Protocol 205_Fusions for mouse hybridoma's

Reagents:

- Mouse myeloma cell line X63.Ag8.653
- IMDM 10% FCS: IMDM+10%FCS + 3,5µl ß-mercaptoethanol (BME)+ 10 ml of P/S/L-glutamine (per 500 ml of medium)
- PEG (polyethylenglycol) solution:
  - 25g PEG4000 (EMD 9727-2) in Erlenmeyer
  - autoclave
  - add 25 ml pre-warmed (37˚C) sterile BSS while PEG is still warm
  - aliquot the PEG solution in aliquots of 3 ml, keep in -20˚C freezer
- HAT 50x stock solution (hypoxanthine, aminopterin, Thymidine) (Invitrogen Cat# 21060-017), aliquots of 3 ml are kept in -20˚C freezer
- HAT-IMDM: dilute HAT50x stock solution in IMDM 10%FCS P/S/LG+ BME
- HT-IMDM: Dilute 50xHT stock solution in IMDM 10%FCS P/S/LG BME medium. Prepare 500 ml.
- IMDM; 10 ml per fusion
- Freezing medium: 90%FCS,10%DMSO.
- 96-,48- and 24-well flat bottom plates
  for spleen: 5 96-well plates per fusion
- Cell culture flasks T25, T75, T125
- 50 ml falcon tubes
- water bath at exactly 37˚C
- sterile pipettes
- mouse macrophages
- table centrifuge
- autoclave

Method:

Approximately a week before fusion:

1. Thaw out a vial of Ag8 cells from liquid nitrogen. Warm the vial in your hand so that it thaws out quickly. Gently pipet the contents of the vial into a 15 ml tube and resuspend the cells in 10 ml of IMDM 10%FCS. Spin the cells down at 1200rpm, 4˚C for 3 min. Remove the supernatant and resuspend the cells in 10 ml IMDM 10%FCS.
   Prepare a 24 well plate with 1 ml IMDM 10%FCS medium per well. Add 6 drops of cell suspension to the first column, 5 drops to the second, etc. Place the remainder of the cell suspension in a T25 and add approximately 5 ml of IMDM 10%FCS medium. Incubate at 37˚C.
2. prepare HAT-IMDM; dilute 50x stock HAT solution in IMDM 10%. Prepare 50 ml per fusion, plus an additional amount (at least 10 ml) to test the HAT medium.
3. Test the HAT-IMDM medium on Ag8 cells; cells should die within 1-2 days.

Day before fusion:
Macrophage culture:

1. peritoneal wash sterily with 10 ml IMDM 10% FCS per mouse (you will need 1 mouse for 2 plates and for spleen: 5 plates per fusion)
2. transfer macrophages into a 50 ml falcon tube and fill to 20 ml with IMDM 10% FCS
3. transfer 100µl/ well macrophages onto sterile 96 flat bottom wells plates; use a multichannel pipetter and a sterile 100 ml boat.
4. Incubate overnight at 37˚C

Ag8 cells:
Split +/- confluent Ag8 cells 1:2 to have them in exponential growing phase. Prepare about one T150 flask per 2 fusions.

Fusion day:
Spleen or LN cell preparation:

1. Smash spleen or LNs in BSS on sterile grid.
2. Fully resuspend the cells by pipetting the whole solution up and down several times.
3. Spin cell suspension for 10 min at 1000 rpm.
4. Remove supernatant and add 10 ml of BSS.
5. Count cells in counting chamber.

Ag8 cell preparation:

1. Transfer the Ag8 culture from the T150 flask into 50 ml tubes and spin for 10 min at 1000 rpm.
2. Remove supernatant and resuspend in 10 ml of BSS.
3. Count cells in counting chamber.

Fusion:

1. Pre-warm the following things to 37˚C in a water bath:
   - PEG solution 1ml/fusion
   - IMDM 10 ml/fusion
   - HAT-IMDM 10%FCS 50 ml/fusion
2. Prepare a beaker with 37˚C water in hood
3. Spleen: mix 2x107 cells of spleen with 2x107 Ag8 cells, mix gently
4. Centrifuge for 10 min at 1000rpm, RT!!
5. Remove supernatant
6. Resuspend pellet by tapping
7. Hold tube in warm water and add PEG drop wise while tapping/swirling in 1 min. *Timer 1 min*
8. Let pellet sit for 90s *Timer 2 min 30 s*
9. Add 10 ml warm IMDM drop wise (first slow, then faster) to pellet during 90s, while gently shaking. *Timer 4 min*

10. Incubate 3 min. *Timer 7 min*.
11. Centrifuge 5 min at 900 rpm.
12. Carefully remove supernatant
13. Resuspend by tapping
14. Add 50 ml pre-warmed HAT-IMDM, place in water bath 37˚C.
15. Proceed with next fusion.
16. Once all fusions have been performed, plate the fused cells on plates containing macrophages: 100µl of cell suspension per well, use multichannel pipette.
17. Incubate at 37˚C, with plates wrapped in saran wrap to prevent evaporation.
18. Check cells at day 7-10 after fusion for colonies.
19. When supernatant is yellow, remove 100µl for ELISA/CBA for isotyping (usually after 14 days).
20. Expand the positive clones into 48-well plates using HT-IMDM medium. (In hard fusions it will be helpful to plate onto macrophages in this stage; plate macrophages, grow them overnight and wash them 3x with IMDM 10%FCS prior to use to remove any other cells than macrophages).
21. Further expansion can be done into 24 well plates, T25 and T75 in IMDM 10% FCS.
22. Once cells are growing confluent in T75; spin the cell suspension down in 50 ml falcon tubes at 1000rpm. Resuspend the cells in a total of 4 ml of freezing medium.
23. Aliquot the cells in aliquots of 1 ml in Cryo vials.
24. Freeze down the cells using The Mr freeze boxes (make sure that the boxes contain isopropanol to slowly freeze the cells) in -80 freezer. Once the cells are frozen, please place the vials in the liquid nitrogen tank.