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Human CD34+ cell isolation from fetal liver, and fetal thymus preparation V.2

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Human Islet Research Ne...



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Protocol status: Working

We use this protocol and it's working

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**Funders Acknowledgement:****NIH****Grant ID: U01 DK123559****Abstract**

This protocol details the steps for isolating human CD34+ cells from human fetal liver. It also explains how to prepare human fetal thymus for immediate use or for freezing, as well as the process for thawing. The CD34+ cells are hematopoietic progenitor cells and can be used to generate humanized mice through reconstitution of immune cells via IV injection after bone marrow ablation. These cells can also be used for mixed lymphocyte reaction experiments.

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Materials






Human tissue:

Order human fetal thymus tissue from Advanced Bioscience Resources (Perrin Larton perrin@garlic.com)

Media:

- BM media:
 - o  500 mL 199 Media (**Corning 10060CV**)
 - o  5 mL 1M Hepes to buffer pH
 - o  5 mL 1mg/ml (2714 units/mg) DNase to prevent cell clumping
 - Use 70 um filter with syringe to filter DNase directly into 199 Media
 - o  40 µL 50mg/ml Gentamycin
- MACS buffer:
 - o  500 mL PBS
 - o  5 g BSA
 - o  2 mL EDTA 0.5M
 - o De-gas overnight with parafilm around the caps
- AIM-V medium (serum free):
 - o Human serum (for use with AIM-V)
 - o Add DNase ( 125 µL) if using AIM-V rather than BM media
- Liberase™ TM (Roche) 1 WU/mL in BM Medium

Material:














- Sterile forceps, scissors
-  10 mL and  25 mL pipette tips for pipette aid
- **70 um nylon cell strainer**
- Styrofoam box for ice– for CD34 isolation kit
- Histopaque or Percoll (contains small plastic beads that separate materials by density centrifugation)
-  10 cm **petri-dish**
- Sterile  6 mL syringe
-  50 mL **Falcon tubes**
- DMSO
- **Human AB serum**
- **CD34+ MACS kit (Miltenyi)**

Before start

All procedures have to be performed under sterile conditions!




Digestion method for CD34+ isolation

- 1 Start water bath and heat to  37 °C if not already on.
- 2 Spray the fetal organ test tube before putting it into the hood
- 3 Fill beaker w/ 70% EtOH. Soak your tools in this.
- 4 Lay out tools onto petri dish to evaporate the EtOH off of them (EtOH will fix the tissue). Open fetal liver test tube and pour out all contents into a big petri dish.
- 5 Separate the white connective tissue from the rest of the tissue and place onto the lid of the original petri dish. Also remove the gall bladder if present (and discard this).
 - a. The white connective tissue should still be saved b/c it can be used for HLA typing (will place this by itself into a cryovial. Do not add media. Then place into  -20 °C freezer into box that says “fetal tissue for HLA typing”)
 - b. Remove the gall bladder and as much connective tissue as possible (and the ligaments), as they cannot be digested and would clot the pipette
- 6 Prepare pre-diluted Liberase solution in BM media or AIM-V media) in a  15 mL Falcon tube:
 - a. Add  5 mL AIM-V medium (or BM media) to a  50 mL falcon tube – will say BM media for rest of protocol, but seems like you can use AIM-V + 10% human serum too.
 - i. Add  125 µL DNase **only** if using AIM-V.
 - b. Add  200 µL Liberase ( 0.5 mg) – found in  -20 °C freezer in  200 µL aliquots.
- 7 Transfer the non-connective tissue liver pieces from the petri dish to a  50 mL Falcon tube with  5 mL Liberase + BM media.
 - a. Take big pieces and cut it into smaller pieces above the  50 mL Falcon tube so that the cut pieces drop directly back into the Falcon tube.



i. This way, you do not need to take more time later to transfer the smaller pieces from the petri dish.


b. Can then use a pipette to transfer the remaining pieces and media from the petri dish back into the  50 mL Falcon tube

i. **Don't add this directly to the Liberase. Put into a different falcon tube, centrifuge, aspirate supernatant, and then resuspend with Liberase (or solution already containing Liberase) to avoid diluting the Liberase.**



