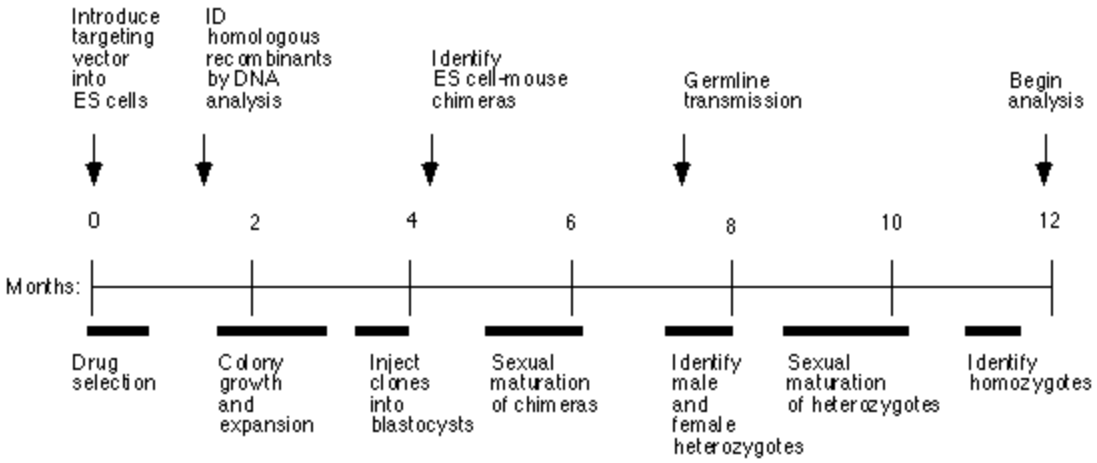
Timeline for Generation of ES Cell-Derived Mice



PROTOCOL FOR CRYOPRESERVING SPERM & EMBRYOS WITH LN2 WITHIN THE BIO5 ANIMAL FACILITY

1. Coordinate with PI the procedure for dissection of cauda epididymus and vas deferens if mice are not in BIO5. All mice subject to this procedure must come from a clean room.
2. If mice are not clean the line must first be rederived before cryopreservation of sperm or embryos.
3. Clean rooms are determined by sentinel mice in each room and are tested by the Pathology Dept. Room reports are sent by email directly to Debbie Stead and are also on UAC's Animal Scoop-listserve. Mice are also tested by the Pathology Dept. and results must come back before the cryopreservation procedure begins. Those reports are emailed directly to Debbie Stead. A call is also made to the Pathology Dept. to verify that "x" room is still clean.
4. Two days before the procedure a call is made to URIC Cryogenics (621-2374) to order a 160L/180L tank of liquid nitrogen which is delivered to the mezzanine level of BIO5. The same day a call is made to Tim Ruddy to let him know a tank is on the way. Once delivered, Tim Ruddy will pick up the tank, disinfect it with Clidox and bring it to room 40 in BIO5.
5. On the day scheduled to proceed with the cryopreservation instructions/training is given to the PI on how to dissect either the cauda epididymus and vas deferens from the male, or the oviducts containing fertilized zygotes from the female.
6. These pieces of tissue are:
   a. put in a tube with the appropriate medium and transported to the pass-thru where the tube is wiped down with a Clorox wipe for Debbie Stead to pick up from inside the facility; or

b. Debbie Stead meets with the PI in the lobby of the Keating Building, retrieves the tube(s), brings them to the dirty side of the changing room of BIO5, sprays them with Clidox, inserts the tube(s) into a plastic bag and wipes the plastic bag down with a Clorox wipe. The plastic bag is then brought into the clean side of the changing room and into the facility.

7. Once tubes are inside the facility the collection of sperm or zygotes is done under a hood. Sperm is collected in straws and the end of the straw is heat sealed according to Jackson Laboratories protocols. (See separate protocol for procedure). Zygotes are distributed in cryopreservation tubes, the Jackson Laboratories protocol is used, and the cryopreservation tubes are placed in our controlled-rate freezer. (See separate protocol for procedure.)
8. All cryopreservation procedures are done in room 40 in BIO5 and all exposed tissue is handled under a hood.
9. At the end of the procedure all tissue is put back into the tube(s) from which they came. Clidox is sprayed into the tube(s), capped and thrown out in the biohazard container.
10. For Sperm Cryopreservation: Filled and sealed straws are placed in a cassette which holds 5 straws which is then placed in a small dewar filled with liquid nitrogen from within the animal facility. This dewar is dedicated to the clean side. The dewar is brought to the door which separates the clean from dirty side of the changing room where the dedicated, unsterilized, dewar is waiting for the transfer of straws. Straws are transferred with forceps which are also dedicated for use within the facility. The dedicated dewar and forceps are brought back into room 40. Debbie Stead then changes from dedicated scrubs into street clothes and brings the unsterilized dewar up to the cryo storage tanks and places the cassettes in sleeves and records their placement within the tank. Sperm is stored in the vapor phase of liquid nitrogen.

