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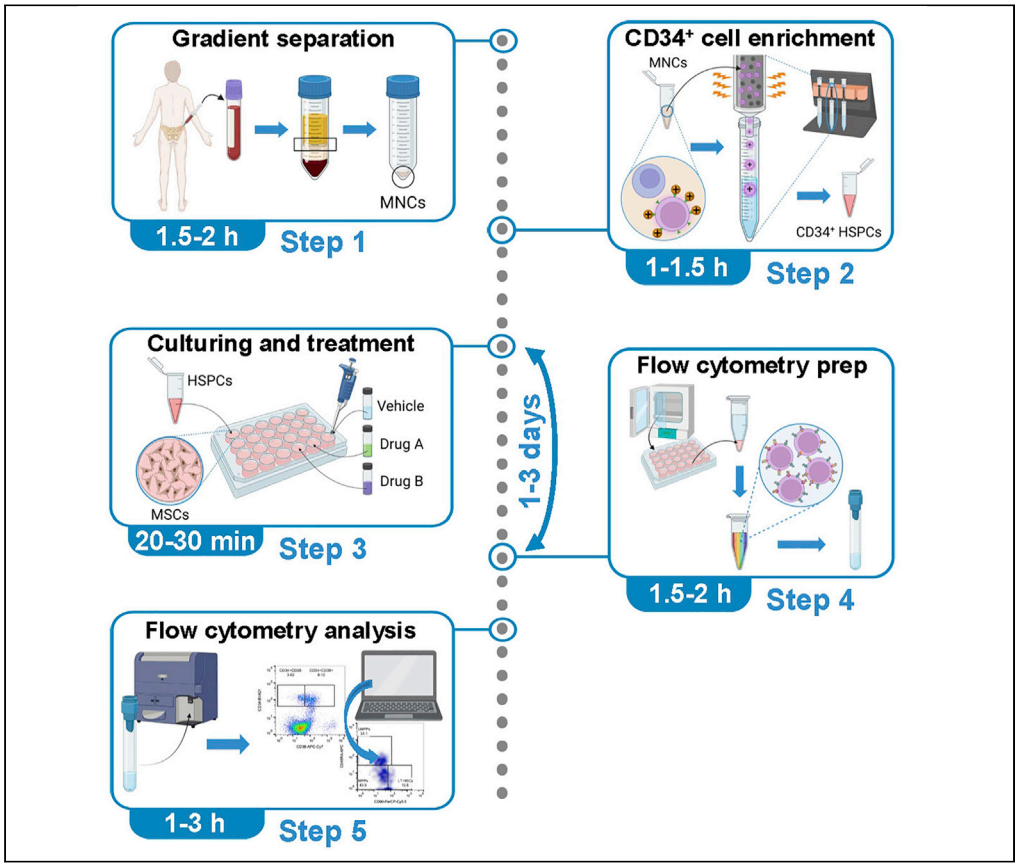
Isolation, culture, and immunophenotypic analysis of bone marrow HSPCs from patients with myelodysplastic syndromes



Drug testing assays in hematopoietic stem and progenitor cells (HSPCs) are fundamental in biological studies of myelodysplastic syndromes (MDS) but have historically entailed a technical challenge. This protocol allows the efficient isolation of MDS HSPCs from bone marrow mononuclear cell fractions and their culturing with the support of stromal cells for improved maintenance during drug testing. Lastly, specific steps are given to quantify surviving cells and assess changes in the HSPC hierarchies.

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

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Highlights

Isolation of mononuclear cells from patient bone marrow aspirates

Magnetic enrichment of CD34$^+$ hematopoietic and progenitor cell fractions

Co-culturing of CD34$^+$ cells with stroma for *in vitro* drug testing assays

Analysis of hematopoietic hierarchies in native and cultured samples by flow cytometry

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Isolation, culture, and immunophenotypic analysis of bone marrow HSPCs from patients with myelodysplastic syndromes

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SUMMARY

Drug testing assays in hematopoietic stem and progenitor cells (HSPCs) are fundamental in biological studies of myelodysplastic syndromes (MDS) but have historically entailed a technical challenge. This protocol allows the efficient isolation of MDS HSPCs from bone marrow mononuclear cell fractions and their culturing with the support of stromal cells for improved maintenance during drug testing. Lastly, specific steps are given to quantify surviving cells and assess changes in the HSPC hierarchies.

For complete details on the use and execution of this protocol, please refer to Ganan-Gomez et al. (2022).

BEFORE YOU BEGIN

1. The protocol below describes the specific steps for HSPC analysis and isolation from fresh bone marrow (BM) aspirates. However, it may also be performed with frozen live BM mononuclear cells (MNCs), in which case investigators can thaw the cells and proceed directly to the magnetic enrichment. Moreover, the protocol may also be performed with frozen live $CD34^+$ cells, whenever available, thus skipping the first two sections of the protocol and proceeding directly to the culturing and treatment of HSPCs after thawing the $CD34^+$ cells. This option may be more affordable if the investigators do not have access to equipment and reagents for magnetic enrichment and do not plan to analyze many samples.
2. Supporting mesenchymal stromal cells (MSCs) from healthy donors for the co-culturing of MDS HSPCs should be pre-seeded in 48-well or 24-well plates between 16 h and 1 week prior to the experiment. Herein we only provide instructions for preparing the co-culture plates. The specific steps and details for establishing and maintaining long-term MSC cultures will depend on the source of the MSCs (e.g., commercially cryopreserved, freshly obtained from donors, etc.) and are not provided in this protocol.
3. Regardless of whether fresh or frozen samples are used, it is critical that the cells are preserved on ice for most of the protocol. Investigators should make sure that the centrifuges are refrigerated to 4°C beforehand.
4. Before starting the protocol, investigators should prepare and cool down the Working, Washing and 1× MACS Rinsing solutions. Before the culturing step, prepare and warm up the culturing medium, trypsin and Dulbecco's Phosphate-Buffered Saline (DPBS) to 37°C.

Before flow cytometry analysis, warm up a small aliquot of Washing solution to 37°C. Thawing SYTOX Green beforehand and preparing a stock dilution of 1:50 in DPBS is recommended.

5. Working under a sterile hood and the addition of antibiotics and antimycotics is advised for solution preparation and for gradient separation steps and is required for the HSPC culturing and treatment step. The rest of the protocol can be carried out on the bench.
6. Investigators should note that multiparameter flow cytometry is a technique that requires set up by an experienced user. It is highly recommended that the flow cytometry panel described here be set up in advance to this experiment, using BM MNCs from fresh or frozen donors or patients. Depending on the user's skills and the instrument characteristics, additional controls other than those described here may be necessary for setup.

Institutional permissions

Research involving human specimens must be performed in accordance with the Declaration of Helsinki. Prior to collection of human specimens, investigators should obtain approval by the corresponding Institutional Review Boards and written informed consent by the patients.

Solution preparation

Timing: 15 min

7. Prepare the Working and Washing solutions by combining the reagents indicated in the respective tables in the materials and equipment section.
8. Prepare the 1× MACS rinsing solution:
   a. In a 0.2 μm bottle-top filter, combine the reagents indicated in the corresponding materials and equipment table.
   b. Vacuum-filter the resulting solution.

Pre-seeding of healthy mesenchymal stromal cells

Timing: 30 min

Note: Before starting, warm up the culturing medium, DPBS and trypsin.

9. Harvest confluent early-passage MSCs from culture:
   a. Aspirate the MSC culturing medium.
   b. Gently wash by adding warm sterile DPBS to the walls and swirling the flask or dish.
   c. Aspirate the DPBS and add a small volume of pre-warmed trypsin, sufficient to cover the surface of the MSCs (e.g., 5 mL).
   d. Incubate at 37°C for 3–5 min.
   e. Add 10 mL of MSC culture medium and pipette up and down to homogenize the cells and fully detach any left on the surface of the flask or dish.
10. Transfer the cells to a centrifuge tube and spin down at 475 × *g* for 5 min.
11. Aspirate the supernatant and resuspend the cell pellet in culture medium:
    a. Homogenize the pellet with 1 mL of MSC Culture medium using a micropipette.
    b. Bring up the volume of the cell suspension to 10 mL.
12. Take a small aliquot for counting (e.g., 10 μL) and combine it with the same volume (1:1) of Trypan blue.
13. Count the cells and assess viability.

