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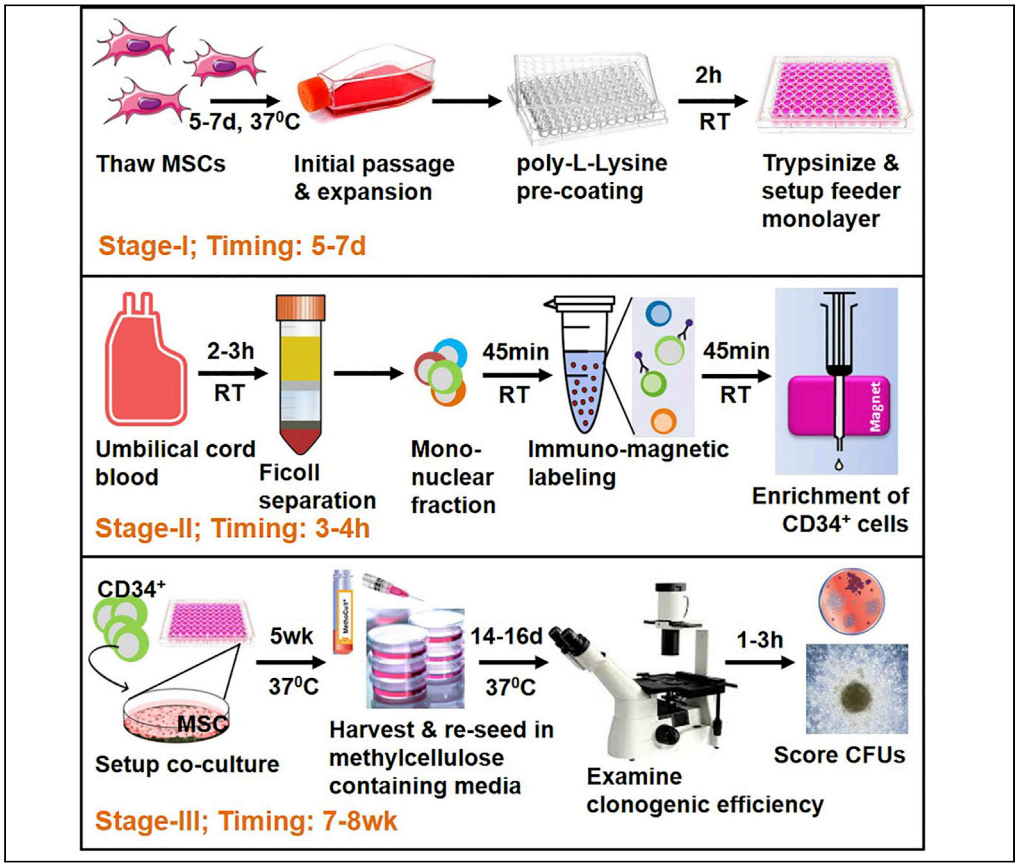
Establishment of a Long-Term Co-culture Assay for Mesenchymal Stromal Cells and Hematopoietic Stem/Progenitors



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HIGHLIGHTS

We report long-term co-culture of mesenchymal stroma and hematopoietic stem/progenitors

End-point colony-forming analysis helps determine myelo-erythroid differentiation

This protocol analyzes mesenchymal stromal cell potential to support hematopoiesis

Long-term culture-initiating cell assay is a surrogate for hematopoietic engraftment

Sinha et al., STAR Protocols 1, 100161
December 18, 2020
https://doi.org/10.1016/j.xpro.2020.100161

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Establishment of a Long-Term Co-culture Assay for Mesenchymal Stromal Cells and Hematopoietic Stem/Progenitors

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SUMMARY

We describe a protocol for a long-term co-culture assay to study the contribution of mesenchymal stromal cells (MSCs) in regulating hematopoietic stem/progenitor cell (HSPC) activity. In addition, we describe the use of a clonogenic assay to determine myelo-erythroid differentiation. This long-term culture-initiating cell assay can be used for qualitative analysis of MSCs capable of supporting hematopoiesis and may also be used as a proxy readout to study HSPC repopulation. For complete details on the use and execution of this protocol, please refer to Sinha et al. (2020).

BEFORE YOU BEGIN

Prepare Tissue Culture Plates

1. Use 96-well flat-bottomed tissue culture (TC) wells to generate adherent mesenchymal stromal (MSC) monolayer.
2. Pre-coat the TC wells with 100 μL of 0.01% poly-L-lysine at 25°C–30°C for 2 h or 12–16 h at 4°C.

   Note: Pre-coating of the plates must be done just prior to use. For 12–16 h coating of the wells using poly-L-lysine, seal the edges of the plate with parafilm and store in a refrigerator maintained at 4°C. Do not keep the plates for more than 24 h in this condition.