11. For Embryo Cryopreservation: Cryopreservation tubes with frozen zygotes are placed in the dedicated, sterilized dewar within BIO5. The procedure is then same as for sperm cryopreservation.
12. After straws or tubes are in the main storage tanks in the vapor phase of liquid nitrogen and results are recorded Debbie Stead goes back into BIO5, cleans the hood and microscope with Clidox. The liquid nitrogen in the small dewar used for transport is left to evaporate and then sprayed down with Clidox.

THAWING PROCEDURE

RNA Microinjection Buffer and Cas9 mRNA Reagents

MATERIALS

- *in vitro* transcribed mRNA or RNA
- Oligonucleotide resuspended in RNAse free water
- DNA donor plasmid prepared with an endotoxin free plasmid kit or long single stranded (lss) DNA prepared with Takara's Guide-it lssDNA Production System
- 0.02 µm Anotop 10 Syringe Filters (Whatman Cat. no. 6809-1002) — *Do NOT filter nucleic acid solutions through the Anotop filters, the 0.02 µm pores will trap nucleic acids and remove them from solution*
- Millipore dialysis filter (Millipore #VMWP02500, pore size 0.05 µm)
- Sterile 10 ml syringe
- Sterile 10cm Petri dish
- Pipetman
- RNAse Free Pipet Tips
- Sterile 1.5 ml Microtubes
- Sterile 15 ml tube
- Sterile 50 ml tube
- Sterile 10 ml pipets
- 1 M Tris-HCL, pH 7.4 (Sigma Cat. no. T263)
- 0.5 M EDTA (Sigma Cat. no. E7889)
- Nuclease Free Water (Sigma cat. no. W4502)

PROCEDURE

RNAse Free Microinjection Buffer (10 mM Tris-HCl, pH 7.4, 0.01 mM EDTA)
Filter buffer through filter for use as dialysis buffer or for use as microinjection buffer.

mRNA/sgRNA/oligo/plasmid Donor Preparation for Microinjection
The GEM Core has used CRISPR/Cas9 to target more than 60 genes in mice and produce CRISPR-based single nucleotide polymorphism (SNP) knockins, tag knockins, knockouts, simple and complex conditional knockouts.

1. sgRNA used by our core are bought as modified from Synthego. Nuclease reagents typically clog glass needles used to microinject reagents into fertilized eggs. Clogged needles lead to decreased egg survival and lower yields of genetically modified mice or rats. To reduce clogging and improve outcomes, the wash buffers and elution buffers used in mRNA and sgRNA purification are pre-filtered through 0.02 um filters. Do not pass nucleic acid solutions through the filters. The pore size on the filters is small enough to trap nucleic acids. Alternatively, Cas9 mRNA or Cas9 protein solutions are purchased from commercial vendors.
2. Oligonucleotide donors are subjected to spot dialysis to remove embryotoxic chemicals. Follow standard guidelines for working with RNA to protect against later RNA degradation.
2.1. Fill a 10cm or 15cm Petri dish with nuclease free microinjection buffer. Place a Millipore dialysis filter on the surface of the buffer so that it floats (place the filter shiny side up).
2. 2. Carefully spot the nucleic acid solution into the center of the filter. Replace the Petri dish lid. Dialyze for 30-60 minutes. Up to 200 ul can be placed on a filter without losing it to the buffer. Leave the dialysis to proceed quietly without any shaking or movement. Do not let the dialysis to go more than 3 hours; otherwise the drop might begin to evaporate.
2. 3. Carefully Pipette off the solution. Place the tip in the middle of the droplet and carefully aspirate as much as possible without stopping. Transfer the nucleic acid solution to a sterile microtube. Quantitate and store at – 80°C. Recoveries between 50-70% of the original volume are normal. The rest remains attached as a very thin liquid layer onto the surface of the filter and is difficult to pipette it off.
3. The GEM Core microinjects nuclease reagents into the pronucleus. This has been demonstrated to be superior to cytoplasmic injection for protein expression from mRNA (Wefers et al., 2013). We use for pronuclear microinjection the following concentrations: 50 ng/ul Cas9 protein + 30 ng/ul sgRNA + 50 ng/ul oligo donor DNA or 50ng/ul lssDNA donor.
4. To prepare for microinjection, mix together Cas9 protein and sgRNA in 0.02 um filtered RNAse Free Microinjection Buffer and incubate for 10-15 minutes at room temperature. Add the oligo or lssDNA and spin down for 10 minutes at 16K rpm. Transfer the supernatant into RNAse free tube.