Note: We suggest counting in a hemocytometer but other methods, such as the use of automated cell counters, are acceptable.

14. Calculate the volume of the cell suspension needed to plate the desired number of wells, at 6,000 live MSCs per well in 48-well plates, or 12,000 live MSCs per well in 24-well plates.

Note: These cell numbers are illustrative and recommended to obtain 50% confluency in 24–48 h. At this density, MSCs will not need medium replacement before the co-culture is established.

15. Aliquot the needed volume of the cell suspension and add enough MSC culture medium to obtain a final density of 12,000 cells/mL.
16. Plate the cell suspension by adding 0.5 mL/well in 48-well plates, or 1 mL/well in 24-well plates.
17. Incubate the plates at 37°C and 5% CO_2 until the time of the experiment (minimum, 16 h).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD2-FITC (5% v/v)	BD Biosciences	Cat#555326; RRID: AB_395733
Anti-human CD3-FITC (10% v/v)	BD Biosciences	Cat#349201; RRID: AB_400405
Anti-human CD4-FITC (5% v/v)	Invitrogen	Cat#MHCD0401; RRID: AB_10392546
Anti-human CD7-FITC (5% v/v)	BioLegend	Cat#343104; RRID: AB_1659216
Anti-human CD10-FITC (5% v/v)	Leinco	Cat#C139; RRID: AB_2828446
Anti-human CD11b-FITC (5% v/v)	Invitrogen	Cat#11-0118; RRID: AB_1582242
Anti-human CD14-FITC (5% v/v)	BD Biosciences	Cat#347493; RRID: AB_400311
Anti-human CD19-FITC (10% v/v)	BD Biosciences	Cat#340409; RRID: AB_400024
Anti-human CD20-FITC (10% v/v)	BD Biosciences	Cat#555622; RRID: AB_395988
Anti-human CD33-FITC (5% v/v)	Invitrogen	Cat#11-0337-42; RRID: AB_1603221
Anti-human CD56-FITC (2.5% v/v)	BD Biosciences	Cat#562794; RRID: AB_2737799
Anti-human CD235a-FITC (2.5% v/v)	BD Biosciences	Cat#559943; RRID: AB_397386
Anti-human CD34-BV421 (5% v/v)	BD Biosciences	Cat#562577; RRID: AB_2687922
Anti-human CD38-APC-Cy7 (5% v/v)	BioLegend	Cat#303534; RRID: AB_2561605
Anti-human CD90-PerCP5.5 (10% v/v)	Invitrogen	Cat#45-0909; RRID: AB_10718245
Anti-human CD45RA-APC (10% v/v)	Tonbo Biosciences	Cat#20-0458; RRID: AB_2621578
Anti-human CD123-PE (5% v/v)	BD Biosciences	Cat#555644; RRID: AB_396001
Chemicals, peptides, and recombinant proteins		
DPBS, no calcium, no magnesium	Gibco	Cat#14190144
Fetal Bovine Serum, qualified, heat inactivated	Gibco	Cat#16140071
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140122
Amphotericin B	Gibco	Cat#15290026
MACS® BSA Stock Solution	Miltenyi Biotec	Cat#130-091-376
AutoMACS® Rinsing Solution	Miltenyi Biotec	Cat#130-091-222
Ficoll-Paque™ PLUS Media	Cytiva	Cat#17144002
OneComp eBeads	Invitrogen	Cat#01-1111-41
SYTOX™ Green Nucleic Acid Stain	Invitrogen	Cat#S7020
Trypsin-EDTA (0.25%), phenol red	Gibco	Cat#25200056
Streck Cell Preservative™	Streck	Cat#213358
RPMI 1640 Medium	Gibco	Cat#11875093
Alpha Modification of Eagle's Medium 1× (AMEM)	Corning	Cat#15012CV
Critical commercial assays		
CD34 MicroBead Kit, human	Miltenyi Biotec	Cat#130-046-702
AccuCheck Counting Beads	Invitrogen	Cat#PCB100
Software and algorithms		
FlowJo	BD Biosciences	www.flowJo.com
Other		
Refrigerated centrifuge for 15–50 mL tubes (18.9 cm rotor radius)	Eppendorf	Cat#5810R

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Refrigerated table top centrifuge for 1.5 mL tubes (9.8 cm rotor radius)	Eppendorf	Cat#5424R
LS Columns	Miltenyi Biotec	Cat#130-042-401
QuadroMACS™ Separator	Miltenyi Biotec	Cat#130-090-976
MACS® MultiStand	Miltenyi Biotec	Cat#130-042-303

MATERIALS AND EQUIPMENT

- Recipes for the Working, Washing and 1× MACS Rinsing solutions are provided for a final volume of 500 mL but investigators may choose to prepare smaller volumes, depending on the number of samples to be processed. These solutions can be stored in the refrigerator for several months if used under aseptic conditions.
- Recipes for the Antibody cocktail and the Viability stain are provided for 1 sample and volumes should be scaled up accordingly by the investigator. A 10% excess volume is recommended in each preparation.

MSC culture medium		
Reagent	Final concentration	Amount
Fetal bovine serum (FBS)	10%	50 mL
Penicillin/Streptomycin	1%	5 mL
Amphotericin B	0.1%	0.5 mL
Minimum Essential Medium (MEM) Alpha (α-MEM)	N/A	444.5 mL
Total	**N/A**	**500 mL**
Store at +4°C for a maximum of 6 months.		

Modified Turk's solution		
Reagent	Final concentration	Amount
Acetic acid, glacial	2%	0.2 mL
Distilled water	N/A	9.8 mL
Total	**N/A**	**10 mL**
Store at +18°C–22°C for a maximum of 1 year.		

⚠ CRITICAL: Acetic acid is flammable liquid and releases a flammable vapor. It is also highly corrosive and causes severe skin burns and eye damage. Keep away from heat, sparks, open flames and hot surfaces. Take precautionary measures against static discharge. Wear protective gloves, protective clothing, eye protection and face protection. Do not breathe dust, fumes, gas, mist, vapors or sprays. Use in a chemical hood and keep the container tightly closed.

Working solution		
Reagent	Final concentration	Amount
FBS	2%	10 mL
Penicillin/Streptomycin	1%	5 mL
Amphotericin B	0.1%	0.5 mL
Sterile DPBS	N/A	484.5 mL
Total	**N/A**	**500 mL**
Store at +4°C for a maximum of 2 months, or 6 months when opened under aseptic conditions.		

Washing solution		
Reagent	Final concentration	Amount
FBS	10%	50 mL
Penicillin/Streptomycin	1%	5 mL
Amphotericin B	0.1%	0.5 mL
Sterile DPBS	N/A	444.5 mL
Total	**N/A**	**500 mL**

Store at +4°C for a maximum of 2 months, or 6 months when opened under aseptic conditions.

1× MACS Rinsing solution		
Reagent	Final concentration	Amount
MACS® BSA Stock Solution	5%	25 mL
Penicillin/Streptomycin	1%	5 mL
Amphotericin B	0.1%	0.5 mL
AutoMACS® Rinsing Solution	N/A	469.5 mL
Total	**N/A**	**500 mL**

Store at +4°C for a maximum of 2 months, or 6 months when opened under aseptic conditions.

MDS HSPC Antibody cocktail (per MNC sample*)		
Reagent	Final concentration	Amount
Anti-human CD2-FITC	5%	5 µL
Anti-human CD3-FITC	10%	10 µL
Anti-human CD4-FITC	5%	5 µL
Anti-human CD7-FITC	5%	5 µL
Anti-human CD10-FITC	5%	5 µL
Anti-human CD11b-FITC	5%	5 µL
Anti-human CD14-FITC	5%	5 µL
Anti-human CD19-FITC	10%	10 µL
Anti-human CD20-FITC	10%	10 µL
Anti-human CD33-FITC	5%	5 µL
Anti-human CD56-FITC	2.4%	2.5 µL
Anti-human CD235a-FITC	2.4%	2.5 µL
Anti-human CD34-BV421	5%	5 µL
Anti-human CD38-APC-Cy7	5%	5 µL
Anti-human CD90-PerCP	10%	10 µL
Anti-human CD45RA-APC	10%	10 µL
Anti-human CD123-PE	5%	5 µL
Total	**N/A**	**105 µL**

Store at +4°C protected from light. A small stock can be prepared in advance and stored for a maximum of 1 month.
*Cultured samples require half of this volume.

