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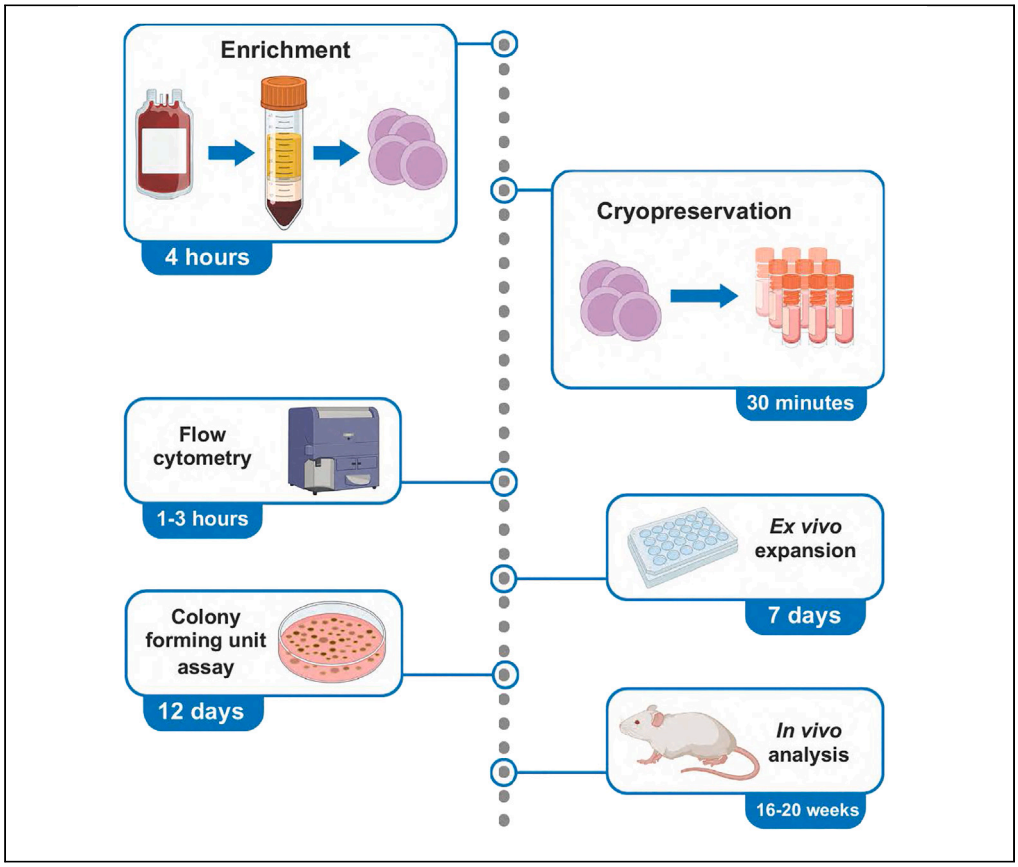
Protocol for enrichment and functional analysis of human hematopoietic cells from umbilical cord blood



Umbilical cord blood (CB) is a donor source for hematopoietic cell therapies. Understanding what drives hematopoietic stem and progenitor cell function is critical to our understanding of the usage of CB in hematopoietic cell therapies. Here, we describe how to isolate and analyze the function of human hematopoietic cells from umbilical CB. This protocol demonstrates assays that measure phenotypic properties and hematopoietic cell potency.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Analysis of umbilical cord blood hematopoietic cell numbers and functional potency

Isolation of hematopoietic cell populations from cord blood units

Ex vivo analysis of hematopoietic cell numbers, proliferation, and differentiation

Measure hematopoietic engraftment and immune reconstitution *in vivo* using mouse models

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Protocol for enrichment and functional analysis of human hematopoietic cells from umbilical cord blood

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SUMMARY

Umbilical cord blood (CB) is a donor source for hematopoietic cell therapies. Understanding what drives hematopoietic stem and progenitor cell function is critical to our understanding of the usage of CB in hematopoietic cell therapies. Here, we describe how to isolate and analyze the function of human hematopoietic cells from umbilical CB. This protocol demonstrates assays that measure phenotypic properties and hematopoietic cell potency.

For complete details on the use and execution of this protocol, please refer to Broxmeyer et al.[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps that can be taken to comprehensively characterize the content and functional potency of human hematopoietic stem (**HSCs**) and progenitor cells (**HPCs**) from umbilical cord blood (**CB**). We have utilized this protocol to determine the potency of unmanipulated cord blood units (**CBUs**), most recently to show that long-term cryopreserved CBUs maintain highly functional HSCs/HPCs.[1] Determining the potency of umbilical CB is crucial to its use, as there is a higher potential for graft failure following use of CB for hematopoietic cell therapies compared to other donor sources.[2–5] Understanding factors that influence potency may therefore lead to improved methods of collection, processing, or treatment to enhance transplantation outcomes. The methods described in this protocol lay the groundwork for these types of analyses. However, this protocol can also be applied to study unmanipulated human bone marrow (**BM**) or mobilized peripheral blood (**PB**), which could be used to test the effects of various health/disease states or environmental exposures on the hematopoietic system of patients. Further, these same concepts can be applied to the study of hematopoietic cells from mouse BM with some slight alterations to the protocol (not discussed here but described in detail in several of our previous publications[6–8]), allowing for the use of a model organism to study *in vivo* effects of diseases, treatments, etc. Finally, given the current interest in using hematopoietic cells for other cellular therapies such as gene editing, gene therapies, and CAR-T/NK cell development, among others, this protocol can also be applied to test the functional competency of HSC/HPC fractions that have been manipulated and/or expanded *ex vivo* from CB, BM, or mobilized PB.

⚠ CRITICAL: When working with human blood products, all steps should be performed in a biosafety cabinet and the units should be treated with BSL2+ precautions.

Note: Many of the sections in this protocol can be considered standalone analyses. For instance, oftentimes investigators can get a preliminary idea of the functional competency

of CB cells by performing just colony forming unit assays and/or expansion assays. The most comprehensive analysis would utilize all of the below subsections, but they can also be used individually or in different combinations to address the overall goals of the current project.

⚠ CRITICAL: Umbilical CB is most often received as fresh whole blood in preservatives, frozen whole blood in DMSO, frozen volume depleted blood in DMSO (which is whole blood with plasma volume removed), frozen red blood cell depleted blood in DMSO (which is whole blood with the majority of high-density red blood cells removed), frozen mononuclear cells in DMSO, or frozen CD34+ enriched cells in DMSO. CD34+ cell cells, which are primarily described here, are highly enriched for HSC/HPC populations. When working with frozen cells, the units should be stored in liquid nitrogen vapor phase or liquid phase immediately after receipt until the day of isolation and analysis.

Institutional permissions

This protocol utilizes human tissue. For the purposes of studying the basic biology of HSC/HPCs derived from CBUs, which are de-identified and considered medical waste, institutional review board approval is typically not required. However, each institute differs and approval should be sought in accordance with their guidelines.

The analysis of truly functional HSC/HPCs requires *in vivo* analysis through use of a model organism, such as mice, as described here. Mice are a vertebrate species and their use in research requires institutional approval. This study was approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (approval number 21071).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Fc block (1:1,000)	BD Biosciences	Cat#553141
Anti-human CD34-APC (clone 581; 5:50)	BD Biosciences	Cat#423104
Anti-human CD38-FITC (clone HIT2; 5:50)	BD Biosciences	Cat#555459
Anti-human CD135-PE (clone 4G8; 3:50)	BD Biosciences	Cat#558996
Anti-human CD45RA-PE-CF594 (clone HI100; 2:50)	BD Biosciences	Cat#562298
Anti-human CD10-PE-Cy7 (clone HI10a; 2:50)	BD Biosciences	Cat#565282
Anti-human CD90-BV421 (clone 5E10; 2:50)	BD Biosciences	Cat#562556
Anti-human CD49f-PerCP-Cy5.5 (clone GoH3; 2:50)	BD Biosciences	Cat#562475
Anti-human CD45-APC (clone HI30; 5:50)	BD Biosciences	Cat#561864
Anti-human CD33-PE (clone WM53; 5:50)	BD Biosciences	Cat#562854
Anti-human CD3 (clone SK7; 3:50)	BD Biosciences	Cat#560176
Anti-human CD19 (clone HIB19; 5:50)	BD Biosciences	Cat#555412
Chemicals, peptides, and recombinant proteins		
Hetastarch	McKesson	Cat #1145300
Human albumin	MilliporeSigma	Cat # SRP6182
Ethylenediaminetetraacetic acid (EDTA)	Fisher	Cat #BP2482-500
Dimethyl sulfoxide (DMSO)	Fisher	Cat #D128-500
Red blood cell lysis buffer	BioLegend	Cat#420301
IMDM (liquid)	Lonza	Cat #12-722F
IMDM (powder)	Gibco	Cat#12-200-036
RPMI1640 (Gibco)	Thermo Fisher Scientific	Cat#11875135
Formaldehyde (16%)	Thermo Scientific	Cat#28908
StemSpan SFEM II	STEMCELL Technologies	Cat#09655
Fetal bovine serum (Corning)	Fisher Scientific	Cat#35015CV
Ficoll-Paque Plus	Cytiva	Cat#17144003

