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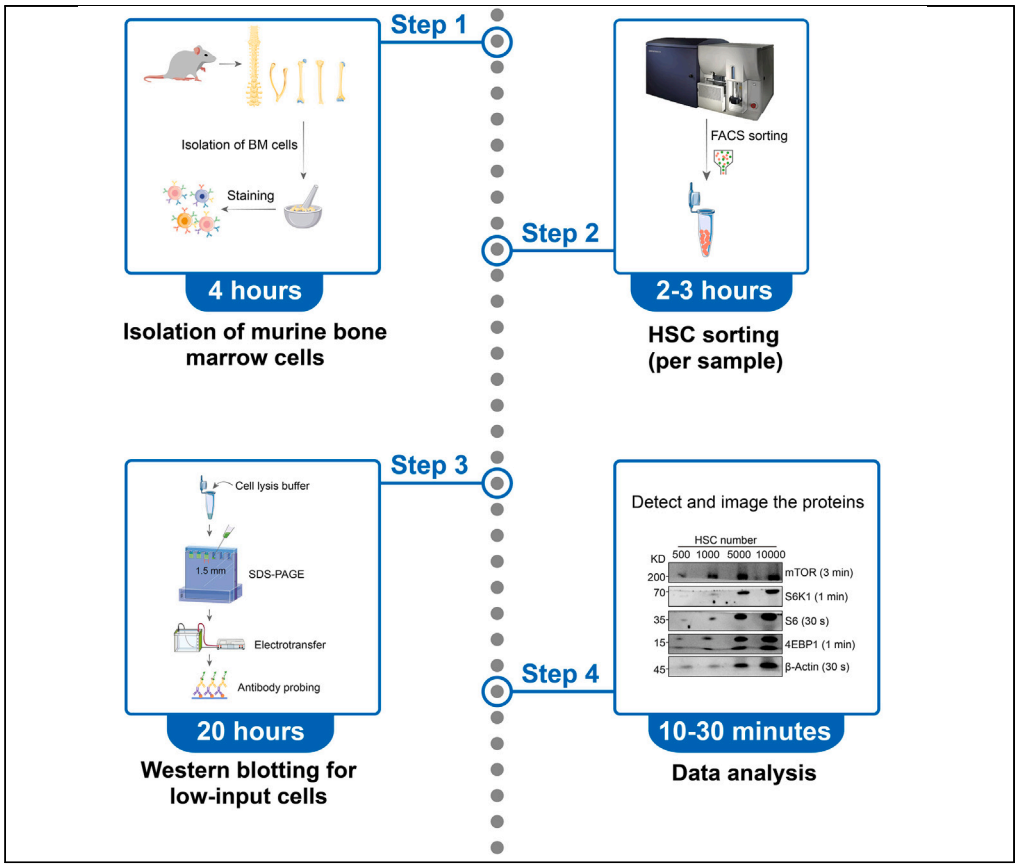
Protocol

Protocol for high-sensitivity western blot on murine hematopoietic stem cells



Hematopoietic stem cells (HSCs) sustain hematopoiesis during homeostasis and regeneration. However, their limited availability poses a challenge for protein analysis. Here, we present a protocol for performing high-sensitivity western blot on HSCs using two techniques that enhance HSC isolation from mice and boost sensitivity for low cell numbers. We describe steps for isolating murine bone marrow cells, antibody staining, and cell sorting and post-sort analysis. We then detail a western blot procedure suitable for low numbers of HSCs.

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

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Highlights

Optimize the procedure for isolating HSCs from murine bone marrow tissues

Standardize the protocol for sorting HSCs

Enhance the sensitivity of western blotting for low cell numbers

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Protocol for high-sensitivity western blot on murine hematopoietic stem cells

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SUMMARY

Hematopoietic stem cells (HSCs) sustain hematopoiesis during homeostasis and regeneration. However, their limited availability poses a challenge for protein analysis. Here, we present a protocol for performing high-sensitivity western blot on HSCs using two techniques that enhance HSC isolation from mice and boost sensitivity for low cell numbers. We describe steps for isolating murine bone marrow cells, antibody staining, and cell sorting and post-sort analysis. We then detail a western blot procedure suitable for low numbers of HSCs.

