EUCOMM JM8 ES cell mouse production protocol (CNR Monterotondo)

ES cell preparation

Materials:
- Foetal Bovine Serum (FBS) for ES Cells from distribution centre, Helmholtz Germany
- KNOCKOUT DMEM x ES cells (GIBCO 10829)
- DPBS w/o CaMg (GIBCO)
- .5% Trypsin-EDTA 10X (GIBCO)
- L-Glutamine 200mM 100X (GIBCO)
- 2-β-Mercaptoethanol 20ml 50mM
- ESGRO® LIF
- Gelatin 2% (Sigma G1393)
- Pen Strep (GIBCO 15140)
- Foetal Bovine Serum (FBS) 100ml (GIBCO)
- DMEM (GIBCO 41966)
- MEM NEAA 100X (GIBCO)

ES CELL Media: 500ml Knockout DMEM, 90ml FBS (from Helmholtz Germany), 6ml L-Glutamine 200mM 100X, 1.2ml 2-β-Mercaptoethanol 50mM, 60µl LIF ESGRO®. Filtered.

¤ Cells arrive from Helmholtz, immediately freeze in liquid nitrogen
¤ SNL are gown in a different media (SNL Media: 500ml DMEM, 50ml FBS, 5ml MEM NEAA, 5ml L-Glutamine 200mM 100X, 1ml 2-β-Mercaptoethanol 50mM, 6ml Pen Strep)
¤ Prior to using media for cells warm between room temp and 37 deg c .

Expansion of ES cells

Monday
-Thaw 1 vial in 1 well/6well + SNL with 4ml of ES media (SNL cells thawed a few days before use- enough to coat dish)
Tuesday
-Change media (4ml ES media)
Wednesday
-Remove media
-Wash with 2ml of PBS
-Add 1,5ml of Trypsin 1X and put in the incubator for 5' (or until the cells are completely detached)
-Add 3ml of ES media, collect all and centrifuge for 3' at 3000rpm
- Remove supernatant
-Resuspend in 1ml of ES cells media until the cells are separated and put in 2x6cm dish + gelatine Sigma 0,1% (500µl x dish)
Thursday
-Change media

Friday
-Remove media
-Wash with 2ml of PBS
-Add 1,5ml of Trypsin 1X and put in the incubator for 5' (or until the cells are completely detached)
-Add 3ml of ES media, collect all and centrifuge for 3' at 3000rpm
-Resuspend in 2.5ml of Freeze media (ES media + 10% DMSO-Sigma) until the cells are separated, and freeze 5 vials of cells (500µl x vial) at -80°C. Transfer after a few days into Liquid Nitrogen.

Preparation of ES cells for DNA extraction for Southern Blot anaylsis

-Thaw 1 vial of previously expanded cells in one 9cm dish + SNL with 13ml of ES media (SNL cells thawed a few days before use - enough to coat dish)
-Change media every day until the cells arrive at the maximum confluence
(leave in the dish 7-14 days)
-Detach the cells and extract DNA and perform southern blot. Correctly targeted cells are used for injection, incorrectly targeted clones are discarded.

Preparation of ES cells for Injection

Monday
-Thaw 1 vial of expanded cells in 1 well/6well + SNL with 4ml of ES media(SNL cells thawed a few days before use- enough to coat dish)
Tuesday
-Change media (4ml ES media)
Wednesday
-if the cells are not to be used for injection change media or use for injection following the protocol below
Thursday (normal day of injection)
-Remove media
-Wash with 2ml of PBS
-Add 1,5ml of Trypsin 1X and put in the incubator for 5' (or until the cells are completely detached)
-Add 3ml of ES media, collect all and centrifuge for 3' at 3000rpm
-Remove the supernatant
-Resuspend the cells in 500µl of ES cell media until the cells are separated and put on a 9cm dish with 8ml of ES media and put in the incubator for 20'(Preplating)
-Remove the supernatant and put in a 50ml falcon tube (labelled S), put on the dish a further 8.5ml of ES cell media, wash the dish gently by rolling the dish side to side 2-3 times ,
-Remove the media (wash) and put it in a separate 50ml falcon tube (labelled W). Put both in the centrifuge for 3' at 3000rpm.
-Remove the supernatant
-Resuspend the cells in 400-500µl of ES cell media and put the falcon tubes in ice. Use the W for injection. (selecting the small round cells where possible)
-In the case of a clone with very few cells you can use the Supernatant.

Superovulation and preimplantation embryo collection protocol (C57BL/6J-Tyr<c-2J>)

C57BL/6J-Tyr<c-2J albino females used at 3-5weeks age

Light/dark cycle 12hrs light/12 hrs dark 7am to 7pm light

PMSG: obtained from national Hormone and Peptide Program 2000 iu concentration diluted to 50 i.u /ml in sterile 0.9% saline and frozen at - 80 deg C

http://www.humc.edu/hormones/

HCG: Obtained from Intervet 1500 i.u concentration and diluted to 50 i.u/ml in sterile 0.9% saline and frozen at - 80 deg C

- **Day 1**: PMS 5iu given IP around 13.00 hrs
- **Day 3**: HCG 2.5iu given IP 48 hours later at 13.00 hrs and mated with stud males: 1 female/male
- **Day 4**: before 11.00hrs plug check females and record stud performance (cull any males that have not plugged a female in 4 to 5 consecutive attempts). Set up CD1 females with Vasectomised males(see below)
- **Day 6**: In the afternoon collect oviducts and uterus in pre-warmed 0.9% saline. Flush embryos from both oviduct and uterus using pre-warmed FHM. Wash embryos in FHM and then pre-equilibrated KSOM and leave in KSOM drops in the CO2 incubator (5% CO2, 37 deg C) and culture overnight. Flush also "negatively" plugged females as often these are false negatives and females contain fertilised morulae.
- **Day 7**: Use expanded blastocysts for ES cell injection