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Methylcellulose	R&D Systems	Cat #HSC003
Glutamine	Gibco	Cat #A29168-01
2-Mercaptoethanol (2-ME)	Sigma	Cat #M-6250
Epogen (Amgen)	McKesson Medical-Surgical	Cat#419193
Recombinant human stem cell factor	R&D Systems	Cat#255-SC
Recombinant human Flt-3 ligand	R&D Systems	Cat#308-FK
Recombinant human interleukin-3	R&D Systems	Cat#203-IL
Recombinant human granulocyte-macrophage colony stimulating factor	R&D Systems	Cat#215-GM/CF
Recombinant human stem cell factor	R&D Systems	Cat#255-SC
Critical commercial assays		
Miltenyi Biotec CD34+ MicroBead Kit UltraPure	Miltenyi Biotec	Cat#130-100-453
Experimental models: Organisms/strains		
Mouse: NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG; 8–12 weeks; male or female)	The Jackson Laboratory	Strain#005557
Software and algorithms		
Extreme limiting dilution analysis user interface	Walter+Eliza Hall Bioinformatics	https://bioinf.wehi.edu.au/software/elda/
Other		
Ziplock bag	N/A	N/A
LS column	Miltenyi Biotec	Cat#130-042-401
5 mL polystyrene tubes	Falcon	Cat#352054
14 mL polystyrene tubes	Falcon	Cat#352051
30 mL syringe	BD Biosciences	Cat#302832
26 3/8-gauge needle	BD Biosciences	Cat#305110
25-gauge needle	BD Biosciences	Cat#305122
18.5-gauge needle	BD Biosciences	Cat#305196
16-gauge needle	BD Biosciences	Cat#305197
15 mL conical tube	Thermo Scientific	Cat#339650
50 mL conical tube	Thermo Scientific	Cat#339652

MATERIALS AND EQUIPMENT

FACS Buffer		
Reagent	Final concentration	Amount
PBS	N/A	498 mL
EDTA (500 mM)	2 mM	2 mL
BSA	0.5% w/v	250 mg
Total	**N/A**	**500 mL**

- Add all ingredients to a flask and mix thoroughly.
- Sterile filter through a 0.45 μm low protein binding filter prior to use.

Note: FACS buffer can be stored at 4°C for several months.

Note: When preparing reagents for culture or transplantation, ensure sterility by performing all steps described here under a tissue culture hood.

2.1% Methylcellulose		
Reagent	Final concentration	Amount
Sterile ddH_2O	N/A	550 mL (boiled to ~490 mL)
Methylcellulose-4000 cP	2.1%	N/A
2× IMDM	1×	500 mL
Penicillin/Streptomycin (10,000 U/mL/ 10,00 μg/mL)	100 U/mL/ 100 μg/mL	10 mL
Total	**N/A**	**1 L**

Note: 1 L of methylcellulose is sufficient for approximately 200 independent units with three replicates each.

- Make 2× IMDM according to manufacturer's instructions (https://www.thermofisher.com/order/catalog/product/12200036)
  - For example, utilizing IMDM powder from Gibco, add entire packet of powdered medium (which is preportioned for 1 L of liquid media) and 3.024 g sodium bicarbonate to 500 mL sterile ddH_2O.

⚠ CRITICAL: Ensure IMDM is at 2×, as it will be diluted with methylcellulose for a final concentration of 1×. Any powdered IMDM can be used, but 1× liquid IMDM cannot be used.

- Add 10 mL pen/strep to 2× IMDM. Using a 22 μm filter, filter sterilize and store at 4°C until needed.
- Weigh 21 g of dry methylcellulose into a sterilized, 2 L flask containing a sterile 7 cm stir bar.
- In a separate flask, bring 550 mL of ddH_2O to a boil, reduce heat to slow boil and continue a slow boil for 3–5 min.
- In a tissue culture hood, add the boiled H_2O to the dry methylcellulose powder. Mix thoroughly making sure that all the powder has been moistened.
- Place flask on a stir plate and mix well until slurry has cooled to 37°C–40°C. Do not let it cool too much and do not let the stir bar stop.
- While the slurry is cooling, warm the 2× IMDM back to 37°C.
- In the tissue culture hood, add 500 mL of warmed 2× IMDM to methylcellulose slurry, cap the flask, and mix vigorously by hand for 1 min.
- Recap and place on stir plate at 20°C–25°C for 1–2 h.
- Transfer to cold room and stir for 16–20 h .
- Aliquot into 100 mL into containers and store at −20°C for 6 months.

Note: Aliquots of methylcellulose can be stored at any volume depending on usage.

Note: Methylcellulose will begin to solidify without agitation as it cools, so the stir bar must always be spinning fast and must be big enough to be mixing the entire volume in the flask.

Alternatives: Pre-made methylcellulose for human hematopoietic progenitor CFU assays can be purchased from companies such as STEMCELL Technologies and R&D Systems, among others.

Colony forming unit assay liquid media component

Reagent	Final concentration	Amount
Fetal Bovine Serum (FBS)	60% v/v	1.5 mL
Glutamine (200 mM)	4 mM	50 μL
2-Mercaptoethanol (10 mM)	0.04 mM	10 μL
Epogen (EPO, 500 U/mL)	2 U/mL	10 μL
Recombinant human GM-CSF (50 μg/mL)	20 ng/mL	1 μL
Recombinant human IL-3 (50 μg/mL)	20 ng/mL	1 μL
Recombinant human SCF (50 μg/mL)	100 ng/mL	5 μL
IMDM	N/A	823 μL
Total	**N/A**	**2.4 mL**

- Add all ingredients to a conical tube. Invert to mix thoroughly.
- Media can be sterile filtered through a 0.45 μm low protein binding filter.

Note: This is enough liquid media for 5 mL methylcellulose, which is enough for three 35 mm dishes from one CBU/cell dose/condition. Scale up for the number of total groups that will be

tested. Final concentrations for the methylcellulose suspensions will be half of what is listed in the table, as 2.5 mL methylcellulose will be added to 2.4 mL media + 0.1 mL cell suspension.

Alternatives: CFU assays can also be plated in 6-well plates.

⚠ CRITICAL: FBS from different lots will differ in composition. It is critical that different lots of FBS be tested, and it is recommended to pre-test lots for capacity to support CFU formation and then order bulk amounts of the same lot to avoid this potentially confounding variable.

STEP-BY-STEP METHOD DETAILS

Thawing frozen whole blood units or red blood cell depleted units

Timing: 45 min

This section describes thawing frozen CBUs. When working with frozen mononuclear or CD34+ cells, refer to "Thawing frozen mononuclear or CD34+ enriched cells" section, step 15. When working with fresh CBUs refer to "CD34+ cell enrichment from whole umbilical CB", step 23.

Note: Due to potential differences in processing from each manufacturer, which may affect cell numbers and function, it is recommended to use the same format and manufacturer for replicates for a given experiment.

1. Prior to thawing the CBU, prepare 75 mL (for each unit) thawing solution by combining 37.5 mL 6% hetastarch solution with 37.5 mL 5% human albumin solution. Keep on ice.
2. Moving quickly, take the frozen CBU out of liquid nitrogen, seal it in a clean Ziploc bag (in case the integrity of the blood bag is compromised) and place directly in a 37°C water bath.

Note: It is recommended to thaw one unit at a time to minimize cellular stress during processing. Once the cells have been thawed and washed, they can be placed on ice until all units are thawed; processing of all units can then continue together.

3. Thaw the unit with gentle manual agitation until it is partially thawed. The unit will have a "slushy-like liquid" state at this point.
4. Remove the unit from the water bath and Ziploc bag and thoroughly sterilize the outside of the unit with 70% Ethanol. Move the unit into a sterile biosafety cabinet.
5. Clean the port (Figure 1) with 70% Ethanol.
6. Using a 30 mL syringe attached to an 18.5-gauge needle, slowly add 25 mL thawing solution over the course of 3–5 min to the thawed blood bag through the access port while gently mixing at 20°C–25°C.
7. Remove the entire volume of blood and thawing solution from the blood bag using the syringe and needle and split into two 50 mL conical tubes.
8. Wash the bag with an additional 25 mL of thawing solution and remove the entire volume, adding equal volumes to the same two 50 mL conical tubes from step 7.
9. Centrifuge cells at 550 × *g* for 10 min at 20°C–25°C with the brake off.
10. Aspirate supernatant down to ~1 mL above the level of the cell pellet.

Note: Red blood cell depleted units will have a much smaller cell pellet. It is important to note that the lysis of red blood cells during freeze-thaw of volume depleted or whole blood units may impact the overall health of the unit; likewise, CD34+ isolated units are less indicative of what is collected and utilized in the clinic. Thus investigators should take care to select the unit type that is most appropriate for their intended use.

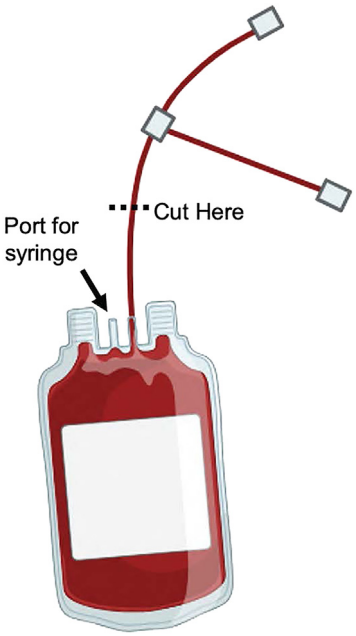

Figure 1. Diagram of CBU blood bag
Shown are the port that can be used to withdraw blood from using a syringe and needle or where the bag can be cut to allow for pouring of the blood into tubes.

⚠ CRITICAL: The cell pellet will be very loose once centrifuged; therefore, the brake must be turned off and tubes should be removed from the centrifuge with caution. Do not decant liquid; aspiration using an electric pipettor or vacuum is required to not disturb the cell pellet.

