Isolation of CD34$^+$ Cells from Human Fetal Liver and Cord Blood

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[Abstract] CD34 is a glycosylated cell surface protein and represents a well-known marker for primitive progenitor cells in various organs, especially cord blood, bone marrow and fetal liver. CD34$^+$ progenitor cells are suitable for a series of studies, *e.g.* cell differentiation, transplantation as well as construction of humanized mouse models. Here, we describe a method to isolate CD34$^+$ cells from the human cord blood and fetal liver.

Materials and Reagents

1. Collagenase, Type IV (Life Technologies, catalog number: 17104019)
2. Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, catalog number: D6546)
3. ACK Lysing Buffer (Life Technologies, catalog number: A1049201)
4. RoboSepTM Buffer (STEMCELL Technologies, catalog number: 20104)
5. Trypan blue solution (Sigma-Aldrich, catalog number:T8154)
6. EasySep^{TM} Human Cord Blood CD34 Positive Selection Kit (STEMCELL Technologies, catalog number:18096)
7. StemSpan^{TM} SFEM (STEMCELL Technologies, catalog number: 09650)
8. DMSO Hybri-Max Sterilefilt (Sigma-Aldrich, catalog number: D2650)
9. Hyclone Fetal Bovine Serum (Thermo Fisher Scientific, catalog number: 30070.03)
10. Liquid nitrogen
11. 1 mg/ml Collagenase IV (see Recipes)
12. Freezing medium (see Recipes)
13. PBS/EDTA (2 mM) (see Recipes)

Equipment

1. Syringe Filter (0.20 µm Blue Rim) (Minisart ®, catalog number: 16534-K)
2. Centrifuge Tube (50 ml Blue Cap) (BD Biosciences, Falcon®, catalog number: 35 2070)

3. T75 cell culture flask
4. Petri dish (90 x 15 mm) (Thermo Fisher Scientific, catalog number: BSN 101VR20)
5. Cell scrapers (L29 cm Blade 2 cm) (SPL Life Sciences Co., catalog number: 90030)
6. Shaker Incubator (New Brunswick Scientific, model: Innova 4000)
7. Sterile wire mesh (100 µm) (cut larger than the size of a 90 x 15 mm Petri dish)
8. Syringe (10 cc Luer Lok) (BD, catalog number: DS-BD-15026C)
9. Tabletop Centrifuge Legend RT (Sorvall)
10. FalconTM Polystyrene Round-Bottom Tubes (14 ml) (BD, catalog number: 352057)
11. Big EasySep® Magnet (STEMCELL Technologies, catalog number: 18001)
12. Hematocytometer (TOMY DIGITAL BIOLOGY, catalog number: DHC-N01)
13. Cryovial
14. Cryo tube (free standing 2 ml) (Corning Incorporated, catalog number: 430488)
15. Mr. Frosty Freezing Container (Cyro 1DEGC) (Thermo Fisher Scientific, catalog number: PLW- FS-00033)
16. Leucosep tube
17. 37 °C incubator
18. Centrifuge

Procedure

Part I: Isolation of $CD34^+$ cells from fetal liver

A. Processing fetal liver

1. Weigh out Collagenase IV for a final concentration of 1 mg/ml Collagenase IV with DMEM.
2. Dissolve Collagenase IV with 10 ml of DMEM and filter the suspension using a 0.2 µm filter. Divide the suspension equally into 2 x 50 ml Falcon tubes.
3. Place the fetal liver in a petri dish filled with 20 ml DMEM.
4. Cut liver into small pieces using cell scrapers at room temperature. Mix the suspension well and divide the volume evenly into the prepared Falcon tubes.
5. Add more DMEM to the petri dish to wash down excess tissue and transfer into the same prepared Falcon tube.
6. Bring the tissue suspension to a total volume of 40 ml per Falcon tube with DMEM.
7. Incubate 37 °C for 30 min with shaking 200 rpm.
8. Place a sterile 100 µm wire mesh in the petri dish.
9. Filter tissue suspension through the 100 µm wire mesh. Grind non-filtered tissue particles with the end of a 10 ml plunger against the mesh. Ensure that there are no remaining clumps.

10. Transfer filtered medium into a fresh 50 ml Falcon tube.
11. Spin at 400 *x g* for 5 min with a tabletop centrifuge.
12. Remove supernatant.

B. Lyse red blood cells

13. Add 10 ml of ACK lysing buffer to the pellet and resuspend well.
14. Incubate for 3 min at room temperature.
15. Neutralise the buffer with 10 ml of DMEM. Resuspend.
16. Spin at 400 *x g* for 5 min at room temperature.
17. Remove supernatant.

