Mouse Embryonic Stem (ES) Cell Isolation

Undifferentiated mouse embryonic stem (ES) cells are necessary for the production of mouse mutants using homologous recombination and blastocyst-mediated transgenesis. Suitable ES cell lines are available from commercial sources (*UNIT 23.3*) and from many investigators. However, investigators planning to use ES cells extensively may find that the isolation of new lines provides a simple and economical method for maintaining stocks of early passage ES cells. In addition, ES cells obtained from mutant mouse lines may facilitate the analysis of mutant phenotypes, particularly when the mutation causes early embryonic lethality.

ISOLATION OF MOUSE ES CELLS

BASIC PROTOCOL

ES cell isolation is straightforward, although success rates can be quite variable. ES cells are derived from the inner cell mass of blastocysts (i.e., 3.5-day-old embryos). Blastocysts are simply cultured for several days, during which time they attach to the surface of the tissue culture plate. Both trophoblast and inner cell mass cells divide after attachment. Inner cell mass outgrowths are picked, dispersed by trypsinization, and replated. Under appropriate conditions a percentage of the isolated outgrowths will continue to divide and maintain an undifferentiated ES cell morphology.

Materials

Modified ES medium (see recipe)
Blastocysts, 3.5-day-old post-coitum embryos (Hogan et al., 1994)
Hanks' balanced salt solution (HBSS), calcium- and magnesium-free (*UNIT 23.2*)
DPBS-EDTA (*UNIT 23.3*)
0.25% (w/v) trypsin-EDTA (*UNIT 23.2*)

Inverted microscope
Gilson-style automatic pipettor with 20-µl pipet tips
96-well U-bottom plate

Additional reagents and equipment for preparing gelatin-coated plates with MEF feeder layers for embryonic stem cell culture (*UNIT 23.3*) and for passaging and freezing embryonic stem cells (*UNIT 23.3*)

NOTE: All cell culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Plate out embryos

1. Prepare gelatinized 24-well tissue culture plates with mitotically inactivated MEF (mouse embryo fibroblast) feeder layers as described in *UNIT 23.3*.

   Plates can be prepared a few hours in advance or the day before.

2. Replace medium in each well with modified ES medium prior to use. Drop individual blastocysts into separate wells and return plates to incubator.

   Aim for the center of the well, otherwise the blastocysts will be difficult to observe with the microscope because of distortion near the edge of wells.

3. Observe embryos daily with an inverted microscope.

   The majority of blastocysts will hatch from the zona pellucida and attach to the plate by the second or third day. Figure 23.4.1A shows an attached blastocyst approximately 48 hr after plating. The zona pellucida is not present. In this early stage, the embryo still retains its original shape.