3. Treating wells with poly-L-lysine increases the adherence of the stromal monolayer and prevents peeling off from the surface during subsequent media exchange.
4. Ensure the wells are dry after coating is complete.
5. Seed OP9 cells at a density of 2.5×10^3 cells/cm^2 per well in 200 μL of DMEM supplemented with 20% FBS, Pens-Strep (1×) and L-glutamine (1×). Incubate the cells at 37°C with 5% CO_2 for at least 5 days.
6. If media is turning yellow hemi deplete the media.
7. The cells should reach 100% confluency after 5–7 days.

   ⚠ CRITICAL: Ensure umbilical cord blood (UCB) derived mononuclear cells (freshly prepared or cryopreserved) are in stock. Once the MSC monolayer forms enrich $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) from UCB for immediate use.

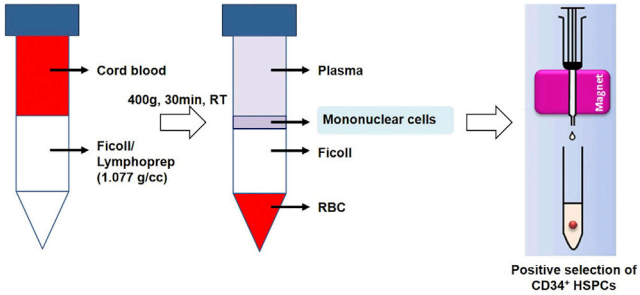

Figure 1. Schema Representing Isolation of Human HSPCs

Establishment of Adherent Feeder Layer Prior to the Addition of HSPCs

Timing: 5–7 days

8. The feeder layer needs to be established 5–7 days prior to the addition of the HSPCs

Isolation of CD34$^+$ Human Hematopoietic Stem and Progenitor Cells (HSPCs)

Timing: 3–5 h

9. Cord blood mononuclear cell preparation from fresh end of term healthy samples
10. Use these mononuclear cells to isolate cord blood-derived CD34$^+$ HSPCs using column-free human progenitor cell isolation kit (Figure 1)

Note: Either process the mononuclear cells for immediate use or cryopreserve them in liquid nitrogen for future use. Cells are cryopreserved in cryogenic media containing 90% FBS and 10% DMSO.

⚠ CRITICAL: Commonly used sources to enrich and isolate human HSPCs are umbilical cord blood, mobilized peripheral blood/apheresis and bone marrow aspiration. This protocol has used human cord blood-derived mononuclear cells as the source of CD34$^+$ cells. For using cryopreserved samples for isolation of CD34$^+$ cells, ensure that cells are viable using hemocytometer and trypan blue before proceeding with progenitor cell isolation. Also ensure that the cells do not form clump during thawing. Avoid clumping by rapid addition of the sample to complete media (IMDM containing 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine). Breakdown the cell pellets before adding resuspension media (IMDM containing 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine along with 10 ng/mL of SCF, FLT3, and TPO). Remove clumps by passing the cell suspension through a 70 μm filter. This leads to loss of viable cells and may significantly reduce overall yield and quality of CD34$^+$ cells.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
rh SCF	PeproTech	Cat# 300-07
rh FLT-3	PeproTech	Cat# 300-19
rh TPO	PeproTech	Cat# 300-18

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ficoll or Lymphoprep	Stem Cell Technology	Cat# 07851
MethoCult	Stem Cell Technology	Cat# H4034
MyeloCult	Stem Cell Technology	Cat# H5100
Hydrocortisone	Stem Cell Technology	Cat# 74142
Horse Serum	Stem Cell Technology	Cat# 06750
β-mercaptoethanol	Sigma	Cat# M3148
DMEM	Thermo	Cat# 11995065
IMDM	Thermo	Cat# 12440053
FBS (heat inactivated)	Thermo	Cat# 10438026
FBS	Thermo	Cat# 16000044
PBS (Ca^{2+} and Mg^{2+} free)	Sigma-Aldrich	N/A
Isopropyl alcohol	Sigma-Aldrich	Cat# I9516
Penicillin/Streptomycin	Thermo	Cat# 15070063
L-Glutamine	Thermo	Cat# 25030081
Trypsin	Thermo	Cat# 25300062
poly-L-Lysine	Sigma-Aldrich	Cat# P4707
Critical Commercial Assays		
Column-Free Human CD34 Positive Selection Kit (for cord blood)	Stem Cell Technology	Cat# 18066A
EasySep™ Buffer	Stem Cell Technology	Cat# 20144
Experimental Models: Cell Lines		
OP9	ATCC	Cat# CRL-2749; RRID: CVCL_4398
Biological Samples		
Human umbilical cord blood	This study	Samples were collected according to CSIR-IICB Human Ethics Committee approval and following guidelines set by Institutional Review Board
Serological pipettes	N/A	N/A
Sterile polystyrene tubes	N/A	N/A
Sterile pipette tips	N/A	N/A
Syringe (5 mL)	N/A	N/A
18 Gauge Blunt-End Needles	N/A	N/A
T-25 tissue culture treated flasks	N/A	N/A
150 mm culture dishes	N/A	N/A
35 mm Gridded Scoring Dish	Thermo	Cat# 174926
Permanent fine-tip marker	N/A	N/A
96-well plates tissue culture treated	N/A	N/A

