**Working with Haploid Mouse Embryonic Stem Cells**

**ES cell culture in chemically defined 2i medium**

ES cells can be cultured in an undifferentiated state in chemically defined 2i medium. Haploid ES cells can be cultured on gelatine coated tissue culture vessels. Feeders can be added optionally 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, pre-plate the feeders the day before.

**100 ml 2i medium:**

100 ml NDiff (e.g. StemCells Inc: NDiff 227; 500mL;Takara Clontech, catalogue number Y40002;

30 μl CHIR99021 (10 mM Stock, store at -20°C; e.g. Reagentsdirect Cat No. 27-H76, 2mg,)

10 μl PD0325901 (10 mM Stock, store at -20°C; e.g. Reagentsdirect Cat No. 39-C68, 2mg)

20ng/ml LIF e.g. eBioscience, 14-8521-80, mouse recombinant LIF, 25 µg)

5 ml 7.5% BSA (Life Technologies, product number: 15260-037) – optional in case of attachment problems

1 ml Pen/Strep (100 x Life Technologies, product number15140-122) optional.

**Passaging of cells cultures in 2i**

For routine culture cells should be passaged every 2 days. Wash cells carefully with PBS. Add 700ul Accutase (Life Technologies, product number 15140A11105-01) to T25 flask and incubate 5 minutes at 37°C. Add 10 ml DMEM/F12 (Gibco) / 0.075% BSA (to prepare wash buffer add 5ml 7.5% BSA to 500ml DMEM/F12) and pipette up and down 5 - 8 times to make a single cells suspension. Spin at 300g for 5 minutes. Take up in 1ml fresh 2i medium and plate into new tissue culture flask (in a ratio of 1:5 to 1:10).

**Alternative: Passage using Trypsin**.

We recommend Trypsin if cells are grown on a feeder layer. Add 500ul Trypsin/EDTA to a T25 flask,

incubate for 3 minutes at 37oC and take up in 5ml serum containing ES DMEM (2i does not contain

serum or Trypsin inhibitors). Pipette up and down 5 to 8 times to generate a single cell suspension.

Then spin down and plate into 2i medium in a 1:5 to 1:10 ratio (note a small amount of serum

containing medium does not disrupt 2i culture).

**Freezing haploid ES cells**

Haploid ES cells can be frozen using standard procedures. 2x Freezing medium is 80% FBS and 20% DMSO and should be pre-cooled on ice.

**Thawing haploid ES cells**

Haploid ES cells should be thawed quickly in a 37oC waterbath. Then re-suspend in 5ml ES medium and spin 5 min at 300g. Re-suspend pellet in appropriate volume of 2i medium and plate. Thawing can induce diploidisation and haploid ES cells should be FACS purified within 2 passages after thawing.

**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 µl/500ml β-mercaptoethanol. Three embryos can be pooled in a

175 cm2 tissue culture dishes. Passage every 3 days 1:3 for a total of 3 passages (per embryo 30 x

cm2 plates). Mitotically inactivate the cells in suspension for 30 minutes using a Gammacell 40

irradiator or similar. Centrifuge and freeze aliquots for later use. 2x Freezing medium is 80% FBS and 20% 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 (e.g. 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 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 trysin/EDTA. Haploid ES cells can be cultured in serum / LIF conditions, but the rate of diploidisation is greatly increased!

**500 ml ES DMEM medium:**

500 ml high glucose DMEM (Gibco), product number 11965-092.

75 ml Fetal bovine serum (FBS, ES cell pretested FBS)

5 ml Glutamine (100 x, Life Technologies, product number 25080-081)

5 ml Pen/Strep (100 x , Life Technologies, product number15140-122)

5 ml NEAA (100 x, Life Technologies, product number 11140-050)

5 ml Sodium Pyruvate (100 x, Life Technologies, product number 11360-039)

182 μ L of 55 mM 2-mercaptoethanol (1:550 dilution), (Life Technologies, product number 21985023)

20 ng/ml LIF e.g. eBioscience, 14-8521-80, mouse recombinant LIF, 25 ug)

**Sorting haploid ES cells from a mixed haploid / diploid population**

Prepare HOECHST 33342 1:10 dilution: Add 10 microliter HOECHST 33342 dye (Life Technologies, product number 62247) to 90ul NDIFF 227 medium.

Trypsinize ES cells and take up in ES DMEM **medium** with serum. Spin down cells (300g, 5

minutes) and take up in 500 microliter 2i/LIF medium per T25 flask (T75 flask in 1.5 ml medium). Add

7.5 microliter (T25; for T75 add 22.5 microliter to 1.5ml cell suspension) 1:10 diluted HOECHST

33342 and incubate in a 15 ml falcon tube for 20 minutes at 37°C in a tissue culture incubator.

Note: The final dilution of HOECHST 33342 for the staining is 15ug/ml.

Pass the cells through a 50 micron cell strainer (FALCON) into a polypropylene FACS tube (BD) for

sorting on a cell sort. Collect cells in a FACS tube containing 1 ml ES DMEM with serum / LIF.

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**Note:** sorting based solely on forward / side scatter parameters is possible (see Figure 1) and can

achieve similar purities as sorting by Hoechst 33342 on a well calibrated MoFlo. Using this strategy

does not require a UV laser. The sorting gates can be adjusted using a diploid control cell line that

was grown in identical growth medium.



Figure 1 – Flow sorting based on forward / side scatter parameters provides sufficient resolution to separate haploid from diploid ES cells with high purity independent of Hoechst staining. Diploid control ES cells grown in parallel in identical medium conditions can be used to define sorting gates.

After sorting spin down cells (300g, 5 minutes), re-suspend in 2i/LIF medium and plate on

feeders with pre-equilibrated 2i/LIF medium. As a rough guide plate between 1x106 and 2x106 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 directly or after one more passage. Usually the 129 mouse strain

derived haploid ES cells lines will be stable with a haploid content of >90% for several passages,

depending on conditions.