Chapter 14

Ex Vivo Expansion of Murine and Human Hematopoietic Stem Cells

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Abstract

Hematopoietic stem cells have the capacity to self-renew and give rise to the entirety of the mature blood and immune system throughout the lifespan of an organism. Here, we describe methods to isolate and culture murine bone marrow (BM) $CD34^{-}ckit^{+}Sca1^{+}Lineage^{-}$ ($CD34^{-}KSL$) hematopoietic stem cells (HSCs). We also describe a method to measure functional HSC content via the competitive repopulation assay. Furthermore, we summarize methods to isolate and culture human $CD34^{+}CD38^{-}Lineage^{-}$ cells which are enriched for human hematopoietic stem and progenitor cells.

Key words Hematopoietic stem cell, Self-renewal, Cell expansion, Regeneration, Reconstitution, Competitive repopulation assay, Cord blood

1 Introduction

Cell surface markers have been utilized for isolation of HSCs. These isolation methods employ a combination of magnetic column separation to enrich for lineage-depleted cells, followed by fluorescence-activated cell sorting (FACS), which facilitates the collection of hematopoietic cells based on multiple surface markers that are tagged with fluorescent probes. The ability to isolate live HSCs provides the capability to measure the effects of specific treatments (i.e., cytokines, genetic modulation) on HSC and progenitor populations [1, 2]. HSCs can be characterized functionally via the competitive repopulation assay, in which limiting dilutions of HSCs are transplanted via tail vein injection into lethally irradiated, congenic mice which also receive host BM competitor cells [1–4]. Over time, donor hematopoietic cell engraftment can be measured via flow cytometric analysis of recipient peripheral blood (PB) or BM cells by distinguishing surface CD 45.1^{+} or CD 45.2^{+} expression. The levels of donor chimerism can be tracked in the peripheral blood through 20 weeks to determine the kinetics of donor cell engraftment and to estimate donor long-term HSC content.

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Limiting dilution analysis can be performed using 3–5 donor hematopoietic cell doses, such that a subset of recipient mice will demonstrate non-engraftment of donor cells. This approach allows for Poisson statistical analysis to estimate the frequency of donor HSCs in comparative donor sources [1, 2]. Our laboratory has utilized these same methods to be employed to estimate the residual frequency of HSCs in the BM of mice following exposure to genotoxic stressors such as ionizing radiation [1, 2].

Like murine HSCs, human hematopoietic cell populations can be enriched via fluorescence activated cell sorting, and then placed in culture for genetic modification or in order to expand subpopulations ex vivo. Cord blood units are a rich source of human HSCs and have been used in HSC transplantation in adults [5–7]. Here, we describe methods to isolate and expand human CB HSCs ex vivo, which has potential implications to improve donor CB engraftment in patients following transplantation.

2 Materials

2.1 Isolation and Culture of Murine CD34$^-$ ckit$^+$Sca1$^+$Lineage$^-$ Bone Marrow Cells

1. Dissecting tools: scissors and forceps. Autoclaved and are sterile.
2. Bone marrow collection media: Iscove's DMEM (IMDM, Cellgro, Manassas, VA), 10 % Fetal Bovine Serum (FBS), 1 % penicillin–streptomycin. Prepare 500 ml. Sterilize with 0.2 μm filter. Store at 4 °C (*see* **Note 1**).
3. Buffer for Lineage Depletion: 10 % FBS, 1 % penicillin–streptomycin in phosphate buffered saline (PBS), pH 7.2.
4. Staining buffer: 1 % FBS in PBS.
5. Plasticware: 28.5 gauge insulin syringes, 40 μm mesh nylon strainer, 15 and 50 ml conical tubes, tubes for FACS, 96-well U-bottom clear polystyrene plate (*see* **Note 2**).
6. ACK Lysing Buffer (Lonza, Walkersville, MD). Store at room temperature.
7. Trypan Blue (Lonza, Walkersville, MD).
8. Magnetic cell sorting: Lineage Depletion Kit, MACS LS columns, and MidiMACS Separator (Miltenyi Biotec, Auburn, CA).
9. Antibodies and reagents for FACS: CD34, ckit, and Sca1 antibodies conjugated with fluorophores, 7-aminoactinomycin D (7-AAD, *see* **Note 3**).
10. Thrombopoietin, Stem Cell Factor, Flt3 Ligand (TSF) Mouse Cytokine media: 20 ng/ml Thrombopoietin, 125 ng/ml Stem Cell Factor, 50 ng/ml Flt3 Ligand in bone marrow collection media (*see* **Note 4**).

