WORKING WITH HAPLOID MOUSE

EMBRYONIC STEM CELLS
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ES cell culture using serum and LIF

ES cells can be grown in serum containing medium in the presence of feeders and LIF. Serum is a natural product and has to be selected for use in ES cell culture. Commercial sources offer ES cell pretested sera and it is advisable that a suitable serum batch is reserved for stable culture conditions. The cultures should be passaged every 2 to 3 days using trypsin/EDTA.

500 ml ES DMEM medium:
500 ml high glucose DMEM (PAA)
75 ml Fetal bovine serum (FBS, eg. PAA ES cell pretested FBS)
5 ml Glutamine (100 x, Life Technologies)
5 ml Pen/Strep (100 x , Life Technologies)
5 ml NEAA (100 x, Life Technologies)
5 ml Sodium Pyruvate (100 x, Life Technologies)
4 ul β-mercaptoethanol (Sigma)
500.000 units LIF (=5 ug; eg. eBioscience, 14-8521-80, mouse recombinant LIF, 25 ug, GBP 145.57)

Preparation of feeder cells
ES cells are grown on a layer of mitotically inactivated mouse embryonic fibroblast (MEF) cells. For preparation dissect embryos at embryonic day 13.5 and remove the head, liver and guts. A suspension of small clumps of tissue is then easily achieved by passing the embryo through an injection needle using a 5ml syringe. Culture the MEFs in high glucose DMEM containing 10% FBS, 1 x Glutamine, 1 x Pen/Strep and 4 ul/500ml β-mercaptoethanol. Three embryos can be pooled in a 150 cm2 Tissue culture dish. Passage every 3 days 1:3 for a total of 3 passages (per embryo 30 150cm2 plates). Mitotically inactivate the cells in suspension for 30 minutes using a Gammacell 40 irradiator. Centrifuge and freeze aliquots for later use. Freezing medium is 3 volumes medium, 2 volumes serum, 1 volume DMSO and should be pre-cooled on ice. For use thaw the inactivated feeders in a 37°C water bath, wash once with medium, and plate onto gelatine treated tissue culture flasks.

Preparation of gelatinized plates
For ES cell culture tissue culture plastic requires coating with gelatine. For this prepare a 0.2% solution of gelatine (eg. SIGMA G1890; gelatine from porcine skin) in water. Dissolve gelatine completely using a microwave and then autoclave the solution (do not sterile filter!). The solution can be stored under sterile conditions at room temperature. For treating tissue culture plastic cover the surface with gelatine solution and let stand for at least 10 minutes in the laminar flow tissue culture work bench. Remove the gelatine solution and replace by medium or plate feeder cells.

ES cell culture using serum replacement

As an alternative to growing ES cells in serum containing medium a serum replacement can be used. Life Technologies offer Knockout™ Serum Replacement (Cat. No. 10828-028, 500 ml) that can be used as to substitute the fetal bovine serum. Feeder cells will need to be preplated into gelatine treated tissue culture flasks in serum containing medium the day before as MEFs will not attach in
serum free medium. Following passaging the cells, trypsin needs to be inactivated either by resuspending the cells in serum containing medium and centrifugation before resuspending in new media.

500 ml ES DMEM medium with serum replacement:
500 ml high glucose DMEM (PAA)
75 ml Knockout™ Serum Replacement
5 ml Glutamine (100 x, Life Technologies)
5 ml Pen/Strep (100 x, Life Technologies)
5 ml NEAA (100 x, Life Technologies)
5 ml Sodium Pyruvate (100 x, Life Technologies)
4 ul β-mercaptoethanol (Sigma)
500,000 units LIF (=5 ug; eg. eBioscience, 14-8521-80, mouse recombinant LIF, 25 ug, GBP 145.57)

ES cell culture in chemically defined 2i medium

ES cells can be cultured in an undifferentiated state in chemically defined 2i medium plus LIF. Feeders can be optionally added and will enhance cell attachment to the tissue culture dish. If feeders are to be used these need to be plated in regular serum containing medium in gelatine coated tissue culture flasks as MEFs will not attach in 2i medium. For using feeders preplate the feeders the day before and wash feeders once with PBS before plating ES cells in 2i medium. It is important to note that 2i medium does not contain serum or Trypsin inhibitors, therefore using Accutase for passaging the culture is advisable. Accutase is removed by centrifugation and resuspension in new 2i medium. For routine culture cells should be passaged every 2 days.