Viability stain (per sample)		
Reagent	Final concentration	Amount
SYTOX Green, 1:50 dilution in DPBS	0.1%	1 µL
Working solution	N/A	0.5 mL
Total	**N/A**	**~0.5 mL**

Prepare extemporaneously and store on ice and protected from light.

HSPC Culture medium		
Reagent	Final concentration	Amount
FBS	10%	50 mL
Penicillin/Streptomycin	1%	5 mL
Amphotericin B	0.1%	0.5 mL
RPMI 1640 medium	N/A	444.5 mL
Total	**N/A**	**500 mL**

Store at +4°C for a maximum of 6 months.

BD Influx Cell Sorter settings				
Laser color	Laser, nm	Band, nm/range	Fluorochrome	Marker
Blue-green	488	530/40	SYTOX Green/FITC	Live/dead, Lineage
Blue-green	488	710/50	PerCP-Cy5.5	CD90
Violet	407	460/40	BV421	CD34
Yellow	561	585/29	PE	CD123
Red	640	670/30	APC	CD45RA
Red	640	750LP	APC-Cy7	CD38

Alternatives: The modified Turk's solution can be replaced by Turk's solution containing 1% of crystal violet. Cell counting with Trypan blue is not recommended as red blood cells may interfere with the visualization of white blood cells.

Alternatives: Regular laboratory chemicals and reagents such as DPBS, FBS, penicillin-streptomycin and amphotericin B may be obtained from other sources than those indicated in the key resources table.

Alternatives: The MACS® BSA Stock Solution may be replaced by a home-made solution of 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

Alternatives: We used a QuadroMACS™ Separator with the LS Columns, but those can also be used with a MidiMACS™ Separator, a SuperMACS™ II Separator in combination with a LS Column Adapter or a MultiMACS Cell24 Separator Plus in combination with the Single Column Adapter.

Alternatives: We specifically set up the flow cytometry panel using the antibodies and concentrations reported above; however, the same antibody conjugates may be obtained from different sources. In that case, we recommend titrating the antibodies.

Alternatives: Different centrifuges can be used than those specified in the key resources table, as long as they can be refrigerated to 4°C–10°C and achieve similar speeds.

Alternatives: The flow cytometry analysis step described here was set up in a BD Influx Cell Sorter (now discontinued), but other instruments with comparable optics may be used.

Alternatives: FlowJo is recommended for data analysis, although any flow cytometry software may be employed.

STEP-BY-STEP METHOD DETAILS

Gradient separation of mononuclear cells

Timing: 1.5–2 h

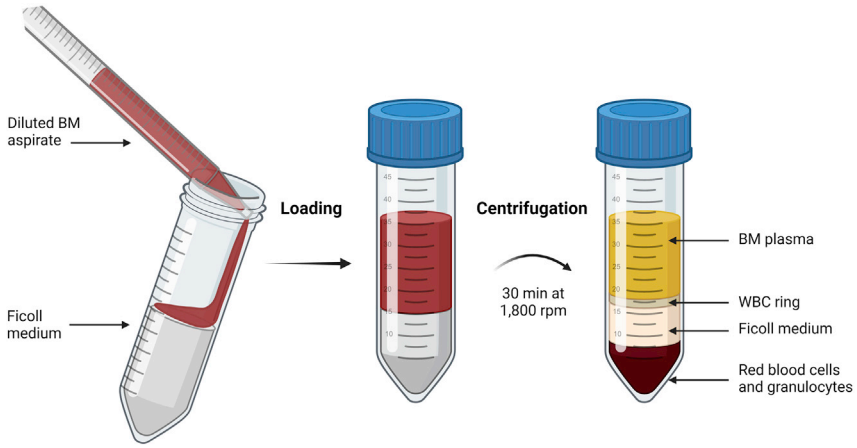

Figure 1. Before and after gradient separation of a BM aspirate

A diluted BM aspirate is loaded onto the Ficoll medium (left) forming a flat and smooth interphase (center). After centrifugation, BM components are separated into layers on the basis of their density (right).

In this step, the components of a BM aspirate will be separated on the basis of their density to allow the isolation of the MNCs. After centrifugation of the BM aspirate with Ficoll medium, a density gradient will have formed, and the components of the BM aspirate will be separated into layers (Figure 1). A cloudy white layer containing the MNCs (the "white blood cell [WBC] ring") should be seen in the interphase, around the center of the tube, and collected.

1. Dilute the BM sample 1:1 in DPBS in a 15 mL or 50 mL centrifuge tube, depending on the final sample volume, and note the volume.
2. Add the same volume of Ficoll medium into a clean centrifuge tube.

   Note: Make sure that there is enough space for the diluted BM aspirate; if not, split the Ficoll into as many tubes as needed.

3. Load the diluted BM aspirate onto the Ficoll:
   a. Tilt the tube containing the Ficoll at an angle of approximately 45°.
   b. Load the pipette with the diluted BM aspirate and position the tip on a 90° angle against the wall of the tube containing the Ficoll.
   c. Slowly let the sample go down the walls of the tube and start forming a layer on top of the Ficoll.

   ⚠ CRITICAL: Be careful to not disturb the interface between the Ficoll and the BM aspirate. Pipetting the diluted BM aspirate directly into the bottom of the tube or using too much pressure will cause the sample and the Ficoll to mix and therefore no gradient will form, and the sample will be irrecoverable.

4. Remove the accelerator and/or brake functions of the centrifuge and spin down the tube(s) for 30 min at 685 × *g* and +18°C–22°C.

   ⚠ CRITICAL: It is crucial that the accelerator and/or brake of the centrifuge are off during the first centrifugation step, and that investigators avoid shaking the tube, as any perturbance in the interphase or gradient may result in the loss of the WBC ring.

5. Carefully aspirate the upper phase (plasma) without perturbing the WBC ring. Troubleshooting 1.

6. In a clean centrifuge tube, add approximately twice the volume of Washing buffer as the volume of the diluted BM aspirate.
7. Collect the WBC ring with a pipette by bringing the tip to the surface of the ring and slowly aspirating while moving it in circular motion along the walls of the tube.
8. Transfer the cells into the tube containing the Washing buffer.
9. Activate the accelerator and/or brake functions of the centrifuge and spin down for 10 min at 685 × *g* and +18°C–22°C.
10. Aspirate the supernatant, wash the sample and count the cells:
    a. Resuspend the pellet in 1 mL of Washing buffer by repeatedly pipetting up and down until there are no clumps.
    b. If the sample had been split in several tubes, or if the pellet was in a 50 mL tube, transfer each of the 1 mL cell suspensions to a 15 mL centrifuge tube and bring them to a volume of 10 mL with Washing buffer.
    c. Take a small aliquot (e.g., 20 µL) and dilute it 1:1 with modified Turk's solution for counting.
    d. Mix briefly with the micropipette and add 2 volumes of DPBS or Working solution to achieve a 1/4 dilution.

    Note: If the cell density is too high for counting, further dilute the suspension as needed.

    e. Count the nuclei in a hemocytometer and calculate the total number of MNCs in the sample.
11. Considering cell counts, take an aliquot containing 0.5–2 million cells for flow cytometry analysis and transfer it to a microcentrifuge tube.
12. Fixate the MNCs in the aliquot for future use by adding 1:1 volume of Streck™ cell preservative and store the aliquot following the equipment-specific manufacturer's specifications.

    Note: The MNCs will be analyzed by flow cytometry alongside the cultured cells and be used as a reference of the HSPCs in their native BM frequencies in order to confidently set the flow cytometry gates for data analysis, as the cellularity in the cultured samples will be significantly lower.

    ⚠ CRITICAL: Do not store fixated samples for longer than 1 week (less than 5 days is preferable).

    Optional: Instead of fixating the MNCs, investigators may choose to preserve the aliquot on ice until the HSPCs have been isolated and cultured (after step 32), and then prepare the MNCs for flow cytometry and analyze them fresh on the same day. The recorded sample will later need to be included in the data analysis along with the cultured cells (step 47).