◆ Centrifuge settings: 4 C | 1650 RPM | 3 minutes


◇◇ Discard supernatant by pipetting off the solution

8 Vortex this tube for 20 seconds.


9 Place in a rack and place in  37 °C water bath. (The rack should already be in the bath)

a. Let it sit for 6 minutes.

b. Vortex for 20 seconds again and pipette up and down with  10 mL pipette. Then place back in  37 °C water bath for another 6 minutes.

i. Repeat this step a few times until you can pipette up and down easily with a  5 mL pipette


(1). Must spray down with EtOH each time before placing it back into the hood. Return to

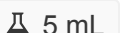
 37 °C water bath each time.

(2). usually completely digested within 10-15 minutes.

ii. The liver should now be disrupted to a single cell solution. The pipette should not clot anymore (except for ligaments, etc).

c. At some point, if not done already, also cut up the connective tissue that you reserved for HLA typing and place it into a yellow/blue top container (without media) while the tissue is digesting in the water bath.

10 Grab at least two 70 um filters and place 1 on top of 2 new  50 mL Falcon tubes.

11 Once solution is easily pipettable through  5 mL pipette and “feels” like a single cell suspension, **top off Falcon tube with BM media.**



















- 12 Transfer  25 mL of the solution to each new  50 mL Falcon tube through the 70 um filter. (should have 2 Falcon tubes with filtered  25 mL cells)
- 13 Centrifuge both of these  50 mL Falcon tubes
- a.** Centrifuge settings: 4 C | 1650 RPM | 3 minutes
- 14 While centrifuging, prepare **four**  50 mL Falcon tubes with  13 mL Histopaque (use a  10 mL pipette)
- 15 When centrifuge done, aspirate supernatant, then resuspend each pellet in  50 mL BM media.
- 16 Add  25 mL of cells + BM media to each tube with  13 mL Histopaque (4 total) by slowly dripping the cells onto the top of the Histopaque. Can tilt the tube and support it on any structure for stabilization (e.g. Styrofoam holder for the  50 mL falcon tubes, a  50 mL falcon tube holder, etc.)
- a.** Weigh the tubes in the centrifuge containers to make sure they are balanced. Add water into the container if it is not balanced.
- b.** Centrifuge all 4 tubes: if using histopaque: 25 °C | 0 prog | 2000 rpm | 25 minutes, accel: 1, brake: 0
- c.** Grab some lunch!
- 17 Collect the buffy coat into two  50 mL Falcon tube (each 2 tubes goes into 1 tube) – then fill up the  50 mL ube with BM media:
- a.** Use  10 mL or  25 mL pipette and suck up the “ring”/layer right above the Histopaque. Will inevitably suck up Histopaque as well, but try to suck up all of the layer right above it.
- b.** Collect all of the layer/Histopaque until you get close to the RBCs to make sure you have obtained all of the buffy coat (which contains PBMCs)

