# Freezing bone marrow for ER-Hoxb8 GMP cell line production

Materials

- RPMI media = RPMI + 10% FBS + P/S
- FACS buffer = PBS + 2% FBS + EDTA (1 mM)
- 50 ml conical tubes
- Ficoll-Paque PLUS density gradient (1.077) (GE 17-1440-02)
- 40-micron cell strainer (e.g. Fisher 22-363-547)
- Acridine orange
- 2x Freezing media = 80% FBS / 20% DMSO (sterile-filtered)
- Mr. Frosty freezing chamber (very important)

Protocol

1. Euthanize mouse.
2. Dip the entire mouse into a beaker filled with 70% ethanol.
3. Dissect out the 2 femurs and 2 tibias being as sterile as possible.
4. Transfer the bones to a 10 cm dish with ~10 ml of FACS buffer.
5. Crush bones using the back of 50 ml conical tube.
6. Mix well with a transfer pipet.
7. Transfer the 10 ml of cells through a 40-micron cell strainer into a fresh 50 ml conical tube.
    a. Try to leave the bones and debris in the 10 cm dish.
8. Add another 10 ml of FACS buffer and crush the bones again.
9. Transfer the 10 ml of cells through the same cell strainer into the 50 ml conical tube.
10. Layer the 20 ml of bone marrow cells gently over 20 ml of Ficoll-Paque Plus.
11. Centrifuge 400g x 25 minutes at RT
12. Transfer the cell layer to a fresh 50 ml conical.
13. Count the cells using Acridine Orange.
    a. Acridine orange highlights nucleated cells and excludes debris and red blood cells.
    b. From an adult mouse, you should have 25 – 50 million cells at this point.
14. Pellet 200g x 10 minutes.
15. Resuspend the cells at 20-40 million / ml in RPMI media.
    a. Resuspend the cells gently but well with a P1000 to make a single-cell suspension.
16. Chill on ice.
17. Slowly add 1 volume of ice-cold freezing media with constant gentle agitation.
18. Distribute 1 ml into each labeled cryovial.
19. Freeze overnight with Mr. Frosty in -80-degrees freezer.
20. Transfer the next morning to liquid N2 prior to shipping.