11. Wash cells one time by adding 45 mL cold PBS or saline and gently resuspend the cell pellet completely by gentle vortexing.
12. Centrifuge the cells at 550 × *g* for 10 min at 20°C–25°C with the brake off.
13. Aspirate PBS using electric pipettor or vacuum.
14. Continue to step 29 in the "CD34+ cell enrichment from whole umbilical CB" section.

Pause point: Thawed/washed CB cells can be placed on ice until all units being used that day are thawed and washed. Then, processing of all units can move forward concurrently.

Thawing frozen mononuclear or CD34+ enriched cells

Timing: 45 min

This section describes thawing frozen mononuclear or CD34+ cells. When working with fresh CBUs, refer to "CD34+ cell enrichment from whole umbilical CB", step 23.

15. Moving quickly, take the frozen cell vial out of liquid nitrogen and move directly to a 37°C water bath. Thaw the vial with gentle agitation in the water bath.
16. When the pellet is mostly thawed, transfer the entire volume to a 15 mL conical tube by gently pipetting.
17. Slowly add 12 mL of sterile IMDM to the conical tube.

Alternatives: Serum free or other media (i.e. RPMI) can be used during thawing.

18. Gently wash the cryovial with 1 mL of IMDM and add to the 15 mL conical tube.
19. Cap the conical tube and gently invert to mix.

20. Centrifuge the cells at 350 × *g* for 10 min at 4°C with the brake on and remove supernatant.
21. Wash one time with 15 mL PBS and centrifuge at 350 × *g* for 10 min at 4°C with the brake on.
22. If starting material is mononuclear cells, continue to step 33 in the "CD34+ cell enrichment from whole umbilical CB" section. If starting material is CD34+ cells, continue to step 37 in the "immunophenotyping to analyze HSC/HPC numbers and frequencies" section.

Note: Viability of cells may be variable from unit to unit depending on freezing efficiency. It is recommended to thaw an extra vial of CD34+ cells for controls during *ex vivo* expansion or other analysis.

CD34+ cell enrichment from whole umbilical CB

Timing: 5 h

This section details the isolation of low-density cord blood (LDCB) and enrichment of the CD34+ cell population from whole blood CBUs, volume depleted CBUs, or red blood cell depleted CBUs. If you are working with mononuclear cells that have already been thawed, continue to step 33. If you are working with thawed CD34+ enriched cells, continue to "immunophenotyping to analyze HSC/HPC numbers and frequencies," step 37.

23. If starting with fresh whole CB, remove whole CB from bag.
    a. Either cut top of bag or insert needle into port of bag (Figure 1).
    b. Pour or syringe transfer 20–25 mL of whole blood into a fresh 50 mL conical tube.
        i. Repeat until all blood is removed from the bag.

Optional: Aliquots of whole CB for later analysis (e.g., to confirm gene or protein expression in cells from a specific unit, etc.) can be cryopreserved at this time by adding 200 μL of blood to a cryovial, then adding 800 μL of 12.5% DMSO, 87.5% FBS (final concentration of 10% DMSO) to the blood. Invert the tube to mix. Freeze at −80°C in a controlled freezing device (i.e. Mr. Frosty freezing container) and within 72 h move to liquid nitrogen for long-term storage.

Optional: Plasma can be collected from the CBU at this time for later analysis (e.g., for plasma proteomic analysis). Transfer 1 mL CB to a 1.5 mL tube. Centrifuge at 1000 × *g* for 15 min at 4°C in a microcentrifuge. Remove supernatant containing CB plasma to a new labeled 1 mL tube. Store at −80°C for up to 1 year.

Optional: To obtain more cells from the CBU, rinse out the bag with 10 mL PBS and distribute into appropriate conical tube.

24. Add 1× PBS to the 50 mL conical tubes containing whole CB so the final volume is approximately 45 mL in order to wash out preservatives the blood is bagged with.
25. Invert to gently mix.
26. Centrifuge diluted CB at 550 × *g* for 10 min at RT with the brake off.

⚠ CRITICAL: The cell pellet will be very loose once centrifuged; therefore, the brake must be turned off and tubes should be removed from the centrifuge with caution. Do not decant liquid; aspiration with electric pipettor or vacuum is required to not disturb the cell pellet.

Note: For all steps involving centrifugation with the brake off, allot approximately 10–15 additional min for the centrifuge to come to a stop depending on centrifuge.

27. While CB is centrifuging, prepare 50 mL conicals containing Ficoll-Paque.

a. For each conical containing diluted CB, prepare one 50 mL conical tube containing approximately 20 mL of Ficoll Paque.

28. Aspirate off supernatant with electric pipettor or vacuum from tubes containing CB, leaving approximately 20 mL remaining in the conical.
29. Tightly cap the tubes and vortex the cell pellet and remaining plasma/PBS to resuspend the cells.
30. Layer the resuspended CB onto the Ficoll Plaque.
    a. Angle the conical containing Ficoll-Paque to 45°. Using a pipette aid set to the slowest setting and a 25 mL serological pipette, gently add the blood to the side of the tube, allowing a layer of blood to form on top of the Ficoll-Paque.

⚠ CRITICAL: Disturb the layering as little as possible, as it will affect the purity of the low-density fraction.

31. Centrifuge at 550 × *g* for 30 min at RT with the brake off.

⚠ CRITICAL: The LDCB layer will be extremely loose; use caution when inserting and removing conicals from the centrifuge.

32. Remove LDCB fraction from centrifuged tubes and transfer into new 50 mL conical tubes.
    a. The LDCB fraction, which contains monocytes, lymphocytes, and HSCs/HPCs, is the middle layer, sandwiched by plasma (top layer) and Ficoll-Paque (bottom layer). There will be a red pellet at the bottom of the tube containing non-nucleated red blood cells and high-density polymorphonuclear cells, such as granulocytes (Figure 2).
    b. Collect the LDCB fraction, which will be primarily white (with some red blood cells present) by pipetting using a 1 mL pipette and a sterile tip. This usually requires drawing up 5–10 mL total volume, 1 mL at a time.

Alternatives: If preferred, a pipette aid and sterile serological pipette can also be used to remove the low-density fraction. Place the serological pipette tip in the center of the low-density layer and gently aspirate the layer, without moving the pipette tip. Cells should naturally move from the outside of the tube towards the middle. After most of the layer is removed, move the pipette tip around to collect remaining cells.

Note: Low density fractions will look different between CB units. Some will have more red cell contamination, and some will contain fatty deposits. Collect all of these cells for highest yield of CD34+ cells.

Optional: The high-density cell pellet (which contains non-nucleated red blood cells and high density polymorphonuclear cells, such as granulocytes) can be used for analysis or cryopreserved for later analysis. As our focus in this protocol is on the LDCB fraction, we direct you to additional publications[9,10] for these types of analyses.

33. After transferring to new tubes, add 1× PBS so the final volume is approximately 45 mL.
34. Invert several times to wash cells. Remove a small aliquot of cells for counting.
    a. Count cells using preferred counting method (e.g., hemocytometer) to ensure proper reactions for enrichment with magnets and beads.
35. Centrifuge at 550 × *g* for 10 min at 4°C with the brake off or on low.

Optional: If using cells for sensitive application (e.g. transplantation or omics studies) wash cell pellet an additional time with 1× PBS to remove any debris or lysed cells.

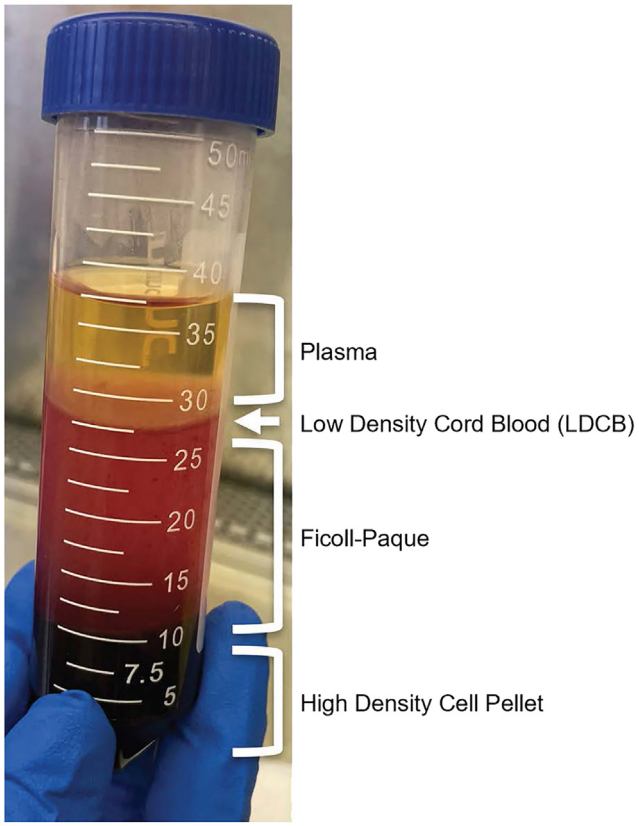

Figure 2. Diagram of LDCB in a Ficoll-Paque conical tube
Shown are the layers that contain the plasma, LDCB, Ficoll, and high-density cell pellet.

Optional: LDCB can be directly plated in colony forming unit assays. To do this, refer to section "*ex vivo* colony forming unit (CFU) assays" step 48.

Optional: LDCB can be cryopreserved at this time for later use (e.g., for transplantation using whole LDCB) by resuspending in 10% DMSO, 90% FBS and freezing at −80°C in a controlled freezing device. The recommended cell density for freezing LDCB is no greater than 1×10^7 cells/mL. It is NOT recommended to freeze LDCB for later enrichment of CD34+ cells.