13. Centrifuge the rest of the cell suspension for 5 min at 475 × *g* and 4°C and aspirate the supernatant.
14. Resuspend the cell pellet in 1 mL of Washing solution and transfer the cell suspension to a microcentrifuge tube.

Magnetic enrichment of the MNC fraction

Timing: 1–1.5 h

To minimize sample processing times and avoid subjecting the cells to high mechanic stress such as the one inherent to fluorescent-activated cell sorting, $CD34^+$ HSPCs will be isolated by magnetic enrichment. BM MNCs will be labeled cells with an anti-CD34 antibody conjugated with magnetic beads and subsequently eluted through a magnetic column which will selectively retain $CD34^+$ cells. This step can be performed following manufacturer's specifications, but here we offer an optimized protocol for working with low cell numbers and preserving their viability.

15. Centrifuge the cell suspension for 5 min at 300 × *g* in a tabletop centrifuge at 4°C and aspirate the supernatant using a micropipette.
16. Stain the sample using the CD34 MicroBead Kit:
    a. Resuspend the pellet in 300 µL of 1× MACS Rinsing solution.
    b. Add 100 µL of FcR block and 100 µL of anti-CD34 beads.
    c. Mix by lightly pipetting up and down.
    d. Incubate at 4°C for 30 min.

Note: Volumes provided in step 16 are for cell numbers below 100 million. If a sample yielded more cells, investigators would need to adjust the volumes accordingly.

17. During incubation, prepare a magnetic column by placing it on the separator and condition it by adding 3 mL of 1× MACS Rinsing solution and letting it elute into a tube or tray (discard).
18. After incubation, wash the cell suspension by adding 500 µL of Washing solution into the tube and mix by pipetting up and down.
19. Centrifuge for 5 min at 300 × *g* and 4°C and aspirate the supernatant using a micropipette.
20. Resuspend the cell pellet in 500 µL of 1× MACS Rinsing solution for every 100 million cells and load the suspension into the column.

Optional: If you would like to isolate other cell types from the same sample, place a 15 mL centrifuge tube under the column and on ice to collect the cell fraction enriched in $CD34^-$ cells. In step 23 and onwards, proceed in parallel with the $CD34^+$ cells.

21. Elute the $CD34^-$ cell fraction:
    a. Let the cell suspension go through the column entirely (until the upper part is completely dry).
    b. Add 2 mL of 1× MACS Rinsing solution to wash and let it elute entirely.
    c. Repeat step b.
22. Elute the $CD34^+$ cell fraction:
    a. Place a clean 15 mL centrifuge tube containing 1 mL of Washing solution under the column and on ice and remove the column from the separator.

⚠ CRITICAL: If using the QuadroMACS™ Separator, do not remove or add any columns while a cell suspension or wash is eluting, as a perturbance in the magnetic field may release the retained positive cells. When working with more than one column, wait until all have finished eluting before removing any of them.

    b. Add 5 mL of 1× MACS Rinsing solution to the column.
    c. Use the provided plunger to apply gentle pressure to the column and quickly elute the cells that had been retained.
23. Add 5 mL of Washing buffer to the tube and centrifuge for 5 min at 475 × *g* and 4°C to pellet the cells.
24. Discard the supernatant by aspiration or decantation and remove any leftovers with a micropipette.
25. Resuspend the $CD34^+$ pellet with 0.5–1 mL (depending on its size) of pre-warmed HSPC Culture medium (or Washing buffer for the $CD34^-$ fraction) and transfer the cells to a microcentrifuge tube.

Culturing and treatment of HSPCs

Timing: 20–30 min + culture time (1–3 days)

In this step, isolated $CD34^+$ cells will be cultured and treated with the drugs of choice of the investigator. To preserve cell viability in culture, isolated $CD34^+$ HSPCs will be seeded over a layer of

previously-plated healthy MSCs. Cells will be cultured in a cytokine-free medium to prevent expansion and differentiation of the cells, which should preserve their original hierarchy for the duration of this step. Thus, the readout of this experiment will be the quantification of the surviving HSPCs in distinct differentiation states by flow cytometry.

⚠ CRITICAL: After magnetic purification, $CD34^+$ cells will be very sensitive to stress. This is a time-sensitive step that should be completed as quickly as possible. Isolated cells should be kept on ice until plating and investigators should avoid performing additional centrifugations.

26. Immediately after homogenizing and transferring the cell suspension, take a 10 µL aliquot and combine it with Trypan blue (1:1).
27. Count the number of live cells and calculate the number of wells that can be plated and/or the final cell density per well, as well as the volume of cell suspension and of fresh medium needed in each well (final volume = 0.5 mL/well in 48-well plates or 1 mL/well in 24-well plates). Troubleshooting 2.

Note: Between 10,000–100,000 cells can be plated per well in 48-well plates; twice as many per well can be plated in 24-well plates. If lower cell numbers are obtained, investigators can also scale down and use 96-well plates seeded with a proportional number of MSCs.

Note: When planning the number of cultured wells per sample, investigators should account for the corresponding controls (solvent, negative controls, inhibitory controls) pertinent to their experiment. Whenever possible, technical replicates should be cultured. Duplicates are sufficient, but triplicates may be plated if enough cells are available. However, we recommend prioritizing on culturing the highest possible number of cells per well instead of many wells per condition. If the number of cells obtained is low, it is preferable to plate single wells per condition and later combine the results with those of biological replicates (other patients' samples) during the statistical analysis.

28. Prepare the culture plates:
    a. Using a micropipette, remove the medium from the MSC cultures without touching the bottom of the well.
    b. Carefully add 0.5–1 mL of pre-warmed DPBS to each well by placing the tip of the micropipette on the walls.
    c. Swirl the plate and aspirate the DPBS with the micropipette to remove any dead cells left.
    d. Add the corresponding volume of pre-warmed HSPC Culture medium calculated in step 27 to each well.
29. Split the cells into the wells as needed.
30. Incubate the cells at 37°C and 5% CO_2 for 2–16 h before treating them.

Note: *De visu* assessment of cellularity and viability under the microscope before treating the cells is recommended.

31. Add the corresponding drug and solvent solutions to each well and mix well by moving the plate horizontally in several directions in a cross-like pattern. Avoid mixing by pipetting to prevent cell loss.
32. Incubate at 37°C and 5% CO2 for 24–72 h (or up to 5 days) as needed before proceeding to assessing the results by flow cytometry analysis.

Flow cytometry preparation

Timing: 1.5–2 h

This step involves the staining of the MNCs aliquoted after gradient separation (step 12) and the cultured $CD34^+$ cell fraction for flow cytometry analysis of the immunophenotypic HSPC

subpopulations (Will et al., 2012; Pang et al., 2013). Counting beads will be added to each of the cultured cell tubes and used as internal standards to normalize the number of cells recorded per sample, which will allow a more precise calculation of the absolute number of live cells per well.

Note: *De visu* assessment of cellularity and viability under the microscope before beginning for comparison with the pre-treatment status is recommended. Troubleshooting 3.

33. Using a micropipette, briefly mix the contents of each well by pipetting up and down 2–3 times and transfer the cell suspensions to microcentrifuge tubes.
34. Wash the wells:
    a. Add 250–500 μL of pre-warmed Washing solution to each well.
    b. Gently pipette up and down 2–3 times.
    c. Transfer the content of each well to the corresponding tube, combining it with the previously collected cell suspension.
35. Centrifuge the tubes, as well as the MNCs fixated in step 12, at 300 × *g* and 4°C for 10 min.

Optional: If an unstained flow cytometry control is needed, collect a small aliquot (10%–20% v/v) of the MNCs before proceeding to step 35 and centrifuge them along with the rest of the MNCs and the $CD34^+$ cells; then, keep them on ice until step 42. Proceed as with single color controls.

36. During centrifugation, prepare enough volume of the HSPC Staining cocktail indicated in materials and equipment, considering that cultured samples require half of the volume of a fresh/fixated sample.

Note: Some of the antigens included in the "dump gate" of the flow cytometry panel (CD7, CD10) are sometimes expressed at low levels by normal HSPCs. Therefore, if investigators are using healthy donor samples as controls in this protocol, a separate antibody staining cocktail without anti-CD7 and anti-CD10 should be used with those samples.