2.2 Isolation and Culture of Human $CD34^+CD38^-Lineage^-$ Cord Blood Hematopoietic Cells

1. Wash Buffer: 10 % FBS, 1 % penicillin–streptomycin in PBS, pH 7.2.
2. Priming Media: PBS without Ca^{+2} and Mg^{+2} at room temperature or degassed (*see* **Note 5**). No serum or protein additives.
3. Separation Media: 2 % FBS in PBS.
4. Cord blood units (*see* **Note 6**).
5. Human Hematopoietic Progenitor Cell Enrichment Kit: Human hematopoietic progenitor cell enrichment cocktail, magnetic colloid, pH 7.0–7.5 (StemCell Technologies, Vancouver, BC, Canada) (*see* **Note 7**).
6. StemSep Magnet, Negative selection columns, and Peristaltic Pump with Pump Tubing (StemCell Technologies, Vancouver, BC, Canada) (*see* **Note 8**).
7. Thrombopoietin, Stem Cell Factor, Flt3 Ligand (TSF) Human Cytokine media: 20 ng/ml Thrombopoietin, 125 ng/ml Stem Cell Factor, 50 ng/ml Flt3 Ligand in 10 % FBS with IMDM.

3 Methods

Carry out all procedures under sterile conditions. Procedures should be done expeditiously to improve cell viability.

3.1 Isolation of Murine $CD34^-ckit^+Sca1^+Lineage^-$ Bone Marrow Cells

1. Euthanize mice using protocols approved by the Institutional Animal Care and Use Committee. Dissect bilateral femurs and tibias. Clip both ends of femurs. Insert insulin syringe filled with bone marrow collection media. Flush bone marrow into 50 ml conical tube filled with collection media. Repeat several times on both ends of femurs until femur appears white. Repeat with tibias.
2. Pellet cells in centrifuge at 350 × *g*, 5 min.
3. Aspirate supernatant.
4. Lyse red blood cells with 3 ml of ACK Lysing Buffer and vortex. Incubate 3 min at room temperature (*see* **Note 9**).
5. Neutralize lysis buffer with greater than 3 volumes of buffer for lineage depletion.
6. Pass cells through 40 μm mesh nylon strainer (*see* **Note 10**).
7. Pellet cells in centrifuge at 350 × *g*, 5 min.
8. Aspirate supernatant.
9. Resuspend cells in 40 μl of buffer for lineage depletion per 10^7 cells (*see* **Note 11**). Volume is dependent on total number of mice in collection.
10. Obtain cell count by trypan blue exclusion.