Pause point: The washed LDCB can be resuspended in FACS buffer and kept at 4°C or on ice for up to 1 h without loss of viability.

36. Enrich for CD34+ cells using Miltenyi separation kits according to the manufacturer's instructions (https://www.miltenyibiotec.com/US-en/products/cd34-microbead-kit-ultrapure-human.html).

Optional: CD34+ enriched cells can be cryopreserved at this time and safely used after thaw for all analyses discussed in this protocol. To freeze the cells, resuspend at a maximum of 1×10^6 cells/mL in a cryovial containing 10% DMSO and 90% FBS. Freeze at −80°C in a controlled freezing device, then move to liquid nitrogen for long-term storage within one week.

Immunophenotyping to analyze HSC/HPC numbers and frequencies

Timing: 1.5 h preparation, 15 min per CBU flow cytometry

This section details immunophenotypic profiling of HSC/HPC subpopulations found in CB CD34+ cells. Immunophenotypically defined HSCs/HPCs, while not defined by their function, are known to be enriched in functional hematopoietic cells. Therefore, determining numbers and frequencies of these cell populations is useful to defining the cellular contents of a CBU.

37. Count cells using preferred counting method to ensure proper antibody dilution.
38. Centrifuge cells at 550 × *g* for 5 min at 20°C–25°C.
39. Prepare antibody cocktail. Example panel for HSC/HPCs:

Cell surface marker	Fluorophore	Clone	Dilution per 2 million cells
CD34	APC	581	5 μL
CD38	FITC	HIT2	5 μL
CD45Rα	PE-CF594	HI100	2 μL
CD10	PE-Cy7	HI10a	2 μL
CD90	BV421	5E10	2 μL
CD49f	PerCP-Cy5.5	GoH3	2 μL
FLT3L (CD135)	PE	4G8	3 μL

Note: This is an example immunophenotyping panel; antibody dilutions may change depending on lot number or clone and should be optimized depending on usage of a specific antibody. There are a wide variety of fluorophore combinations that are possible, and this is one that has been validated and used in published studies.[1,8,9,11,12] There are also additional immunophenotypic markers of HSCs/HPCs that are not listed here but can be found in other studies.[12–14] For catalog numbers, refer to key resources table.

⚠ CRITICAL: Single color staining controls should also be made for flow cytometry analysis for the purposes of setting voltages, compensation, and gating. See Maecker and Trotter 2006[15] for detailed flow cytometry methods.

40. After appropriate volume of all antibodies is added to the cocktail, adjust volume by adding PBS so that 50 μL of antibody solution can be added to each individual sample.
41. Add 50 μL of antibody cocktail to each sample and stain for 15 min at 20°C–25°C in 5 mL polystyrene flow cytometry tube in a low light environment.
42. Add 1 mL of PBS to each tube and centrifuge at 550 × *g* for 5 min at 20°C–25°C. Decant supernatant.
43. Wash cells 2× with 1 mL PBS. Centrifuge at 550 × *g* for 5 min at 20°C–25°C.

Note: Volume of PBS for washes should be between 1–2 mL per wash to properly remove excess antibody.

Optional: Cells can be fixed in 500 μL of a 1.5% paraformaldehyde (PFA) solution for 20 min at 20°C–25°C or in a 1% PFA solution for 1–24 h at 4°C. After fixation, cells should be washed 1× with 1 mL PBS and resuspended in an appropriate volume of PBS for flow cytometry analysis. Cells can be stored at 4°C for 48 h with minimal loss of signal. It is not recommended to store at 4°C longer than 48 h.

⚠ CRITICAL: As cells isolated from CBUs may be considered BSL2, inquire with the appropriate regulatory body regarding the status of running unfixed CB cells on the cytometer. It may be a requirement to fix cells for flow cytometry, but this will not affect acquisition if analyzed within the appropriate amount of time.

44. Resuspend cells in an appropriate volume of PBS or FACS Buffer for flow cytometry analysis.

Optional: Keep stained cells protected from light to preserve intensity of fluorophore by wrapping in foil.

45. Example populations for flow cytometry analysis:

Cell population	Population markers
Hematopoietic stem cell	CD34+ CD38- CD45Rα- CD90+ CD49f+
Multipotent progenitor cell	CD34+ CD38- CD45Rα- CD90- CD49f-
Multilymphoid progenitor	CD34+ CD38- CD45Rα+ CD10+
Common myeloid progenitor	CD34+ CD38+ CD45Rα- CD10- FLT3L+
Granulocyte-macrophage progenitor	CD34+ CD38+ CD45Rα+ CD10- FLT3L+
Megakaryocyte-erythroid progenitor	CD34+ CD38+ CD45Rα- CD10- FLT3L-
HSC/HPCs (broad)	CD34+ CD38-
Myeloid progenitors (broad)	CD34+ CD38+

46. As flow cytometry acquisition is dependent on the type of cytometer, user, and institutional regulations, refer to Maecker and Trotter, 2006[15] on basics for voltage selection, compensation, and gating.
47. An example gating strategy for the analysis of HSC/HPC subpopulations is found in Figure 3. HSC/HPC subpopulations can be calculated as the following:
    a. Frequency of parent gate.
    b. Frequency of CD34+ cells.
    c. Frequency of total cells.
    d. Total number in CBU.

Note: A minimum of 100,000 events is recommended to properly identify and assess hematopoietic cell populations.

Ex vivo colony forming unit (CFU) assays

Timing: 2 h

This section describes how to perform colony forming unit (CFU) assays. CFU capacity is a measure of the ability of progenitor cells to proliferate and differentiate into single or multiple lineages. The number of CFUs in a given CBU is used as a standard measure of predicted potency when selecting units for clinical transplantation. CFU assays can be performed using whole blood, LDCB, CD34+ enriched cells, or sorted subpopulations of HSCs/HPCs. For simplicity, we describe using LDCB and CD34+ cells.

⚠ CRITICAL: If any other analysis of the CBU of interest will be performed using post freeze-thaw cells, it is highly recommended to perform CFU assays after the freeze-thaw as well. CFU capacity is the only metric we have found to be consistently affected by cryopreservation.

48. Pre-warm 2.1% methylcellulose in a 37°C water bath.
49. Determine the number of cell groups/doses/conditions you will use. Each lab will determine an optimal number, but the recommended starting numbers are as follows:
    a. For CD34+ cells:
        i. 1000 cells.
        ii. 500 cells.
        iii. 250 cells.
    b. For LDCB:
        i. 1×10^5 cells.

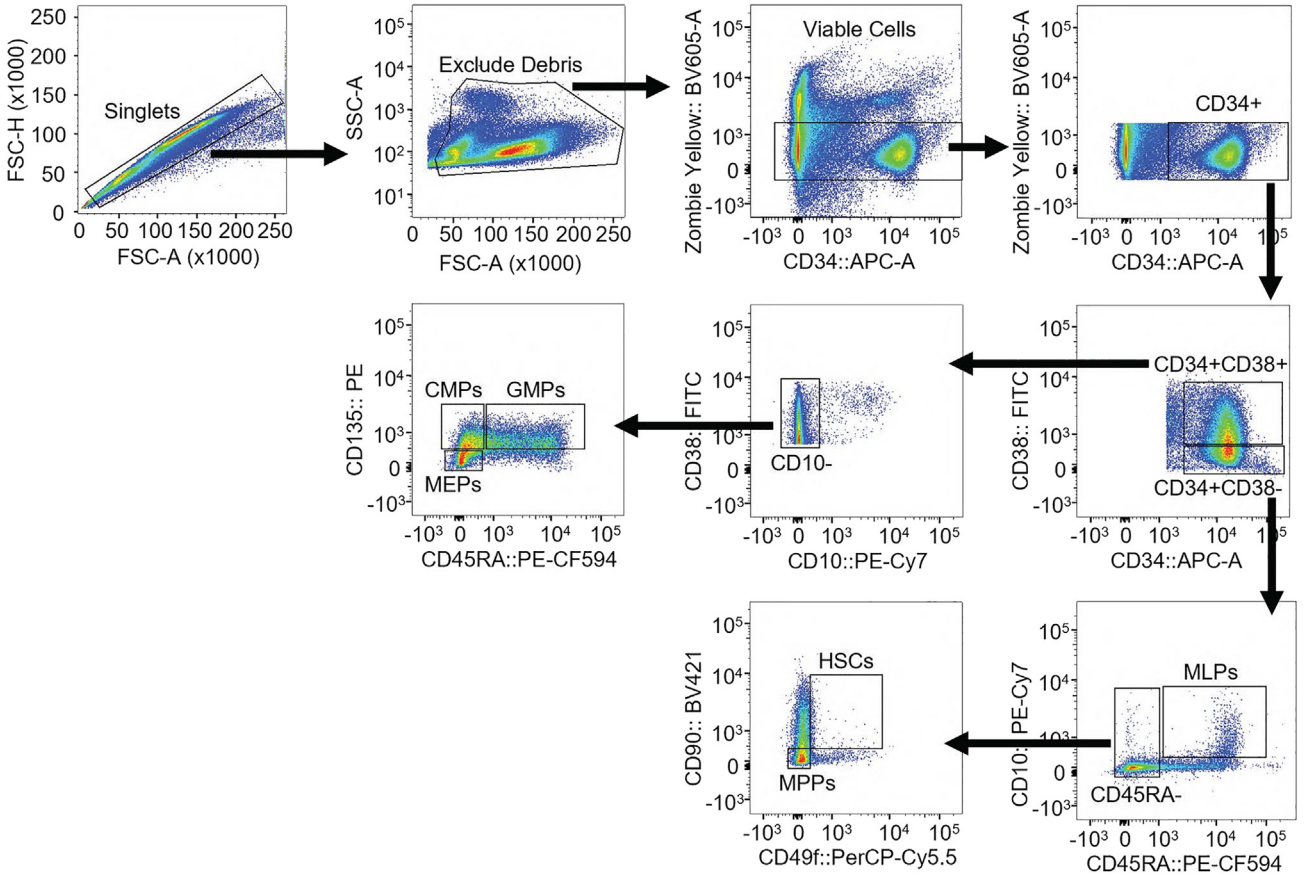

Figure 3. Gating strategy for immunophenotypic analysis of HSC/HPC subpopulations

Shown are the steps taken to define increasingly stringent subpopulations of HSCs/HPCs. Arrows indicate a parent gate that is being further subdivided in the next panel using additional markers.

ii. 5×10^4 cells.
iii. 2.5×10^4 cells.