37. Carefully aspirate the supernatants using a micropipette.

⚠ CRITICAL: After centrifugation, $CD34^+$ cell pellets will be very small, so a marginal volume of supernatant can be left in the tube to avoid touching the pellet with the tip.

38. Add 100 μL of HSPC Staining cocktail to the MNCs sample, or 50 μL to each cultured sample, and mix briefly with the tip.
39. Incubate samples for 30–45 min at 4°C and protected from light.
40. During incubation, prepare single-color controls:
    a. Vortex OneComp eBeads thoroughly and aliquot 500 μL in a microcentrifuge tube.
    b. Pipette 50 μL of beads into microcentrifuge tubes pre-labeled with the names of the 6 fluorochromes used in this panel.
    c. Add 50 μL of Working solution to each tube, for a final volume of 100 μL.
    d. In each tube, add the following volumes of the corresponding antibodies and mix by vortexing.

Antibody	Volume
Anti-CD14-FITC (or any other FITC-conjugated antibody)	5 μL
Anti-CD34-BV421	5 μL
Anti-CD38-APC-Cy7	5 μL
Anti-CD90-PerCP-Cy5.5	10 μL
Anti-CD45RA-APC	10 μL
Anti-CD123-PE, 1:10 dilution in DPBS	2 μL

e. Incubate for 15–30 min at 4°C and protected from light and proceed to step 42 in parallel with the MNCs and $CD34^+$-enriched cells.

41. During incubation, prepare flow cytometry tubes for the cultured $CD34^+$ samples by adding 5 μL of counting beads, thoroughly mixed for 1 min, to each tube using reverse pipetting (see manufacturer's specifications). If using strainer caps, remove the caps prior to adding the beads and then recap the tubes. Keep tubes at 4°C and protected from light.

Note: Do not add counting beads to unstained and single-color control tubes.

⚠ CRITICAL: Precise pipetting is essential for the calculation of absolute cell numbers using counting beads. Investigators should make sure to use well calibrated pipettes.

42. After incubation, add 100 μL of Washing solution to each tube and centrifuge for 5 min at 300 × *g* and 4°C.
43. During centrifugation, prepare enough volume of the SYTOX Green solution indicated in materials and equipment.

Note: The SYTOX Green solution will only be needed to stain the MNCs and $CD34^+$ cells but not the unstained and single-color controls.

44. Aspirate the supernatants with a micropipette. A marginal volume can be left in $CD34^+$ cell tubes.
45. Resuspend cell pellets, except for the unstained control, in 250–500 μL ($CD34^+$ cells, in 250–300 μL; MNCs, in 350–500 μL) of SYTOX Green solution, and bead pellets, as well as the unstained control, in 350–500 μL of Working solution and transfer the corresponding suspensions into pre-labeled flow cytometry tubes.

⚠ CRITICAL: Keep flow cytometry tubes at 4°C and protected from light until analysis.

46. Proceed to flow cytometry analysis following instrument-specific instructions.

Note: Set the recording limit to 200,000–500,000 events (or the entire sample) in order to ensure the recording of enough cells in each HSPC gate, particularly in cultured samples.

⚠ CRITICAL: Use the total BM MNCs fixated in step 12 and stained in parallel with the cultured samples to set up or adjust the flow cytometry template, not the cultured samples containing counting beads. Record cultured samples entirely. To record counting beads, set a low value for threshold or discriminator in the FSC channel. To ensure accuracy in cell number quantification, make sure to collect a minimum of 1,000 beads while recording each sample.

Flow cytometry data analysis

Timing: 1–3 h

This step involves setting up the gates for analysis and quantification of the HSPC hierarchies in fresh and cultured MDS BM samples. Information on how to set up a flow cytometry template or manipulate the equipment for recording are not detailed here as steps will vary with the instrument and user-specific circumstances.

Our flow cytometry panel contains 5 antibodies against HSPC-specific markers and 12 antibodies against lineage markers, all of which are conjugated with FITC and will serve to discriminate all lineage-positive (Lin^+) cells from the Lin^- HSPCs. A green viability dye, SYTOX Green, will be detected in

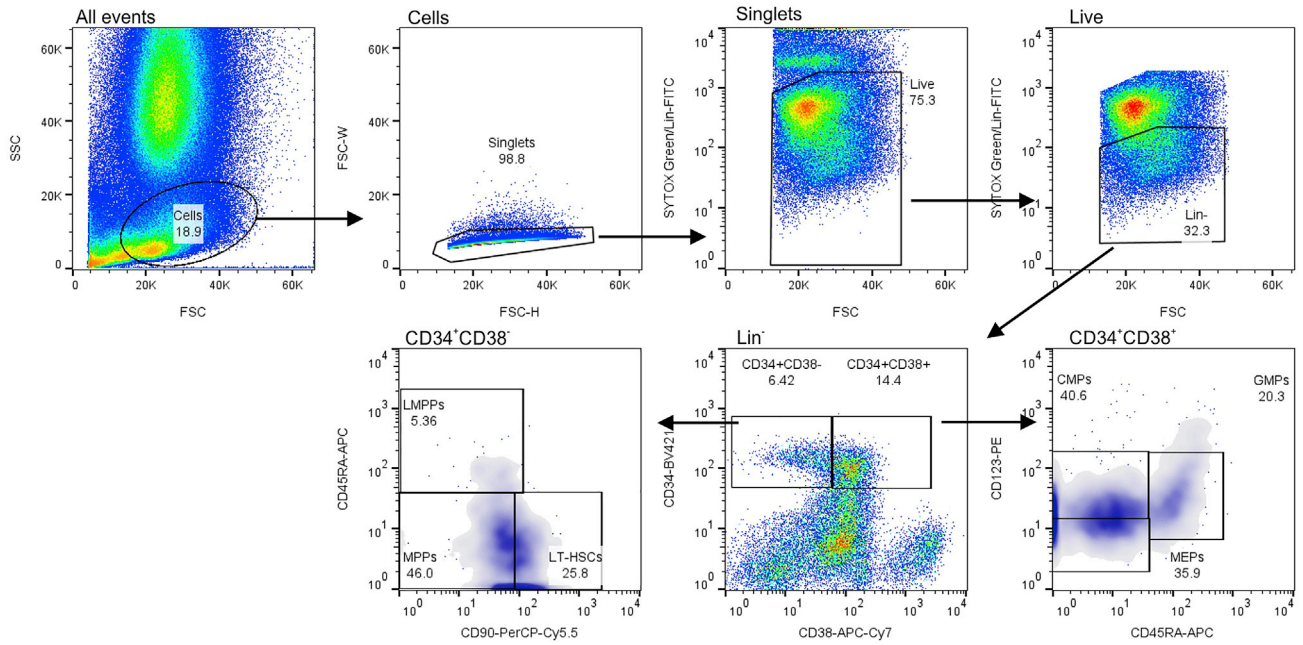

Figure 2. Example flow cytometry analysis of BM MNCs from a patient with MDS

Flow cytometry plots displaying a representative analysis of immunophenotypic HSPC subpopulations in a sample of BM MNCs. Arrows represent the steps of the suggested gating strategy.

the same channel as FITC to simultaneously exclude dead cells (we refer to the FITC$^+$ gate as the "dump gate"). Therefore, this particular staining is not useful for the analysis of mature BM cell populations. Troubleshooting 4.