REDERIVATION

April 13, 2007
Besselsen protocol amendment:

The purpose of rederivation by embryo transfer is to eliminate undesirable pathogens from infected mouse lines. Early embryos are harvested from female mice recently bred to male mice (male or female mice may be infected). The fertilized eggs are harvested from oviducts and washed to remove any infectious agents. The embryos are then surgically implanted into uninfected pseudopregnant female mice recently bred to uninfected vasectomized males. The mice are maintained separately until the offspring and/or recipient dam are determined to be free of the undesirable pathogen. We will be specifically evaluating the ability of this procedure to eliminate various murine parvoviruses from infected mouse lines.

Vasectomized studs & recipient foster mothers (we will be using Swiss Webster mice but potentially any strain of immunocompetent mouse could be used) will fall under Pain category D. We estimate we will use 6 vasectomized male mice and 12 recipient female mice for these studies (one vasectomized male per 2 recipient females to induce pseudopregnancy). Other mice that will be infected or used as breeding partners for infected mice are approved for use under our existing protocols, as is the superovulation procedure for the donor female mice. This will provide up to 6 litters per group (infected male and infected female origin) to assess the efficacy of the rederivation procedure for elimination of murine parvoviruses. Four litters is considered the minimum sample size per group, with extra litters requested to account for potential cannibalization and poor embryo implantation.

Embryo rederivation procedures include: Super ovulation of female mice with PMS and HCG intraperitoneal injections followed by mating to males. The next day the females undergo CO2 euthanasia to harvest day one embryos. Vasectomized stud males are mated with Swiss Webster females to produce pseudo pregnant females. The next day these pseudo pregnant Swiss Webster females undergo survival surgery when embryos are implanted into the oviducts. Surgeries will be performed by Stephanie Munger or Dr. Besselsen. Both have completed the IACUC certification required for rodent surgery and anesthesia.

Anesthetic Regimen:
Drug: avertin Dose: 0.025 ml of 1.5% avertin/gm. body weight .
Route of Administration: I.P. injection Duration: approximately 30 minutes .
Monitoring Processes: paw pinch and respiration rate will be used to assess depth of anesthesia.

Surgical procedures: Embryo transfers will be done under sterile conditions under anesthesia in a designated location in Dr. Besselsen's laboratory (CAF room 22). Pseudo pregnant mice are anesthetized by intra peritoneal injection (I.P.) of 0.025 ml/g body weight of 1.5% avertin. Embryo transfer of micro manipulated embryos involves surgical implantation into anesthetized pseudo pregnant mice. A small transverse incision (5-8 mm) is made through the skin along the lower spine approximately 1 cm caudal to the hip. Skin

is moved until the incision is directly over one ovary and fat pad. A small incision is then made through the overlying muscle; and the ovarian fat pad, ovary and oviduct are exteriorized and held in place with a serafine clamp. Embryos are taken up into a special transferring pipette and injected into the oviduct. The procedure is repeated for the contra lateral oviduct. The skin is closed with two 9mm Michel wound clips. Wound clips are removed after 6-10 days. These surgeries take 3-8 minutes for each mouse. Blastocyst implantation is identical to the above procedure except that the blastocysts are injected into the uterine horn of the avertin anesthetized pseudo pregnant female mice.

Vasectomies will be performed under sterile conditions under anesthesia. Two month old males are anesthetized by intra peritoneal injection (I.P.) of 0.025 ml/g body weight of 1.5% avertin. A single transverse incision is made in the skin of the scrotum with sterile micro-dissection scissors. The vas deferens is located attached to the epididymus. A short (10mm) length of each vas deferens is cut with the scissors and removed without exteriorizing the testes, to ensure that sperm cannot move between the severed ends. The skin is closed with two 9mm Michel wound clips. Wound clips are removed after 6-10 days.