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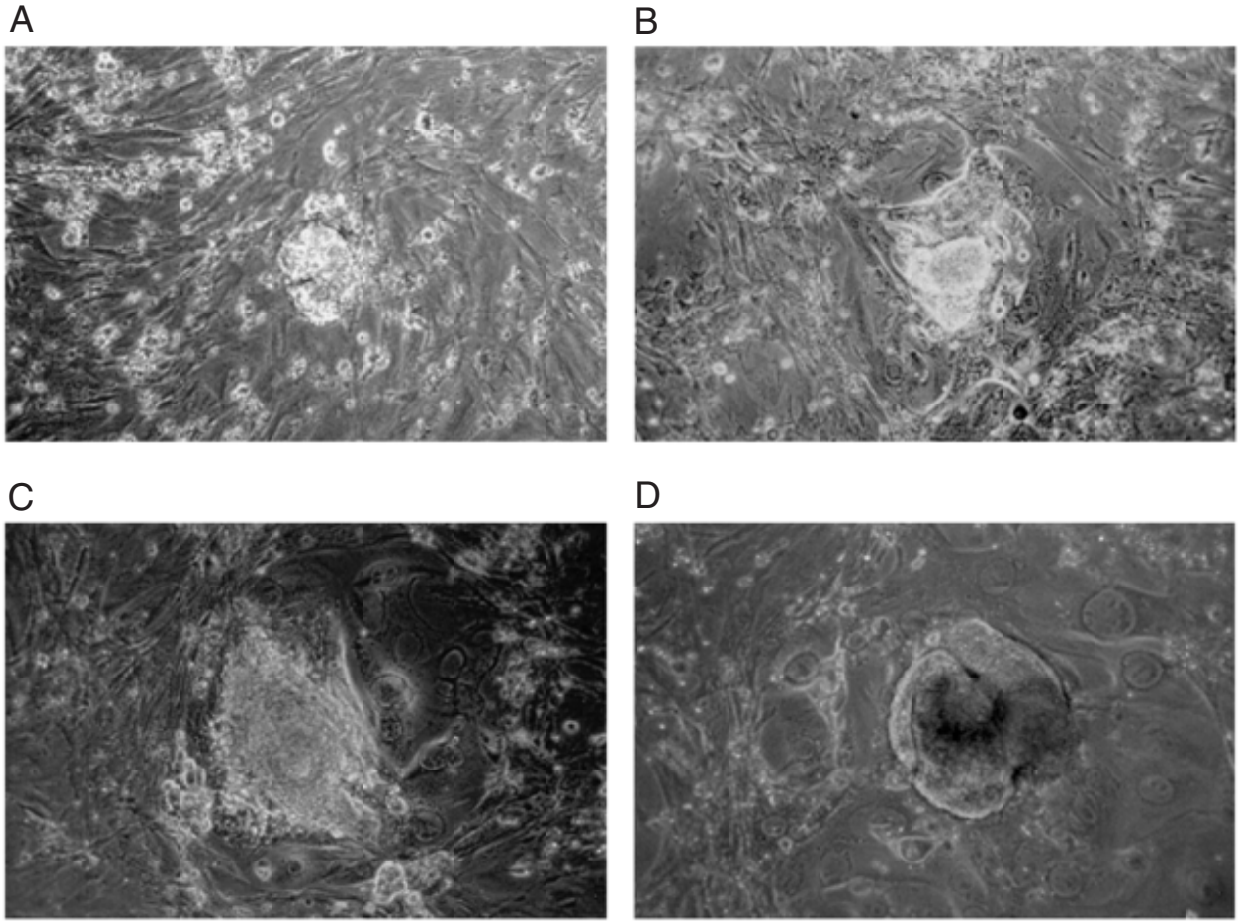

Figure 23.4.1 Morphology of plated blastocysts as shown in phase-contrast images of blastocysts at various time points after plating on MEF feeder cells. (**A**) Appearance of a blastocyst shortly after attachment to the plate ~48 hr after plating. (**B**) Typical appearance of a blastocyst 3 to 4 days after plating. Note the expansion of inner cell mass and trophoblast cells spreading out beneath. (**C**) Typical appearance of blastocyst ready to pick. (**D**) Appearance of a blastocyst that has been left on the plate 1 day too long. Note the pigmentation on the top surface indicating substantial differentiation.

4. Change medium after embryos have attached to the plate.

   The trophoblast cells will spread out quickly under the embryo. Over the next several days the inner cell mass will begin to expand. Figure 23.4.1B shows the inner cell mass at an early stage of expansion. Note the large flattened trophoblast cells under the inner cell mass. The inner cell mass is expanding as a multilayered colony.

Pick inner cell mass outgrowths

Approximately 5 or 6 days after plating, the inner cell mass outgrowths will be ready to disperse. Figure 23.4.1C shows an outgrowth ready to pick. Figure 23.4.1D shows an outgrowth that has been left too long. If the outgrowth is left on the plate for too long, the cells begin to differentiate. This is typically observed as an accumulation of pigmented cells on the top of the colony. Round, loosely attached cells may also appear on the top of the outgrowth as differentiation occurs. Outgrowths should be picked before significant pigment accumulates. Outgrowths with a small amount of differentiation should still be dispersed, because they can yield decent lines.

