin C treated, Strain C57/BL6	2.5 x 10 ⁷ cells	PMEF-HL
EmbryoMax PMEF, Strain CF1	2.5 x 10 ⁷ cells	PMEF-CF
EmbryoMax PMEF, Not Mitomycin C Treated, Strain CF1	2.5 x 10 ⁷ cells	PMEF-CFL

ESGRO Complete Cell Culture System

Description	Quantity	Cat. No.
ESGRO Complete Clonal Grade Medium	100 mL	SF001-100
ESGRO Complete Clonal Grade Medium	500 mL	SF001-500
ESGRO Complete Basal Medium	100 mL	SF002-100
ESGRO Complete Basal Medium	500 mL	SF002-500
ESGRO Complete Derivation Kit	1 kit	SF003
ESGRO Complete Switch Kit	1 kit	SF004
Kit Components:		
• ESGRO Complete Clonal Grade Medium (100 mL)		
• ESGRO Complete Basal Medium (100 mL)		
• ESGRO Complete Accutase (100 mL)		
• ESGRO Complete Gelatin (100 mL)		
• PBS (100 mL)		
• ESGRO Complete Freezing Medium (50 mL)		
ESGRO Complete Freezing Medium	50 mL	SF005
Accutase Solution for ESGRO Complete Medium	100 mL	SF006
ESGRO Complete Trypsin	100 mL	SF007
ESGRO Complete Gelatin	500 mL	SF008
ESGRO Complete Enzyme Free Dissociation Solution	100 mL	SF009
ESGRO Complete G418 Antibiotic		SF011
ESGRO Complete Puromycin Antibiotic		SF012
ESGRO Complete Hygromycin B Antibiotic		SF013

ES Cell Qualified Culture Media & Supplements

Description	Quantity	Cat. 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
RESGRO Culture Medium	250 mL	SCM001
RESGRO Culture Medium	500 mL	SCM002
EmbryoMax ES Cell Qualified FBS, gamma irradiated	500 mL	ES-010-B
EmbryoMax ES Cell Qualified FBS	500 mL	ES-009-B
EmbryoMax ES Cell Qualified DMEM, Low-bicarb, w/o glutamine & sodium pyruvate	500 mL	SLM-220-B
EmbryoMax ES Cell Qualified DMEM, Low-bicarb, w/o sodium pyruvate	500 mL	SLM-120-B
EmbryoMax ES Cell Qualified DMEM, w/o glutamine & sodium pyruvate	1 Liter	SLM-021-A
EmbryoMax ES Cell Qualified DMEM, w/o glutamine & sodium pyruvate	500 mL	SLM-021-B
EmbryoMax ES Cell Qualified Cell Culture, Freezing Medium (2x)	50 mL	ES-002-D
EmbryoMax ES Cell Qualified Cell Culture, Freezing Medium (2x)	10 x 10 mL	ES-002-10F
EmbryoMax ES Cell Qualified Cell Culture, Freezing Medium (2x)	5 x 10 mL	ES-002-5F

EmbryoMax ES Cell Qualified Culture Reagents

Description	Quantity	Cat. No.
EmbryoMax ES Cell Qualified 0.1% Gelatin in Sterile Water	500 mL	ES-006-B
EmbryoMax ES Cell Qualified Electroporation Buffer	50 mL	ES-003-D
EmbryoMax ES Cell Qualified Nucleosides (100x)	50 mL	ES-008-D
EmbryoMax ES Cell Qualified MEM Non-Essential Amino Acids (100x)	100 mL	TMS-001-C
EmbryoMax ES Cell Qualified-2-Mercaptoethanol (100x)	20 mL	ES-007-E
EmbryoMax ES Cell Qualified Filtered Light Mineral Oil	100 mL	ES-005-C
EmbryoMax ES Cell Qualified L-Glutamine Solution (100x)	100 mL	TMS-002-C
EmbryoMax ES Cell Qualified Penicillin-Streptomycin Solution	100 mL	TMS-AB2-C
EmbryoMax ES Cell Qualified Filtered Silicon Oil	100 mL	ES-004-C
EmbryoMax ES Cell Qualified DPBS (no Mg ²⁺ or Ca ²⁺)	1 L	BSS-1006-A
EmbryoMax ES Cell Qualified DPBS (no Mg ²⁺ or Ca ²⁺)	500 mL	BSS-1006-B
EmbryoMax ES Cell Qualified DPBS	1 L	BSS-1005-A
EmbryoMax ES Cell Qualified DPBS	500 mL	BSS-1005-B