MATERIALS AND EQUIPMENT

Solutions required

- 0.01% poly-L-Lysine solution
- PBS (Ca^{2+} and Mg^{2+} free)
- 0.05% Trypsin containing 0.05% EDTA
- 1.077 g/mL Ficoll or Lymphoprep
- RBC lysis buffer (optional)

- PBS containing 2% FBS and 1 mM EDTA (Recommended media for $CD34^+$ cell isolation in place of EasySep™ Buffer)

Recipe for Co-culture of Isolated HSPCs with OP9 Cells

Reagent	Final Concentration (mM or μM)	Volume (μL)
Hydrocortisone (10^{-3} M) (2.42 mg)	10^{-5} M (To prepare 10^{-3} M solution of hydrocortisone dissolve 2.42 mg in 5 mL of α-MEM media. Dilute 1:100 to obtain a final concentration of 10^{-5} M)	5 mL of α-MEM media
β-mercaptoethanol	100 μM	N/A
Horse serum	5%	N/A
FBS (Heat Inactivated, HI)	10% (used during co-culture)	N/A

Maintain OP9 cells in DMEM media containing 20% non-heat inactivated FBS and supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine

⚠ CRITICAL: Use β-mercaptoethanol with necessary precaution. Freshly prepare hydrocortisone just before use. Store the stock solutions at 2°C–8°C for up to 1 week. Use hydrocortisone for long-term culture and long-term culture-initiating cell assays

Alternatives: Use MyeloCult in place of IMDM containing 10% FBS during co-culture

Equipment for Isolation of $CD34^+$ HSPCs

Equipment	Source	Identifier
EasySep™ Magnet	Stem Cell Technology	Cat# 18000

STEP-BY-STEP METHOD DETAILS

Establishment of Adherent Cell Layer

Timing: 5–7 days

This step involves adherent feeder layer formation for subsequent co-culture with HSPCs over a period of 5 weeks

1. Culture of OP9 cells
2. Purchase parental OP9 cells from ATCC. Maintain these cells in DMEM media with supplements as mentioned below.

⚠ CRITICAL: It is essential to maintain the cells at 70% confluency for two to three passages in T-25 cm^2 flasks at 37°C with 5% CO_2 and 100% humidity prior to seeding for adherent layer formation (Figure 2).

Reagents for Culturing OP9 Cells

Components	Final Concentration
DMEM	N/A
Non-Heat Inactivated FBS	20%
Penicillin	100 U/mL
Streptomycin	100 mg/mL
L-Glutamine	2 mM

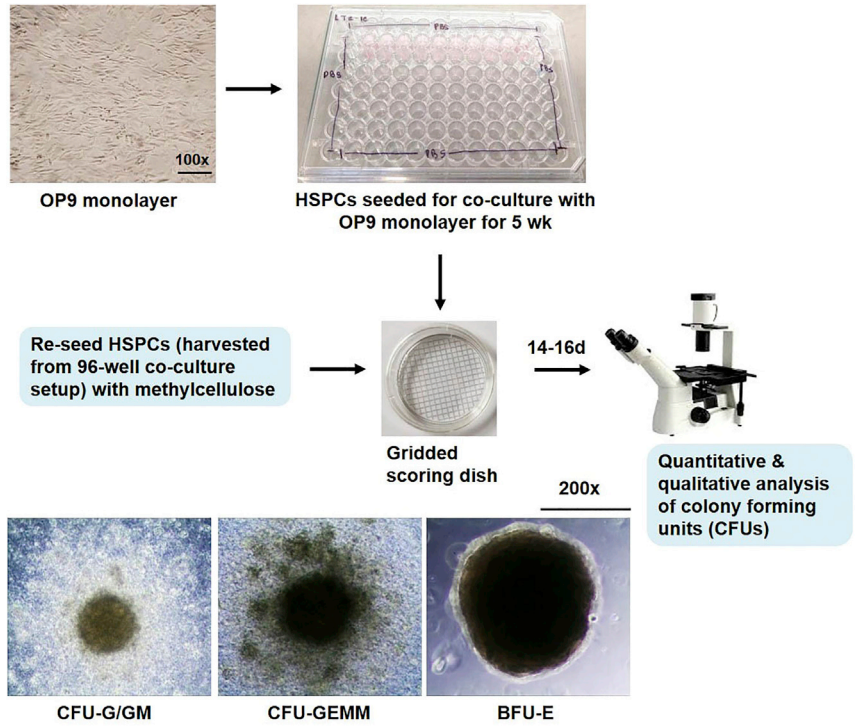

Figure 2. Working Model of Long-Term Co-culture of MSCs and HSPCs and Clonogenic Assay

a. Remove culture media carefully without disrupting the cellular layer.
b. Wash the cellular layer with 1× PBS.
c. Trypsinize the cells using 500 µL of 0.05% Trypsin containing 0.05% EDTA for 2–3 min at 37°C. Observe the flask under microscope to check that the cells have detached. Gently tap the flask incase still there are attached cells in the flask. Add equal volume of OP9 culture media after trypsinization for neutralization. Collect the cell suspension in fresh 15 mL tubes.
d. Pellet down the cells at 125 × *g* for 5 min at 25°C–30°C.
e. Resuspend cells in 5 mL of fresh culture media.
f. Count viable cells using trypan blue and hemocytometer. Mix 10 µL of trypan blue to 10 µL of media containing cells in suspension. Mix carefully and add 10 µL of the mix to hemocytometer for counting trypan blue negative (live) cells using an inverted microscope.
    i. Viable cell count is essential to support the HSPCs for a period of 5 weeks
    ii. Reseed unused cells for subsequent use or cryopreserve

