Dave LePage: Protocol for derivation of embryonic stem cell lines from mouse embryos

References

I used a pastiche of three original sources:
Manipulating the Mouse embryo, 3 ed. Protocol 4.11: “Collecting Blastocysts” was employed for isolation of 3.5dpc blastocysts.

Manipulating the Mouse Embryo, 3 ed. Protocol 8.5: “De novo isolation of embryonic stem cells from blastocysts” was employed for disaggregating the embryo explants. {The most difficult part of the procedure}

Bryja, V. et al. “An efficient method for derivation of mouse embryonic stem cells”, Stem Cells 2006; 24:844-849, was employed for all the media formulations and timing of the disaggregation steps.

Protocol for derivation of embryonic stem cell lines from mouse embryos

Schedule of Timed Matings

The method involves close collaboration with an investigator who will do all the mouse breeding to generate 4-5 plugged females by natural mating. The investigator should use the following breeding regime:

You will generate 5 females plugged on the same day, on a day agreed to in advance with us. Because the matings are done without hormone stimulation, you will need 10 stud males and a large cohort of 25 young, sexually mature females (6-8 weeks). If you can identify estrus females, you can use a smaller number of males. The males should be experienced in mating but rested for a few days prior to attempting these critical matings.

After coordinating with us, you will place 2-3 females with each male on the Sunday. On the Monday morning, you will check plugs. If one quarter of the females were in estrus, we would expect 5-6 females to have mated. Remove all the females and house the plugged females together. On Thursday, bring the plugged females up to your lab.

The females are brought up to the investigator's lab and I do the dissection, flushing, embryo culture, embryo disaggregation, and culture of any resulting ES cell lines.

A rough outline of a single mating and derivation cycle is detailed. If a second round of mating is required, it is staggered by a week following the first round.

(Week 1) Sunday: Investigator's lab sets-up matings
Monday: Check plugs. Set mated females aside. Unmated females can be used again (0.5 d.p.c.).
Tuesday: 1.5 d.p.c.
Wedneday: 2.5 d.p.c. Plate out PMEFs for embryo culture.
Thursday: Mice are brought up to investigator's laboratory (3.5 d.p.c.).

I euthanize mice and dissect uteri.

Return to transgenic lab and flush out 3.5 d.p.c. embryos.

Embryos put into culture in individual 4-well PMEF using SR-medium (day 0).

Depending on yield, decide if a second round of matings is required, based on goal of 25-40 embryos.

Friday: day 1.
Saturday: day2.
(Week 2) Sunday: day 3.

Monday: day 4.
Tuesday: day 5.
Wednesday: day 6--Primary embryo outgrowth disaggregation. (Hardest part).
Disaggregations put into 4-well PMEFs with FBS-medium.
Thursday: day 7. Change medium to SR-medium.
Friday: day 8.
Saturday: day 9.
(Week 3) Sunday: day 10.
Monday: day 11. Critical decision day. Is there anything worth passaging?
Secondary passaging day for most outgrowths, using FBS-medium onto 4-well PMEFs.
Each secondary becomes an individual cell line.
Tuesday: day 12. Switch to SR-medium and feed daily thereafter. Stragglers?
Wednesday: day 13. Stragglers?
Secondaries from Monday (d.11) may be ready to passage soon.
Begin passaging onto 35mm PMEFs as required.
Passage in FBS-medium. Next day switch to SR-medium and feed daily thereafter.
Thursday: day 14. Passaging? Stragglers?
Friday: day 15. Passaging? Stragglers?
Saturday: day 16. Passaging? Stragglers?
(Week 4) Sunday: day 17. Passaging? Stragglers?
Monday: day 18. Last chance for any stragglers. Passaging?

Freeze individual lines in vials as appropriate, removing aliquots for DNA.
Evaluate DNA aliquots for likelihood of adaptation to growth on gelatinized plastic.

Protocol for derivation of embryonic stem cell lines from mouse embryos

Initial culture conditions

Matings are set-up on Sunday and plugs are checked on Monday for delivery of mice on Thursday. On the **Wednesday** prior to dissecting embryos, prepare 4-well plates with PMEFs as follows:

Establishing monolayers of PMEFs

Components:

Iscove's modified dulbecco's medium (IMDM); Gibco/Invitrogen #12440-053 for 500ml; 12440-046 for 1 L.