5. Prepare gelatinized 24-well plates with feeder cells in advance with enough wells for each of embryo (*UNIT 23.3*).

6. Replace medium in embryo cultures 2 to 4 hr prior to picking.

   This is thought to maximize plating efficiency.

7. Rinse wells with Hanks' balanced salt solution (HBSS, calcium- and magnesium-free). After aspirating, add a small volume of HBSS to wells to keep the cell layer from drying out.

   Process only a few wells at a time.

8. Pick up inner cell mass outgrowth using an automatic pipettor with a 20-µl tip and place the cell clump in a well of a 96-well U-bottom plate containing 25 µl DPBS-EDTA in each well.

   Try to transfer the cell mass in a minimal volume (i.e., ≤5 µl). Some investigators find that picking is more easily performed using an inverted microscope with a low-power objective or a dissecting microscope. However, the cell mass can be seen by eye at this stage. With some practice, picking by eye is less tedious than picking with a microscope. In either case, the outgrowth can be dislodged by scraping the tip of the pipet around the cell mass to tear the trophoblast and MEF layer. Then, the inner cell mass can be picked up by simultaneously nudging the clump with the pipet tip and releasing the pipet plunger to aspirate the cells. It does not matter if some fibroblasts and trophoblasts are transferred with the inner cell mass. After placing the cells in the 96-well plate, check under the microscope to verify that the picking procedure was successful.

9. Add 50 µl of 0.25% trypsin-EDTA to each cell containing well of the 96-well plate and place in incubator for 5 to 10 min.

   Do not pick too many at one time. The first outgrowth picked should not sit in PBS-EDTA for longer than 10 min prior to addition of trypsin.

10. Add 100 µl modified ES medium to each well to inhibit trypsin. Triturate 3 to 10 times using a Gilson-style pipettor with 200-µl tips. Transfer cells to a new 24-well feeder plate.

    Try to disperse the outgrowth into several small clumps. Check with microscope to determine the degree of dispersal. Differentiation will occur if cells are not dispersed. However, do not try to achieve a single-cell suspension; trypsinization and trituration sufficient to achieve single-cell suspension usually kills cells at this stage.

Screen and expand putative ES cell lines

11. Monitor plates daily (within 2 days after plating, colonies should be visible). Mark the wells with ES cell–like colonies and observe their morphology on successive days.

    Look for ES cell colonies like those depicted in Figure 23.4.2A and B. These will appear as small rounded clumps of cells with sharp refractal edges; nuclei are usually distinct, but cell borders are not (see UNIT 23.3 for more detail and pictures). Most wells will contain some embryo-derived cells. Figure 23.4.2, panels C through E, shows common morphologies of non-ES cell colonies. 5% to 15% of the wells may contain ES cell–like colonies when using embryos from the 129 mouse strain (see Background Information and see Troubleshooting regarding the effects of mouse strain on ES cell yields).

12. After 3 to 6 days, passage ES cell colonies as follows:

    a. In wells with only a few ES cell colonies, pick cells by pipet as described for the initial inner cell mass outgrowths (steps 5 to 10) and replate on gelatinized 24-well feeder plates.