3. Seed cells in poly-L-Lysine coated wells for formation of feeder layer
    a. Pre-coat the TC 96-wells by adding 100 µL of 0.01% poly-L-Lysine for 2 h at 25°C–30°C.
    b. Remove poly-L-lysine completely as residual amount can become toxic for the cells.
    c. Ensure the wells are dry before seeding the cells for adherent layer formation.
    d. Count the number of viable OP9 cells (1f) and seed at a density of 2.5 × 10^{-3}/cm^2 per well in 200 µL of DMEM supplemented with 20% FBS, Pen-Strep (1×) and L-glutamine (1×) per well so that the cells reach confluency of 100% in 5–7 days.
        i. For seeding cells in a 96-well plate, use the inner 60 wells and avoid the peripheral 36 wells.
        ii. Add sterile water or PBS to the unused peripheral 36 wells in order to maintain humidity and preventing evaporation from the wells containing media.
4. Hemi deplete (half media change) after 3 days when the media color partially changes to yellow and cells are 50% confluent.

Note: Adding excess fresh media can lead to over proliferation and detachment of the monolayer. It is essential to maintain the stromal cells as a monolayer, and hemi-depletion helps to maintain an even monolayer. An even, adherent cell monolayer also prevents HSPCs from migrating and adhering to the culture surface of the wells.

Note: Using OP9 cells as stromal support usually does not require the irradiation process. However, use of primary MSCs or FBMD-1 stromal cell line may require further irradiation in order to prevent excessive growth of stroma causing withdrawal of the stromal sheet from the well periphery. Irradiation process commonly involves subjecting nearly confluent stromal layers to 20 Gy radiation using a ^{137}Cs or ^{60}Co γ source. Replace the culture media one day after irradiation with IMDM media containing hydrocortisone and 20% horse serum. Alternatively use Mitomycin C to inhibit excessive growth of the adherent cell layer for long-term culture assays (Ponchio et al., 2000).

Isolate HSPCs once the adherent layer is ready around day 6.

⚠ CRITICAL: Start a fresh experiment if the adherent OP9 layer is not 100% confluent at the end of 7 days. There are many reasons for this: 1. It indicates that cells are not sufficiently healthy; 2. Cells with lower confluency will not be able to support the HSPCs for 5 weeks; 3. If there are empty spaces without the adherent stromal layer, HSPCs will tend to adhere to the TC surface.

Note: Viable cell count at the time of seeding can ensure healthy status of the cells. Live cells will proliferate easily and reach the desired confluency in the stipulated time frame. Essentially this reflects the growth kinetics of OP9 cells (*sh-Control*) that we have recently reported (Sinha et al., 2020; Toksoz et al., 1992). Primary MSCs or FBMD-1 cell line may take longer to reach full confluency.

⚠ CRITICAL: If it takes more than 14 days to reach 100% confluency, we do not advise using these cells for the assays. It is not advisable to keep the cells in culture for more than 7 days (for OP9) and 14 days (for primary MSCs and FBMD-1), without co-culturing once the confluent adherent monolayer is formed.

Isolation of CD34$^+$ HSPCs

Timing: 3–5 h

This step describes processing of fresh umbilical cord blood samples to obtain mononuclear cells and subsequent enrichment of HSPCs. Collect cord blood samples from term pregnancies after informed consent and strictly following human ethics committee guidelines. In the clinical setting the umbilical cord is clamped, wiped with antiseptic, and needle inserted into the vein to withdraw the desired volume of blood. Typically one term pregnancy will help collect about 50 mL of cord blood specimen. With fresh samples, perform density gradient centrifugation and HSPC enrichment on the same day. Otherwise thaw cryopreserved samples for HSPC isolation. Isolate HSPCs one day prior to seeding for the co-culture as they may require 12–16 h pre-stimulation with of recombinant SCF, FLT3L and TPO (10 ng/mL each).

5. Isolation of mononuclear cells from cord blood samples

   a. Gently layer 25 mL of undiluted cord blood sample on top of 25 mL of Lymphoprep or Ficoll (1.077 g/mL) to form the density gradient (Figure 3A).
   b. Thus, for a 50 mL cord blood unit, evenly distribute the sample into two 50 mL centrifugation tubes to perform the density gradient.

