I. Preparation of irradiated primary embryo fibroblasts

1. Prepare timed matings of homozygous mating pairs from a knockout line which expresses the neo gene (the resulting fibroblasts will be $G418^r$). Alternatively, homozygous males can be mated with wild-type females to produce heterozygous embryos. In either case, the resulting fibroblasts should be tested for sensitivity to G418, since heterozygous and homozygous fibroblasts will most likely have different sensitivities to the antibiotic treatment.

2. At E14.5, isolate embryos into PBS. Wash 2x in PBS.

3. Remove the head and internal organs from each embryo, using fine forceps (sterilized with ethanol). Rinse the carcasses in PBS (2x).

4. Mince the carcasses into small pieces in trypsin/EDTA (use a pair of scalpels with fresh sterile blades). Use just enough trypsin/EDTA to cover the embryos. Add more trypsin/EDTA to bring the volume to 2 ml/10 embryos. Mix well and incubate at 37°C for 30 min.

5. Inactivate trypsin with an equal volume of DMEM/10% FCS. Dissociate tissues by vigorous pipetting (use a sterile pasteur pipette). Allow large tissue pieces to settle out, then transfer supernatant to a fresh tube. Add more trypsin/EDTA to the large, settled tissue fragments, wait 3-5 min, add equal volume of DMEM/10% FCS, then dissociate by vigorous pipetting. Allow large pieces to settle again, then pool supernatant with previous supernatant. Repeat this cycle 3-4 times.

6. Combine all supernatants, adjust volume with DMEM/10% FCS and plate into 175 cm^2 flasks (1 embryo/flask). When confluent, trypsinize and freeze cells in two tubes /175 cm^2 flask. Freezing media is DMEM/15% FCS/10% DMSO. Freeze slowly at -80°C, then transfer to -150°C.

7. To prepare irradiated feeder cells, thaw one vial of frozen cells into three 175 cm^2 flasks. When these flasks are confluent (after approximately 2-3 days), split into 9 flasks. When the 9 flasks reach confluency, split 1:3 again to obtain 27 flasks.

8. When the 27 flasks reach confluency (usually 2-3 days after the split from 9 flasks), harvest all cells by trypsinization, pellet, and combine all pellets in 50 ml DMEM/10% FCS.

9. Irradiate, using between 2000-3000 rads.

10. Freeze irradiated cells after addition of DMSO to 10% final concentration (prepare 6 freezing vials from each flask - each vial will have the equivalent of approximately a T25 flask). Freeze slowly at -80°C, then transfer to -150°C.