Figure 1

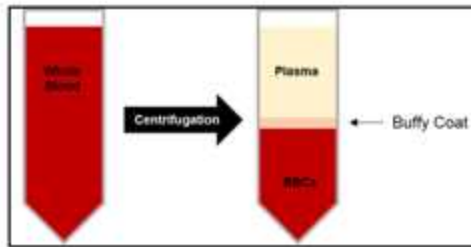
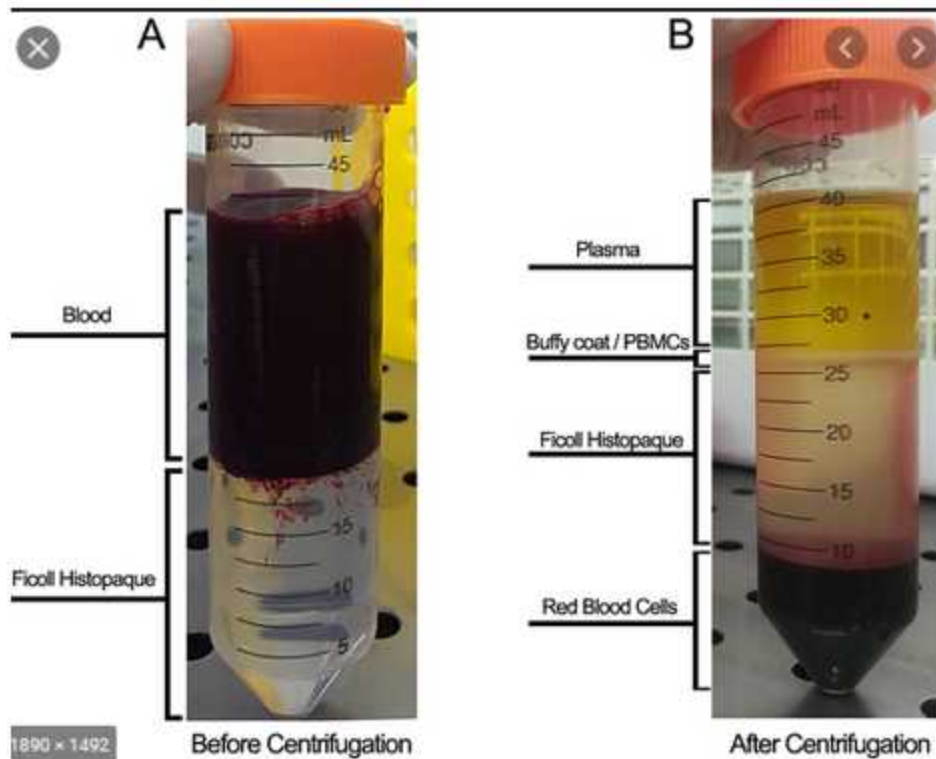
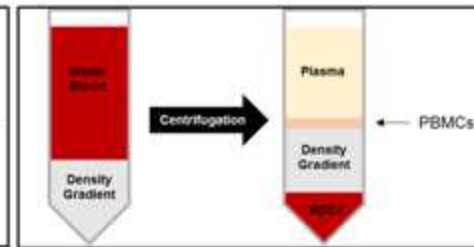


Figure 2



18 Centrifuge the tubes that you have just collected

a. 4 C | 1650 RPM | 4 minutes

b. Discard the supernatant by aspirating with pipette

c. Optional: RBC Lysis – Add 5ml ACK Lysis buffer, incubate 5min RT.







i. If RBC lysis, wash with BM media, then centrifuge and resuspend in MACS buffer (step 19)

19 Resuspend in  15 mL MACS buffer.

20 Take  100 μL and add to a 1.5 round bottom blank tube labeled “Pre” for flow cytometry

21 Count the cells: do serial dilution to 1:25 dilution factor


a.  40 μL Trypan blue +  10 μL cells (1:5 dilution factor). Then  10 μL of this combination (1:5 dilution factor) into another  40 μL Trypan blue = 1:25 dilution factor




MACS isolation:

22 Centrifuge cells 4 C | 1650 RPM | 3-4 minutes




a. While centrifuging, grab CD34+ microbeads + FcR blocker (MACS Miltenyi Biotec CD34 Microbead kit)

b. Calculate volumes needed for addition of FcR Block and Beads

- 1 volume FcR Block= _____ul (1x volume of beads)
 - 1 volume CD34 microbeads= _____ul (10ul per 10^7 cells)
 - 8 volumes Macs Buffer= _____ul (8x volume of beads)
 - Max volumes: 200 uL FcR blocker, 200 uL CD34 microbeads, 1.6 mL MACS buffer
- ♦ Example calculation: for 339.375E6 cells à 33.9E7 cells =  339 μL . But since max =

 200 μL , we used  200 μL CD34+ microbeads,  200 μL FcR blocker, and

 200 μL MACS buffer.

