MMRRC Protocol for Microinjection of JM8A ES cell clones into blastocysts

August 26, 2009

Materials and Reagents:

Item	Vendor	Catalog Number
PMSG	National Hormone & Pituitary Program	1190
HCG	Sigma	CG-10
M2 medium	Speciality Media	MR-015
KSOM+AA	Specialty Media	MR-106-D
hepes buffered DMEM	Gibco	12430-054
FBS (ES cell tested)	Hyclone	SH300 70.03
Mineral Oil	Sigma	M-8410

Selection of Donor strain:

JM8A is derived from C57BL/6N strain. It has agouti gene engineered into the agouti locus. Inject these cells into blastocysts from B6D2F1 x C57BL/6 donors will get agouti and black chimeras.

Microtool Preparation:

Holding pipettes are prepared from glass capillaries (custom glass tubing #9-000-3000), injection pipettes from glass capillaries (custom glass tubing #9-000-2155) using a horizontal micropipette puller (Model: 97, Sutter Instrument co.). The holding pipette has an external diameter of 60-80 µm and an opening of 10-15 µm. The injection pipette has an external diameter of 12-17µm and an internal diameter of 10-15 µm.

Superovulation of Donor Females:

1. Day 0: Give PMSG (5 I.U.) to 3-4 weeks old C57BL/6 females by I. P. injection at 2 pm.
2. Day 2: Give HCG (5 I.U.) to the females at 1 pm by I. P. injection. Mate the females to B6D2F1 stud males.
3. Day 3: Check plugs.

Preparation of Blastocyst:

1. Day 6 - Sacrifice plugged female mice by CO_2 asphyxiation or cervical dislocation at 3.5dpc at 8:00 am.

2. Dissect open the abdomen, locate the complete uterine horn and remove, taking as much fat from the uteri off as possible.
3. Flush the uterine horn into a petri dish by inserting a 26g needle (attached to a 3ml syringe) into the oviduct/uterine junction and flush approximately 0.5ml of M2 through. Fluid should be visible flushing through the uterus and out of the cervical opening.
4. Collect all the eggs using a mouth transfer pipette and sort blastocysts, morulae and undeveloped embryos into separate 40µl drops of KSOM+AA covered with oil (preequilibrated) into the incubator (5% CO_2 at 37^oC). Record the number of embryos.

ES cell preparation:

Follow "Expansion of JM8A ES Cell Clones for Microinjection and Future Use" section under "MMRRC Culturing Protocol for JM8A (Agouti) ES Cell Clones".

Blastocytst Microinjection:

1. Prepare a 60mm petri dish to place injected blastocysts into. Pipette four 40µl drops of KSOM+AA, cover with oil and place in a 5% CO_2 incubator to equilibrate.
2. Prepare a cavity slide by making a large (~40µl) drop of ES injection buffer into the center of the well and cover with mineral oil.
3. Place an appropriate number of blastocysts onto the slide, then add the prepared embryonic stem cells. Lower the holding pipette into the small drop of medium and fill to the shoulder, by capillary action. Place into the main drop.
4. Fill the tip of the injection needle with medium from the other small drop, by capillary action; place the needle into the main drop. Collect about 100 round and small ES cells with the injection pipette.
5. Hold a blastocyst using the holding pipette, with the inner cell mass closest to the holding pipette. Gently push the injection pipette into the blastocyst. Once inside release 10 to 15 ES cells and gently withdraw the needle. Continue until all blastocysts on the slide have been injected. Try not to exceed 30 min for each set of blastocysts.
6. Place injected blastocysts into the pre-equilibrated KOSM-AA drops in a 5% CO_2 incubator.

Embryo transfer:

Transfer 6 to 7 injected blastocysts into each uterine horn per 2.5 dpc pseudopregnant CD1 female.

MMRRC Germline Testing Protocol for C57BL/6N (agouti) derived ES cells (JM8A3, JM8A3.N1, JM8A1.N3)

Rev. 06/15/10

Strain Background:

1. The chimera's donor blastocyst C57BL/6 and the heritage is a/a (black) on the "Agouti gene" and has no *Tyr^c^* (non-albino) on the "Color gene".
2. The chimera's ES cell is created from C57BL/6N with a repair at the non-agouti locus by gene targeting, and the heritage is A/a (agouti) on the 'Agouti gene', and has no *Tyr^c^* (non-albino) on the "Color gene"[1].
3. The female (C57BL/6N) breeding with the chimera is *a/a* (black) on the "Agouti gene" and no *Tyr^c^* on the "Color gene".
4. Chimera derived from injection of the JM8 sub-line agouti (A/a) ES cells into a black host embryo (a/a) will be agouti and black. Percent chimerism is based on the % of agouti coat color on the mouse.
5. These ES cells are XY and you will want to breed the male chimera. On rare occasion we encounter cells that are XØ, meaning they have lost the Y chromosome. We will notify you if this is the case for the clones or chimeras you receive. If cells are XØ you will breed the female chimera to C57BL/6N males.

Breeding chimeras with C57BL/6N females:

1. Set chimeras (6 weeks of age), no more than one male per breeding cage, to breed with C57BL/6N females (6-8 weeks of age), and no more than 3 females per cage.
2. If there is no sign of plug at 7 days, or pregnancy at 10 -14 days if you do not perform plug checks, remove females and give males 2-3 days rest and then replace with new females. Repeat 3 times before retiring chimera, and/or consider an artificial reproductive technique (ART) for testing chimera.
3. If plugs or pregnancies are observed, leave one (or both if your vivarium allows it) of the pregnant females in with male, await the birth, and allow for re-mating.
4. If plugs are detected but no pregnancy ensues, repeat step 1.

Germline testing:

1. At ten days of age, number pups (toe clip, ear tag, etc.) and take tissue samples (tail snips, ear punch, etc.) from ***both*** agouti and black pups and submit for genotyping analysis (PCR, Southern, etc.).
2. Use germline-positive mice to establish breeding colony, and discard wild type mice. (Note: Wild type littermates should not be used for breeders as these may be host embryo derived rather than cell line derived. New C57BL/6N breeders should be requested for mating to the heterozygous positive mice for colony build, or sibling mating of heterozygous mice.)

---

[1] *Pettitt et al.*: Agouti C57BL/6N embryonic stem cells for mouse genetic resources. Nature Methods, 2009.