Note: It is recommended to use 2–3 different cell doses (e.g. 250 cells/mL and 500 cells/mL) per sample until an optimal number has been decided upon by each individual. Variability in cell counting methods can lead to plating variable cell numbers. Plating too many cells can lead to overcrowding of the CFU plate and reduced accuracy in counting. Plating too few cells can result in low CFU numbers and loss of rare CFU subpopulations.

50. Prepare the liquid component of the methylcellulose media (see materials and equipment section). It is recommended to plate CFU assays in technical triplicates. Some volume will be lost to bubbles in later steps, therefore prepare enough for 5 CFU plates per cell group/condition.

Note: This media can be prepared in bulk for multiple groups/conditions/cell doses of cells and aliquoted for use in the different CFU assays.

Note: Adjusting the growth factors in the CFU mixture will change the types and distributions of CFUs formed. We consider the mixture provided in the materials and equipment section to be "maximum stimulation" conditions and it is ideal for enumerating CFU-granulocyte, macrophage (CFU-GM) and CFU-granulocyte, erythroid, macrophage, megakaryocyte

(CFU-GEMM) progenitors. Low numbers of burst forming unit-erythroid (BFU-E) will be observed in maximal stimulation conditions. To better enumerate BFU-E cells, remove the SCF and GM-CSF.

51. Distribute 2.4 mL media for each group to labeled 14 mL polystyrene tubes.
52. Prepare cells that will be plated so that there are enough cells for 5 CFU plates in 100 μL of IMDM.
    a. For example, if you are plating 1000 CD34+ cells per CFU plate, they should be resuspended at a concentration of 50,000 cells/mL. 100 μL of this cell suspension will contain 5000 CD34+ cells, which will then be added to enough media for 5 CFU plates, meaning that 1000 CD34+ cells will be on each plate.
53. Add 100 μL of cell suspension to 14 mL polystyrene tube containing liquid media components.
54. Add 2.5 mL pre-warmed methylcellulose to each 14 mL tube using a 5 mL syringe.
55. Vortex vigorously for 30 s to fully mix the cells into the semi-solid methylcellulose mix. The tubes should appear extremely bubbly.
56. Allow tubes to sit for 20 min at 20°C–25°C to allow the bubbles to rise to the top of the tube.

Note: Depending on the amount of bubbles, this time may change. Allow enough time for bubbles to be removed from methylcellulose mixture.

57. Using a 5 mL syringe with a 16-gauge needle, collect methylcellulose/cell suspension and plate in pre-labeled 35 mm plastic dishes (not culture-treated).
    a. With syringe attached, pull up a very small volume from the top of the methylcellulose and then expel it all on the side of the tube to remove any air inside the syringe and eliminate bubbles from CFU plate.
    b. Move the needle to the bottom of the methylcellulose suspension. Allow it to sit there for 30 s to let bubbles rise off the needle tip.
    c. Slowly pull up the volume you will plate, usually 3 mL at a time.
    d. Expel 1 mL to each 35 mm dish.

⚠ CRITICAL: Methylcellulose mixture is too viscous to pipette accurately. Using a syringe and needle is highly recommended. The needle cannot be smaller than 18-gauge or the cells can begin to shear.

58. Put all 35 mm dishes on a large (150 cm) dish with at least two 35 mm dishes filled with sterile water in the middle. Label large dish with date and any other pertinent information.

⚠ CRITICAL: The methylcellulose plates must be properly humidified. If you do not add water dishes as described in step 58, most incubators (even those with humidity control) will cause the methylcellulose CFU plates to dry out by day 12, making them difficult or impossible to score.

59. Incubate for 12 days in humidified incubator at 37°C and 5% CO_2.

Optional: CFU assays can be performed in low oxygen (5% O_2) incubators. Higher numbers of CFUs have been observed in low oxygen incubators.

60. On Day 12, remove CFU plates from the incubator.

Pause point: CFU plates can be stored in a 4°C cold room for up to 2 days with no effects on CFU appearance. After 2 days, the red appearance of erythroid cells will begin to fade and may be completely gone after 1 week.

61. Score the CFU plates using a 4× objective lens on an inverted microscope.
    a. To maintain your place on the plate while counting, either draw lines using a marker on the bottom of the plate or use a grid template scored on a piece of clear plastic.
    b. Plates can be counted for total CFU and for specific CFU subtypes, which are identified by morphology of the colonies.
62. Shown in Figures 4A–4C are representative 4X images of the different subtypes of CFUs with corresponding 300μm scale bars that can be identified from this protocol. Additional representative images from independent labs can be found at https://www.stemcell.com/colony-enumeration-and-identification-for-custom-hematopoietic-training-courses.html.

Note: It is important to note that CFU scoring has a certain degree of subjectivity when distinguishing different lineages of CFUs. One scientist's CFU-GEMM may be another's BFU-E. It is therefore recommended to have more than one person score CFU plates separately to confirm results.

Note: Here we show representative images of CFU-GM, CFU-GEMM, and BFU-E. We do not distinguish between CFU-G, CFU-M, and CFU-GM colonies and we do not score CFU-E, though procedures for this are described elsewhere.[16,17]

Ex vivo expansion of frozen or fresh CD34+ CB cells

Timing: 1 h

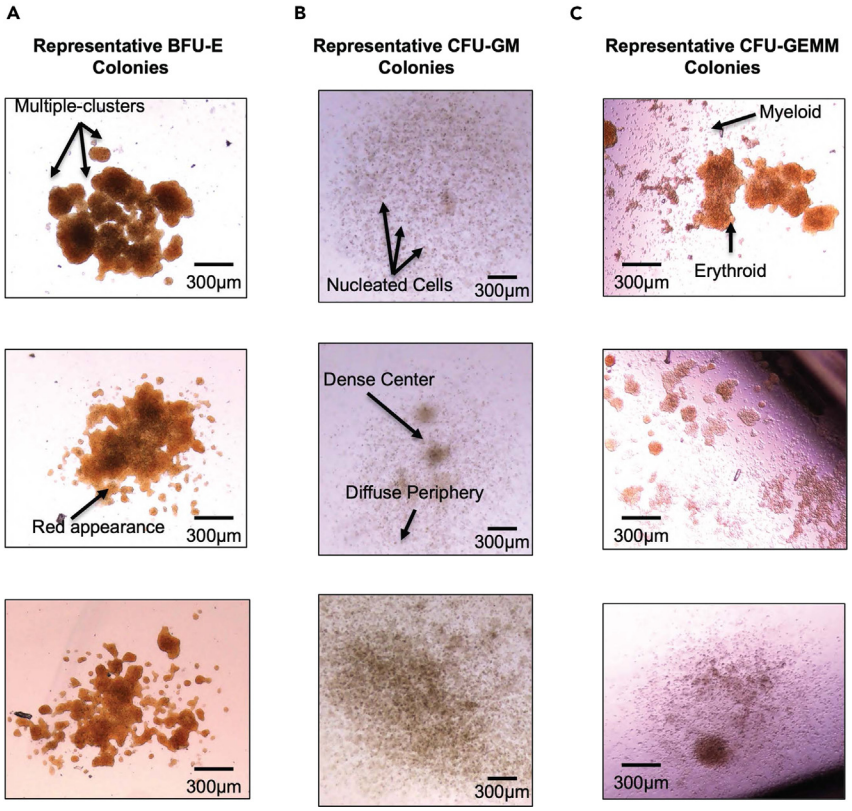

Figure 4. Representative images of HPC CFUs
(A–C) The indicated subtype of CFU is shown at a 4× magnification. Scale bars represent 300μm.

This section describes how to perform *ex vivo* expansion assays using CD34+ enriched cells, which gives a measure of the proliferative capacity of HSCs/HPCs.

63. Centrifuge enriched cells at 550 × *g* for 10 min at 20°C–25°C.
64. Resuspend cells at 50,000 cells/mL for expansion. Human CD34+ cells grow efficiently in serum-free media with 100 ng/mL recombinant human stem cell factor (SCF), FLT3L, and thrombopoietin (TPO).

Note: Cell densities ranging from 25,000 cells/mL to 300,000 cells/mL can be used. Depending on length of culture and post-expansion assays, this number will need to be optimized. 50,000 cells/mL works well for all addressed applications.

Optional: Additional cytokines can be added to the growth media. Depending on the cytokines that are added, specific distributions of hematopoietic cell subpopulations can be acquired.

Alternatives: RPMI+10% FBS can be used as a cost effective alternative media for growing CD34+ cells. Due to the batch variability in FBS products that has been previously discussed, serum free media is highly recommended for sensitive applications, while generic media with FBS can be used for pilot studies and experiments where strong differences between samples are expected.