Ordering Information *continued*

EmbryoMax ES Cell Qualified Culture Reagents *continued*

Description	Quantity	Cat. No.
EmbryoMax ES Cell Qualified 1M HEPES Buffer Solution	100 mL	TMS-003-C
EmbryoMax ES Cell Qualified Ultra Pure H ₂ O	1 L	TMS-006-A
EmbryoMax ES Cell Qualified Ultra Pure H ₂ O	500 mL	TMS-006-B
EmbryoMax ES Cell Qualified Ultra Pure H ₂ O	100 mL	TMS-006-C

EmbryoMax Cosmid Genomic Libraries

Genomic DNA libraries from various murine strains cloned into cosmid vectors containing inserts from 30–46 kbp. An average of 4–5 × 10⁶ primary clones generated. Libraries have been amplified 1x in soft agar and are supplied as 1.0 mL aliquots of glycerol stocks. Typical titers are 10⁴ to 10⁵/μL.

Description	Quantity	Cat. No.
EmbryoMax Cosmid Genomic Library, Strain C57-Bl6	1.0 mL	CGL-C57
EmbryoMax Cosmid Genomic Library, Strain DBA-1	1.0 mL	CGL-DBA
EmbryoMax Cosmid Genomic Library, Strain FVB	1.0 mL	CGL-FVB
EmbryoMax Cosmid Genomic Library, Strain NOD	1.0 mL	CGL-NOD
EmbryoMax Cosmid Genomic Library, Strain PlJ	1.0 mL	CGL-PlJ

EmbryoMax Targeting Vectors

Description	Quantity	Cat. No.
EmbryoMax Targeting Vector, Hygromycin Resistant	1 vial	ESTV-HYGRO
EmbryoMax Targeting Vector, G418 (Neo) Resistant	1 vial	ESTV-NEO

EmbryoMax Mouse Embryo Culture Reagents

Description	Quantity	Cat. 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	5 × 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
Modified M16 Medium (1x), Powdered Media Kit	5 × 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 × 10 mL*	MR-020P-5F

*1 × 50 mL & 5 × 50 mL sizes are also available.

EmbryoMax Rat Embryo Culture Reagents

Description	Quantity	Cat. No.
EmbryoMax m-RECM Rat 1-cell Embryo Culture Medium, w/ HEPES	50 mL	MR-169-D
EmbryoMax m-RECM Rat 1-cell Embryo Culture Medium	50 mL	MR-168-D
EmbryoMax m-RECM Rat 2-cell Embryo Culture Medium	50 mL	MR-166-D
EmbryoMax m-RECM Rat 2-cell Embryo Culture Medium, w/ HEPES	50 mL	MR-167-D

EmbryoMax Cryopreserved Mouse Embryos

Description	Quantity	Cat. No.
EmbryoMax Cryopreserved Mouse Embryos	1 straw	CRY-BL6-8

Cell Dissociation Reagents

Description	Quantity	Cat. 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

Kits for Detection of ES Cell Differentiation

Description	Quantity	Cat. No.
ES Cell Characterization Kit	1 kit	SCR001
Alkaline Phosphatase Detection Kit	1 kit	SCR004

ESGRO Medium Supplement Compatible ES Cell Lines

Table 20.1: ESGRO mLIF Medium Supplement alone

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

Table 20.2: ESGRO mLIF Medium Supplement & feeder cell layer

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

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