Post surgically the mice will be returned to a clean cage except they will be placed on paper toweling. The cage will be placed on a warming pad set to 37 degrees centigrade until the mice are active. At that time, the paper towel is removed. The mice will be observed until they resume normal ambulation. The incision will be inspected daily for healing progress. Wound clips will be removed at 6-10 days. Post-operative analgesic will be Buprenex (0.1 mg/kg) S.C. given once on the day of surgery.

GEMM Core Frozen Embryo Rederivation Procedure

1. Arrival of vials containing frozen embryos is coordinated with the consigner. GEMM Core will allow the use of its dry shipper with all charges for shipping incurred by the PI. There is no 'rental' charge for the use of the dry shipper. The PI signs a waiver to pay for replacement of the shipper in the event that it is damaged or not returned within two weeks.
2. Upon arrival at the U of A, the vials of frozen embryos is stored in the two vapor-phase liquid nitrogen storage container.
3. On day one, Swiss Webster females are mated to vasectomized males.
4. On day two, females showing a post-coital plug are separated from the males.
5. Embryos that are at the 2-cell stage are thawed according to the protocol received from the consigner. Oviduct implant surgeries are done the same day.
6. Embryos that are at the 8-cell stage are thawed on day three according to the protocol received from the consigner.
7. This is a typical thaw protocol. Timing and media may differ according to protocol:
    - Control vials are thawed slowly, with media being added drop-wise to dilute the cryoprotectant (DMSO).
    - Once thawed, embryos are collected and washed through 3 wells of KSOM under oil.
    - This culture dish with the embryos is cultured overnight in a 37 degree 5% CO2 incubator.
    - Embryos are monitored over the next two days for development to blastocyst stage.
8. On day four, embryos are implanted into 2.5 day post-coital (dpc) pseudopregnant females as a uterine transfer. [If there are not sufficient 2.5 dpc females, 0.5 dpc females can be used in an oviduct transfer surgery.]*

*Some protocols may specify that 8-celled embryos be transferred on the thaw day; they are implanted into 0.5 dpc female oviducts.

EMBRYO DERIVATION TIMELINE SOP

Important Information:

1. Plan to have at least five 3.5-4 week old females ready for superovulation. (More females would be better, but no more than 10 if you have them.)

2. Males should be proven studs between the ages of 10-20 weeks. A proven stud is a male who has successfully impregnated a female. You can start mating the males at 8 weeks of age.

3. The males provided for embryo derivation should be mated with a female two weeks prior to the move into my derivation room. The female should be removed 1 week prior to the move. You can use a wild-type female for this. She can be PTS'd once you know she is pregnant. It is just a way to optimize the males so they have enough fresh sperm to fertilize the eggs of the females you are using for the derivation.

It is better to mate the males twice before leaving them without females the week prior to the move. **It is important that the males do not have females with them the week prior to the move.**

4. Timeline:

a) 45 days prior to the move to my derivation room, mate males with females. Give approximately 4 days for the females to conceive.

b) Twenty days after plugging she will have a litter (or a maximum total of 24 days).

c) Those litters can be weaned at 21 days and the females will be moved to my derivation room, along with your males, the week prior to being used.

d) Mate the males with either one of your females or a wild-type female 2 weeks before the move.

e) **Remove any female with the male 1 week before the move.**

This is a total of approximately 9 weeks (4a-e). I need between 4-10 males and the same number of females. If you are using wild-type females to mate with your males then they can be ordered 2 weeks prior to superovulation and delivered into Room 1218 in AHSC. Please order 3 week old females.

If you are mating your males to your females the males should be between the ages of 10-20 weeks of age. This means that you need to start your matings an additional 8-10 weeks prior to the above timeline. This insures that I am using 10-20 week old males from the first litter and 3-4 week old females from the second litter. Again, if you are using your males and ordering 3-4 week old wild-type females then this step can be skipped and you can go with just #4a-e above.

Purification of Gene Targeting Vector DNA for Electroporation

1. PURIFY PLASMID FROM BACTERIA.

We recommend the Qiagen EndoFree Plasmid Maxi kit for the purification of the targeting vector plasmid from bacteria. Please follow the directions in the kit. Electroporation of Qiagen purified DNA has been used successfully by a number of labs. Alternatively, plasmid DNA can be purified by CsCl banding.