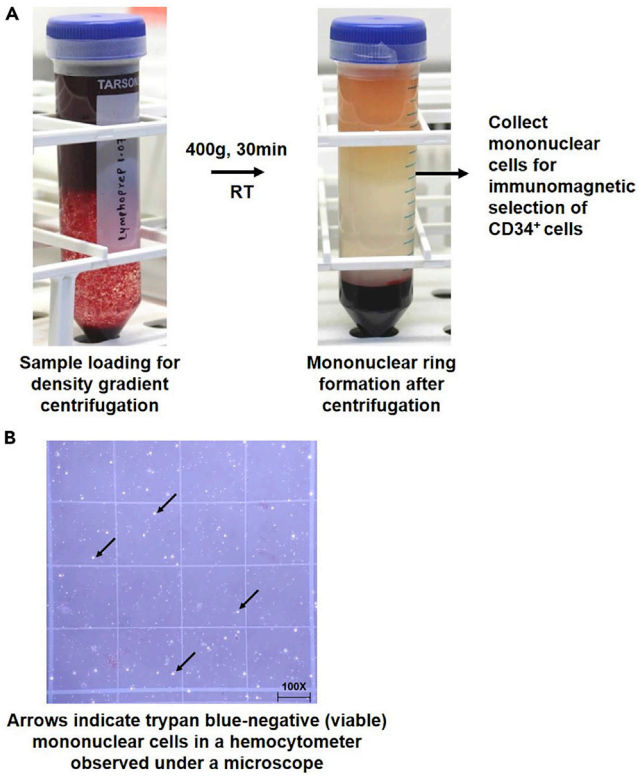

Figure 3. Density Gradient Centrifugation and Viable Cell Counting Analysis

Image showing (A) cord blood density gradient centrifugation and (B) cell counting of mononuclear cells.

c. Perform density gradient centrifugation at 400 × *g* using a horizontal rotor, for 30 min at 25°C–30°C, with an acceleration set at 9 and deceleration at 0. This usually takes around 1.5 h.

d. After the centrifugation carefully collect the mononuclear cells that forms a white ring between the Lymphoprep layer and the plasma using a serological pipette without disturbing the gradient (Methods Video S1). RBCs should have accumulated at the bottom of the tube.
Methods Video S1. Density Gradient Centrifugation, Related to Step 5d

e. Repeat the density gradient centrifugation once more for a total of two times to sufficiently remove RBC contaminants.

f. Wash the mononuclear cells with 40 mL of PBS at 500 × *g* for 5 min at 25°C–30°C to remove residual amount of Lymphoprep.

g. Take viable cell counts using trypan blue and hemocytometer. Mix 10 μL of trypan blue to 10 μL of PBS containing cells in suspension in a separate microcentrifuge tube. Mix rapidly and add 10 μL of the mix to hemocytometer for cell counting using an inverted microscope (Methods Video S2). Live cells should appear as trypan blue negative (Figure 3B).
Methods Video S2. Counting of Trypan Blue Negative Hematopoietic Cells Using a Hemocytometer, Related to Step 5g

h. Proceed with isolation of $CD34^+$ cells or immediately freeze the mononuclear cells. Cryopreserve cells in multiple vials using a cryogenic solution containing 90% FBS and 10% DMSO as the final concentration. Do not freeze more than 10–15 × 10^6 cells per vial. At the beginning resuspend the cell pellets in 100% FBS, total resuspension volume will depend on the number of vials to be used for freezing in accordance with the total number of mononuclear cells obtained from the sample. For each 1.8 mL cryogenic vial add 500 μL of cell suspension, and on the top add 500 μL freezing solution containing 20% DMSO and mix gently. Immediately store

the cryogenic vials, placed within a freezing container, at −80°C. The freezing containers carrying 100% isopropyl alcohol ensure achieve a rate of cooling near −1°C/min, which is the optimal rate for cell preservation. For long-term storage transfer the vials into liquid nitrogen containers in another 24–48 h.

i. For using cryopreserved specimens for isolation of HSPCs, thaw the cells in sufficient quantity (use at least 10–20 mL per vial) of complete media (IMDM containing 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine). Ensure that the cells do not form clump during the process by rapidly adding of the sample to complete media with gentle tapping at 25°C–30°C. Centrifuge the cell suspension at 1,000 × *g* for 5 min at 25°C–30°C, aspirate out the media containing DMSO, and wash twice with 50 mL of PBS. Finally resuspend the cells in 1 mL PBS, remove cell clumps if any by passing the cell suspension through a 70 μm filter, and take viable cell count before proceeding for immunomagnetic selection. We did not use DNase to avoid clumping.

Alternatives: Remove RBC contamination by performing RBC lysis after completion of first density gradient centrifugation. Resuspend mononuclear cells in 25 mL of 1× RBC lysis buffer and incubate at 25°C–30°C for 5 min to a maximum of 10 min. After the incubation, top up the tube with sufficient volume of PBS and centrifuge cells at 800 × *g* for 10 min at 25°C–30°C. Wash the cells with PBS to remove residual volume of RBC lysis buffer. Take viable cell counts. Cryopreserve mononuclear cells or immediately proceed for $CD34^+$ cell isolation. Alternatively, perform a second round of density gradient centrifugation. Performing two consecutive rounds of density gradient centrifugation can be a better method to obtain good quality of cells.