For complete details on the use and execution of this protocol, please refer to Li et al (2022).[1,2]

BEFORE YOU BEGIN

Hematopoietic stem cells (HSCs) are multipotent stem cells responsible for the maintenance and replenishment of the blood system throughout the lifetime by giving rise to all blood cell types, including red blood cells, white blood cells, and platelets. HSCs are a rare population, representing only about 0.01% of cells in the bone marrow of mice, and traditional fluorescence-activated cell sorting (FACS) methods typically isolate only around 5,000 HSCs from the hind limb bone marrow of an individual mouse.[3] Given that a substantial quantity of protein is typically required for a western blot (1×10^5 to 1×10^6 cells), it becomes challenging to perform routine analysis with HSCs, as a considerable number of mice would be needed.[4–6] The limitations of the methodology for detecting protein expression in HSC studies hinder mechanistic analysis using biochemistry. Hence, there is an urgent need to enhance current methods to enable western blot analysis in HSCs. To tackle this challenge, we have developed an integrated strategy that enhances the yield rate of HSCs in mice while improving the sensitivity of western blot analysis.[1,2,7] Consequently, this development enables more efficient and effective protein analysis for this critical stem cell population.

Institutional permissions (if applicable)

Housing and handling of all mice, as well as experimental procedures, were approved by the Animal Care and Use Committee of the Sun Yat-sen University or Ruiye Bio-tech Guangzhou Co., Ltd.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD3 ε PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#100310; RRID: AB_312675
Anti-mouse CD4 PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#100410; RRID: AB_312695
Anti-mouse CD8a PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#100710; RRID: AB_312749
Anti-mouse/human CD11b (Mac-1) PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#101210; RRID: AB_312793
Anti-mouse Ly-6G/Ly-6C (Gr-1) PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#108410; RRID: AB_312793
Anti-mouse/human CD45R/B220 PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#103210; RRID: AB_312995
Anti-mouse IgM PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#406544; RRID: AB_2876730
Anti-mouse TER-119 PE-Cy5 (working dilution: 1 in 200)	BioLegend	Cat#116210; RRID: AB_313711
Anti-mouse Ly-6A/E (Sca-1) PE-Cy7 (working dilution: 1 in 50)	BioLegend	Cat#108114; RRID: AB_493596
Anti-mouse CD117 (c-Kit) APC (working dilution: 1 in 50)	BioLegend	Cat#105812; RRID: AB_313221
Anti-mouse CD48 APC-Cy7 (working dilution: 1 in 50)	BioLegend	Cat#103432; RRID: AB_2561463
Anti-mouse CD150 PE (working dilution: 1 in 50)	BioLegend	Cat#115904; RRID: AB_313683
Anti-mouse CD45 PE-Cy5 (working dilution: 1 in 100)	Thermo Fisher Scientific	Cat#15-0451-82; RRID: AB_468752
Anti-mouse CD45 PE-Cy7 (working dilution: 1 in 100)	Thermo Fisher Scientific	Cat#25-0451-82; RRID: AB_2734986
Anti-mouse CD45 PE (working dilution: 1 in 100)	Thermo Fisher Scientific	Cat#12-0451-82; RRID: AB_465668
Anti-mouse CD45 APC (working dilution: 1 in 100)	Thermo Fisher Scientific	Cat#17-0451-82; RRID: AB_469392
Anti-mouse CD45 APC-Cy7 (working dilution: 1 in 100)	BioLegend	Cat#103116; RRID: AB_312981
FITC Annexin V (working dilution: 1 in 100)	BioLegend	Cat#640945; RRID: AB_2629519
DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) (working concentration: 0.01 μg per μL)	Thermo Fisher Scientific	Cat#D1306; RRID: AB_2629482
FITC anti-mouse Ki-67 antibody (working dilution: 1 in 100)	BioLegend	Cat#652410; RRID: AB_2562141
Anti-mTOR (working dilution: 1 in 1000)	Proteintech	Cat#66888-1-Ig; RRID: AB_2882219
Anti-S6K1 (working dilution: 1 in 1000)	Proteintech	Cat#14485-1-AP; RRID: AB_2269787
Anti-S6 (working dilution: 1 in 1000)	Cell Signaling Technology	Cat#14662; RRID: AB_2798559
Anti-4EBP1 (Ser51) (working dilution: 1 in 1000)	Cell Signaling Technology	Cat#9644S; RRID: AB_2097841
Anti-β-actin (working dilution: 1 in 1000)	Cell Signaling Technology	Cat#4970S; RRID: AB_2223172
Chemicals, peptides, and recombinant proteins		
10× PBS buffer	Sangon Biotech	Cat#E607016-0500
Fetal bovine serum Australian origin	HyClone	Cat#XYM-Hyclone-SH30084
Na_2EDTA	Sangon Biotech	Cat#A100105-0500
NH_4Cl	Damao Chemical Reagent Factory	Cat#12125-02-9
$KHCO_3$	Sangon Biotech	Cat#A501195-0500
7-AAD viability staining solution (working concentration: 0.5 μg per mL)	BioLegend	Cat#420404
BSA	Sigma	Cat#A1933-5G
30% Acr/Bis (29:1) glue solution	Meilunbio	Cat#MA0071