Blastocyst Injection and Embryo transfer

Media:

FHM for flushing and handling and blastocyst injections(Millipore MR 122 D) + 1mg/ml Bovine serum albumin (sigma A3311 embryo tested) filtered through 0.22u filter

KSOM for preimplantation embryo culture (Millipore MR 107 D) + 1mg/ml Bovine serum albumin (sigma A3311 embryo tested) filtered through 0.22u filter

Paraffin Oil for embryo culture and blastocyst injection (Sigma M5310 embryo tested)

Blastocyst Injection:

- Harvest ES cells as above in 400- 500ul ES media and place on ice ready for injection.
- Set up an FHM drop on the lid of a 10 cm petri dish and overlay with paraffin oil.
- Set up microinjection apparatus and programme positions of Eppendorf NK2 manipulators
- Place up to 20 blasts into the injection drop or as many as can be injected in 30-40 minutes. Drops used and injections performed at room temperature.
- Add 50-100ul ES cells (from the wash) to the dish and inject each blast with 10 to 15 ES cells. Use small, round ES cells where possible.
- Return injected blasts to the incubator (5% CO2, 37 deg C)in pre-equilibrated KSOM under oil or transfer to a drop of FHM and immediately embryo transfer to a recipient.
- Repeat this as necessary. A fresh batch of ES cells should be harvested after 2- 3 hours.
- When embryos are ready to be embryo transferred place them in a drop of FHM in a 60mm petri dish

Embryo Transfer:

- **Day 4** CD1 females 26-34g are selected for oestrus and mated with vasectomised males.
- **Day 5** before 11.00hrs plug check females and record stud performance

On the day of transfer prepare anaesthetic as follows:

Anaesthetic:
Make up a Ketamine/medatomidine mixture as follows:

0.19mls of Ketamine (Imalgene 100mg/ml Ketamine)
0.25mls of Medetomidine (Domitor 1mg/ml Medetomidine)
2.06mls of Sterile water

dosage rate : females 75mg/kg Ketamine - 1mg/kg Domitor

Doses of above anaesthetic mix given I/P Females = 0.1ml/10g

Weight -Female 15g= 0.15ml
-Female 20g= 0.2ml
-Female 25g= 0.25ml
-Female 30g= 0.3ml

Reversal agent:
0.04mls of Atimazepole Hydrochloride (Antisedan 5mg/ml Atimazepole)
make up to 1ml with Sterile water
Antisedan dose rate :1mg/kg

- Once anaesthetised put a drop of pre-warmed 37 deg C 0.9% saline on each eyeball to avoid dehydration.
- Soak instruments and the work surface with 70% alcohol
- Transfer blastocysts up to 10 per uterine horn to 2.5dpc CD1 recipient weight 26-34g bilaterally.
- If 2.5dpc recipients are not available 0.5dpc recipients can also be used to transfer the embryos to the oviduct, 10 blasts per oviduct.
- Post-op give 1ml of pre-warmed saline S.C to each female and 0.2ml of reversing agent IP.
- Place the recipient in the heated recovery chamber. Once recovered transfer to stock room

Weaning and breeding of Chimeras

- Male chimeric mice are weaned at c.21 days post birth and are given a subjective % coat colour score (Chimeras are black/agouti (from ES cells) and white (from host blastocyst). If there is a high contribution from the ES cells they will be more black/agouti than white (Fig.1).

Fig. 1 showing variation of coat colour chimerism in a litter born from a blastocyst injection from JM8N4 cells into C57BL/6J-Tyr<c-2J> blastocyst. Blackest mice have the strongest ES cell contribution with albino mice having none at all.

- At 6 weeks of age the 4 strongest % coat colour male chimeras are mated with 2 x C57Bl6NTac mice to try and establish gem line transmission (GLT).

- Each chimeric mice is bred for up to 5 litters and 50 pups to establish if the mutation is being transmitted. With JM8.N4 (Black C57BL/6N) ES cells all F1 offspring, both wild type and mutant are black and it's therefore necessary to perform PCR analysis from an ear clip on all F1 offspring produced.

- With JM8A (Agouti C57BL/6N) ES cells F1 offspring will be black (wildtype and mutants) and agouti (mutants only). Fig 2.

- Once we have accumulated 4 male F1 mutants these are passed to EMMA for cryopreservation and the breeding is terminated.

Fig 2. Coat colour genetics influencing the colour of the F1 offspring:

A = agouti a= non agouti

B= black b = brown

C= colour c= albino

JM8N4 Es cells = aaBBCC (black)

C57BL/6J-Tyr<c-2J> blastocyst = aaBBcc (Albino)

JM8A Es cells = AaBBCC (Agouti)

C57Bl6N mice = aaBBCC (Black)

Therefore:

JM8N4 x C57Bl6N = offspring aaBBCC (black). Possible GLT

JM8A x C57Bl6N = offspring AaBBCC (agouti) or aaBBCC (black). Possible GLT

C57BL/6J-Tyr<c-2J x C57Bl6N = offspring aaBBCc (black). Wildtype.