# SELECTION FOR DOUBLE KNOCK-OUT ES CELLS

## 1. Determination of drug resistance level

- Seed single knockout ES cells onto a gelatin-coated 6-well plate at a density of $10^5$ cells per well in 3 ml of 60% medium.
- Refeed the cells the next day with fresh 60% medium, adding to each well increasing concentrations of the drug to be tested:
  - G418:              0, 0.4, 0.8, 1.2, 1.6, 2.0 mg/ml
  - Hygromycin B:    0, 0.3, 0.6, 0.9, 1.2, 1.5 mg/ml
  - Puromycin:       0, 4, 8, 12, 16, 20 mg/ml
- Culture the cells for at least 7 days. Determine the drug concentration at which complete cell death is observed. If cells are surviving the highest concentration, a new targeting vector with another selectable marker should be used.

## 2. Selection for spontaneous double knockouts at high drug concentration

- Culture a subcloned single knockout ES cell line in complete medium + β + LIF on one 6-well plate with MEFs. A confluent well contains approximately $3 \times 10^6$ cells.
- Trypsinize the cells with TVP (10x) and seed onto four 10-cm petridishes, coated with gelatin, in 60% medium.
- Refeed the cells the next day with fresh 60% medium, and start selection:
  · add to two dishes the concentration of drug at which all cells died within seven days
  · add to one dish a somewhat lower, to the other a somewhat higher concentration.
- Refeed the cells with selective medium every 2-4 days. Surviving colonies arise after 8-12 days.

## 3. Generation of double knockouts by consecutive targeting

- Prepare a new targeting vector with another selectable marker gene
- Electroporate single knockout cells as described.

NOTES:

> · In some cases cross resistance can occur. E.g. in the presence of a *hyg* marker, selection for *neo* requires a somewhat higher G418 concentration (250  g/ml).
>
> · Double selections are not recommended as this would obscure lethality of complete gene inactivation.

4. Generation of double knockouts by combining 2 and 3
- Electroporate single knockout cells with the same targeting vector as was used before.
- Select cells at high drug concentration.