Hyclone FBS tested for ES cells; heat inactivated; 40ml aliquots; stored -20. Characterized Fetal Bovine Serum, Catalog #SH30071, Lot#ARH27145.

100x Pen/strep: Gibco/Invitrogen #15070-063 for 100ml; 10ml aliquots; store -20.

Primary mouse embryonic fibroblast feeder layers (PMEFs), Mitomycin-C treated. Purchased from Specialty Media (now Millipore), PMEF-CF.

Gelatinized tissue culture dishes:
.1% gelatin (typeB from bovine skin, Sigma G9382); .3g in 300ml ddH2O autoclaved; cover dishes with solution and aspirate off; allow to air dry in hood--approximately 1/2 hour (alternatively can allow to air dry overnight): use within 24 hours.

4-well plates: Nunc multidish 4wells (176740).

PMEF-medium/50 ml:
44.5ml Iscove's Modified Dulbecco's Medium
5ml FBS (10% final)
0.5ml 100x PEN/STREP (50 u or μg/ml final)

(1) Rapidly thaw 1 vial of PMEF-CF cells in a waterbath and transfer to 10ml of PMEF-medium. Spin down and resuspend into 25ml PMEF-medium.

(2) Transfer 0.5ml/well onto several gelatinized 4-well plates. {Depending on the expected yield of embryos, you will want 6-10 plates total}

(3) Incubate overnight. Verify the presence of a mono-layer prior to use. {Mono-layers are good for about 2 weeks}

Preparing PMEF plates for embryo culture
Prior to culturing embryos, replace PMEF-medium with **SR-medium**.

Recipe for SR-medium

Components:
Iscove's modified dulbecco's medium (IMDM); Gibco/Invitrogen #12440-053 for 500ml; 12440-046 for 1 L; store 4 degrees and protect from light.

Knockout SR serum replacement for ES cells (SR); Gibco 10828-028; 10ml aliquots; stored -20. DO NOT HEAT INACTIVATE.

100x 2-mercaptoethanol (Sigma M7522, 14.3 M stock): dilute 70 microliters into 100ml of sterile ddH2O and filter sterilize; 10ml aliquots; store 4 degrees.

100x MEM nonessential amino acids: Gibco/Invitrogen #11140-050 for 100ml; 10ml aliquots; store 4 degrees.

100x Pen/strep: Gibco/Invitrogen #15070-063 for 100ml; 10ml aliquots; store -20.

Recombinant LIF protein, produced as per the V. Prideaux protocol; filter sterilized; put into 30 microliter aliquots; flash frozen in liquid nitrogen and stored -80; each batch tested for efficacy against older batches. Alternatively LIF can be purchased from Chemicon (ESGRO 10^7 units: ESG1107; enough for 10L of medium) and supplemented at 10^3units/ml.

SR-medium/50 ml:
40ml Iscove's MDM (contains 4 mM L-glutamine and 1 mM sodium pyruvate)
To an aliquot of IMDM add in **exactly** the order listed:
10ml SR (20% final)
0.5ml 100x (10 mM) 2-mercaptoethanol (.1mM final)
0.5ml 100x MEM nonessential amino acids (.1 mM final)
0.5ml 100x PEN/STREP (50 u or μg/ml final)
30 μl of LIF (one -80 aliquot)

(4) Aspirate PMEF-medium from 4-well plates and replace with 1.0ml **SR-medium**. Return to incubator and allow to equilibrate for several hours prior to culturing embryos. {Do this prior to fetching your uteri}

Protocol for derivation of embryonic stem cell lines from mouse embryos

Dissection of 3.5dpc mouse embryos

4-5 plugged females should be delivered to the investigator's lab on **Thursday** morning.

Prior to dissecting embryos prepare the following:
Procure some FHM or M2 medium from microinjection colleagues. {I have used both types of flushing medium with success}

Blunt 2x30-gauge needles and load onto 1.0ml syringes with FHM or M2 medium.

Pull a small collection of embryo handling pipets from 4 inch stock.

Prepare a 35mm dish with 4 drops of FHM or M2 medium for collecting and washing the embryos.

(5) Take a bunny suit, mask, gloves, and appropriate tools with you to the investigator's lab. Gown up.