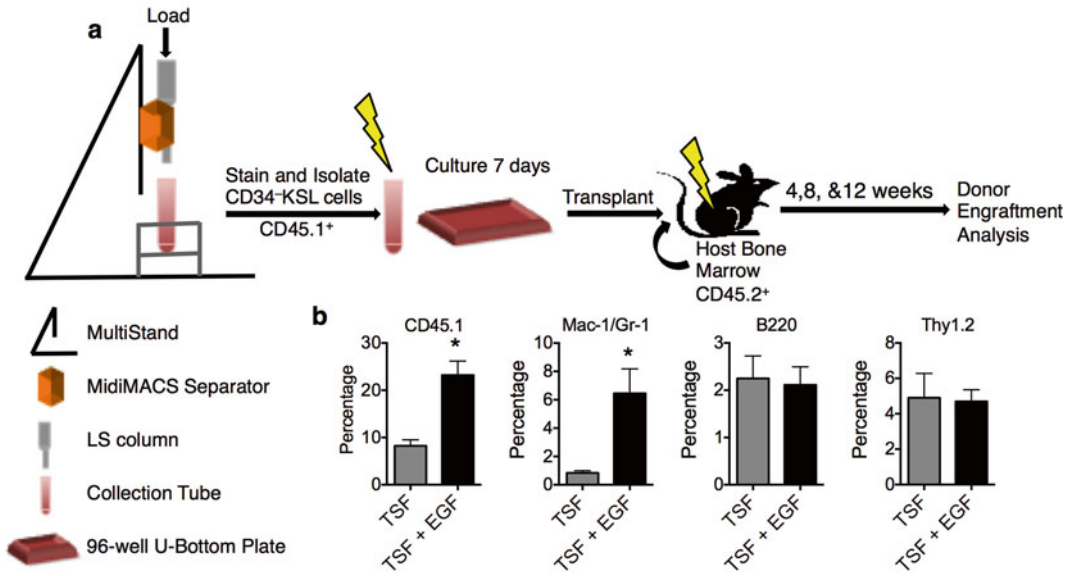

Fig. 1 Isolation of murine CD34⁻KSL cells followed by irradiation, ex vivo culture, and competitive repopulation assay. (**a**) Schematic diagram of method to isolate BM CD34⁻KSL cells. Red blood cell-depleted bone marrow from CD 45.1⁺ mice is labeled for magnetic enrichment of lineage-negative cells. Labeled sample is loaded into a primed LS column. Lineage-negative cells are collected, stained, and FACS-sorted for CD34⁻, ckit⁺, and Sca1⁺ (CD34⁻ KSL) cells. To measure the regenerative capacity of these cells, cells were exposed to 300cGy ionizing radiation (Cs137 source). Cells were cultured with recombinant cytokines (thrombopoietin, stem cell factor, and flt3 ligand = TSF) or TSF supplemented with epidermal growth factor (TSF + EGF). Following 7 days in culture, CD34⁻KSL cells and their progeny were collected and transplanted by tail vein injection into lethally irradiated (950cGy) CD45.2⁺ recipients along with 2×10^5 host BM cells as competitors. Total and multi-lineage donor engraftment in the blood were measured by flow cytometric analysis of the PB between 4 and 20 weeks following transplantation. (**b**) Peripheral blood donor engraftment at 8 weeks of CD45.2⁺ recipient mice following transplantation of 100 CD34⁻KSL cells exposed to 300cGy followed by 7-days in culture with TSF + EGF or TSF alone. Total CD 45.1⁺ donor engraftment, myeloid (Mac-1/Gr-1), B cell (B220), and T cell (Thy1.2) engraftment are shown. *$p < 0.05$. (*see* ref. 2, Fig. 1g)

11. Add 10 μl of Biotin-antibody cocktail per 10^7 cells. Mix well and incubate on ice or at 4 °C for 10 min (*see* **Note 12**).
12. Add 30 μl of buffer per 10^7 cells.
13. Add 20 μl of Anti-biotin Microbeads per 10^7 cells (*see* **Note 13**). Mix well and incubate on ice or at 4 °C for 15 min.
14. Wash cells by adding at least 2 ml per 10^7 cells of buffer for lineage depletion and centrifuge at $300 \times g$ for 10 min.
15. Aspirate supernatant completely.
16. Resuspend up to 10^8 cells in 500 μl of buffer for lineage depletion (*see* **Note 14**).
17. Place LS column(s) in the magnetic field of MACS Separator (*see* **Note 15**, *see* Fig. 1).