- Usually we get around 500E6 cells. For this amount, we **don't** use 500 uL of beads (it's too much). Usually between 100 to 200 uL is enough.
- ♦  150 μL CD34+ beads
- ♦  150 μL FcR blocker
- ♦  1.2 mL MACs buffer

23 Resuspend cells with amount of MACS buffer first.

a. Then add FcR blocker (must add FcR blocker before microbeads!!)

b. Then add CD34 microbeads






- c. Pipette up and down
- 24 Incubate for 30 minutes at 4 °C
- 25 Wash cells by adding 5-10ml MACS buffer. Centrifuge cells 1650 RPM, 3-4 min, 4C. Aspirate and discard supernatant. **Important to stick to this centrifuge speed because at higher speeds beads that have NOT bound cells will also be spun down into pellet.*
- a. Prepare MACS set up while centrifuging:
- b. LS filter placed into MACS set up “multi stand” with fin tips pointing toward you; place 15 mL falcon tube underneath LS filter; place 70 uM filter on top of the LS filter
- i. Label the falcon tube “CD34-”
- c. a. Add 3 mL MACS to the 70 uM filter + LS filter after you put it together to wet the filter
- 26 Resuspend the sample in 1 mL MACS and add this to the MACS filter tube **through the 70 uM filter**
- 27 Once liquid has flowed through the filter, wash 3 times with 3 mL MACS each
- a. For the first wash, put the 3 mL into the original 15 mL tube that held the tissue. Pipette up and down and add to the LS filter
- b. Can then toss the original tissue tube and the 70 uM filter on top of the LS filter
- c. For the next 2 washes, add 3 mL MACS straight to the LS filter
- 28 Remove the CD34- tube, and switch it with a tube labeled CD34+.
- 29 Remove the LS filter from the magnet, place it over the CD34+ tube, **add 5 mL MACS buffer**, take the plunger, and force out the CD34+ cells.
- 30 **Add** 100 µL **of CD34+ and** 100 µL **CD34- to separate round bottom, blank tubes for flow cytometry later**



- 31 Count the CD34- and CD34+ cells
 - a. For CD34-: use 1:125 dilution (12-13 mL)
 - b. For CD34+: use 1:25 dilution (5 mL)
- 32 Determine how many vials to freeze
 - a. For CD34+: freeze 2 million per vial. Use AIM-V + 10% HS in 10% DMSO
 - b. For CD34-: freeze ~50 million per vial. Use AIM-V + 10% HS in 10% DMSO
- 33 Stain the small aliquots taken before for purity check by flow cytometry:
 - a. hCD3 BV650
 - b. hCD19 APC
 - c. hCD45 PE-CF594
 - d. hCD34 PE
 - e. DAPI
 - f. Human serum as blocker


Alternative: CD34+ isolation by smashing the fetal liver

- 34
 1. Put fetal liver in a petri dish and smash it gently with a sterile plunger of a 3 or 6ml syringe. Transfer cell suspension into  50 mL conicals through a 40 um cell strainer
 2. Place cell suspension on  14 mL of Histopaque 1.077 of a  50 mL tube.
 3. Centrifuge 600 g, 25 min, 25 C, acc 3, break 0
 4. Collect buffy coats and filter through 40 um cell strainer. Fill up with MACS buffer
 5. Count cells
 6. Spin 6 min 400 g, 4 C,
 7. Resuspend in MaCs buffer according to protocol







8. Perform MACS

Fetal thymus isolation and freezing

- 35
1. Cut thymus in \pm 2 mm 3 big pieces in a petri dish
 2. Transfer counted pieces (e.g. 10) into a cryotube. Remove medium
 3. Fill up with  1 mL of cryomedium
 4. Let the cells incubate for 5 minutes (DMSO has to diffuse into the tissue in order to mediate cryoprotection)
 5. Alternatively, thymus can immediately be transplanted

Thymus thaw

- 36
1. Take vials and transfer them to a  37 °C water bath. Let them sit there for 10 minutes
 2. Pour the thymic pieces into  15 mL BM medium (warm) and let them sit for 10 minutes
 3. Before transfer to a new Falcon, vigorously pipette the thymic pieces up and down to remove excess of thymocytes. Transfer the cells to new  15 mL BM
 4. Let the cells sit for 10 minutes. Before transfer into a new falcon, pipette vigorously up and down to remove excess of thymocytes. Transfer the cells to new  15 mL BM
 5. The cells are now ready for transplantation