65. Culture in a 37°C humidified incubator with 5% CO_2 for a desired length of time.
66. Harvest expanded cells for subsequent analysis via flow cytometry, colony assays, or *in vivo* transplantation.

Note: Typical time for culture is 7 days, but analysis of expanded cells can be performed at any time point. Every 4 days, culture media should be replenished with fresh cytokines. This can include adding fresh media (containing 2× concentration of cytokines) on top of existing media or centrifuging and resuspending cells in fresh media (containing 1× concentration of cytokines) and replating.[1]

In vivo analysis of engraftment capacity using mouse modeling

Timing: 3 h for initial transplantation (16–20 weeks for entire procedure)

This section describes mouse models of cord blood transplantation. *In vivo* engraftment and multi-lineage reconstitution of the hematopoietic system are the best metrics to date to measure the potency of hematopoietic cells. Increased engraftment capacity and SCID repopulating cell frequency indicate enhanced stem and progenitor cell potency. Human myeloid and lymphoid cell populations in recipient mice can be measured to determine the multilineage reconstitution capacity of cells from a given CBU. The accepted standard humanized mouse model utilizes NOD.Cg-*Prdkc*^{scid} *Il2rg*^{tm1Wjl}/*SzJ* (NSG) mice.

Note: Alternative approaches to mouse models of cord blood transplantation have been previously discussed.[18]

67. Determine the appropriate number of mice needed for the given transplantation analysis and order them from in-house breeding at your institution or from an outside company (e.g., The Jackson Laboratory).
    a. When quantitatively determining SCID repopulating frequency, which is an accepted standard measure of true HSC potency, it is recommended to use a minimum of 3 cell concentrations and 5 mice per cell concentration, thus a minimum of 15 mice per group.

b. It is recommended to order mice so that they will be 8–12 weeks old at the time of hematopoietic cell transplantation.

Note: Simple engraftment capacity of the CBU can be tested using one cell dose, which will give a qualitative "yes/no" answer to whether the cells are capable of hematopoietic reconstitution *in vivo*.

68. Mice should be equilibrated to the new institution/room for a minimum of 1 week, but 2–3 weeks is ideal.
69. Up to 1 month prior but at least 1 week prior to transplantation, mice should be given an antibiotic chow and acidified water according to their institutional regulations to reduce the risk of infection and gastrointestinal complications.

Alternatives: Mice can be given antibiotics in their drinking water instead of in their feed.

⚠ CRITICAL: NSG mice are immune deficient. They must be handled with extreme care, as they are very sensitive to infection and stress. Best practice includes cleaning gloves or changing gloves between cages of NSG mice when handling them.

70. The day prior to thawing CBUs (or receiving fresh CBUs), processing, and transplantation, irradiate enough mice for transplantation using your institution's approved irradiation method.
    a. Use a sublethal 350 Gy dose of radiation.
    b. It is best practice to irradiate at least 1 extra mouse per experiment to keep as a non-transplanted control.
    c. Return mice to original cage with original cage mates.
    d. Wash mouse restrainers with soap and warm water between each use and dry completely.
71. Determine cell doses of CD34+ cells to use for transplantation.
    a. For accurate SCID repopulating frequency calculations, the lowest cell dose must be limiting so that at least a fraction of the mice do not exhibit engraftment.
    b. Cell doses must be optimized by each lab, but recommended doses of CD34+ cells per injection are:
        i. 1×10^4 cells for the highest dose.
        ii. 2.5×10^3 cells for the intermediate dose.
        iii. 5×10^2 cells for the lowest dose.
72. Collect enough cells from each CBU for the desired amount of mouse transplantations and resuspend in 20°C–25°C sterile PBS for the appropriate number of injections (1 volume per mouse recipient).
73. Warm up NSG mice using an approved heating lamp. When the tail veins are clearly visible, they are ready to inject.
74. Inject irradiated NSG mice via tail vein using a 26–28 g needle with the approved volume of cell suspension from the appropriate cell dose.

⚠ CRITICAL: Cells will fall out of solution over time. Make sure to thoroughly mix the cell suspension between each injection.

75. Return mice to normal chow diet 4 weeks after transplantation.
76. Monitor human cell chimerism in PB of the transplanted recipient mice over time.
    a. Using an approved method, such as submandibular vein bleeding, collect 50 μL of blood in 10 μL of heparin in a 5 mL polystyrene round bottom tube from injected mice at weeks 4, 8, 12, 16 post-transplantation.
    b. Lyse red blood cells (RBC) by adding 2 mL 1× RBC lysis buffer.
    c. Incubate at 4°C for 15 min. Add 2 mL PBS to each tube and invert to mix.
    d. Centrifuge at 400 × *g* at 4°C for 5 min to pellet nucleated cells.

e. Wash once with 1 mL PBS.
f. Resuspend the cells in 50 μL of PBS containing a 1:1000 dilution of mouse FC block (BD Biosciences cat. #553141).
g. Prepare the following antibody cocktail in PBS:

Cell surface marker	Fluorophore	Clone	Dilution per 50 μL of blood
Human CD45	APC	HI30	5 μL
Human CD33	PE	WM53	5 μL
Human CD3	APC-H7	SK7	3 μL
Human CD19	FITC	HIB19	5 μL

Note: This is an example immunophenotyping panel; antibody dilutions may change depending on lot number or clone and should be optimized depending on usage of a specific antibody. For catalog numbers, refer to key resources table.

h. Adjust the final volume of the cocktail up to 50 μL per staining reaction.
i. Add 50 μL to each cell suspension and stain at 20°C–25°C for 15 min.
j. Adjust volume up to 4 mL with PBS.
k. Pellet cells by centrifuging at 400 × *g* at 4°C for 5 min.
l. Wash 2× more with 1 mL PBS.
m. Resuspend cells in appropriate volume of PBS or FACS buffer for flow cytometry analysis.
n. Analyze cells as described in section "immunophenotyping to analyze HSC/HPC numbers and frequencies." Example gating strategies are shown in Figure 5.
    i. Human chimerism should be measured as CD45+ cell percentage of total cells (size excluding debris and red blood cells).
    ii. Myeloid (CD33+) and lymphoid (CD19+ or CD3+) cells should be measured as a percentage of human CD45+ cells.

Optional: Cells can be fixed in 500 μL of a 1.5% PFA solution for 20 min at 20°C–25°C or in a 1% PFA solution for 1–24 h at 4°C. After fixation, cells should be washed 1× with 1 mL PBS and resuspended in 300 μL PBS. Cells can be stored at 4°C for 3 days with minimal loss of signal and up to 1 week with slight loss of signal.

77. Examine human cell chimerism and determine SCID repopulating cell frequency in the bone marrow at the experimental end point.
    a. Using approved method, euthanize recipient mice at week 16 or 20 post transplantation.
    b. Harvest BM in a sterile tissue culture hood from one femur by flushing.
        i. Sterilize a pair of forceps and a pair of scissors for dissection with 70% Ethanol.
        ii. Spray stomach of mouse with 70% EtOH.
        iii. Snip skin on stomach to allow for pulling the skin down the legs of the mouse.
        iv. Cut off one leg of mouse by cutting at the hip joint or cutting into pelvis.
        v. Clean off large muscles from femur.
        vi. Bend knee backward to cleanly remove tibia.
        vii. Bend kneecap backward to cleanly remove it from the femur.
        viii. Fill a 5 mL syringe with PBS. Attach a 25-gauge needle.
        ix. Put the needle into the femur lengthwise going through where the kneecap was. Slowly push and twist the needle forward until it comes out the other side of the femur. Pull the needle back so that it is just inside the femur.
        x. Flush BM from femur into a 5 mL polystyrene round bottom tube using by depressing the syringe.

            Press the syringe slowly at first, scraping the inside of the femur with the needle. After 2.5 mL of flushing, depress the plunger with slightly more force to push the remaining BM into the tube.

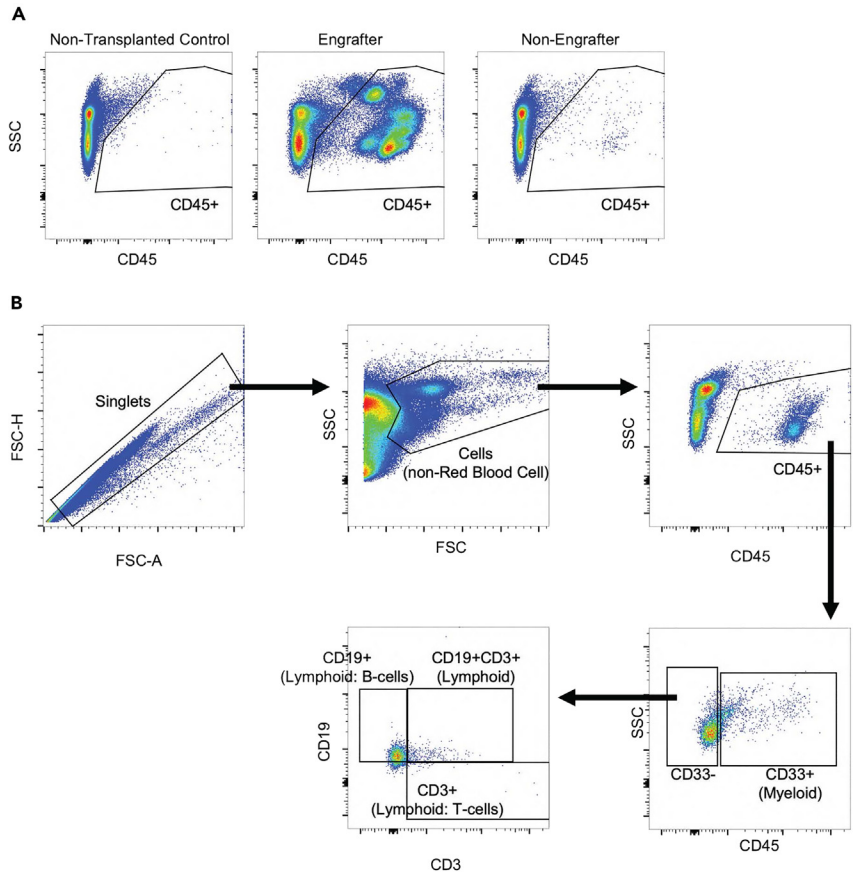

Figure 5. Gating strategy for human chimerism analysis in a transplantation mouse model

(A) Representative FACS plots showing non-transplanted control, an engrafted mouse, and a non- engrafted or low-engrafted mouse following transplantation.