47. Use the MNCs sample to set the gates as follows (Figure 2): troubleshooting 5.
    a. In FSC vs. SSC, select the cell population with low FSC and low SSC (lymphocytes).
    b. Display the cells in FSC-H vs. FSC-W and select the singlets.
    c. Display singlets in FSC vs. FITC and select live cells: SYTOX Green$^-$ (FITC may be dim or negative).
    d. Within live cells, select lineage negative (Live Lin$^-$): SYTOX Green$^-$, FITC$^-$.
    e. Display live Lin$^-$ cells in CD38-APC-Cy7 vs. CD34-BV421 and select hematopoietic stem cells (CD34$^+$, CD38$^-$) and myeloid progenitor cells (CD34$^+$, CD38$^+$).
    f. Display the CD34$^+$, CD38$^-$ stem cell compartment in CD90-PerCP vs. CD45RA-APC and gate:
        i. Long term (LT)-HSCs: CD90$^+$, CD45RA$^-$.
        ii. Multipotent progenitors (MPPs): CD90$^-$, CD45RA$^-$.
        iii. Lymphoid-primed MPPs (LMPPs): CD90$^-$, CD45RA$^+$.
    g. Display the CD34$^+$, CD38$^+$ myeloid progenitor compartment in CD45RA-APC vs. CD123-PE and gate:
        i. Common myeloid progenitors (CMPs): CD123$^+$, CD45RA$^-$.
        ii. Granulomonocytic progenitors (GMPs): CD123$^+$, CD45RA$^+$.
        iii. Megakaryocytic progenitors (MEPs): CD123$^-$, CD45RA$^-$.
48. Using any of the recorded CD34$^+$ samples, set gates for the counting beads according to manufacturer's specifications, or as indicated below and shown in Figure 3:
    a. In FSC vs. SSC, select the population with low FSC and medium-high SSC.
    b. Display the selected population in FSC vs. FITC, exclude the dead cell debris (scattered, FITC-high) and gate the two well-defined bead populations with low and medium-intensity FITC signals. This gate ("Beads") will be used for quantification.

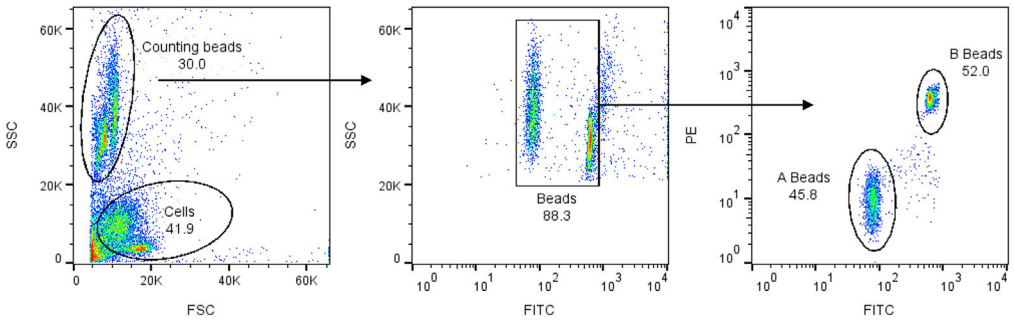

Figure 3. Gating of counting beads

Flow cytometry dot-plots showing the approximate location of counting beads in a FSC vs. SSC plot, as well as the suggested gating strategy for A and B beads.

c. Display the Beads in FITC vs. PE and create independent gates for A Beads and B Beads. The proportions of each type of beads will serve as a reference for quality assessment by comparison with lot-specific percentages.

49. Collect quantitative information on the number of live cells in each population of interest, and the total number of beads recorded from each sample.
50. Calculate the absolute number of cells in the populations of interest by using the following formula: *Absolute cell count = (Number of cells in gate/Number of recorded beads) x Bead concentration (n/μL) x 5 μL*; where the bead concentration is lot-dependent and given by the manufacturer and 5 μL is the volume of beads added to each tube.
51. Normalize the data to the number of cells in the control (vehicle) sample.

EXPECTED OUTCOMES

Investigators should be able to successfully isolate BM $CD34^+$ HSPCs, characterize their immunophenotype and assess drug sensitivity of specific HSPC subpopulations. For reference, the expected frequencies of all the gated populations in the BMs of healthy adults over 50 years of age are summarized in Table 1. Investigators should note that MDS patients have fewer HSPCs than age-matched healthy individuals and have profoundly altered HSPC hierarchies (Ganan-Gomez et al., 2022), so the values obtained from patient samples may not always fall within the provided ranges. The number of live $CD34^+$ cells obtained after enrichment should be, roughly, around 50,000 $CD34^+$ cells per 1 million MNCs (5%) but will vary significantly between patients and may be affected by other factors such as the quality of the BM aspirate (e.g., hemodilution), treatments received by the patient at the time of BM aspiration, sample preservation conditions prior to processing and sample manipulation during the protocol.

Table 1. Gating strategy and expected frequencies of human HSPCs in healthy adults >50 y.o

Population	Marker expression	% Of live MNCs, [range]
Lineage negative (Lin^-)	$CD2^-$, $CD3^-$ $CD4^-$, $CD7^-$, $CD10^-$, $CD11b^-$, $CD14^-$, $CD19^-$, $CD20^-$, $CD33^-$, $CD56^-$, $CD235a^-$	20 [5–40][a]
HSCs	Live/Lin^-/$CD34^+$, $CD38^-$	1.2 [0.5–4]
LT-HSCs	Live/Lin^-/$CD34^+$, $CD38^-$/$CD90^+$, $CD45RA^-$	0.1 [0.5–1.5]
MPPs	Live/Lin^-/$CD34^+$, $CD38^-$/$CD90^-$, $CD45RA^-$	0.9 [0.5–2]
LMPPs	Live/Lin^-/$CD34^+$, $CD38^-$/$CD90^-$, $CD45RA^+$	0.1 [0.03–0.3]
Progenitors	Live/Lin^-/$CD34^+$, $CD38^+$	4.5 [1–10]
CMPs	Live/Lin^-/$CD34^+$, $CD38^+$/$CD123^+$, $CD45RA^-$	2 [0.5–5]
GMPs	Live/Lin^-/$CD34^+$, $CD38^+$/$CD123^+$, $CD45RA^+$	2 [0.2–4]
MEPs	Live/Lin^-/$CD34^+$, $CD38^+$/$CD123^-$, $CD45RA^-$	0.3 [0.05–3]

[a]The percentage of Lin^- cells in total live MNCs may vary significantly depending on how much peripheral blood is aspirated with the marrow.

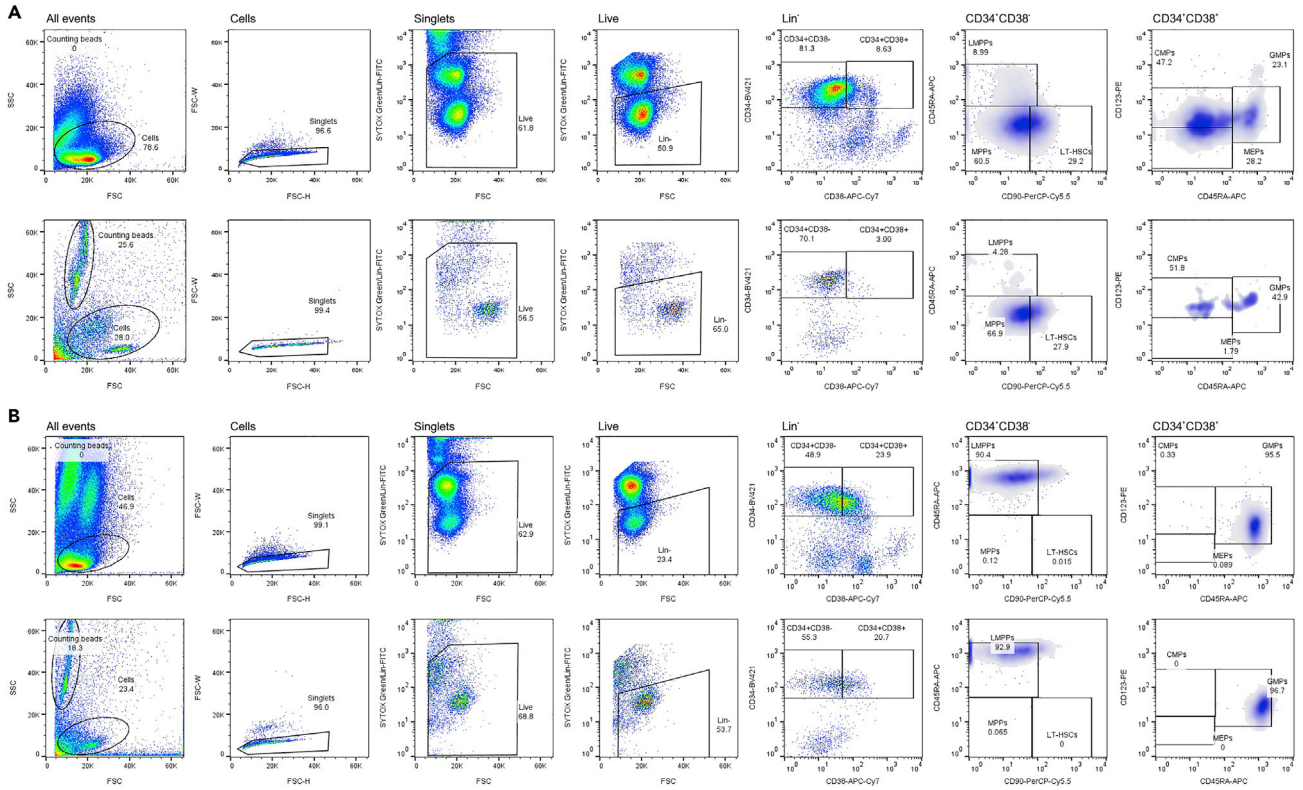

Figure 4. Compared frequencies of MDS HSPCs in total BM MNCs and after CD34$^+$ enrichment and culturing