2. LINEARIZE 200 MICROGRAMS OF PLASMID DNA WITH THE APPROPRIATE RESTRICTION ENZYME DIGEST.

Run a DNA on a minigel to verify that digestion is complete. Extract the DNA with phenol-chloroform, then with chloroform and precipitate by adding NaCl and ethanol. Make sure you use fresh phenol with neutral pH for maximum DNA recovery and highest cell viability in electroporation. Wash the DNA pellet in 70% ethanol and allow to it air dry. Resuspend the DNA in sterile TE (10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA) at 2 mg/ml and deliver it to the Transgenic Core for electroporation. Prior to electroporation, we will verify the concentration and run it on a minigel to check the size and look for degradation.

Mouse Tail Digest Protocol

1. Place 300 ul **Cell Lysis Solution** with 1.5 µl **Proteinase K** (20mg/ml) per sample in 50 ml tube. Invert tube gently to shake but not create bubbles.
2. Add 300 µl Lysis/ProK mix to each tail sample.
3. Tape samples to rocker platform, and place in 55°C oven overnight to inactivate ProK.

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1. Add 1.5 µl **RNase A** (4mg/ml) to each sample using a 10 µl pipettor; fresh tips for each sample. Invert tubes 25 times and incubate at 37°C water bath for 15-60 minutes.
2. Label new 1.5 ml tube for each sample and add 300 µl Isopropanol (2-propanol) to each.

3. Cool samples to room temperature on ice for 1 minute.
4. Add 100 µl **Protein Precipitation** buffer to each sample.
5. Vortex for 20 seconds, and centrifuge at 10,000 rpm for 1 minute.

6. Pour supernatant into new tubes with isopropanol; invert to mix 50 times.
7. Centrifuge at 10,000 rpm for 1 minute.

8. Pour off supernatant leaving DNA pellet and drain tube on clean absorbent towel.
9. Add 300 µl **70% Ethanol** and invert to wash DNA pellet.
10. Centrifuge at 10,000 rpm for 1 minute.

11. Pour off supernatant leaving DNA pellet and drain tube on clean absorbent towel.
12. Air dry samples for 5-10 minutes.
13. Add 200 µl **DNA Hydration** (TE solution) solution.
14. Place in 65°C oven for 1 hour to expand DNA; then refrigeration or leave samples at room temperature overnight prior to refrigeration.

Cell Lysis Solution, Protein precipitation buffer, RNAse and DNA Hydration buffer can be bought as a kit from 5Prime. Otherwise regular lysis buffer can be used, RNAse could be bought from Roche or other company and DNA Hydration buffer can be substituted with TE buffer. I do not have idea what is in the Protein Precipitation buffer but is seems to work well for purifying DNA from tail tips while it does nothing for cell lysates.

Microinjection DNA Purification

1. Perform restriction digest to liberate transgene from plasmid vector sequences. Final yield should be 10 – 20 micrograms of transgene insert.
2. Separate restriction digest products on agarose gel using either TBE or TAE.
Use either low- or standard- melting temperature agarose.
3. Place gel on transilluminator. Cut out band(s) of interest. Use a clean razor blade or scalpel.
Remove as much excess agarose as possible. Minimize DNA exposure to UV light to prevent photochemical damage (less than 1 minute).
4. Transfer agarose slice(s) to a preweighed tube. Reweigh tube to determine weight of agarose in tube.
5. For each 100 mg of gel, add 200 microliters buffer NTI. If agarose concentration is greater than 1%, add proportionately more buffer. For example, if a 2% agarose gel is used, add 400 microliters buffer NTI for each 100 mg of gel.
6. Dissolve at 50 degrees Centigrade for 10 minutes, vigorously vortexing every 2 to 3 minutes until the agarose is completely dissolved.
7. Place a NucleoSpin cartridge in a 2 ml micro tube and load 750 microliters dissolved gel slice onto the cartridge. Spin at 11,000xg for 60 seconds in a microcentrifuge. Discard the flowthrough. The cartridge has a capacity of 15 micrograms DNA, so you can run several 750 microliters loads of dissolved gel slice through a single cartridge.
8. Filter 750 microliters of buffer NT3 through Anotop 10 0.02 micron filters (GE Healthcare Life Sciences, Catalog number 6809-1002). Add buffer to the cartridge and spin at 11,000xg for 60 seconds in a microcentrifuge. Discard the flow-through.
9. Replace tube with a fresh micro-tube. Spin the empty cartridge at 11,000xg for 60 seconds in a microcentrifuge to completely remove buffer NT3.
10. Elute the DNA from the cartridge: Replace tube with a fresh micro-tube. Add 50 microliters of preheated (60 degrees Centigrade) elution buffer to cartridge and incubate one minute. Spin at 11,000xg for 60 seconds in a microcentrifuge.
Elution buffer: 10 mM Tris-HCl, pH 8.5, 0.02 micron filtered. Check the pH of the elution before just before you use it, best yields are obtained at a pH of 8.5 or greater. The 0.02 micron Anotop syringe filters are available from Whatman (GE Healthcare Life Sciences).
If you have problems with particulates plugging the micoinjection needles, pre-filter the wash and elution buffers with the 0.02 uM filters. Do not filter the DNA through the filters, the small pore size will trap DNA molecules.
11. If desired, repeat step 10 to increase yield. We obtain 90% of the DNA in the first elution.
12. Quantitate DNA solution.
13. Verify size and intact condition of DNA on minigel.
14. Store eluted DNA at -20 degrees Centigrade.