Composition of RBC Lysis Buffer (10×)

Components	Amount (For 100 mL)
NH_4Cl	8.02 g
$NaHCO_3$	0.84 g
EDTA (disodium)	0.37 g
H_2O	100 mL

Filter and store at 4°C for up to 6 months, and warm before use. The pH of the buffer should be between 7.1 and 7.4.

6. Isolation of $CD34^+$ cells

a. Resuspend the mononuclear cells in 500 μL of EasySep™ buffer or the recommended media (PBS containing 2% FBS and 1 mM EDTA). Medium should be free of $Ca^{2+}$and Mg^{2+}.
b. Isolate $CD34^+$ cells using Column-free human CD34 progenitor isolation kit.
c. Add $CD34^+$ selection cocktail from Column-Free Human CD34 Positive Selection Kit (for cord blood) to the cell suspension at a concentration of 100 μL/mL.
d. Mix and incubate at 25°C–30°C for 15 min.
e. Mix magnetic particles thoroughly to obtain even distribution by pipetting up and down at least five times.
f. Add magnetic particles at a concentration of 50 μL/mL of sample.
g. Incubate at 25°C–30°C for 10 min.
h. Add EasySep™ buffer or the recommended media to the tube up to 2.5 mL and mix thoroughly 2–3 times. Use either EasySep™ buffer or recommended media for the process.
i. Place the tube in the magnet and incubate for 5 min.
j. Pick up the magnet and in one continuous motion invert the magnet and tube to discard the supernatant. Leave the tube in inverted position for an additional 2–3 s. Do not shake of the drops adhered to the side walls of the tube. This might lead to loss of enriched cells (Methods Video S3).

Methods Video S3. Immunomagnetic Selection of CD34+ HSPCs, Related to Step 6j

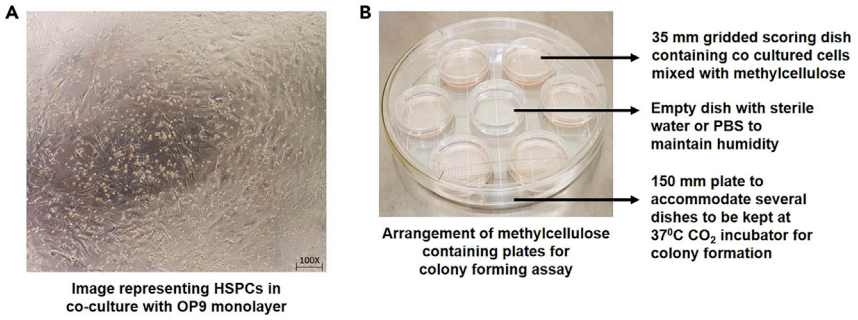

Figure 4. Co-culture and Clonogenic Assay Setup

Photomicrograph images for (A) HSPCs in co-culture with OP9 monolayer and (B) clonogenic assay setup.

k. Repeat steps (h) to(j) for a total of five times.

l. Remove the tube from the magnet. This contains isolated $CD34^+$ cells.

m. Top up with 4 mL of recommended media (defined in 5a as per manufacturer's instruction) and centrifuge at 300 × *g* for 10 min at 25°C–30°C, keeping acceleration 9 and deceleration 0.

n. Resuspend the cell pellet in IMDM media supplemented with 10% heat inactivated FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine.

o. Pre-stimulate cells 12–16 h with 10 ng/mL of each of recombinant human SCF, FLT3L and TPO in IMDM complete media (supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine). We usually pre-stimulate cord blood-derived $CD34^+$ cells prior to *in vitro* co-culture experiments. However, we avoid pre-stimulation for gene expression analysis of $CD34^+$ cells. Absolute number of viable cells does not significantly change after 10–12 h of pre-stimulation.

7. Co-culture assay set up

a. Take viable cell counts immediately after immunomagnetic separation and also after overnight pre-stimulation. Absolute number of viable cells does not significantly change after 10–12 h of pre-stimulation.

b. Ensure that the adherent cell layer is 100% confluent.

c. Seed 25 × 10^3 $CD34^+$ cells per well over the stromal monolayer in 200 μL of IMDM supplemented with 10% heat inactivated FBS, β-mercaptoethanol, 5% horse serum, 10^{-5} M hydrocortisone, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine (co-culture media), and co-culture at 37°C in presence of 5% CO_2 for 5 weeks (Figure 4).

d. Add PBS or sterile water to the peripheral wells to prevent evaporation of media and maintain humidity.

e. Check plate every three days under the microscope to ensure that cells appear healthy and enough volume of media is present in all the wells.

f. Replenish one half of the co-culture media (100 μL) every week without disturbing the adherent feeder layer and the HSPCs. Collect media from the wells in microfuge tubes, spin down at 500 × *g* for 5 min at 25°C–30°C to avoid loss of HSPCs during media change. Resuspend in 100 μL of fresh co-culture media and gently add back to the wells, so that each well has a total of 200 μL of co-culture media.