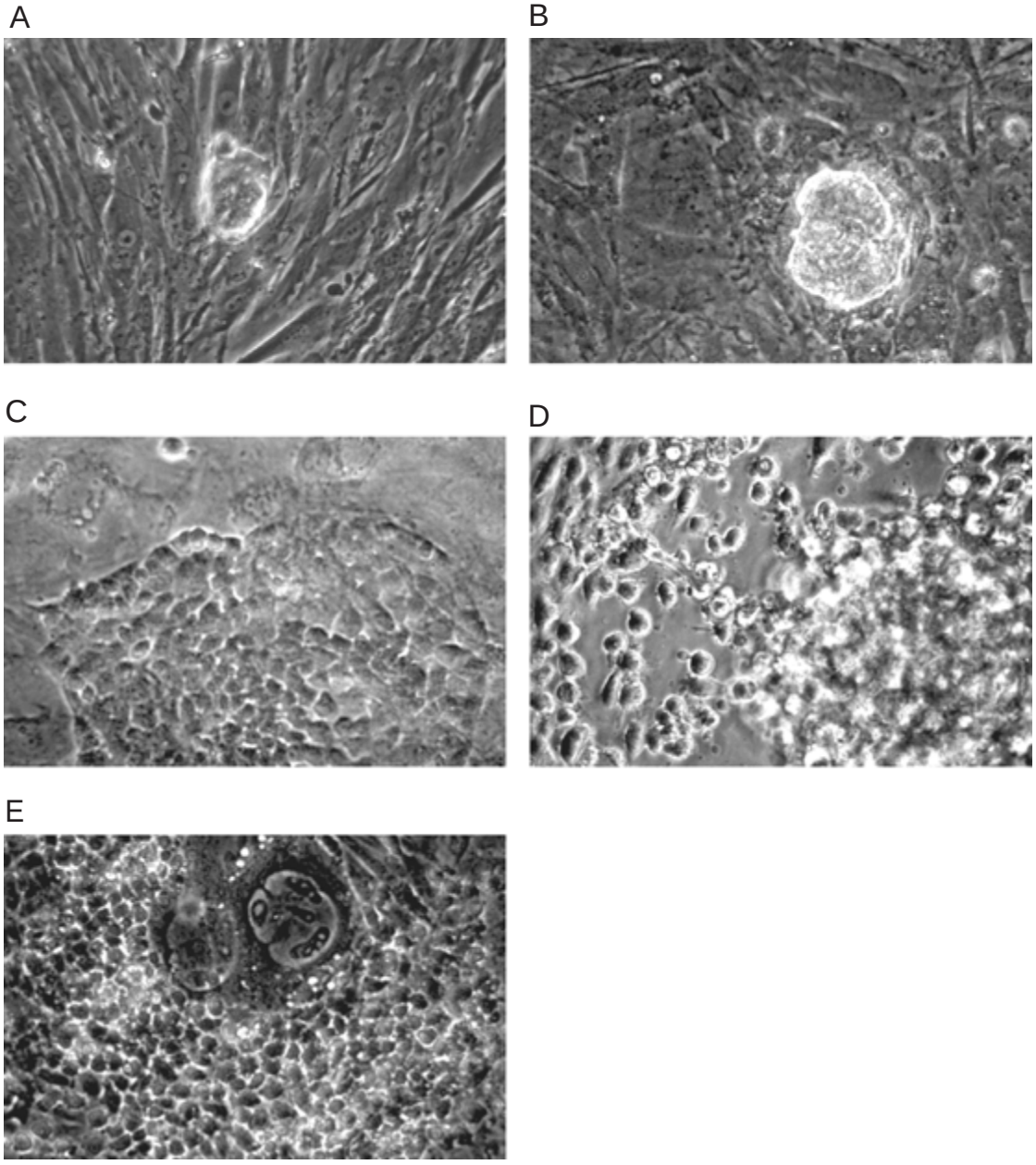

Figure 23.4.2 Cell types observed after inner cell mass disaggregation. Phase-contrast images of some of the different cell morphologies seen 2 to 3 days after disaggregation. (**A, B**) Appearance of putative ES cells. (**C,D,E**) Appearance of common non-ES cell–like colonies. The cells in panel C resemble ES cells to some degree because of the distinct edge. However, unlike ES cells they are growing as a monolayer and the individual cells have distinct borders. Colonies of this type with more tightly packed cells look exactly like ES cells. After passage these misleading colonies will spread out and become easy to distinguish from true ES cells. The round, highly refractal cells in panel D look like the cells that often accumulate on the top of differentiating ES cell colonies. Note that the colony does not have a sharp, refractal edge. The colony in panel E has a diffuse border with giant trophoblast cells visible in the center.

b. In wells with many ES cell colonies, trypsinize the entire well and replate on gelatinized feeder plates with a larger surface area (e.g., 6-well or 25-cm^2 plates) using the protocol described for passaging established ES cell lines (*UNIT 23.3*).