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tris	Beijing Dingguo Changsheng Biotechnology Co., Ltd	Cat#BT350-500g
SDS	Beijing Dingguo Changsheng Biotechnology Co., Ltd	Cat#GS286-500G
APS	Sangon Biotech	Cat#A100486
TEMED	Beyotime	Cat#ST728
Glycine	Sangon Biotech	Cat#A610235
10× RIPA Lysepuffer	Sigma-Aldrich	Cat#20-188
cOmplete, EDTA-free Protease Inhibitor Cocktail	Roche	Cat#4693132001
PhosSTOP (inhibitor tablets for phosphatase)	Roche	Cat#4906845001
PMSF (phenylmethylsulfonyl fluoride)	Sigma-Aldrich	Cat#P7626
5× Loading buffer	Beyotime	Cat#P0015L
PVDF (polyvinylidene difluoride)	Millipore	Cat#IPFL00010
Methanol	Sangon Biotech	Cat#A601617
Non-fat powdered milk	Sangon Biotech	Cat#A600669
Primary antibody dilution buffer	Beyotime	Cat#P0023A
10× TBST buffer	Sangon Biotech	Cat#C520009
Immobilon Western Chemiluminescent HRP Substrate	Millipore	Cat#WBKLS0500
Experimental models: Organisms/strains		
C57BL/6J mouse strain, male or female, 3–4 months old	Jackson Laboratory	N/A
Software and algorithms		
FlowJo	Tree Star Inc.	https://www.flowjo.com/solutions/flowjo
ImageJ	GNU General Public License	https://imagej.net/software/fiji/
GraphPad Prism	GraphPad Software	https://www.graphpad.com/
Other		
QSP pipette tip 1–200 μL for gel loading	Thermo Fisher Scientific	010-Q
40 μm cell strainer	Falcon	352340
Attune NxT flow cytometer	Thermo Fisher Scientific	N/A
BD FACSAria III Cell Sorter	BD Biosciences	N/A
10 μL Maxymum Recovery Microvolume Pipet Tips	Axygen	T-300-L
ChemiDoc Touch Gel Imaging System	Bio-Rad	1708370

MATERIALS AND EQUIPMENT

Cell Isolation buffer

Reagent	Final concentration	Amount
Phosphate buffered saline (PBS) 10×, sterile, pH 7.4	1×	5 mL
Fetal bovine serum (FBS), heat-inactivated and sterile-filtered	2% (v/v)	1 mL
ddH_2O	N/A	44 mL
Total	**N/A**	**50 mL**

Storage: Store at 4°C until use. Cell isolation buffer can be stored at 4°C for up to 7 days, but we recommend making fresh buffer for each new sample.