8. Harvest cells for clonogenic assay

a. Remove media and add to fresh microfuge tubes as this media can contain non-adherent hematopoietic cells.

b. Rinse each well with PBS and add this PBS to the tubes containing media previously removed from the wells. Collect PBS that is used to rinse the wells as it might contain hematopoietic cells.

c. Trypsinize the wells with 100 μL of 0.05% trypsin containing 0.05% EDTA per well for 3–5 min at 37°C. Check the wells under microscope to ensure that the adherent layer has started to detach from the surface.
d. Stop trypsinization by addition of 100 μL of IMDM supplemented with 20% heat inactivated FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine.
e. Collect the cell suspension in tubes which already contain media and PBS collected from the wells prior to trypsinization and spin down at 500 × *g*, for 7–10 min at 25°C–30°C.
f. Remove the media.
g. Wash with PBS and spin at 500 × *g*, for 7–10 min at 25°C–30°C.
h. Resuspend the cell pellet in 200 μL of basal IMDM media.
i. Meanwhile thaw MethoCult and aliquot 3 mL in 14 mL round bottom tubes.
j. Add 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mM L-glutamine to each tube containing 3 mL of MethoCult.
k. Add the entire single cell suspension from each well to the tube containing 3 mL of Methocult using cut tips. We do not use blunt-end needles in this step. [In this long-term co-culture assay we are only interested to understand qualitative effect of stromal cells in regulating HSPC clonogenic potential. Therefore, we did not perform limiting dilution-based LTC-IC analysis].
l. Vortex the tube thoroughly.
m. Allow the tube to stand for 5 min so that the bubbles rise up to the top.
n. Aliquot the MethoCult containing the cells into 35 mm gridded tissue culture scoring dish using 5 mL syringe fitted with 18-gauge blunt-end needles (Figure 4).
o. Ensure minimum bubble formation by slowly adding MethoCult to the gridded dish and even spreading of the MethoCult.
p. Place the plates in a 150 mm dish along with a 35 mm dish containing sterile water to reduce evaporation.
q. Incubate at 37°C in 5% CO_2 with $\geq$ 95% humidity for 14–16 days.
r. Score the number and type of colonies at the end of the incubation period.
s. Count the well as positive if you can detect one or more CFU-G/GM, CFU-GEMM or BFU-E colonies or score as negative if no colonies are present (Figures 2 and 4).

EXPECTED OUTCOMES

After incubation with methylcellulose-based media different types of colonies should form. The frequency of the types of colonies formed can vary according to the treatment of the HSPCs during co-culture or due to the influence of the stromal layer on the HSPCs. The types of colonies formed usually include CFU-G/GM, CFU-GEMM, and BFU-E (Figure 2). CFU-G/GM colonies are smaller and more scattered while CFU-GEMM colonies are larger and more compact. BFU-E colonies are compact, with well-defined boundaries and dark in color as they differentiate into erythroid lineage. Flow cytometry (FACS) analysis using single cell suspension of these colonies can determine cell surface marker expression. In addition, one can determine frequency of long-term colony-initiating cells (LTC-IC) using limiting dilution assay during co-culture and Poisson statistics (Cancelas et al., 2005; Liu et al., 2013; Sengupta et al., 2010).

Note: Figure 2 shows representative images for CFU-G/GM, CFU-GEMM, and BFU-E. We did not observe CFU-Es in our assays since CFU-Es are more frequently obtained for cultures established from peripheral blood than human umbilical cord blood samples.

CFU-G/GM (Colony-Forming Unit-Granulocyte, Macrophage)

20 or more granulocytes and/or macrophages form these colonies. Cells in these colonies are not hemoglobinized and hence do not appear red in color. Individual cells can be identified along the periphery of the colony. One or more dark dense core can be observed in case of larger colonies. They do not require erythropoietin to support their growth. Colonies obtained from cord blood samples are usually larger in size than those obtained from bone marrow or mobilized peripheral blood.

CFU-GEMM (Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte)

A colony that is formed of erythroid cells (containing hemoglobin) and higher number of non-erythroid (do not contain hemoglobin) cells that includes megakaryocytes, granulocytes, and macrophages. Usually the core region of these colonies is made up of erythroid cells and peripheral regions are made up of non-erythroid cells. In certain cases, non-erythroid cells can accumulate on one side of the erythroid cells. Size of CFU-GEMM colonies are usually larger than CFU-GM or BFU-E. The frequency of CFU-GEMM type of colonies is higher in case of cord blood samples than in case of bone marrow. However, variation is usually observed in between samples.

BFU-E (Burst Forming Unit-Erythroid)

More than 200 erythroblasts either singly or in multiple clusters accumulate to form this type of colony. They are hemoglobinized and thus appear deep brown to red in color. Individual cells cannot be identified within the cluster. IL-3, SCF, and EPO containing media support their growth. Cord blood-derived colonies have higher frequency and are larger in size than peripheral blood-derived colonies.