When picking individual colonies, all colonies from a single well can be combined. Deciding when to disperse the colonies depends on colony size and total plate density as described in UNIT23.3. Passage all wells that have colonies that look remotely like ES cells and observe them daily afterwards; non-ES cells will become apparent after passage. In addition, colonies from wells with mixed morphologies should be split. ES cells will usually outgrow other cell types after several passages.

13. Expand each line that maintains an ES cell morphology until there are enough cells to freeze down 3 or more vials at a cell density per vial of $1–3 \times 10^6$ cells/ml (see *UNIT 23.3*).

Typically, enough cells should be present on a 25-cm^2 flask at medium density. The protocols for expansion and freezing are described in UNITS 23.2 & 23.3. Samples of new cell lines should be analyzed for mycoplasma contamination. Investigators may want to karyotype new lines; however, most early-passage lines will have a normal complement of 40 chromosomes.

ES CELL SEX DETERMINATION

SUPPORT PROTOCOL

Male ES lines are used most commonly, primarily because of concerns of X chromosome instability in female ES lines (Robertson et al., 1983). This protocol describes a simple polymerase chain reaction (PCR) screen to determine the sex of an ES cell line.

Additional Materials (also see Basic Protocol)

Digestion buffer (*UNIT 23.5*)
Saturated NaCl
95% ethanol
10× amplification buffer (*UNIT 15.1*)
25 mM 4dNTP mix (*UNIT 15.1*)
Primers (Kunieda et al., 1992):
Set 1: SRY2: TCTTAAACTCTGAAGAAGAGAC
SRY4: GTCTTGCCTGTATGTGATGG
Set 2: NDS3: GAGTGCCTCATCTATACTTACAG
NDS4: TCTAGTTCATTGTTGAGTTGC
5 U/μl *Taq* DNA polymerase
3% (w/v) agarose gel
Molecular weight markers

1.5-ml microcentrifuge tubes
55°C incubator

Additional reagents and equipment for PCR amplification (*UNIT 15.1*), culture of ES cells (*UNITS 23.2 and 23.3*), and agarose gel electrophoresis (*UNIT 2.5*)

Prepare DNA

1. Plate ES cells onto gelatinized 24-well plates without feeder cells. Grow these cells for 3 to 5 passages without feeder cells. Dilute cells at least 1:10 at each split.

The cells must be diluted out to prevent contamination of the PCR reactions from any residual feeder cells. ES cells can be grown in standard ES cell medium (UNIT 23.3) without antibiotics at this stage. Some differentiation will occur in the absence of the feeder layer.

2. Remove medium. Rinse well with HBSS.

3. Add 300 µl digestion buffer, transfer to a 1.5-ml microcentrifuge tube, and incubate overnight at 55°C.

   Plenty of DNA can be obtained from a single well of a 24-well plate, but it is often more convenient to use a larger surface area, such as that of a 25-cm^2 flask.

   Use 1.5 ml of digestion buffer for a 25-cm^2 flask and adjust the volume of subsequent solutions proportionally.

4. Add 150 µl saturated NaCl and vortex vigorously (the solution will turn milky white). Add 2 vol of 95% ethanol (the solution will turn clear except for precipitated DNA).

   Some investigators precipitate the DNA using 2 vol ethanol (or 1 vol isopropanol) without adding salt. However, the DNA pellet resuspends more easily if salt is added.

