Human hematopoietic stem cells isolation from umbilical cord blood.

Umbilical cord blood (CB) is obtained from healthy full-term newborns (Department of Gynecology and Obstetrics, UNMC). The University of Nebraska Medical Center Institutional Review Board has determined that the collection of CB samples does not constitute human subject research, as defined at 45CFR46.102, as the samples are collected without identifiers from discarded tissue. UNMC obtains consent from patients to use any leftover biological material for research without identifiers.

Reagents and instruments.

CD34 MicroBead Kit UltraPure Human from Miltenyi Biotec (catalog no. 130-100-453) for isolation of human CD34+ hematopoietic stem cells (HSC)

Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS BSA Stock Solution (#130-091-376) 1:20 with autoMACS® Rinsing Solution (#130-091-222). Keep buffer cold (2−8 °C). Degas buffer before use, as air bubbles could block the column.

MACS Columns and MACS Separators. CD34+ cells can be enriched by using MS or LS Columns (positive selection from $2x10^8$ or $2x10^9$ cells).

CD34 Stem Cell Cocktail (# 130-093-427) for flow cytometric analysis of separated cells.

Note: *All procedures are performed in sterile conditions in a biosafety cabinet.*

Procedure

1. Collection of umbilical cord blood in 10 ml heparinized tubes (BD Vacutaner). Gently vortex and keep at room temperature. Blood should be picked up within 24 hours after collection.
2. Dilute blood with phosphate buffered saline (PBS) in 1:2 ration in 50 ml conical tube. Mix gently.
3. Aliquot lymphocyte separation media (Ficoll-Paque™) and overlay diluted blood in 50 ml conical tubes carefully.
4. Centrifuge 35 minutes to 400g without brake at room temperature.
5. Collect “buffy coat” in 50 ml conical tube and wash in total 50 ml with PBS/BSA/EDTA buffer for 10 minutes at 1500 rpm.
6. Wash cells once with buffer and resuspend in a final volume of 300 µL of buffer for up to 10^8 cells. Proceed to magnetic labeling.
7. Add 100 µL of FcR Blocking Reagent for up to 10^8 total cells.
8. Add 100 µL of CD34 MicroBeads UltraPure for up to 10^8 total cells.
9. Mix well and incubate for 30 minutes in the refrigerator (2−8 °C).
10. Wash cells by adding 20 mL of buffer for up to 10^8 cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
11. Resuspend up to 10^8 cells in 500 µL of buffer. Place cells in ice.
12. Place column in the magnetic field of a MACS Separator.
13. Prepare column by rinsing with the appropriate amount of buffer: MS: 500 µL LS: 3 mL.
14. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
15. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the flow- through from step 15. MS: 3×500 µL LS: 3×3 mL
16. Remove column from the separator and place it on a suitable collection tube.

17. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. MS: 1 mL LS: 5 mL
18. To increase the purity of CD34+ cells, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 13 to 18 by using a new column without collecting flow-through.

Purity of isolated $CD34^+$ cells are evaluated by flow cytometry using FACS BD array instrument (representative plot is shown on **Figure 1**). Additional staining for CD3+ cells should be included. MHC I characterization for HLA-A2-positive samples could be done on negative fraction of cells. Analysis of CD34+ cells can be accomplished by direct immunofluorescent staining using an antibody recognizing an epitope different from that recognized by the CD34 monoclonal antibody QBEND/10 (e.g. CD34-PE, clone: AC136, # 130-081-002). Use the antibodies in appropriate concentrations as recommended by the manufacturers. Typically, staining for 15 minutes at 2−8 °C should be sufficient. After fluorescent staining, cells should be washed and resuspended in PBS buffer. Samples with greater than 90% purity are what we use for transplantation. When we do not have newborn pups, isolated CD34+ cells should be frozen, and information of samples are recorded. Our weekly yield of CD34+ cells is greater than $4x10^6$ cells.

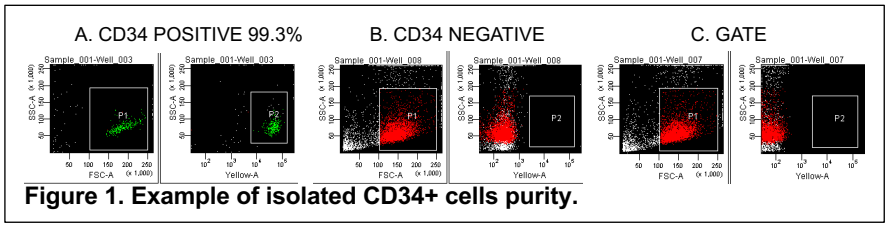

Figure 1. Example of isolated CD34+ cells purity.