18. Prime columns with 3 ml of buffer for lineage depletion per column. Discard effluent. Place clean 15 ml conical tubes underneath columns.
19. Add cell suspension to column(s) and collect effluent in 15 ml conical tubes.
20. Wash columns with 3 ml of buffer for lineage depletion three times. Collect all effluent (*see* **Note 16**).
21. Pellet cells in centrifuge at 350 × *g*, 5 min.
22. Obtain cell count by trypan blue exclusion (*see* **Note 17**).
23. Label cells with CD34, ckit, and Sca1 antibodies in staining buffer (*see* **Note 18**). Incubate 30 min at 4 °C. Rinse cells and resuspend cells at a density of approximately 300 μl per 10^7 cells.
24. Add 15 μl of 7-AAD and proceed to cell sorting (*see* **Note 19**).
25. Pellet cells and resuspend cells in TSF cytokine media (*see* **Note 20**).
26. Seed cells into a 96-well U-bottom plate. Incubate plate at 37 °C, 5 % humidity for the time point of interest (*see* **Note 21**).
27. Collect cells and pellet. Perform cell counts by trypan blue exclusion.
28. Prepare host bone marrow competing cells using **steps 1–8**.
29. Perform cell counts by trypan blue exclusion of host bone marrow cells.
30. Aliquot competing cell dose into sterile eppendorf tubes (*see* **Note 22**).
31. Add donor cells from culture to each eppendorf tube (*see* **Note 23**).
32. Perform tail vein injections of donor and competing host cells into recipient mice that have been lethally irradiated (*see* **Note 24**, *see* Fig. 1).
33. Starting 4 weeks following transplantation and at 4-week intervals, collect peripheral blood by maxillary vein puncture and label for total donor engraftment and multi-lineage engraftment for flow cytometric analysis (*see* **Note 25**).

3.2 Isolation and Culture of Human $CD34^+CD38^-Lineage^-$ Cord Blood and Bone Marrow Hematopoietic Cells

Cord blood and bone marrow cells arrive in the laboratory with all patient information de-identified. Methods will describe procedure for cord blood stem cell isolation. Identical methods can be applied for human bone marrow stem cell isolation.

1. Transfer cord blood into sterile container(s) (*see* **Note 26**).
2. Aliquot 15 ml of ficoll into 50 ml conical tubes.
3. Overlay ficoll with 30 ml of cord blood per tube (*see* **Note 27**).

4. Centrifuge at 350 × *g*, 35 min at 25 °C (*see* **Note 28**).
5. Carefully aspirate upper layer of serum without disturbing buffy coat layer, which contains mononuclear cells.
6. Collect mononuclear cell layer and transfer to new 50 ml conical tubes (*see* **Note 29**).
7. Bring total volume in tubes to 50 ml with wash buffer.
8. Centrifuge at 400 × *g*, 10 min. Discard supernatant.
9. Add 20 ml ACK Lysing Buffer. Vortex (*see* **Note 30**). Incubate cells at 37 °C in water bath for 15–30 min.
10. Bring volume to 50 ml with wash buffer. Centrifuge at 400 × *g*, 10 min. Discard supernatant. Repeat.
11. Resuspend cells in wash buffer and perform cell counts with trypan blue exclusion (*see* **Note 31**).
12. Add StemSep enrichment cocktail at 100 µl/ml. Mix and incubate on ice for 30 min or at room temperature for 15 min (*see* **Note 32**).
13. Add magnetic colloid at 60 µl/ml. Mix and incubate on ice for 30 min or at room temperature for 15 min (*see* **Note 33**).
14. Assemble column into magnet. Assemble pump tubing and prime column with priming media (*see* **Note 34**, *see* Fig. 2).
15. Wash from the top down with the appropriate volume of separation media (*see* **Note 35**).
16. Load sample and repeat **step 15**. Collect sample volume and wash volume (*see* **Note 36**).
17. Pellet cells by centrifugation 350 × *g*, 5 min. Perform cell count by trypan blue exclusion (*see* **Note 37**).
18. Label cells with CD34 and CD38 antibodies in staining buffer (*see* **Note 38**). Incubate 30 min at 4 °C. Rinse cells and resuspend cells at a density of approximately 300 µl per 10^7 cells.
19. Add 15 µl of 7-AAD and proceed to cell sorting (*see* Fig. 2).
20. Pellet cells and resuspend in human TSF cytokine media. Aliquot cells into 96-well U-bottom plate for culture.
21. Perform tail vein injections of cultured cells into NOD-SCID or NOD-scid *Il2rg* recipient mice that have been conditioned with radiation.
22. Starting 4 weeks following transplantation and at 4-week intervals, collect peripheral blood by maxillary vein puncture and label for total donor engraftment and multi-lineage engraftment for flow cytometric analysis (*see* **Note 39**, *see* Fig. 2).