100 ml 2i medium:
100 ml N2B27 (eg. StemCells Inc: NDiff N2B27; 500mL; Cat. No. SCS-SF-NB-02;)
30 µl CHIR99021 (10 mM stock, store at -20°C; eg. reagentsdirect Cat No. 27-H76, 20mg,)
10 µl PD0325901 (10 mM stock, store at -20°C; eg. reagentsdirect Cat No. 39-C68, 20mg)
100,000 units LIF (=1 ug; eg. eBioscience, 14-8521-80, mouse recombinant LIF, 25 ug,)
5 ml 7.5% BSA (Life Technologies)
1 ml Pen/Strep (100 x Life Technologies)

Passaging of cells cultures in 2i
(Optional: wash once with Accutase (Invitrogen) - this step is not necessary and possibly risky when cells are not well attached)
incubate with Accutase 5 minutes at 37°C
add 10 ml DMEM/F12 (PAA) with 3 ml 7.5% BSA per 500 ml and pipette up and down 5 times to make a single cells suspension
spin at 900 rpm for 5 minutes
take up in fresh 2i medium and plate into new tissue culture flask (in a ratio of 1:5 to 1:10)

Alternative:
passage with Trypsin and take up in serum containing ES DMEM. Then spin down or directly plate into 2i medium (note a small amount of serum containing medium does not disrupt 2i culture).
Sorting the haploid G1 population of ES cells by HOECHST 33342 sorting

Prepare HOECHST 33342 1:100 dilution:
Add 10 microliter HOECHST 33342 dye (Invitrogen) to 1 ml ES DMEM medium. DO NOT FILTER as the dye will bind to the filter and staining does not work! Keep away from direct light!

Trypsinize ES cells and take up in ES DMEM medium with serum. Spin cells down (900 rpm, 5 minutes) and take up in 500 microliter 2i LIF medium per T25 flask (T75 flask in 1.5 ml medium). Add 100 microliter (T25; for T75 add 300 microliter) HOECHST 33342 1:100 diluted in ES-DMEM and incubate in a 15 ml falcon tube for 30 minutes at 37°C in a tissue culture incubator.
Note: The final dilution of HOECHST 33342 for the staining is 1:500

Pass the cells through a 30 micron cell strainer (FALCON) into a polypropylene FACS tube (BD) for sorting on a MoFlo cell sorter. Collect cells in a FACS tube with 1 ml ES DMEM with serum.

After sorting collect the cells in a 15 ml FALCON tube with 10 ml ES DMEM with serum and spin down (800 rpm, 5 minutes). Resuspend in 2i LIF medium and plate on feeders with pre-equilibrated 2i LIF medium. As a rough guide plate between 1x10^6 and 2x10^6 cells into a T25 flask (not all will survive, avoid plating at a too low density). Cells should be passaged after 2 or 3 days and can then be used for transfection with genetrap vectors directly or after one more passage. Usually 129/Sv mouse strain derived haploid ES cells lines will be stable with a haploid content of >80% for two to three passages.
Oocyte collection from mice and activation

Prepare Hyaluronidase (10 mg/ml stock) in M2 medium:
Dissolve 30 mg Hyaluronidase (Type IV-S, from bovine testes, embryo tested, SIGMA, Prod No: H4272) in M2 medium (SIGMA M7167, 50 ml add 500 microliter 100 x Pen/Strep). Filter sterilize and freeze in 200 microliter aliquots, store at -20°C.

Harvest unfertilized oocytes from superovulated female mice on E0.5. Dissect oviducts and isolate egg masses in M2 medium (3 cm dish with 3 ml M2) using a stereomicroscope. Digest cumulus masses with Hyaluronidase, wash three times in M2 medium.

Oocyte activation:
Add 150 microliter 100 mM SrCl2 and 12 microliter 0.5M EGTA (pH=8) to 3 ml M16 medium (SIGMA M7292, 50 ml add 500 microliter 100x Pen/Strep) or KSOM (Millipore, MR-020P-5F). Filter sterilize to a 3.5 cm dish and pre-equilibrate to 37°C in a tissue culture incubator set to 5% CO2. In the following detail will be given for using M16 medium but KSOM can be used interchangeably.

Prepare 3 dishes with M16 medium and M16 microdrops (place in a 6 cm dish 4 drops of 80 microliter M16 covered with 5 ml mineral oil; SIGMA M8410, 100 ml) equilibrate in tissue culture incubator.

Incubate oocytes for 90 minutes in activation medium in tissue culture incubator.

Wash oocytes through 3 dishes M16 medium and then culture groups of 30-60 oocytes in M16 microdrops for 3 days. Avoid moving dishes not to perturb haploid embryo development.

ES cell derivation:
Inspect cultures every day and follow embryo development. Transfer 8 cell embryos into M16 (or KSOM) supplemented with CHIR99021 (3μM), and PD0325901 (1 μM). Optional: On the next day, transfer blastocysts to N2B27+2i medium and culture another 24h. The resulting blastocysts will vary with some large ones having hatched and smaller blastocysts with a just visible blastocoel cavity. Remove the zona by incubation in prewarmed (37°C) acidic tyrode's solution (SIGMA T1788, 100 ml; store in 1 ml aliquots at -20°C). Observe zona dissolving (should take around 30-60 seconds) and immediately move to dish with M2 medium. Wash through two more dishes with M2 medium and then plate individual blastocysts into a 96 well plate with feeders in 2i+LIF medium. Leave plates untouched for 3 days to allow embryo attachment and ICM outgrowth. Inspect for growth and trypsinize positive wells using 25 μl Trypsin. Add 75 μl ES DMEM with 15% serum and transfer to a new 96 well plate with feeders and pre-equilibrated 2i + LIF medium (200 μl per well). ES cell colonies should then emerge and can be passaged after 3 days to a 24 well plate.