INTRODUCTION OF SUBTLE MUTATIONS USING IN-OUT TARGETING

This procedure allows, via gene targeting, mutations to be introduced into the mouse genome without the presence of a selectable marker gene. It is based on a two-step drug selection procedure using both positive- and negative selection functions of the *Hprt* gene.
Hprt$^-$ 129Ola ES cell line HM-1, that has been shown to give germ line transmission is suitable for this purpose.

1. Preparation of the targeting construct

- Prepare an isogenic insertion-type DNA targeting vector carrying the desired mutation and an *Hprt* cassette (e.g. PGK-*Hprt*).
- Linearize the targeting vector in the region of homology. The ds break must be as far as possible (>1.5 kb) from the mutation that has to be introduced (this to prevent loss of the mutation by DNA degradation and repair). Make sure however, that sufficient homology remains on both sides of the ds break (>1 kb).
- Purify targeting vector according to established protocols.

2. Electroporation and HAT selection

- Electroporate 4×10^7 ES cells with 75 μg of targeting vector.
- Reseed cells at 1×10^7 cells /100 mm dish in 60% BRL-conditioned medium.
- Start HAT (positive) selection next day.
- Continue selection for 8-12 days.
- Pick colonies and expand on MEF feeder layers without selection.
- Freeze clones in 10% DMSO and isolate DNA according to standard procedures.

3. 6-TG counter-selection for revertants

Revertants due to intrachromosomal homologous recombination (*Hprt*$^+$ → *Hprt*$^-$) can be selected for in 6-Thioguanine.
Selection must be performed at low cell density to avoid metabolic co-operation (crossfeeding of metabolites) between *Hprt*$^+$ and *Hprt*$^-$ cells.

NOTE: Spontaneous inactivation of the *Hprt* mini-gene occurs at a frequency $<1 \times 10^{-8}$).

- Reseed a targeted clone at a density of 4×10^5 cells / 10 cm dish in 60% BRL conditioned medium supplied with 10 μg/ml of 6-TG (start with a total 4×10^6 cells).
- Refresh medium every 2 days.
- Continue selection for 8-10 days.
- Pick colonies and expand without 6-TG on MEF feeders.
- Store part in N_2 and analyse DNA for presence of the mutation.