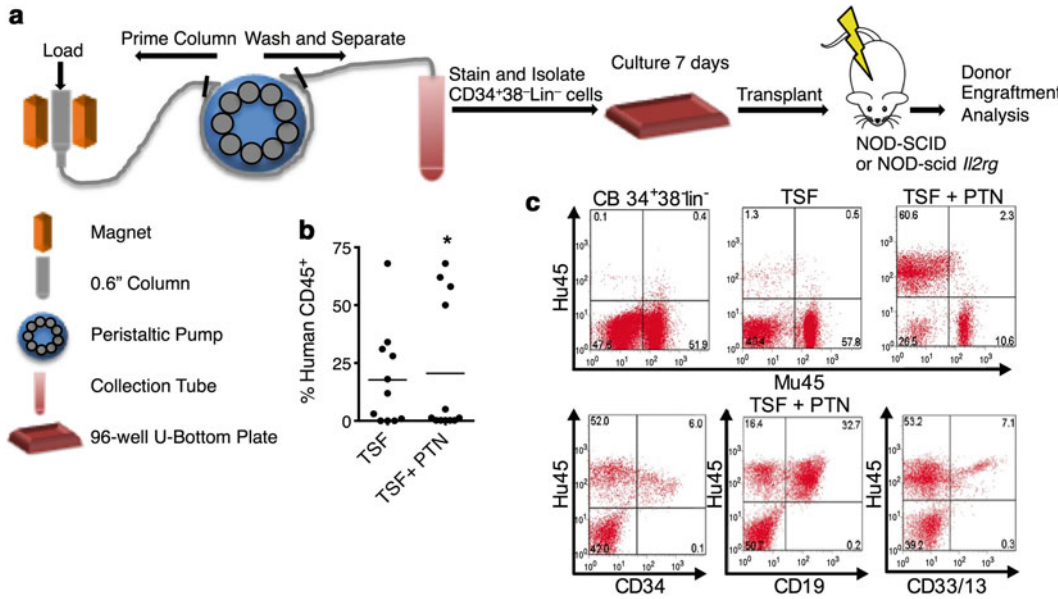

Fig. 2 Isolation and ex vivo culture of human CB $CD34^+CD38^-Lin^-$ cells followed by tail vein injection into NOD/SCID mice. (**a**) StemSep TAC Magnetically labeled cord blood was loaded into a primed and washed 0.6″ magnetic column. The action of the peristaltic pump separated lineage-negative cells into a collection tube. Lineage-negative cells were stained and FACS-sorted for $CD34^+CD38^-Lin^-$ cells. Following 7 days in culture with 500 ng/ml pleiotrophin and human TSF cytokine media (TSF + PTN) or TSF alone, $CD34^+CD38^-Lin^-$ cells and progeny were transplanted via tail vein injection into NOD-SCID or NOD-scid *Il2rg* mice that were conditioned with 300cGy total body irradiation. (**b**) Bone marrow engraftment at 8 weeks is shown following transplantation of the progeny of 2,500 $CD34^+CD38^-Lin^-$ cells following 7 days of culture with TSF or TSF + PTN. $*p < 0.05$. (**c**) Top, representative FACS plots of total human $CD45^+$ hematopoietic cell engraftment at 8 weeks following transplantation of CB $CD34^+CD38^-Lin^-$ cells or their progeny following culture with TSF or TSF + PTN; at bottom, myeloid (CD 33/13) and B cell (CD 19) engraftment in NOD-SCID mice at 8 weeks after transplantation with the progeny of 2,500 cord blood $CD34^+CD38^-$Lin- cells following culture with TSF + PTN (*see* ref. 1, Fig. 3d, e)