ACK Lysis buffer		
Reagent	Final concentration	Amount
NH_4Cl	150 mM	8.02 g
$KHCO_3$	10 mM	1.00 g
Na_2EDTA	50 μM	37.2 mg
ddH_2O	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

Dissolve all reagents in 850 mL of ddH_2O. Adjust pH to 7.2–7.4, add ddH_2O to 1,000 mL.
Storage: Store at 4°C until use. ACK Lysis buffer can be stored at 4°C for up to 6 months.

Antibody panel for FACS-sorting		
Reagent	Volume of antibody per sample (500 μL)	Dilution
Anti-lineage markers-PE-Cy5	20 μL	1: 25
anti-mouse Ly-6A/E (Sca-1) PE-Cy7	10 μL	1: 50
anti-mouse CD117 (c-Kit) APC	10 μL	1: 50
anti-mouse CD48 APC-Cy7	10 μL	1: 50
anti-mouse CD150 PE	10 μL	1: 50
Total	**60 μL**	**N/A**

Storage: Store at 4°C in the dark until use. This master mix can be stored at 4°C for up to 24 h.

10% Acrylamide gel solution		
Reagent	Final concentration	Amount
ddH_2O	N/A	2.05 mL
30% Acr/Bis (29:1) glue solution	10% (v/v)	1.65 mL
Tris (1.5 M, pH 8.8)	373 mM	1.25 mL
SDS (10%)	1.0% (v/v)	50 μL
APS (10%)	0.5% (v/v)	25 μL
TEMED	0.05% (v/v)	2.5 μL
Total	**N/A**	**5.0325 mL**

Storage: Prepare the solution freshly.

Acrylamide stack solution		
Reagent	Final concentration	Amount
ddH_2O	N/A	1.425 mL
30% Acr/Bis (29:1) glue solution	5.07% (v/v)	0.425 mL
Tris (1.0 M, pH 6.8)	249 mM	0.625 mL
SDS (10%)	1.0% (v/v)	25 μL
APS (10%)	0.05% (v/v)	12.5 μL
TEMED	0.1% (v/v)	2.5 μL
Total	**N/A**	**2.515 mL**

Storage: Prepare the solution freshly.

⚠ CRITICAL: The acrylamide stacking solution should be used immediately. 30% Acr/Bis (29:1) glue solution and TEMED are added immediately before usage.

⚠ CRITICAL: Acr/Bis glue solution and TEMED are toxic. Operators should wear masks and operate in a fume hood.

10× Running buffer		
Reagent	Final concentration	Amount
Tris	250 mM	30.3 g
Glycine	1.92 mM	144 g
SDS	1% (g/v)	10 g
ddH_2O	N/A	Up to 1,000 mL
Total	**N/A**	**1,000 mL**
Dissolve all reagents in 850 mL of ddH_2O. Add ddH_2O to 1,000 mL.		
Storage: Store at 4°C for up to 24 h.		

1× Running buffer		
Reagent	Final concentration	Amount
10× Running buffer	1×	100 mL
ddH_2O	N/A	900 mL
Total	**N/A**	**1,000 mL**
Storage: Store at 4°C before use. Prepare fresh each time.		

10% ammonium persulfate (APS)		
Reagent	Final concentration	Amount
APS	10% (g/v)	0.1 g
ddH_2O	N/A	Up to 1 mL
Total	**N/A**	**1 mL**
Dissolve 0.1 g APS into ddH_2O for a final volume of 1 mL.		
Storage: Store at 4°C. Keep fresh and use within 7 days.		

10% sodium dodecyl sulfate (SDS)		
Reagent	Final concentration	Amount
SDS	10% (g/v)	1 g
ddH_2O	N/A	Up to 10 mL
Total	**N/A**	**10 mL**
Dissolve 1 g SDS into ddH_2O to get a final volume of 10 mL.		
Storage: Store at 25°C–30°C. Solution remains viable for weeks to months, no need to make fresh each time.		