C. CD34 selection

18. Resuspend cell pellet with 4-16 ml of Robosep buffer depending on the gestation age of the fetal liver and do a cell count using hematocytometer.
19. Place cells in a 14 ml polystyrene tube (up to 4 ml per tube) and prepare cells to a concentration of 2 x 10^8 cells/ml.
20. Add EasySep™ Positive Selection Cocktail at 120 µl/ml cells (*e.g.* for 5 ml of cells, add 600 µl of cocktail). Mix well and incubate at room temperature for 15 min.
21. Pipette EasySep™ Magnetic Particles vigorously more than 5 times. Do not vortex.
22. Add the particles at 50 µl/ml cells (*e.g.* for 5 ml of cells, add 250 µl of nanoparticles). Mix well and incubate at room temperature for 10 min.
23. Bring the cell suspension to a total volume of 10 ml with Robosep buffer. Gently resuspend cells before placing the tube (without cap) into the magnet. Set aside for 5 min.
24. Pick up the EasySep Magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant fraction. The magnetically labelled cells will remain inside the tube, held by the magnetic field of the EasySep Magnet. Leave the magnet and tube in inverted position for 2-3 seconds, then return to upright position. Do not shake or blot off any drops that may remain hanging from the mouth of the tube.
25. Remove the tube from the magnet and add 10 ml of Robosep buffer. Gently resuspend the cells and place the tube back into the magnet. Set aside for 5 min.
26. Repeat step 24-25, and do a total of 4 washes.
27. After the last wash, resuspend cells and combine cells from different tubes with 5 ml of Robosep buffer.
28. Perform a cell count using hematocytometer.
29. Spin cells down at 400 *x g* for 5 min.

D. Cryopreservation

30. Prepare freezing medium (1.2 ml per 5 million cells).
31. Resuspend cells with freezing medium and aliquot 1.2 ml into each cryovial.
32. Place cryovials in Mr Frosty and leave it overnight.

33. Transfer cryovials to liquid nitrogen the next day.

Part II: Isolation of cord blood $CD34^+$ cells

A Pre-enrichment

1. Pour the cord blood sample into the T75 cell culture flask.
2. Add RosetteSep Cord Blood CD34 Pre-enrichment cocktail at 5 µl/ml of cord blood and gently pipette until thoroughly mixed.
3. Incubate at room temperature for 10 min.
4. Dilute the blood sample with equal volume (1:1) of PBS + 2 mM EDTA and mix gently.

B Isolation of mononuclear cells

5. Add 15 ml of RT Ficoll-Plaque Plus into Leucosep tube.
6. Centrifuge at 1,000 *x g* for 30 seconds at RT to allow the Ficoll-Plaque Plus get into the bottom part of the filter in Leucosep tube.
7. Add 30 ml of diluted blood sample into the LeucoSep + Ficoll tubes.
8. Turn Off The Centrifuge Brake!!! And centrifuge at 1,000 *x g* for 20 min at RT.
9. Remove the plasma layer and collect the buffy coats layer into a new 50 ml falcon tube.
10. Top up 40 ml of PBS + 2 mM EDTA to wash the cells.
11. Turn On The Centrifuge Brake!!! And centrifuge for 15 min at 400 *x g*.
12. Discard the supernatant and resuspend the cell pellet with 10 ml of ACK lysis buffer.
13. Incubate at room temperature for 5 min for RBC to lyse.
14. Quench the sample with 40 ml PBS + 2 mM EDTA (1:4 dilution).
15. Centrifuge for 15 min at 400 *x g*.
16. Resuspend in 1 ml of Robosep buffer and perform cell counting using hematocytometer.

C CD34 selection and cryopreservation

Repeat step 18 to 33 in Part one.

Recipes

1. 1 mg/ml Collagenase IV
   On average use a final volume of 80 ml for a 16-19 weeks old foetus
   Or 160 ml for a 20-24 weeks old foetus
   Make fresh before use
2. Freezing medium
   For every 5 million cells/cryovial, volume of 1.2 ml:
   a. 600 µl StemSpan
   b. 510 µl Heat induced-FBS
   c. 90 µl dimethylsulfoxide DMSO

Ensure mixture is homogenous before resuspending with cell pellet.

3. PBS/EDTA (2 mM)
   Add 2 ml of 0.5 M EDTA stock to 500 ml 1x PBS (Filtered)

Acknowledgments

This protocol was developed and adapted from the previous publication Chen *et al.* (2013).

References

1. Chen, Q., Khoury, M., Limmon, G., Choolani, M., Chan, J. K. and Chen, J. (2013). Human Fetal Hepatic Progenitor Cells Are Distinct from, but Closely Related to, Hematopoietic Stem/Progenitor Cells. *Stem Cells* 31(6): 1160-1169.