5. Resuspend DNA pellet in 50 µl water. Determine DNA concentration by measuring the absorbance at 260 nm (*APPENDIX 3D*).

Perform PCR

6. Perform 2 PCR reactions with each sample: one with the SRY primers and the other with the NDS primers. Mix the following in a 0.5-ml thin-walled PCR tube for each primer set.

   2.5 µl 10× PCR buffer (final $MgCl_2$ concentration 1.5 mM)
   0.2 µl 25 mM 4dNTP mix
   0.5 µM primer 1
   0.5 µM primer 2
   0.2 to 1.0 µg DNA template
   0.5 U *Taq* DNA polymerase
   up to 25 µl H_2O

7. Using the following parameters, run the PCR.

35 cycles	30 sec	94°C	(denaturation)
	30 sec	50°C	(annealing)
	60 sec	72°C	(extension)

Analyze the product

8. Run 10 µl of the products on a 3% agarose gel with the appropriate molecular weight markers.

   The SRY primers are derived from the sex-determining region of the Y chromosome. Male cells will show the 404-bp product; female cells will have no product. The NDS primers span a microsatellite dinucleotide repeat on the X chromosome and serve as a positive control; both male and female cells should show the 244-bp product.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

NOTE: Prepare all cell culture solutions from tissue culture-grade reagents. Use tissue culture-grade water (high resistance and endotoxin free). Sterilize all final solutions by filtration or prepare from sterile stocks. Use disposable sterile plasticware to prevent microbial and detergent contamination.

Modified ES medium

Dulbecco's modified Eagle medium (DMEM), with high glucose (4500 mg/liter), L-glutamine, sodium pyruvate, and pyridoxine (or pyridoxal) hydrochloride (e.g., Life Technologies, JRH Biosciences, or Sigma) containing:
20% (v/v) FBS, heat inactivated for 30 min at 56°C
1× MEM nonessential amino acids (Life Technologies)
2 mM glutamine (from 100× stock; Life Technologies)
0.1 mM 2-mercaptoethanol
20 mM HEPES, pH 7.3
500 to 1000 U/ml murine leukemia inhibitory factor (LIF; Life Technologies, Chemicon)
1× penicillin/streptomycin (from 100× stock; Life Technologies)
1× nucleoside stock (see recipe; optional)
Store at 4°C for up to 1 week

The quality of FBS should be screened by assessing plating efficiency, morphology, and toxicity on ES cells plated at low density in 10%, 15%, and 30% heat-inactivated FBS. Plating efficiency should be ~10% and should be similar for all FBS concentrations. The author has had good success with FBS from Hyclone. Several companies sell FBS that has been screened for ES cell growth using the same techniques. Although it is expensive, laboratories that are new to ES cell culture may benefit from prescreened FBS. In addition, prescreened FBS provides a standard of comparison for other FBS lots.

100× nucleoside stock

In 100 ml of tissue culture grade water dissolve:
80 mg adenine
73 mg cytidine
85 mg guanosine
24 mg thymidine
73 mg uridine

Dissolve by warming at 37°C and filter sterilize. Aliquot and freeze at −20°C. Thaw at 37°C and mix vigorously to redissolve nucleosides prior to use.

The addition of a nucleoside stock to the modified ES medium is recommended by Robertson (1987).

COMMENTARY

Background Information

Pluripotent mouse embryonic stem cells were first isolated by Evans and Kaufman (1981) and Martin (1981). The protocol outlined in this unit is essentially that of Axelrod (1984) and Robertson (1987) with some modifications. The primary differences are related to the culturing conditions including the use of MEFs as feeder layers instead of STO (SIM mouse embryo fibroblasts resistant to thioguanine and ouabain) cells and the addition of recombinant leukemia inhibitory factor (LIF) to inhibit differentiation. Evans and Kaufman (1981) used delayed blastocysts, reasoning that the increase in the number of cells in these embryos would increase the likelihood of successful stem cell isolation. Martin (1981) used immunosurgery to isolate the inner cell mass and cultured on STO feeder cell layers with conditioned medium from embryonal carcinoma cells. Many of these ingenious tricks are no longer necessary because of a more refined understanding of the cell culture conditions required to maintain undifferentiated ES cells. Indeed, ES cell lines capable of contributing to the germ line have been isolated in media supplemented with LIF in the absence of feeder cell layers (Pease et al., 1990).