Tris (1.0 M, pH 6.8)		
Reagent	Final concentration	Amount
Tris	1.0 M	121.1 g
ddH_2O	N/A	Up to 1,000 mL
Total	**N/A**	**1,000 mL**
Dissolve 121.1 g Tris into ddH_2O, pH to 6.8 for a final volume of 1,000 mL.		
Storage: Store at 25°C–30°C for up to 1 month.		

Tris (1.5 M, pH 8.8)		
Reagent	Final concentration	Amount
Tris	1.5 M	181.7 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
ddH_2O	N/A	Up to 1,000 mL
Total	**N/A**	**1,000 mL**

Dissolve 181.7 g Tris into ddH_2O, pH to 8.8 for a final volume of 1,000 mL.
Storage: Store at 25°C–30°C for up to 1 month.

50× stock solutions

- 100× Protease Inhibitor.

Reagent	Final concentration	Amount
Protease Inhibitor	100×	1 tablet
ddH_2O	N/A	500 μL
Total	N/A	500 μL

Storage: Store at −20°C for up to 1 month, relevant experimental steps should be performed on ice.

- 100× Phosphatase inhibitor.

Reagent	Final concentration	Amount
Phosphatase inhibitor	100×	1 tablet
ddH_2O	N/A	500 μL
Total	N/A	500 μL

Storage: Store at −20°C for up to 1 month, relevant experimental steps should be performed on ice.

- 100× PMSF.

Reagent	Final concentration	Amount
Phenylmethanesulfonyl fluoride (PMSF)	100× (100 mM)	174 mg
Ethanol	N/A	10 mL
Total	N/A	10 mL

Storage: Store at −20°C for up to 1 month, relevant experimental steps should be performed on ice.

2× lysis buffer		
Reagent	Final concentration	Amount
10× RIPA lyse buffer	2×	200 μL
100× Protease Inhibitor	2×	20 μL
100× Phosphatase Inhibitor	2×	20 μL
100× PMSF	2×	20 μL
ddH_2O	N/A	740 μL
Total	N/A	1 mL

Storage: Store at 4°C until use. This master mix should be used immediately.

Other solutions	
Name	Reagents
10× PBS	Phosphate buffered saline; 1.37 M NaCl, 27 mM KCl, 80 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4
10× Tris buffered saline with Tween 20 (TBST)	Tris buffered saline with Tween 20; 50 mM Tris-HCl, 1.5 M NaCl, 0.5% Tween 20, pH 7.4-7.6
Total	N/A
Storage: Store at 4°C for up to 1 month.	

STEP-BY-STEP METHOD DETAILS

Isolation of murine bone marrow cells

Timing: ~ 2.5 h

This protocol describes the process for isolating mouse HSCs from a pooled sample of bone marrow obtained from tibias, femurs, humerus, pelvises, and vertebrae[8,9] (Figure 1).

1. Tissue collection.

⚠ CRITICAL: We crushed long bones and flat bones to collect bone marrow cells. We recommend removing the bone fragments after standing for 5 min and filtering the bone marrow cells with 40 μm cell strainers.

a. Euthanize mice with a method approved by the supervisory institution.
b. Sterilize the dissection areas and the external fur of the surgical sites with 70% (v/v) ethanol to reduce the risk of contamination.
c. Use scissors and scalpels to make a midline incision through the skin to expose the forelimbs (axillary to forefoot), hindlimbs (inner thigh to hindfoot), ilium, pelvises, and vertebras.
d. Use curved forceps to dislocate the tibias, femurs, humerus, pelvises, and vertebras in cold cell isolation buffer (1× PBS with 2% FBS).
e. Wash the bones with 70% (v/v) ethanol for 3–5 s and then transfer them into 2 mL of cold cell isolation buffer.
f. Use sterilized surgical instruments and tissues to remove muscle tissue from the bones.

⚠ CRITICAL: The number of mice required per replicate may vary based on the number of proteins to be detected and the sensitivity of antibodies. Typically, one to three 3–4-month-old mice are used for a single replicate.