Keating BIO5 Vivarium Operations

1. Personnel
    a. All (UAC, Research, Custodial, Pest Control, Visitors)
        i. Will receive training and/or be escorted by UAC personnel to enter and work in vivarium
        ii. Personnel working in the vivarium should not own rodents outside the vivarium (e.g. pet rodents, rodents bred or purchased for reptile food)
    b. UAC
        i. Dedicated UAC husbandry personnel
        ii. UAC personnel will clean animal rooms
        iii. Veterinary care provided with assistance from BIO5 husbandry staff
    c. Custodial services
        i. Dedicated custodian and backup
        ii. Will service floors of hallways, lounges, offices, bathrooms, procedure rooms
        iii. Will not enter animal rooms
2. Clothing
    a. Uniforms
        i. Dedicated scrubs to enter vivarium
            1. initial sterilization to enter vivarium
            2. do not leave the facility
            3. laundered in BIO5 vivarium
    b. Shoes and Socks
        i. Dedicated shoes for high use employees/users
            1. Initial sterilization to enter vivarium
            2. do not leave the facility
        ii. Range of shoe sizes provided for general use to other users
        iii. Shoes washed periodically (weekly) in BIO5 vivarium
        iv. Socks provided
    c. PPE
        i. Gowns, gloves, cap, mask required to handle mice or cages in animal rooms
        ii. PPE changed completely between each animal room
        iii. Gloves only if entering room but not handling animals directly
        iv. N95 masks recommended if allergic to mice
        v. No lab coats allowed
3. Personnel entry policies and procedures
    a. Wet shower requirement
        i. A wet shower in the BIO5 vivarium locker room is required if earlier in the day you entered other UAC facilities (e.g. AHSC, CAF, BIO5 external suite, Psychology, UAC support offices) or UA lab areas where there is potential contamination by rodents or BIO5 excluded pathogens.
        ii. A wet shower and clean clothing change at the person's residence is acceptable if you enter the BIO5 vivarium first, without exposure to potentially contaminated areas (UAC facilities or UA Lab areas as described above).
    b. Facility entry procedure for personnel
        i. Enter dirty locker room, disrobe to undergarments, place shoes/clothes in locker, don robe (if desired).

ii. Disinfect any tangible items entering facility (Catcard, locker key, eyeglasses, cell phone, etc.), then rinse items with water as needed. Signs are posted in the dirty side of the locker room with directions on how to properly disinfect items prior to entry.
iii. Wash hands.
iv. If wet shower entry is required (see section 1.g.i above) remove robe and undergarments, enter wet shower area, take wet shower, towel dry, don undergarments.
v. Cover feet with clean socks as entering clean side of locker room. Note: Socks should be placed on as each foot crosses over the delineation between the dirty and clean side of locker room.
vi. Don scrubs.
vii. Proceed through air shower.
viii. Obtain clogs in animal facility.

c. Animal room entry procedure for personnel:
   i. Don PPE at animal room entry (no chemical disinfection of floor when entering)
   ii. Discard PPE when exiting animal room (change PPE completely between each animal room)