CFU-E (Colony-Forming Unit-Erythroid)

One to two clusters are formed by erythroblasts that are lesser than 200 in number. Colonies appear red or brown in color due to accumulation of hemoglobin. Individual cells cannot be identified within the colony. Presence of EPO in media is essential for its growth. This is more frequently obtained for cultures established from peripheral blood than human umbilical cord blood samples.

LIMITATIONS

The protocol described above is suitable for studying myeloid differentiation of HSPCs when co-cultured with stromal feeder layer. Adapt newer protocols to study lymphoid and NK cell clonogenic efficiencies (Bock, 1997; Lemieux and Eaves, 1996; Lemieux et al., 1995; Miller et al., 1998; Punzel et al., 1999). It is essential to use low cell numbers for culture assay and during colony formation. Using high cell density can lead to formation of large number of colonies that will be difficult to score. Also, if cell density is higher the Methocult may not be sufficient to support the growth of the colonies and they may undergo senescence before analysis. Always use freshly prepared cytokines at recommended concentration for best results.

TROUBLESHOOTING

Problem

50 mL of cord blood sample usually yields 5 × 105 CD34+ cells. However, CD34+ cell number is low.

Potential Solution

Pool more than one umbilical cord blood specimens.

Problem

There is overgrowth of macrophages during co-culture.

Potential Solution

Always use horse serum *by default* during co-culture as it restricts macrophage proliferation.

> *Note:* OP9 cells do not produce M-CSF, which can help reduce macrophage proliferation in the co-culture setup.

Problem

Feeder layer is not confluent.

Potential Solution

OP9 cells may not be healthy, thaw another frozen vial of OP9 and start afresh.

Problem
HSPCs are not healthy.

Potential Solution
Use fresh set of cytokines at recommended concentration for pre-stimulation and co-culture. In addition, freshly prepare hydrocortisone every week and use at defined concentration. Changing concentration can alter possible outcomes.

Problem
Feeder layer is detached during co-culture.

Potential Solutions
During media change keep a residual volume of media in the wells and add fresh media on top of the residual volume.

Ensure that the pointed end of the tips do not come in direct contact with the feeder layer, thus disrupting the continuity and leading to detachment of the monolayer.

Problem
Wells become contaminated.

Potential Solution
In case any of the wells become contaminated, add 200 μL of 1 N NaOH solution to the contaminated well (to prepare 1 N NaOH solution, add 40 g of NaOH to 100 mL of distilled water). Dispose of the well contents using aspiration device. Refill the well with 1 N NaOH. Identify the well on top of the lid. Keep checking the plate for possible contamination.

Problem
Absolute number of CFUs is too low or high. Typically 1 mL of human umbilical cord blood specimens generate between 13,000 and 24,000 CFU-GM (which is 15 times higher than that present in the bone marrow or peripheral blood), between 1,000 and 10,000 of CFU-GEMM, and about 8,000 BFU-E (3 times more than that present in the bone marrow or peripheral blood) (Hordyjewska et al., 2015).

Potential Solution
Ensure proper trypsinization while harvesting cells for the clonogenic assay. It is essential to collect all the cells to avoid loss of positive colonies. Single cell suspension ensures that the colonies have developed from clonogenic precursors.

It is essential to include additional wells or dish containing PBS/H_2O to maintain proper humidity. Do not disturb the plates for the first 10 days. One can check the dish after 10 days to see if colonies have formed. This is an optional step, which is to ensure that the culture system is sufficiently hydrated and there is no contamination.

If stromal layer is not fully confluent then HSPCs will start adhering to the culture well surface. So final colony obtained may not reflect the real clonogenic potential of the HSPCs.

While scoring the plates ensure that colonies formed from the feeder layer are not included.

RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by Amitava Sengupta (amitava.sengupta@iicb.res.in; amitava.iicb@gmail.com).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate/analyze any datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100161.

ACKNOWLEDGMENTS

This study is supported by funding from Council for Scientific & Industrial Research (CSIR) (NWP/BIOCERAM/ESC-0103 and Sickle Cell Anemia-Mission Mode Program/HCP-0008 to A.S.), Department of Biotechnology (DBT) (BT/RLF/RE-ENTRY/06/2010), Ramalingaswami Fellowship (to A.S.), DBT (BT/PR13023/MED/31/311/2015) (to A.S.), and Science & Engineering Research Board-Department of Science & Technology (SERB-DST) (SB/SO/HS-053/2013), Government of India (to A.S.). A.S. is a recipient of Indian Council of Medical Research-Department of Health Research (ICMR-DHR) International Fellowship for Indian Biomedical Scientists (INDO/FRC/452/S-11/2019-20-IHD). S.S. and S.C. acknowledge research fellowships from CSIR and UGC, Government of India, respectively. The authors thank Dr. Prasanta Mukhopadhyay for providing umbilical cord blood samples.

AUTHOR CONTRIBUTIONS

Experiments and analysis: S.S. and S.C.; Manuscript design, writing, and editing: S.S. and A.S.; Conception, data interpretation, illustration, and overall direction: A.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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