Flow cytometry plots displaying two representative immunophenotypic analyses of MDS patient BM samples before (top; MNCs) and after (bottom) CD34$^+$ enrichment and subsequent culturing for 72 (A) or 48 h (B). (A) "CMP pattern" MDS patient with a prevalence of LT-HSCs, MPPs and CMPs. (B) "GMP pattern" MDS patient with a prevalence of LMPPs and GMPs.

After culturing, a degree of cell death is expected, but in general investigators should expect to retrieve similar cell numbers as those plated, and similar HSPC frequencies to those in total BM MNCs in the sample of origin (Figure 4). After analysis, investigators should be able to detect changes in the frequencies and absolute numbers of live HSPCs (Figure 5).

LIMITATIONS

The efficacy of this protocol highly relies on the quality and quantity of the BM samples. Hemodiluted or small-volume BM aspirates may yield insufficient MNCs to perform the CD34$^+$ enrichment and/or insufficient CD34$^+$ cells for culturing. If the protocol is performed with cryopreserved MNCs, the viability of the frozen cells will be an important limiting factor. If the viability of thawed cells is 60% or less, performing a gradient separation to remove dead cells is recommended. Note that this step will significantly decrease the cell number, so it may only be taken in the starting cell number is high.

Another factor that may prevent the success of this protocol is the fitness of the stromal support layer. If MSCs are high-passage or have been in culture for too long at high confluence, they will become senescent and induce the depletion of the co-cultured HSPCs.

TROUBLESHOOTING

Problem 1

WBC ring not visible.

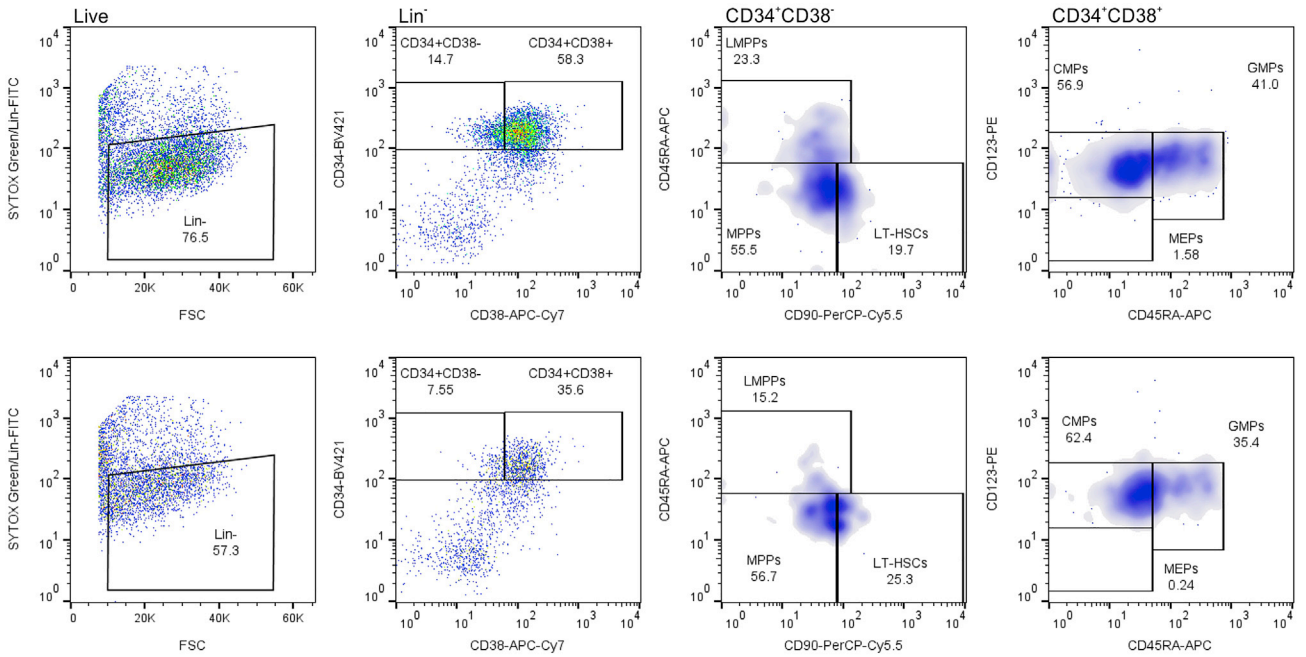

Figure 5. Analysis of live HSPCs in cultured MDS CD34$^+$ cells

Flow cytometry plots showing differences in the frequencies of live HSPC populations in a representative experiment in which BM CD34$^+$ cells from a "CMP pattern" MDS patient were cultured and treated with vehicle (0.05% DMSO; top) or with a combination of the BCL-2 inhibitor venetoclax (50 nM) and the hypomethylating agent 5-azacytidine (0.5 μM) for 72 h (bottom). Numbers represent the frequency (%) in the parent population indicated above each set of plots.

The WBC ring may be difficult to visualize in samples from patients with low BM cellularity or low-volume samples that were diluted in excess and/or loaded in too large centrifuge tubes.

Potential solution

Collect half of the plasma fraction.

In step 5, aspirate only the upper half of the plasma fraction. Then, in step 7, Locate the interphase between the plasma and Ficoll, which should be at approximately half of the total volume, and aspirate the rest of the plasma starting at the interphase and moving up in circles. This should collect any scattered MNCs that are too diluted to form a visible ring. Wash the collected supernatant as indicated in steps 6 and 8 but use a lower volume of Washing buffer and downsize the centrifuge tube as much as possible to improve pellet formation and visualization.

Problem 2

Dead cell clumps after centrifugation.

Elution of the CD34$^+$ cell fraction using the plunger and subsequent centrifugation may decrease the viability of the sample (particularly if the specimen was previously frozen) and dead cell clumps may be observed during counting. If the clumps are too abundant or too big, they may affect the viability of the cultured cells during the incubation period.

Potential solution

Filter cells before culturing.

To eliminate dead cell clumps, use a sterile 40 μm strainer to filter the cell suspension before pipetting it into the culture plate.