4. Caging
   a. Sterilized (autoclaved) upon entry into vivarium
   b. Sterile caging
      i. Autoclaved with bedding in cage
      ii. Irradiated food (sterile)
      iii. Automated water with Hydropaks available for use
5. Husbandry Supplies
   a. Irradiated food only
   b. Heat treated bedding
   c. Hyperchlorinated autowater or hydropak water
   d. All feed/bedding bags externally autoclaved or disinfected by VHP upon entry into vivarium
   e. Hydropak unit at BIO5
6. Equipment
   a. Researcher equipment (computers, etc.) disinfected upon entry via feed/bedding entrance
      i. Hydrogen peroxide gas vaporizer for sterilization of equipment and rooms
      ii. Once equipment enters it should remain in facility until no longer needed for experiments (i.e. no equipment trafficking back and forth between vivarium and lab)
   b. Dedicated tools in tool area for repairs
   c. Dedicated floor machine
7. Mice
   a. Entry
      i. Entry procedures for various sources of mice outlined in separate document
      ii. Non-approved source mice
         1. Quarantine and embryo collection from NAS mice at AHSC or CAF with embryo culture and reanimation at Keating BIO5 vivarium GEMM Core facility
         2. Maintained in holding room for rederived NAS mice. Mice held in room until health monitoring is completed approximately 6 weeks after litter born; animals are then released into vivarium if health report is clean
      iii. Approved source mice
         1. Health status checked prior to ordering to ensure no excluded pathogens
         2. Enter vivarium directly (first stop for vendor shipping trucks)

3. -Shipping containers placed in receiving room and disinfected externally
4. Health status of each shipment confirmed before unboxing
5. Mice unboxed directly into cages in receiving area then transported to animal rooms

iv. Approved source mice, surgically modified
1. Should be avoided if either the surgical procedure can be performed within the BIO5 vivarium after arrival or the experiment can be performed in a UAC facility other than BIO5. EMSS and UAC can provide surgical support for many routine mouse surgeries.

b. Room designation list (two levels)
i. Closed breeding colonies and incoming vendor mice ("A" rooms)
1. May have subdesignations (A1, A2, etc.)
ii. Holding room for rederived NAS mice ("B" room)

c. Breeding colony management
i. EMSS, UAC, or Investigator can provide breeding colony management

d. Health Monitoring
i. Quarterly for prevalent pathogens
ii. Yearly comprehensive screening for all excluded pathogens

e. Humane traps will be placed throughout the facility to collect feral and escaped mice.
i. Checked daily by BIO5 husbandry staff with results recorded in vermin logbook (as in other facilities)

f. Experimental limitations (policy statement approved by VPR)
i. Infectious biohazards are not allowed within main BIO5 vivarium
1. Current UAC facilities that have areas capable of supporting infectious biohazardous mouse studies include the AHSC animal facility, the Central Animal Facility, and the BIO5 external suite (ABSL-2/3 and cubicle rooms).
2. Laboratories with access to the BIO5 vivarium that work with BIO5 excluded pathogens must routinely decontaminate lab areas exposed to pathogens with an appropriate disinfectant.
ii. Use of other biological materials (e.g. sera, antibodies, nucleic acids, cell lines, etc.) and non-infectious biohazardous materials in mice will be evaluated on an individual case basis to ensure there is no introduction of infectious agents through these materials.

Mouse ES Cell Prep for Blastocyst Injection:

Use p100 of 2-day post-thaw ES cells which have been in 2i for previous 48 hr.

Feed cells with ESDMEM (no 2i differentiation inhibitor) 2 hrs before prep.

After 2 hr feed, rinse 3x (5 mls each) with PBS.

Trypsinize 5min with 2ml 0.05% Trypsin.

Resuspend by adding 4 ml ESDMEM (no 2i).

Spin #3 for 3 min. Aspirate off sup.

Resuspend in 5 ml ESDMEM (no 2i) and put on gelatinized p100 for 30 min.

After 30 min collect unattached cells and spin #3 for 3 min.

Resuspend gently in 1.0 ml blastocyst injection medium (ESDMEM + 5% Hepes stock in DMEM) added to 20µl DNase.

Put in Eppendorf on ice during transport to blastocyst injection room and until injection.

SOP FOR ESC INJECTIONS

HORMONES

PMS and hCG stock are in white freezer boxes on the left hand side of the -20° freezer in room 40.

They each need to be diluted with 1000 µL (1 ml) of PBS. PBS is in the Purple capped bottles above the incubator. The Pipetman is located in the drawer just below the incubator. Pipet tips, syringes and needles are also on the shelf above incubator.

