Mouse Adoptive Transfer

General Surgery Lab SOP

9-18-2013

Phosphate Buffered Saline (PBS)

- Lonza Catalog #: 17-516F

70% Ethanol

- 200 proof Ethanol 70mL
- diH_2O 30mL

10% FCS RPMI media

- RPMI 1640 500mL
- Fetal Calf Serum 50mL
- HEPES (2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid) 5mL
- L-glutamine 5mL
- Penicillin-streptomycin 5mL
- Sodium pyruvate 5mL
- non-essential amino acids (NEAA) 5mL
- 2-mercaptoethanol (2-ME) 0.5mL
- Store at 4ºC

RBC Lysis Buffer

- diH_2O 400mL
- Ammonium chloride (NH_4Cl) - 150mM 4.1g
- Potassium bicarbonate ($KHCO_3$) - 1mM 0.5g
- Na_2EDTA (0.1mM) 18.6mg
    - Alternatively use 100µl 500mM EDTA pH8
- Adjust pH to 7.2 to 7.4 with conc. HCl if needed
- Bring volume up to 500mL
- Sterile filter using 0.2µ filter
- Store at 4ºC long term
- Use at room temperature

10% FCS RPMI with 1000units/ml GM-CSF and IL-4

- RPMI 1640 500mL
- Fetal Calf Serum 50mL
- HEPES (2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid) 5mL
- L-glutamine 5mL
- Penicillin-streptomycin 5mL
- Sodium pyruvate 5mL
- non-essential amino acids (NEAA) 5mL
- 2-mercaptoethanol (2-ME) 0.5mL
- GM-CSF 56µL
- IL-4 28µL

- Store at 4ºC

Rabbit Complement

- diH_2O 1mL
- 10% FCS RPMI media 7mL
- Dissolve powder in the diH_2O (Cedarlane; Catalog #CL3051)
- Add to media in 50ml conical
- Filter through 2µm syringe filter into new 50mL conical
- Aliquot 4mL into 15mL conical
- Store at -20ºC

Bone Harvest

- Supplies
    1. Sterile scissors
    2. Sterile forceps
    3. 50mL conical filled with 70% EtOH
    4. Sterile field
    5. Styrofoam board or corkboard
    6. 4 ï 20g needles or pushpins
    7. Alcohol (spray)
    8. 50mL conical with ~10-15mL PBS
    9. Sterile gloves
    10. Ice
- Procedure
    1. Wrap Styrofoam board/corkboard with sterile field
    2. Euthanize mouse with CO_2
    3. Pin mouse to board using the needles/pushpins through the paws, keeping the legs in a 180º angle ï pull forlegs up before pinning for optimal tension
    4. Spray mouse well with 70% EtOH
    5. Cut skin from mouse
    6. Separate muscle from tibia and fibula ï do not break the bones
    7. Remove as much muscle from the bones as possible
    8. Place bones in PBS on ice
    9. Repeat with second leg
    10. Rinse instruments in 70% EtOH conical
    11. Repeat process with second mouse
    12. Store instruments in 70% EtOH conical (needed again while under the hood)

Cell Harvest (Perform all work in hood)

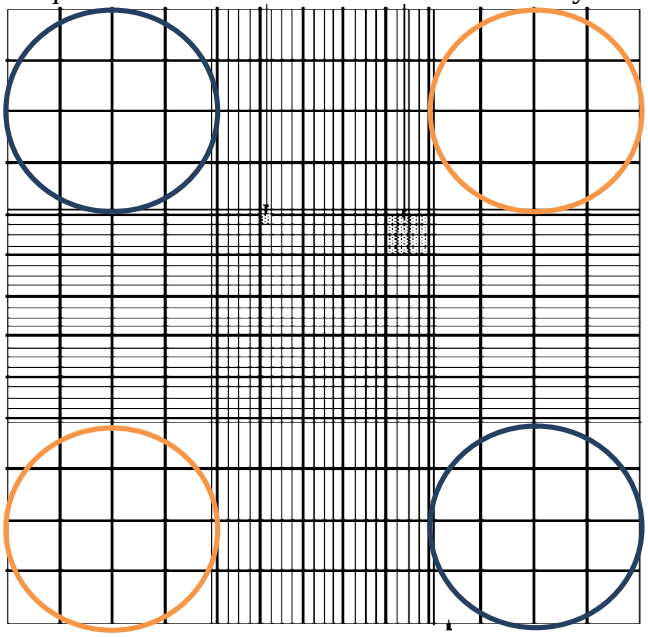
1. Decant PBS from tube and sterilize bones with 70%EtOH for 1 min
2. Transfer bones to lid of petri dish in fresh PBS
3. Remove remaining muscle from bones and cut off ends of bone