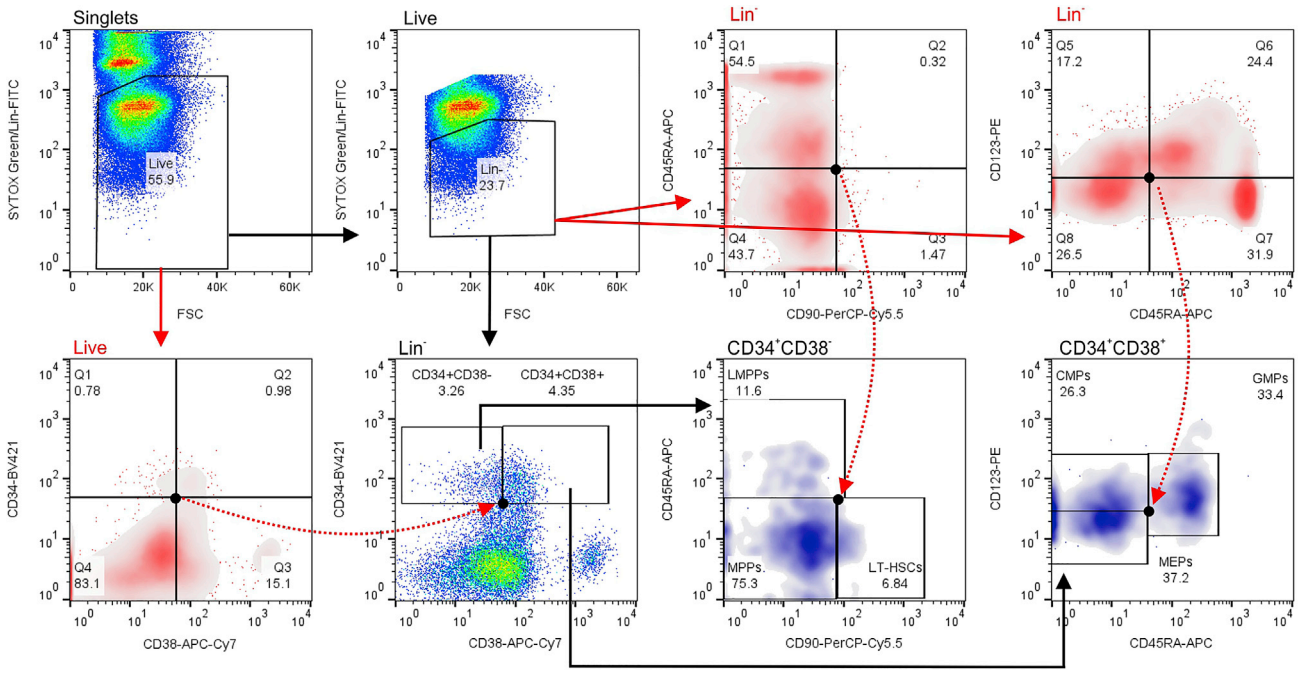

Figure 6. Gating strategy in a sample with poorly resolved populations

Flow cytometry plots displaying a representative analysis of immunophenotypic HSPC subpopulations in MNCs from a BM sample with poorly resolved stem ($CD34^+CD38^-$) and progenitor cells ($CD34^+CD38^+$). Black arrows represent the steps of the suggested gating strategy. Solid red arrows represent additional gating steps taken to visualize marker distributions in larger cell populations (red density plots) and use them as a reference (black dots) to set the gates in populations with fewer cells (dotted red arrows).

Problem 3

Low viability or low cell numbers after culturing.

If, before starting step 33, investigators observe a significant depletion in the number of cultured cells, or if the number of cultured cells was very low from start (e.g., <10,000 cells/well), every following step will pose increased risk of cell loss, as pellets will be hardly visible.

Potential solution

Downsize and optimize cell collection and processing.

In addition to collecting the cells as indicated in steps 33–35, add a short (3 min) trypsinization step before washing the wells to collect as many HSPCs as possible (potentially, those more strongly attached to the stroma), as well as the MSCs. MSCs will help in pelleting the HSPCs and will make the pellet easily visible. Owing to their large size, MSCs will be easily excluded from the flow cytometry analysis in the FSC vs. SSC plot. Also, they are $CD90^+$ but $CD34^-$ so, if gates are properly set, they should not interfere with the HSPC quantification.

Problem 4

Poor resolution of dead and Lin^+ populations.

Dead and Lin^+ cells are detected in the same channel and excluded in the "dump gate". However, in order to calculate the frequency of each HSPC population in total live MNCs, the Live cells gate must be set and dead cells must be well distinguished from Lin^+ cells, which may seem challenging at first.

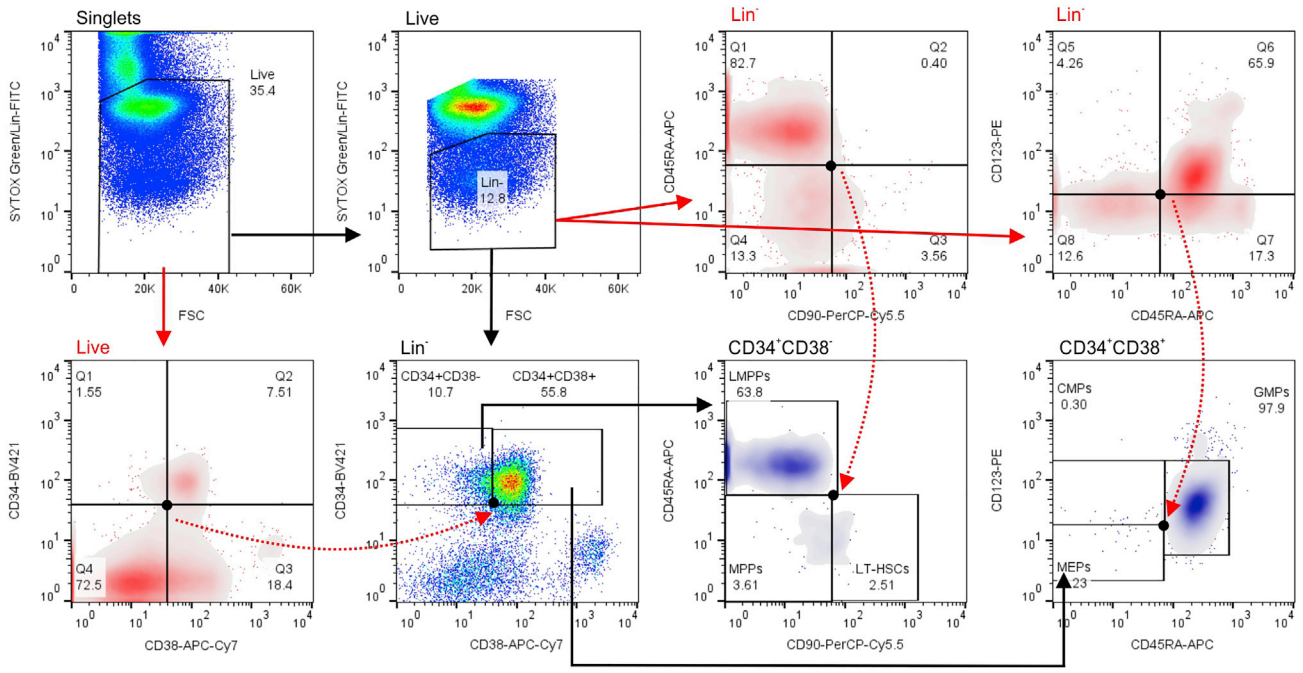

Figure 7. Gating strategy in a sample with underrepresented populations

Flow cytometry plots displaying a representative analysis of immunophenotypic HSPC subpopulations in MNCs from a BM sample with underrepresented HSPC populations (LT-HSCs, MPPs, CMPs, MEPs). Black arrows represent the steps of the suggested gating strategy. Solid red arrows represent additional gating steps taken to visualize marker distributions in larger cell populations (red density plots) and use them as a reference (black dots) to set the gates in populations with fewer cells (dotted red arrows).

Potential solution

Adjust voltage of the green laser and titrate SYTOX Green.

To obtain a good resolution of the two green-positive populations during template set up, you may prepare a fully-stained MNCs sample and resuspend it in Working solution without SYTOX Green to first map Lin^+ cells in the plot, and then add SYTOX Green extemporaneously to visualize both dead and Lin^+ cells. It may also help to prepare a control (MNCs from the same sample or from a different one) stained just with SYTOX Green to adjust its concentration and/or the blue-green laser voltage so that the signal will be as bright as possible for a better separation from the Lin^+ cells.

Problem 5

Difficulty setting gates for low frequency populations.

Patients with MDS have lower frequencies of HSPCs than age-matched healthy individuals and one or more of the stem or progenitor populations may be depleted (Ganan-Gomez et al., 2022), which could potentially hinder gate setting during flow cytometry analysis.

Potential solution

Use other cell populations as a reference.

In cases where there are few cells in one or more HSPC populations, it is useful to look at marker expression in total live cells or live Lin^- cells to discriminate negative versus positive and set the gates accordingly. Some examples are shown in Figures 6 and 7.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Irene Ganan-Gomez (iganan@mdanderson.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate a dataset or code.

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AUTHOR CONTRIBUTIONS

I.G.G. and S.C. designed the experiments. I.G.G. developed the co-culture system and readout assay. K.C.D. developed and set up the flow cytometry panel.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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