In addition to methodology, genetic background can affect the efficiency of ES cell isolation. The majority of ES cells used for gene targeting are derived from 129 substrains, in part because of the ease with which ES cells can be established from this strain. The nomenclature of the 129 strain has been revised recently; refer to Festing et al. (1999) to clarify strain names. Lines have been isolated with more difficulty from some other common

strains such as C57BL/6 (Ledermann and Burki, 1991), Balb/cJ (Noben-Trauth et al., 1996), and C3H/He (Kitani et al., 1996). The factors that influence strain permissiveness are not known.

Critical Parameters and Troubleshooting

The isolation of ES cell lines is not a complicated procedure; however, this does not mean that the procedure is always successful. Several factors are important to consider. High-quality cell culture reagents and proper aseptic technique are necessary for success. Consult *UNITS 23.2 & 23.3* for more detail on these issues. As indicated above, the strain of mouse from which the embryos are derived can have a dramatic effect on success. If a strain other than a standard 129 substrain must be used, first consult the literature for reports of successful ES cell derivation and for any strain-specific approaches. Second, increase the number of starting embryos, because the yield will probably be lower. Third, verify the technique and reagents used by isolating lines from a permissive strain. The procedure may not be successful even with these modifications. Consider more elaborate approaches such as that reported by McWhir et al. (1996).

The most common problems during isolation of ES cell lines are encountered during the initial dispersion of the inner cell mass outgrowth. Under-trypsinization or very limited trituration will result in transfer of the whole cell clump without dispersion. The large colony will differentiate rapidly. At this stage, the well can sometimes be saved by immediately picking the outgrowth a second time, trypsinizing, and replating. Over-trypsinization or extensive trituration also creates a problem. Single cells do not clone well at this stage. Dispersing the outgrowth into a single-cell suspension will usually result in cell death or differentiation. Both of these problems can be avoided by working with only a few outgrowths at one time and by monitoring the procedure with a microscope. In addition, after dispersal, the wells should be scanned carefully for several days. Single ES cell–like colonies are easy to miss, especially if they sit near the edge of the well, where the image is distorted.

Anticipated Results

With embryos derived from 129 strains (e.g., 129X1/SvJ or 129S6/SvEvTac), 5% to 15 % of the inner cell mass outgrowths should give rise to ES cell lines. Half of the resultant lines should be male.

Time Considerations

Initial inner cell mass outgrowths can be picked 5 to 6 days after plating the embryos. Within 3 to 5 days, ES cell–like clumps can be picked and dispersed; 3 to 5 days later wells containing ES cells can be expanded, and subsequently frozen 2 to 4 days later. For sex determination, aliquots of the cell lines should be expanded without feeder cells for 1 to 2 weeks (i.e., 3 to 5 passages).

Literature Cited

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Key References

Hogan, B., et al., 1994. See above.

Robertson, E.J. 1987. See above.

Wurst, W. and Joyner, A.L. 1993. Production of targeted embryonic stem cell clones. *In* Gene Targeting: A Practical Approach (A.L. Joyner, ed.) pp. 33-61. IRL Press, Oxford.

These three references, written by experts in the field, represent the best compilations of ES culture and isolation methods.

Internet Resources

http://www.biosupplynet.com

Search this web site for "embryonic stem cell reagents" to obtain a current list of suppliers that provide medium and ES cells.

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