(6) Using a separate set of tools, dissect out the uteri from all the females, one at a time. {Make these dissections as clean as possible: the less fat and debris, the easier it will be to see the embryos after you flush them out}

(7) Place the uteri into a large drop of FHM or M2 medium in a 35mm dish. Discard gown and return to transgenic lab.

(8) Back in the transgenic lab, put on a new gown, mask, and gloves. Flush out the embryos in a surgery hood with dissecting microscope.

(9) Using separate tools, cut a single half of a uterus just above the cervix. Place it dry into a clean 35mm dish. Visualize the infundibulum and flush with 0.1-0.2ml of medium toward the cut end of the uterus.

(10) Remove the flushed half of a uterus and visualize embryos under the dissecting microscope. Collect embryos and place in the collection plate (drop1).

(11) Continue with all the other halves of uteri. Collect all embryos into drop1 of the collection plate.

(12) Gently transfer the embryos through the other 3 drops on the collection plate. All the embryos will be together in drop4.

(13) Discard gown and transfer the collection plate into the tissue culture lab.

(14) Visualize embryos under the tissue culture microscope (4x objective). Remove appropriate number of 4-well PMEF plates from incubator (each well with 1ml of SR-medium).

(15) Transfer an individual embryo to an individual well. Number all wells that contain an embryo. Return all plates to the cell culture incubator and leave undisturbed for **6 days**. {Count the Thursday of culture as **Day 0**}

(16) The goal is 25-40 embryos. Depending on the yield of embryos from the first collection, contact the investigator's lab if you need them to go through another cycle of breeding. Mating, preparation of PMEF plates, dissection, and collection of embryos will be repeated exactly as previously described. After two cycles of breeding, stop. Do not continue embryo collection until the ES cell derivation procedure has been completed.

Protocol for derivation of embryonic stem cell lines from mouse embryos

Primary disaggregation (1^{o}) of embryo explants

On **Day 6** of incubation (**Wednesday**) the embryo explants are ready for disaggregation. Each disaggregation is done individually. {This is the single hardest and touchiest part of the entire procedure and it normally takes ALL DAY}

For each embryo to be disaggregated, prepare a single receiving well of a 4-well PMEF plate. Replace the PMEF-medium with **ES cell culture medium**.

Recipe for ES cell culture medium

Components:

Iscove's modified dulbecco's medium (IMDM); Gibco/Invitrogen #12440-053 for 500ml; 12440-046 for 1 L; store 4 degrees and protect from light.

Hyclone FBS tested for ES cells; heat inactivated; 40ml aliquots; stored -20. Characterized Fetal Bovine Serum, Catalog #SH30071, Lot#ARH27145.

100x 2-mercaptoethanol (Sigma M7522, 14.3 M stock): dilute 70 microliters into 100ml of sterile ddH2O and filter sterilize; 10ml aliquots; store 4 degrees.

100x MEM nonessential amino acids: Gibco/Invitrogen #11140-050 for 100ml; 10ml aliquots; store 4 degrees.

100x Pen/strep: Gibco/Invitrogen #15070-063 for 100ml; 10ml aliquots; store -20.

Recombinant LIF protein, produced as per the V. Prideaux protocol; filter sterilized; put into 30 microliter aliquots; flash frozen in liquid nitrogen and stored -80; each batch tested for efficacy against older batches. Alternatively LIF can be purchased from Chemicon (ESGRO 10^{7} units: ESG1107; enough for 10L of medium) and supplemented at 10^{3}units/ml.

ES cell culture medium/50 ml:

40ml Iscove's MDM (contains 4 mM L-glutamine and 1 mM sodium pyruvate)
To an aliquot of IMDM add in **exactly** the order listed:
10ml FBS (20% final)
0.5ml 100x (10 mM) 2-mercaptoethanol (.1mM final)
0.5ml 100x MEM nonessential amino acids (.1 mM final)

0.5ml 100x PEN/STREP (50 u or μg/ml final)
30 μl of LIF (one -80 aliquot)

(17) For each well that will receive a disaggregated embryo explant, aspirate the PMEF-medium and replace with 1.0ml **ES cell culture medium**. {Do this first and allow the receiving plates to equilibrate in the incubator for a little while prior to use}

Prior to each individual disaggregation prepare the following:
Pull 1 or 2 pasteur pipets. Go for a fairly wide bore and straight line (no kinks). {The explants are sticky and a wide bore prevents trapping}

Pull a small collection of embryo handling pipets, but use **6-inch stock**. {I like these to have a little longer back end, in case I suck up too hard: 1^{o} disaggregations can get rough and these explants are very precious}

Put a 50microliter drop of trypsin (Trypsin (1x), SAFC Biosciences [thru Sigma], 59428C-500ML) on a 35mm dish. Cover with sterile filtered mineral oil.