PMS is the first hormone given. It is given IP at 2:00 pm. I put all the mice into one cage so I am left with an empty pan. As I give PMS I put each mouse into the empty pan. When done giving injections I separate them out to 4 mice in each of 2 pans, and 2 mice in the last pan. They go back on the rack.

hCG is the second hormone given. It is also given IP. This injection is done at 12:00 pm the second day following PMS, i.e., PMS at 2:00 Monday, hCG at 12:00 Wednesday. The females are immediately mated after hCG.

Plug checking should be done the following morning and females are put back on the rack in their original cages until eggs are collected. The back of the cage cards for the males are dated when plug checking and a check mark is placed next to the date if he plugged, a minus sign if not.

Donor females are always located on the rack just above the B6D2F1 males (green cage cards).

ESTRUS

ND4 females are located on the rack across from the vaz males (both have yellow cage cards). Set up 16-20 females in good estrus - one female per male. There is a card behind the males' cage cards that says "Start Here." Whichever cage has that card is the first male to be used. Use only the males that have an ID number written on the card.

Description of Estrus: The labia for females in estrus will appear swollen and pink with striations. Do not use females that have an opening that is gaping or moist, or is dark rose in color, or if there is any gray coloring around it.

Plug checking is the morning after. If the female is plugged mark the back of the males' cage card with date and a check, or minus mark if not. When plug checking is finished, move the "Start Here" card to the cage after the last male that was checked.

Plugged females should go in a separate row under the ND4 ♀ colony. The bottom of the cage card should read "Plugged day & date for ES on day."

(continued on back)

Electroporation of ES Cells with Targeting Vector

Culture 129/S6 ES cells with ESDMEM (15% FBS in high glucose DMEM with Hepes, glutamine, pyruvate, and minimal essential amino acid supplements + 2i differentiation inhibitor).
Change to fresh medium 4 hours before electroporation.
Trypsinize and resuspend 10[7] cells in 0.8 ml ESDMEM.
Electroporate as follows:

Electroporation Cuvette holds 0.8ml and has 4mm distance for electric field.
Electroporator: Exponential decay, 250V, 500uF, Infinite Resistance. Record time and voltage for that electroporation.
Two EP cuvettes: 1 for Control EP (1/5 of ES cells in the plate-no vector DNA) and 1 for DNA EP (4/5 of cells).
After electroporation, let cells rest for 5 min undisturbed before plating into 8 p100 plates with ESDMEM.
Change-out medium next morning.

48 hrs after electroporation add selection medium, usually 300 ug/ml G418 until colonies have formed. For negative selection add GANC to 2uM for 4 days only. Change medium as needed.

When selected colonies are the diameter of the opening of a 20uL pipette tip (~9 days after electroporation), pick 288 colonies with 20uL pipette tip and place each into 50uL 0.05% Trypsin for 10 minutes. Resuspend and culture each colony in 500uL of ESDMEM in one well of a 24well culture plate. Change-out medium next morning.

After 3-4 days, freeze 4/5 of each colony in 10% DMSO, 90% FBS.
Replate 1/5 of colony.
After 3-4 days lyse cells for genotyping analysis to determine which clones are targeted clones.

Calculating gene copy/genome equivalent

The mouse diploid genome has a mass of 6.42×10^{-12} g

The amount divided of NM(normal mouse)DNA used by this number gives the equivalent number of mouse diploid genomes. If, for example, a PCR assay was set up with 100 ng mouse DNA per sample,

$$100\text{ng} = 1 \times 10^{-7}\text{ g}$$

$$\frac{1 \times 10^{-7}\text{ g}}{6.42 \times 10^{-12}\text{ g/diploid genome}} = 15{,}576 \text{ diploid genomes}$$

The size of the construct in bp multiplied by 1.07×10^{-21} g/bp = the mass of the construct in grams.

The mass of the construct multiplied by the number of diploid genome equivalents = the single gene copy equivalent. For example, if the construct is 5000 or 10,000bp and we are using 100ng of DNA in our PCR, then the mass of the construct is:

$$5000 \times (1.07 \times 10^{-21}\text{g/bp}) = 5.35 \times 10^{-18}\text{g}$$
$$10{,}000\text{bp} \times (1.07 \times 10^{-21}\text{g/bp}) = 10.70 \times 10^{-18}\text{g}$$

Therefore, for 100ng (or 1.5576×10^4 genome equivalents), the single gene copy equivalent is"

5000bp: $(5.35 \times 10^{-18}\text{g}) \times (1.5576 \times 10^4) = 8.33 \times 10^{-14}\text{g}$, or 0.0833pg