2. Collecting bone marrow cells.
a. Mince the tibias, femurs, humerus, pelvises, and vertebrae into bone fragments.
b. Gently crush using a pestle and mortar with 2 mL of cold cell isolation buffer.
c. Transfer the supernatant to a 50 mL conical tube.
d. Re-suspend the bone fragments in 2 mL cold cell isolation buffer and repeat the steps 2b and 2c for 3–4 times until the bone fragments turn white.
e. Stand the 50 mL conical tube on ice for 5 min to allow the bone fragments to settle.
f. Carefully pipette the cell suspensions into a 15 mL conical tube passed through a 40 μm cell strainer (Falcon, Cat#352340) to obtain single cells.
g. Stand the 15 mL conical tube for 5 min to allow the bone fragments to settle.
h. Carefully collect the supernatant containing the bone marrow cells into a new 15 mL centrifuge tube, leaving 50–200 μL leftover with the bone fragments in the bottom of the original tube.

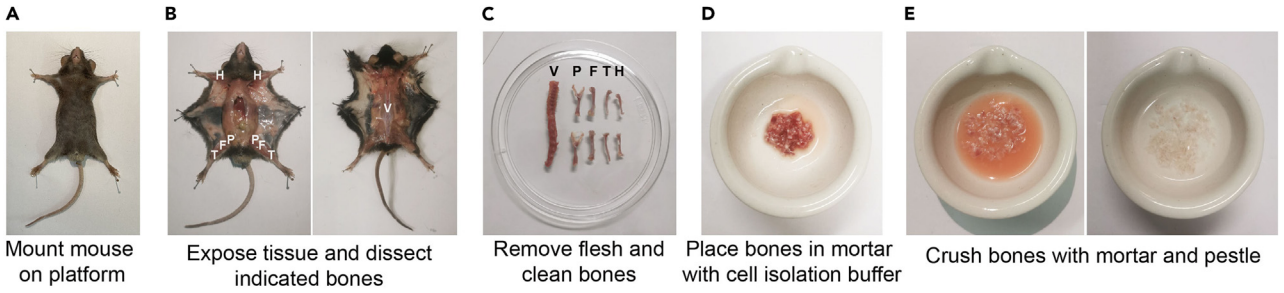


Figure 1. Schema for bone isolation and marrow digestion

(A) The euthanized mice were pinned to a dissection plate, and the surgical sites were sterilized with 70% (v/v) ethanol.

(B) The mice were dissected along the dorsal and ventral midline and length of the limbs to expose the underlying skeletal structures.

(C) The bones were cleaned and presented. V (vertebrae), P (pelvis), F (femur), T (tibia), and H (humerus).

(D) The bones were minced by scissors in 2 mL cell isolation buffer.

(E) The bone fragments (left) are crushed by pestle and mortar for 3–4 rounds to release bone marrow cells until the bone fragments turn white (right).

⚠ CRITICAL: The spinal cord should be removed from the vertebrae. To harvest bone marrow cells from bone fragments, we recommend crushing instead of grinding to obtain higher cell viability.

3. Lysis of red blood cells.
   a. Centrifuge the bone marrow suspension at 550 g for 5 min at 4°C.
   b. Carefully discard the supernatant and add 2 mL of ACK lysis buffer to the cell pellet. Incubate the suspension for 2 min at 25°C–30°C to lyse any remaining red blood cells.
   c. Add 10 mL cold cell isolation buffer to stop the lysis.
   d. Filter the cell suspension into a 15 mL conical tube through a 40 μm filter strainer to remove any remaining cell clumps or debris.

⚠ CRITICAL: Remove the supernatant with 50–200 μL leftover after 5-min-stand to help remove the bone fragments.

   e. Centrifuge the cell suspension again at 550 g for 5 min at 4°C.
   f. Discard the supernatant carefully not to disturb the cell pellet.
   g. Discard the supernatant and resuspend bone marrow cells in 500 μL cold cell isolation buffer. Count cell numbers for antibody staining.