4. Place 70µm strainer on 50mL conical
5. Fill 5mL syringe with 10% FCS RPMI media (use 10% FCS RPMI media until it says otherwise)
    a. Pour media into second conical and fill syringe from here rather than sticking syringe inside media bottle to prevent contamination
6. Use a 27g needle on media filled syringe to flush marrow out of bones onto the cell strainer
7. Place flushed bones into bottom of petri dish
8. Rinse bones with media and pipet onto strainer to collect extra cells ï do this only if you are certain the bones have remained sterile (i.e. if a bone ñescapedò your grasp and was flung into hood, do not rinse!)
9. Remove plunger from 3mL syringe ï use black rubber part of plunger to push leftover tissue through the strainer
10. Centrifuge @ 4ºC for 5mins at 2000rpm
11. Decant and re-suspend pellet
12. Add 2-3mL of RBC Lysis Buffer for 3 mins
    a. Vortex every minute
13. Add 20-25mL of media
14. Centrifuge @ 4ºC for 5mins at 2000rpm
15. Decant and re-suspend pellet
16. Add 500uL media
17. Add 5µl each of 5 antibodies (1µl/100µl of media)
    a. Erythroid precursors ï ter119
    b. T cells ï anti CD3
    c. B cells ï antiB220
    d. Natural killer ï NK1.1
    e. Granulocytes ï gr-1
18. Incubate 30 mins on ice (min 20 mins ï max 60 mins)
19. Wash with media
20. Centrifuge @ 4ºC for 5mins at 2000rpm
21. Decant and re-suspend pellet
22. Add 4mL of Rabbit complement
23. Incubate for 45 mins @ 37 ºC (never longer than 60 mins)
24. Add media to 20 mL
25. Centrifuge @ 4ºC for 5mins at 2000rpm
26. Decant and re-suspend pellet
27. Add 10 mls 10% FCS RPMI with 1000units/ml GM-CSF and IL-4 and count cells

<u>Count cells with hemocytometer</u>

1. Add 190εL Trypan blue to 10εL of the above cell suspension into an 1.5mL eppendorf tube (1:20 dilution)
    a. Thoroughly vortex the suspension before removal of cells and also after addition to the trypan blue

2. Put 10ɛL of this suspension into each side of the hemocytometer.
3. Count 2 opposite ósquaresôof cells on each side of the hemocytometer.



4. Average your counts together and multiply by 10 to obtain your cell count.

Cell Culture Maintenance

- There are different ways to keep BM-DC in culture, the minimum time required to generate BM-DC is 5 days.
- Number of cells to be plated at day 0 (depends on the size of the flask):
    - 75cm^2 tissue culture flasks use 10-15 x 10^6 cells in 22 mL
    - 175cm^2 flask double the amount of cells and media
    - 24 well plates use 0.5 x 10^6 in 2mL

Day	Flask size	Final amt of media/flask	Floating cells (mostly DC)	GM-CSF	IL-4
Day 0 (plating)	175 cm^2	48-50 mL	----------	1000 U/mL (4ng/mL)	500U/mL (2ng/mL)
Day 2	175 cm^2	48-50 mL	Plate them back	1000 U/mL (4ng/mL)	500U/mL (2ng/mL)
Day 4	175 cm^2	48-50 mL	Plate them back	1000 U/mL (4ng/mL)	500U/mL (2ng/mL)
Day 6	175 cm^2	48-50 mL	Cells ready to use		

- Changing the media (every two days)

- If making large amounts of BM cultures add GM-CSF and IL4 to a full bottle of media. If you donôt have a lot of culture, or are at the end of culture, just make up what you need

1. Remove all but ~14mL of media and put in 50mL conical flask
2. Centrifuge for 5 mins at 2000 rpm @ 4ºC
3. Decant and re-suspend
4. Add cells back to flask with 10% FCS RPMI with 1000units/ml GM-CSF and IL-4 media and fill to 48-50mL
    - For the plates, slowly remove 1.5mL of media and then add 2mL fresh 10% FCS RPMI with 1000units/ml GM-CSF and IL-4 media.
        - Do this slowly so the cells are not disturbed.
5. On day 6, remove all cells and isolate CD11c cells with magnetic beads
    - Hit flask 3 times to knock loose adherent DCôs
    - Remove all media and place in 50mL conical
    - Centrifuge at 2000 RMP for 5 mins @ 4ºC
    - Using PBS, place all cells into one conical vial
    - Wash with PBS
    - Centrifuge at 2000 RMP for 5 mins @ 4ºC
    - Follow the Miltenyl instructions for positive isolation
    - Dilute concentration for $10x10^6$ cells per 200µL per sterile PBS
    - Inject into mice