4 Notes

1. Reagents are made on the day of experiment.
2. All plasticware should be sterile.
3. We routinely use CD34 FITC, ckit PE, and Sca1 APC-cy7.
4. Cytokines are lyophilized and require resuspension in IMDM, 1 % FBS, 1 % penicillin–streptomycin and are filter-sterilized through a 0.2 μm Nalgene bottle. Store at 100× concentrations at −80 °C.
5. Degassed media reduces the introduction of air bubbles into columns, which can cause channeling and a decrease in the cell capacity of the column. If cell density exceeds the cell capacity of the column, then cell purity can be compromised.
6. Note the cord unit cell count, volume, identification number, and expiration date and time. We process cord units within

48 h of collection to improve cell viability. Wear personal protective equipment (i.e., lab coat, gloves, goggles) when handling human specimens. Working with human specimens may require approval from the Institutional Review Board.

7. Enrichment kit comes with enough reagents to label 5×10^9 cells.
8. Columns come in a variety of sizes. We use 0.6″ columns, which has a column capacity to lineage deplete 10^8–1.5×10^9 cells. While cell separation could be performed with gravity alone, we use a peristaltic pump to both prime and wash cells during separation.
9. To improve cell yield, it is critical to ensure cell pellet is fully dispersed through vigorous vortexing or aggressive pipetting.
10. This filtration step is critical to remove bone fragments following flushing of bone marrow.
11. After red blood cell lysis, we routinely obtain about 25 million whole bone marrow cells per mouse. For fewer than 10^7 cells, use same volume as indicated for 10^7 cells. When working with larger number of cells, increase volumes accordingly. For example, lineage depletion of 2×10^7 cells requires 80 μl of buffer.
12. Working on ice may increase incubation times. Longer incubation times may increase nonspecific cell labeling. Biotin-antibody cocktail includes monoclonal antibodies to CD5, CD45R (B220), CD11b, Anti-Gr-1, anti-7-4, and Ter119.
13. Vortex Anti-biotin Microbeads immediately prior to adding to labeled cells since beads may settle in container. Microbeads are conjugated to a monoclonal anti-biotin antibody (clone: Bio3-18E7.2; mouse IgG1).
14. Scale volumes up according for greater than 10^8 cells. It is critical that cells are in single-cell suspensions.
15. LS columns should fit snugly into MidiMACS Separator. Each LS column has the capacity to isolate a maximum of 10^8 labeled cells and a maximum of 2×10^9 total cells. They are packaged sterilely and come individually wrapped. Columns are "flow stop" and will not run dry. They have a void volume of 400 μl and a reservoir volume of 8 ml. They are intended for single use only.
16. Add buffer when reservoir is empty. This effluent is the enriched lineage-negative cells. The enrichment rate is between 50- and 1,000-fold, depending on the specificity of magnetic labeling. Lineage-positive cells are retained in columns within the magnetic field. If lineage-positive cells are required, remove the LS column from the magnet and place into a new 15 ml conical tube. Add 5 ml of buffer for lineage depletion to reservoir and immediately apply firm pressure to plunger supplied with column to flush out the lineage-positive cell fraction.