(B) Gating strategy to determine frequencies of human lymphoid and myeloid cell populations in a mouse model of transplantation.

xi. Pipette cells up and down vigorously to dislodge as many cells as possible from debris.

xii. Let debris settle 5 min on ice and transfer cell suspension to fresh tube, leaving any large tissue debris behind.

xiii. Centrifuge cells at 350 × *g* for 5 min at 4°C. Decant supernatant.

c. Count BM cells from each mouse.

d. Distribute 3 × 10^6 BM cells into two separate 5 mL polystyrene round bottom tubes for staining with two different immunophenotyping panels.

e. Centrifuge cells at 350 × *g* for 5 min at 4°C. Decant supernatant.

f. Resuspend the cells in each tube in 50 μL of PBS containing a 1:1000 dilution of mouse FC block.

g. Prepare the antibody cocktail (as well as single stain controls) to measure human chimerism and myeloid/lymphoid cell frequencies as described in 76.g.

h. Add 50 μL of the above cocktail to one tube from each mouse.

i. Prepare the antibody cocktail (as well as single stain controls) to measure human HSC/HPC cell frequencies as described in step 39.

j. Add 50 μL of the above cocktail to the other tube from each mouse.

k. Stain for 15 min at 20°C–25°C in 5 mL polystyrene flow cytometry tube in a low light environment.

l. Bring volume up to 4 mL with PBS.

m. Pellet cells by centrifuging at 400 × *g* at 4°C for 5 min.

n. Wash 2× more with 1 mL PBS.
o. Resuspend cells in appropriate volume of PBS or FACS buffer for flow cytometry analysis.

Optional: Cells can be fixed in 500 μL of a 1.5% paraformaldehyde (PFA) solution for 20 min at 20°C–25°C or in a 1% PFA solution for 1–24 h at 4°C. After fixation, cells should be washed 1× with 1 mL PBS and resuspended in an appropriate volume of PBS for flow cytometry analysis. Cells can be stored at 4°C for 48 h with minimal loss of signal. It is not recommended to store longer than 48 h.

p. Analyze cells as described in section "immunophenotyping to analyze HSC/HPC numbers and frequencies." Example gating strategies are shown in Figure 5.
    i. Human chimerism should be measured as CD45+ cell percentage of total BM cells.
    ii. Myeloid (CD33+) and lymphoid (CD19+ or CD3+) cells should be measured as a percentage of human CD45+ cells.
    iii. Human HSC/HPC numbers should be measured as a frequency of total BM cells.
q. Use the publicly available Extreme Limiting Dilution Analysis software (ELDA) from Walter+Eliza Hall Bioinformatics to calculate SCID repopulating cell frequency.[19]
    i. Using the non-transplanted control to demonstrate where a negative population exists, set a positive gate for human CD45+ cells.
    ii. Mice that exhibit at least two times as much CD45+ cells as a percentage of total cells compared to the non-transplanted control are considered engrafted (Figure 5).
    iii. Enter the number of mice engrafted as instructed on the ELDA software page (https://bioinf.wehi.edu.au/software/elda/) to calculate the SCID repopulating cell frequency.

Optional: BM cells can be used for secondary transplantation to measure the durability of long-term HSCs from a given CBU. To do this, pool 5 × 10^6 BM cells into appropriate volume of PBS per injection for each for the desired number of secondary recipients, then repeat this section using these cells instead of freshly isolated or recently thawed CBUs.

EXPECTED OUTCOMES

Immunophenotyping and colony formation capacity

Cord blood units contain variable numbers of hematopoietic stem and progenitor cells, leading to variable yields of immunophenotypic hematopoietic stem and progenitor cells as well as variable numbers of colony forming units. The yield of these units can be affected by the volume of collection, maternal factors that affect the biological/biochemical composition of the cord blood unit, or collection, cryopreservation, and/or enrichment of the unit. Generally, a single CBU should yield between 5 × 10^5–5 × 10^6 CD34+ cells. HSCs will make up approximately 0.5–1.5% of the CD34+ fraction and different progenitor cell populations can range from 0.5–15% of the CD34+ fraction. Colony forming units are expected to range in numbers from 1 × 10^4 to 1 × 10^5 per 1 × 10^6 CD34+ cells. Ideally, CFU plates contain no more than 150 colonies per plate to make counting as precise as possible.

Expansion

As with the immunophenotyping and colony formation assays, it is expected that expansion will be variable based on the inherent biological variability of the CBU. Expansion capacity typically ranges from 5-fold to 50-fold. Expansion can be dramatically influenced by selection of cytokines used to stimulate growth and the choice of media used to culture the cells. All cord blood units are expected to demonstrate some capacity for proliferation in *ex vivo* culture systems. A lack of proliferation is usually indicative of deficient hematopoietic cells, poor culture conditions, or infection (see troubleshooting).

Transplantation

Most cord blood units should exhibit at least minimal capacity for engraftment in NSG mice. Successful engraftment should be measured compared to a non-transplanted control. When delivering

low cell doses, engraftment can range from undetected to 5% human chimerism. When delivering high cell doses, engraftment can range from undetected to 80% human chimerism. A lack of engraftment does not necessarily indicate a problem in the experimental set up, but could be indicative that the cord blood unit lacked functionally potent stem and progenitor cells. Generally speaking, most transplantations into NSG mice will result in neutrophil (CD33+) and B-cell (CD19+) reconstitution. CD3+ cells are often rare in these models, as the thymus and cytokine environment (or lack thereof) in NSG mice is not conducive to T-cell development. HSCs/HPCs should be found in the bone marrow of recipient mice at low frequencies of total bone marrow cells.

LIMITATIONS

The major limitation of these methods is that they are not fully indicative of hematopoietic cell function in a patient transplantation. The only true measure of hematopoietic cell potency in the context of human transplantation are the outcomes yielded in a patient setting. While immunophenotyping and CFU assays are valuable tools, we have previously shown that there are specific instances when immunophenotypic numbers of HSCs/HPCs do not correlate well with *in vivo* engraftment[20] and CFU assays are sensitive to additional factors such as cryopreservation.[1,21] Expansion assays are valuable metrics of hematopoietic cell proliferation, but it is well established that HSCs/HPCs undergo biochemical and functional changes while growing in *ex vivo* culture systems. Finally, NSG mice, while a gold standard for modeling transplantation outcomes in the lab, do not harbor the same hematopoietic niches or factors as humans do. Whether it is possible to better model hematopoietic cell function in cost effective manners or not should be addressed in future studies.

TROUBLESHOOTING

Problem 1

CD34+ cells do not expand in number during *ex vivo* expansion. See "*ex vivo* expansion of frozen or fresh CD34+ CB cells" section.

Potential solution

- For individual CBUs, a lack of expansion can be a true read out of the proliferative capacity of the unit.
- If several different CBUs from the same experimental day do not expand in number, use fresh cytokines and media. CD34+ cells are acutely sensitive to cytokine levels.
- If using FBS containing media, consider switching to serum-free media (Miltenyi: cat. #130-100-473 (100 mL) (130-100-463 for 500 mL) or STEMCELL: cat. #09605 (100 mL) (09655 for 500 mL)), which more robustly expands CD34+ cells.
- Check/calibrate gas levels on cell culture incubators.

Problem 2

Contamination of *ex vivo* assays. See "CD34+ cell enrichment from whole umbilical CB" and "*ex vivo* expansion of frozen or fresh CD34+ CB cells" section.

Potential solution

- Bacterial infections sometimes travel with CBUs if they are research, rather than clinical, grade. If this is a frequent occurrence, consider adding antibiotics such as penicillin/streptomycin at a concentration of 100 U/mL to the cell culture media. Important note: antibiotics can affect hematopoietic cell subpopulation growth, so care must be taken to know these effects in the population of interest.
- Use fresh media, cytokines, and other reagents that come into contact with the cells. If you believe any of these are contaminated, discard them.

Problem 3

Immunophenotyping of various cell surface markers does not show clear positive population. See "immunophenotyping to analyze HSC/HPC numbers and frequencies" section.

Potential solution

- Titrate antibody amount for any markers exhibiting poor staining.
- Check laser configuration to make sure that the cytometer you are using works with the selected panel of fluorophores.
- Use full minus one (FMO) control to clearly establish a negative/positive gate for that particular antibody-fluorophore combination.

Problem 4

Few or no colonies form in CFU plates. See "*ex vivo* colony forming unit (CFU) assays" section.