(18) Wash an individual embryo explant twice with PBS. Leave the second wash on the well.

(19) Visualize the explant under the tissue culture microscope (4x objective). Score around the explant with a pasteur pipet. Gently transfer the explant with an underlying fragment of monolayer into the drop of trypsin under the oil. Return the original 4-well plate to the incubator. {The explants look VERY DIFFERENT from conventional ICMs: much smaller and sometimes they consist of more than one rounded mass}

(20) Place the dish in the incubator and incubate for 5 min. {Prepare the pipets and trypsin you will need for the next explant during this time}

(21) Remove the trypsin dish and a fresh 4-well with ES cell culture medium from the incubator. Label the receiving well with the embryo's number and 1^{o}.

(22) Take a long embryo handling pipet with a bore that is no wider than the explant. Suck up some ES cell culture medium from the receiving well. Visualize the trypsinized explant under the microscope and expel a small amount of FBS medium into the drop of trypsin, swirling it around the explant.

(23) Take the embryo handling pipet and suck up the trypsinized explant back and forth several times. The explant will start to disaggregate. This will often take a lot of suction, as the explant is very resistant to disaggregation. Reduce the

explant into several smaller clumps of cells, but do not attempt to reduce it completely to single cells. {In fact, you probably couldn't break it down to single cells, even if you tried}

(24) Transfer the clumps of cells to the receiving well, gently distributing the clumps all over the monolayer, as visualized under the microscope. Return plate to incubator.

(25) Repeat these steps for each individual embryo explant. Alternate the culture and receiving plates so that no 4-well plate stays out of the incubator for a long time. Return all 1^{o} disaggregations to the incubator. {**Day 6**}

(26) The next day (**Day 7**), replace the ES cell culture medium with 1.0ml **SR-medium**.

(27) Leave primary disaggregations undisturbed for 4-5 days. Monitor daily.

(28) Around **Day 11** you should start to see clear nests of ES cells in some of the 1^{o} wells. These can be passaged (see below). However, do not discard anything yet. Keep and eye on the primary disaggregations for 4-5 more days, as some of them will often begin to display ES cells. Passage these later lines as appropriate. After a week, if nothing shows up, discard the primaries.

Protocol for derivation of embryonic stem cell lines from mouse embryos

Secondary passaging (2^o) of ES cells from primary disaggregations

Further passaging is done into **ES cell culture medium** on PMEFs. Following overnight incubation, the medium is replaced with **SR medium**. Feed daily thereafter, until confluent.

(29) Around day 11, wash an individual ES cell containing primary well with PBS.

(30) Apply 0.2ml of trypsin and return to incubator for 5 min.

(31) Apply 1.0ml of **ES cell culture medium**. Break up the well with a blue tip. Transfer entire contents of the well to the well of a fresh 4-well PMEF plate. {This 2^o passaging step should seem very easy compared to the primary disaggregations}

(32) The next day, aspirate the medium and replace with **SR-medium**.

(33) Feed daily with **SR-medium** until confluent. At this point the well will contain an established individual ES cell line, and it should be monitored and passaged just like a normal ES cell line.

(34) Passage to 1 or 2 fresh 35mm PMEF dishes as appropriate. Employ **ES cell culture medium** for all trypsinization steps. Following overnight incubation, replace with **SR-medium** and feed daily until confluent.

(35) Generate at least ½ a confluent 35mm dish to create a ½ vial of cells for freezing. {That is an absolute minimum, the preference would be one or two full vials, each containing a confluent 35mm dish on PMEFs}

(36) At freezing take a small aliquot for DNA. Passage the aliquot onto gelatinized plastic with ES cell culture medium. Replace with SR-medium the next day and grow the DNA aliquot until confluent. Carefully observe the DNA aliquot. Cell-lines that **will** adapt off of feeders should retain normal ES cell morphology and should grow quickly. Cell-lines that **will not** adapt are very obvious when taken off of feeders, and should grow slowly.