17. We typically obtain between 8×10^5 and 10^6 lineage negative cells per mouse.
18. The concentrations of antibodies used for staining require titration based on the specific antibody clone and cell density. In general, we use 1 μg/ml per 10^6 cells.
19. 7-AAD allows for flow cytometric exclusion of dead cells, which will be 7-AAD positive. Note the number of 34^-KSL cells collected from the sorting procedure. If the anticipated number of 34^-KSL cells to be collected is less than 5×10^5 cells, then we collect in 1.5 ml eppendorf tubes containing 300 μl of TSF cytokine media. If collecting greater than 5×10^5 cells, then we collect into flow tubes containing 1 ml of TSF cytokine media.
20. Cultures of 34^-KSL cells in TSF cytokine media serve as controls for testing hematopoietic stem cell expansion with other cytokines (i.e., Pleiotrophin, PTN, or epidermal growth factor, EGF) (*see* ref. [1, 2]). To measure the regenerative capacity of 34^-KSL cells following injury such as radiation, cells are irradiated immediately following cell sorting prior to seeding into the 96-well U-bottom plates.
21. We typically seed between 30 and 300 cells per well in 200 μl of TSF cytokine media. For culture of KSL cells, we have maintained these cultures at 5,000 cells per well for 7 days without exhausting the TSF cytokine media. Most of our in vitro hematopoietic stem cell assays are analyzed at 72 h and 7 days.
22. Competing cell dose is usually 2×10^5 whole bone marrow cells (*see* ref. 4). We tend to use $CD45.1^+$ donor cells and $CD45.2^+$ recipient mice.
23. We determine both the cell dose(s) and number of replicates for competitive transplantation prior to setting up cultures. For example, if we intend to transplant 100 34^-KSL cells and progeny donor cells, we seed each well with 100 cells. If we want to have 15 mice per group, then 15 wells per group are seeded with 34^-KSL cells. At time of transplantation, the entire contents of each well are transplanted into one mouse. Injection media should contain 2–10 % FBS in IMDM. Total volume of injection may range from 100 to 250 μl per mouse.
24. Mice are irradiated 6–24 h prior to transplantation. We maintain all mice on water with trimethoprim-sulfamethoxazole following total body irradiation.
25. We collect 100–200 μl of peripheral blood and perform a RBC lysis as described in Subheading 3.1, **steps 4**, **5**. Cells are labeled for flow cytometric analysis as follows: total engraftment ($CD\ 45.1^+$), myeloid (Mac-1/Gr-1), B cell (B220), and T cell (Thy1.2).

26. We use sterile T175 flasks. Autoclaved, sterile 1 l bottles would work as well.
27. We use 2 volumes of cells to 1 volume of ficoll.
28. Be careful to not disturb interface between cells and ficoll when handling tubes. We disable the brake mechanisms on the centrifuge for this step.
29. Adjust hand pipet to the slowest speed for collection to provide better control. We combine mononuclear cells from several tubes (i.e., 3–4) into new conical tubes for ease of handling fewer tubes.
30. As in **Note 9**, aggressive vortexing to disrupt cell pellet will improve cell yield.
31. Cell density should be about 5×10^7 cells per ml, or within the range of $2–8 \times 10^7$ cells per ml. If cells are still clumped, we recommend that cell suspension be passed through a 40 μm mesh nylon strainer.
32. Enrichment cocktail contains mouse monoclonal IgG_1 antibodies bound to a bispecific tetrameric antibody complex directed against both dextran and the following human hematopoietic surface markers: CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A. Cocktail is in PBS and is stable between 2 and 8 °C for 2 years. Longer incubation times may lead to nonspecific binding.
33. Colloid is stable at 2–8 °C for 6 weeks or at –20 °C for 1 year. Colloid should be vortexed prior to refreezing.
34. Column should be inserted from above down into the gap of the magnet. Do not insert column from front of magnet. Connections between columns and pump tubing should be checked to ensure no leaks are present. Priming speed depends on column size. For 0.6″ column, column should be primed at 0.6 ml/min with a pump setting of 3.0. Priming is complete when priming media is visible above the magnetic layers of the column.
35. For 0.6″ columns, 25 ml of separation media is added to the columns. The direction of flow for the peristaltic pump should be reversed and changed to speed of 2 ml/min at a setting of 10. At no point should the column run dry or air bubbles will be introduced.
36. These cells are lineage-depleted cells and are ready to use. Sample purity could be measured by flow cytometric analysis with anti-CD34 antibody. We routinely achieve greater than 90 % purity with these methods.
37. Lineage-negative cells could be stored in liquid nitrogen until ready to use. Alternatively, they may be stored at 4 °C overnight with excellent cell viability.

38. Volume of antibodies for staining will need to be titrated depending on the antibody clone and fluorophore used.
39. We collect 100–200 μl of peripheral blood and perform a RBC lysis as described in Subheading 3.1, **steps 4**, **5**. At the end of the study, usually between 8 and 16 weeks, bone marrow cells may be collected and analyzed for donor engraftment. Cells are labeled for flow cytometric analysis as follows: total engraftment (human CD45), myeloid (human CD 33/13), B cell (human CD 19), and T cell (human CD 3). We also stain for a phenotypic hematopoietic stem cell population with CD 34 and CD 38.

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