Potential solution

- Adjust cell doses to increase numbers per CFU plate.
- Ensure CD34+ purity by using the appropriate number of CD34+ enrichment beads per cell number and using fresh reagents for the enrichment.
- Cell purity can be confirmed by simple flow cytometry analysis by staining the cells with just anti-CD34 antibody prior to plating CFUs.
- Plates may have dried out. Add more dishes of water to surround the CFU plates with humidity. Ensure the humidity control is working in the incubator.
- Use a different lot of FBS.

Problem 5

Mice die before the endpoint of a transplantation experiment. See "in vivo analysis of engraftment capacity using mouse modeling" section.

Potential solution

- If mice die within 2 weeks of transplantation, this is likely due to radiation poisoning. If this happens in a significant number of mice, reduce the dose of radiation and/or introduce wet antibiotic feed and acidified water for 2 weeks following irradiation.
- If mice die between 2–16 weeks post transplantation, it may be due to infection or other stresses. Use increased care when handling mice and change personal protective equipment between each cage of mice. Consult with your animal facility leadership to determine if antibiotic resistant infections such as *C. bovis* (which are common in NSG mice) are present in your animal room and take steps to separate your animals from the infected cages.
- If mice die consistently 16 weeks post transplantation or later, make the endpoint of the experiment 16 weeks. While it is sometimes useful to get a measure of chimerism at 20 weeks post transplantation to check long-term engrafting capacity, NSG mice can become unhealthy this long after irradiation.
- If mice in your highest cell dose are consistently dying, reduce the cell dose.

RESOURCE AVAILABILITY

Lead contact

Further information about any aspect of the protocol and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, James Ropa (jropa@iu.edu).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to the technical contacts, Sarah Gutch (ssgutch@iu.edu) or James Ropa (jropa@iu.edu).

Materials availability

This study did not generate new resources.

Data and code availability

Further information about data is available upon request to the lead contact. This protocol did not involve new codes.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.G. and J.R.; formal analysis, S.G. and J.R.; funding acquisition, J.R., M.L.C., and M.H.K.; methodology, S.G., L.B., S.C., M.L.C., and J.R.; resources, J.R., M.L.C., and M.H.K.; supervision, M.L.C., M.H.K., and J.R.; writing – original draft, S.G. and J.R.; writing – review and editing, S.G., L.B., S.C., M.L.C., M.H.K., and J.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Broxmeyer, H.E., Luchsinger, L.L., Weinberg, R.S., Jimenez, A., Frenet, E.M., Van't Hof, W., Capitano, M.L., Hillyer, C.D., Kaplan, M.H., Cooper, S., et al. (2023). Insights into highly engraftable hematopoietic cells from 27-year cryopreserved umbilical cord blood. Cell Rep. Med. *4*, 101259. https://doi.org/10.1016/j.xcrm.2023.101259.

2. Ruggeri, A., Labopin, M., Sormani, M.P., Sanz, G., Sanz, J., Volt, F., Michel, G., Locatelli, F., Diaz De Heredia, C., O'Brien, T., et al. (2014). Engraftment kinetics and graft failure after single umbilical cord blood transplantation using a myeloablative conditioning regimen. Haematologica *99*, 1509–1515. https://doi.org/10.3324/haematol.2014.109280.

3. Gutman, J.A., Riddell, S.R., McGoldrick, S., and Delaney, C. (2010). Double unit cord blood transplantation: Who wins-and why do we care? Chimerism *1*, 21–22. https://doi.org/10.4161/chim.1.1.12141.

4. Ponce, D.M., Hilden, P., Devlin, S.M., Maloy, M., Lubin, M., Castro-Malaspina, H., Dahi, P., Hsu, K., Jakubowski, A.A., Kernan, N.A., et al. (2015). High Disease-Free Survival with Enhanced Protection against Relapse after Double-Unit Cord Blood Transplantation When Compared with T Cell-Depleted Unrelated Donor Transplantation in Patients with Acute Leukemia and Chronic Myelogenous Leukemia. Biol. Blood Marrow Transplant. *21*, 1985–1993. https://doi.org/10.1016/j.bbmt.2015.07.029.

5. Wagner, J.E., Eapen, M., Carter, S., Wang, Y., Schultz, K.R., Wall, D.A., Bunin, N., Delaney, C., Haut, P., Margolis, D., et al. (2014). One-unit versus two-unit cord-blood transplantation for hematologic cancers. N. Engl. J. Med. *371*, 1685–1694. https://doi.org/10.1056/NEJMoa1405584.

6. Naidu, S.R., Capitano, M., Ropa, J., Cooper, S., Huang, X., and Broxmeyer, H.E. (2022). Chromatin remodeling subunit BRM and valine regulate hematopoietic stem/progenitor cell function and self-renewal via intrinsic and extrinsic effects. Leukemia *36*, 821–833. https://doi.org/10.1038/s41375-021-01426-8.

7. Capitano, M.L., Mohamad, S.F., Cooper, S., Guo, B., Huang, X., Gunawan, A.M., Sampson, C., Ropa, J., Srour, E.F., Orschell, C.M., et al. (2021). Mitigating oxygen stress enhances aged mouse hematopoietic stem cell numbers and function. J. Clin. Invest. *131*, e140177. https://doi.org/10.1172/JCI140177.

8. Trinh, T., Ropa, J., Aljoufi, A., Cooper, S., Sinn, A., Srour, E.F., and Broxmeyer, H.E. (2021). Leptin receptor, a surface marker for a subset of highly engrafting long-term functional hematopoietic stem cells. Leukemia *35*, 2064–2075. https://doi.org/10.1038/s41375-020-01079-z.

9. Ropa, J., Cooper, S., Capitano, M.L., Van't Hof, W., and Broxmeyer, H.E. (2021). Human Hematopoietic Stem, Progenitor, and Immune Cells Respond Ex Vivo to SARS-CoV-2 Spike Protein. Stem Cell Rev. Rep. *17*, 253–265. https://doi.org/10.1007/s12015-020-10056-z.

10. Chen, Y., Huang, J., Guo, Z., Zhu, Z., Shao, Y., Li, L., Yang, Y., Yu, Y., Liu, L., and Sun, B. (2022). Primitive genotypic characteristics in umbilical cord neutrophils identified by single-cell transcriptome profiling and functional prediction. Front. Immunol. *13*, 970909. https://doi.org/10.3389/fimmu.2022.970909.

11. Ropa, J., Cooper, S., and Broxmeyer, H.E. (2022). Leukemia Inhibitory Factor Promotes Survival of Hematopoietic Progenitors Ex Vivo and Is Post-Translationally Regulated by DPP4. Stem Cell. *40*, 346–357. https://doi.org/10.1093/stmcls/sxac004.

12. Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: a human perspective. Cell Stem Cell *10*, 120–136. https://doi.org/10.1016/j.stem.2012.01.006.

13. Jaatinen, T., Hemmoranta, H., Hautaniemi, S., Niemi, J., Nicorici, D., Laine, J., Yli-Harja, O., and Partanen, J. (2006). Global gene expression profile of human cord blood-derived CD133+ cells. Stem Cell. *24*, 631–641. https://doi.org/10.1634/stemcells.2005-0185.

14. Fares, I., Chagraoui, J., Lehnertz, B., MacRae, T., Mayotte, N., Tomellini, E., Aubert, L., Roux, P.P., and Sauvageau, G. (2017). EPCR expression marks UM171-expanded CD34+ cord blood stem cells. Blood *129*, 3344–3351. https://doi.org/10.1182/blood-2016-11-750729.

15. Maecker, H.T., and Trotter, J. (2006). Flow cytometry controls, instrument setup, and the determination of positivity. Cytometry A. *69*, 1037–1042. https://doi.org/10.1002/cyto.a.20333.

16. Kaufman, D.S., Hanson, E.T., Lewis, R.L., Auerbach, R., and Thomson, J.A. (2001). Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. USA *98*, 10716–10721. https://doi.org/10.1073/pnas.191362598.

17. Pamphilon, D., Selogie, E., McKenna, D., Cancelas-Peres, J.A., Szczepiorkowski, Z.M., Sacher, R., McMannis, J., Eichler, H., Garritsen, H., Takanashi, M., et al. (2013).

Current practices and prospects for standardization of the hematopoietic colony-forming unit assay: a report by the cellular therapy team of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Cytotherapy *15*, 255–262. https://doi.org/10.1016/j.jcyt.2012.11.013.

18. Cooper, S.H., Capitano, M.L., and Broxmeyer, H.E. (2023). Experimental models of mouse and human hematopoietic stem cell transplantation. Methods Mol. Biol. *2567*, 205–232. https://doi.org/10.1007/978-1-0716-2679-5_14.

19. Hu, Y., and Smyth, G.K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J. Immunol. Methods *347*, 70–78. https://doi.org/10.1016/j.jim.2009.06.008.

20. Broxmeyer, H.E., Ropa, J., Capitano, M.L., Cooper, S., Racioppi, L., and Sankar, U. (2022). Camkk2 knockout bone marrow cells collected/processed in low oxygen (physioxia) suggests camkk2 as a hematopoietic stem to progenitor differentiation fate determinant. Stem Cell Rev. Rep. *18*, 2513–2521. https://doi.org/10.1007/s12015-021-10306-8.

21. Page, K.M., Zhang, L., Mendizabal, A., Wease, S., Carter, S., Gentry, T., Balber, A.E., and Kurtzberg, J. (2011). Total colony-forming units are a strong, independent predictor of neutrophil and platelet engraftment after unrelated umbilical cord blood transplantation: a single-center analysis of 435 cord blood transplants. Biol. Blood Marrow Transplant. *17*, 1362–1374. https://doi.org/10.1016/j.bbmt.2011.01.011.