Protocol for derivation of embryonic stem cell lines from mouse embryos

Growing de-novo mouse embryonic stem-cell lines (general guidelines)

These cells were derived using the basic protocol described in Bryja, et al. [An efficient method for derivation of mouse embryonic stem cells. Stem Cells 2006; 24:844-849.], with modification. This method requires alternating the media employed. All trypsinization steps employ standard FBS containing **ES cell culture medium** (see below) onto primary mouse embryonic fibroblast feeder layers (PMEFs). After setting down overnight the medium is changed to **SR-medium**, with the FBS replaced 1:1 with Serum Replacement (SR). Cells are fed daily thereafter with **SR-medium** until confluent, then trypsinized using FBS medium for all passaging and cryopreservation steps.

For the initial thawing, passaging, and cryopreservation employ only the TMF-house FBS that I will provide. A standard vial is the contents of a confluent 35mm dish on PMEFs. It should be thawed 1:1 onto PMEFs as follows:

(1) Thaw vial rapidly in water bath and put into 8mls of FBS containing **ES cell culture** medium.

(2) Recover cells by centrifugation and plate out onto PMEFs with **ES cell culture** medium.

(3) The next day replace medium with **SR-medium** and feed daily thereafter until confluent.

(4) Once confluent, passage cells 1:3 or 1:4. Alternate the media as appropriate.

(5) Once the passaged cells are confluent the majority can be cryopreserved with FBS medium. However, passage a small aliquot of cells using your own serum to verify that the cells will accept different serum. Assuming they hold up in your own serum you can stop using the TMF-house FBS.

Components:

Iscove's modified Dulbecco's medium (IMDM); Gibco/Invitrogen #12440-053 for 500ml; 12440-046 for 1 L; store 4 degrees and protect from light.

Hyclone FBS tested for ES cells; heat inactivated; 40ml aliquots; stored -20. Characterized Fetal Bovine Serum, Catalog #SH30071, Lot#ARH27145.

OR

Knockout SR serum replacement for ES cells (SR); Gibco 10828-028; 10ml aliquots; stored -20. DO NOT HEAT INACTIVATE.

100x 2-mercaptoethanol (Sigma M7522, 14.3 M stock): dilute 70 microliters into 100ml of sterile ddH2O and filter sterilize; 10ml aliquots; store 4 degrees.

100x MEM nonessential amino acids: Gibco/Invitrogen #11140-050 for 100ml; 10ml aliquots; store 4 degrees.

100x Pen/strep: Gibco/Invitrogen #15070-063 for 100ml; 10ml aliquots; store -20.

Recombinant LIF protein, produced as per the V. Prideaux protocol; filter sterilized; put into 30 microliter aliquots; flash frozen in liquid nitrogen and stored -80; each batch tested for efficacy against older batches. Alternatively LIF can be purchased from Chemicon (ESGRO 10^7 units: ESG1107; enough for 10L of medium) and supplemented at 10^3units/ml.

Gelatinized tissue culture dishes:
.1% gelatin (typeB from bovine skin, Sigma G9382); .3g in 300ml ddH2O autoclaved; cover dishes with solution and aspirate off; allow to air dry in hood--approximately 1/2 hour (alternatively can allow to air dry overnight): use within 24 hours.

Primary mouse embryonic fibroblast feeder layers (PMEFs), Mitomycin-C treated. Purchased from Specialty Media (now Millipore), PMEF-CF.

ES cell culture medium **or** SR-medium/50 ml:
40ml Iscove's MDM (contains 4 mM L-glutamine and 1 mM sodium pyruvate)
To an aliquot of IMDM add in **exactly** the order listed:
10ml FBS **or** SR (20% final)
0.5ml 100x (10 mM) 2-mercaptoethanol (.1mM final)
0.5ml 100x MEM nonessential amino acids (.1 mM final)
0.5ml 100x PEN/STREP (50 u or μg/ml final)
30 μl of LIF (one -80 aliquot)