The Laboratory of Jason Butler, PhD
Center for Discovery and Innovation
Hackensack Meridian Health

Defined by Mike Poulos, PhD
Lab Manager – Mike Gutkin, MS
October 2020

Media/Reagents

mEC Complete Media

See **ButlerLab_mEC-Media_2020.pdf** for recipe and instructions

8X Digestion Solution

Prepared in Hank's Balanced Salt Solution + 20 mM HEPES (pH 7.4). Store aliquots at -20ºC.

A. 20 mg/mL Collagenase A – (Roche 11088793001)
B. 8 U/mL Dispase II – (Roche 04942078001)

MACS Buffer

Store solution at 4°C. During EC isolations, MACS Buffer should always be cold

A. 1X PBS (pH 7.4) – (Corning 21-040-CV)
B. 0.5% BSA (Fraction V) – (Fisher BP1605-100)
C. 2mM EDTA – (Corning 46-034-CI)

Dynabeads/αCD31 Enrichment

A. Dynabeads Sheep Anti-Rat IgG – (Thermo Fisher 11035)
B. Purified anti-mouse CD31 Antibody – (Clone MEC13.3; Biolegend 102502)
C. DynaMag-2 Magnet – (Thermo Fisher 12321D)

Fibronectin

See **ButlerLab_mEC-Media_2020.pdf** for use and instructions

A. Fibronectin human plasma – (Millipore-Sigma F0895)

Magnetic-activated Cell Sorting (MACS)

A. Lineage Cell Depletion Kit, mouse – (Miltenyi Biotec 130-090-858)
B. QuadroMACS Separator – (Miltenyi Biotec 130-092-857)
C. LS Columns – (Miltenyi Biotec 130-042-401)
D. Pre-Separation Filters, 30μm – (Miltenyi Biotec 130-041-407)

Isolation of Murine Bone Marrow Endothelial Cells (mBMECs)

Dynabeads/αCD31 prep (antibody/bead prep enough for 10 BMEC isolations)

1. Wash Dynabeads 3X in MACS Buffer
   a. Add 1mL cold MACS Buffer to 100μL beads (10X prep) in a 1.5mL round-bottom microfuge tube and wash by gently pipetting several times
   b. Capture beads on magnet for 1 minute and remove supernatant with a P1000
   c. Repeat 2X
2. Resuspend beads in 1mL MACS Buffer and add 20μL αCD31 Antibody (10 μg)
3. Incubate beads/antibody cocktail at 4°C rocking/rotating for 30 minutes
4. Wash beads 3X in 1mL MACS Buffer (same as step 2)
5. Resuspend beads in 1mL MACS Buffer and store at 4°C until use

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EC Isolation

1. Euthanize the mouse via CO_2 asphyxiation
2. (Optional, not necessary) Using a 26.5G needle, perfuse (cardiac) the mouse with 1X PBS to remove excess blood
3. Remove hindlegs, denude all long bones (humerus/femur/tibia) and place on ice
4. Gently disassociate the bone with a mortar and pestle
5. Digest the tissue in 7mL Hank's Balanced Salt Solution + 20 mM HEPES (in a 15 mL conical tube) + 1mL 8X Digestion solution (1X concentration: 2.5 mg/mL Collagenase A, 1 U/mL Dispase II) for 20 minutes at 37°C with gentle agitation/rotating
6. Filter the digested tissue through a 40μm cell strainer (into a 50 mL conical tube)
7. Add 10mL MACS Buffer to bones and pipet repeatedly to wash remaining cells from bone tissue. Filter through same 40μm cell strainer (combining like samples)
8. Bring final volume to 50mL with cold MACS Buffer
9. Centrifuge 400Xg for 5 minutes at 4°C – Carefully aspirate supernatant
10. Perform Lineage Depletion of the single cell suspension (Miltenyi 130-090-858) according to the Miltenyi Mouse Lineage Cell Depletion Protocol
11. Resuspend cell pellet in 900μL cold MACS Buffer
12. Add cells to 100μL prepared Dynabeads/αCD31 (1mL total)
13. Incubate cells at 4°C for 20 minutes (rotating) in a 1.5mL round bottom microfuge tube
14. Capture beads/cells on magnet for 1 minute and remove supernatant with a P1000
15. Wash beads/cells 6X
    a. Add 1mL cold MACS Buffer to beads/cells in a round-bottom microfuge tube and wash by gently pipetting several times
    b. Capture beads/cells on magnet for 1 minute and remove supernatant with a P1000
    c. Repeat wash 4X with cold MACS Buffer
    d. Repeat wash 1X with cold 1X PBS (pH 7.4)
16. Resuspend the beads/cell pellet in 1mL mEC Complete Media and plate in a single well of a 12-well plate (wells should be coated with fibronectin prior to plating)
17. Incubate cells at 37°C 5%CO_2
18. 24 hours after plating, add 4uL myrAkt virus (16,000pg virus) in mEC Complete Media for 2 additional days.
19. 4 days after plating, aspirate virus/media (using P1000), and add 1 mL fresh mEC Complete Media – grow to confluency, changing media every 2-3 days as needed