⚠ CRITICAL: The total nuclear bone marrow cells from one 3–4 month-old mouse is ~1.5×10^8. The cell numbers were counted by Flow Cytometer Attune NxT (Thermo Fisher Scientific).

Antibody staining

Timing: ~ 1.5 h

HSCs are Lineage$^-$Sca1$^+$c-Kit$^+$ (LSK) CD48$^-$CD150$^+$ cells. The lineages cocktails include anti-CD3, anti-CD4, and anti-CD8 for T cells, anti-B220 and anti-IgM for B cells, anti-CD11b and anti-Gr-1 for myeloid cells, and anti-Ter-119 for erythroid cells. The color compensation and isotype controls were used to set up the instrument and gating strategies in a standard way.

4. Resuspend the cells in cell isolation buffer at a 1×10^5 cells per μL, and aliquot 500 μL cells in each 1.5 mL microcentrifuge tube.

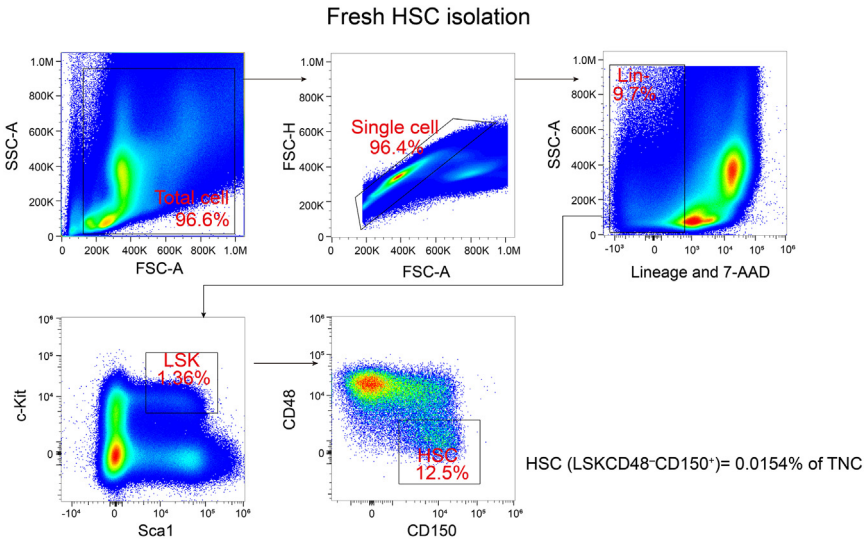


Figure 2. Gating Strategy for HSCs

The bone marrow Lineage$^-$Sca1$^+$c-Kit$^+$ (LSK)CD48$^-$CD150$^+$ HSCs were gated. TNC, Total Nucleated Cells.

5. Add 20 μL of lineage (PE-Cy5) antibody mix, 10 μL of anti-Sca-1 (PE-Cy7), 10 μL of anti-c-Kit (APC), 10 μL of anti-CD48 (APC-Cy7), and 10 μL of anti-CD150 (PE) antibodies into each tube.
6. Incubate the tubes at 4°C on a shaker in the dark for 1 h.

Optional: The Annexin V (FITC), DAPI, and anti-Ki67 (FITC) may be added in the HSC staining panel for cell cycle and apoptosis analysis.

7. After staining, add 1 mL cell isolation buffer to each tube, and centrifuge them at 550 g for 5 min at 4°C.
8. Discard the supernatant, and resuspend the cells in cell isolation buffer to a final concentration of 5 × 107 cells per mL. The stained cells can be stored at 4°C in the dark before sorting.
9. Add 7-AAD at final concentration 0.5 μg per mL, detected in the PE-1 Cy5 channel, for 5 min to 2 exclude dead and lineage-positive cells before sorting.

Optional: Lineage cell depletion or c-Kit$^+$ cell enrichment can be performed to save sorting time. However, the cell enrichment may result in up to 30% cell loss.

Cell sorting

Timing: 2–3 h per sample

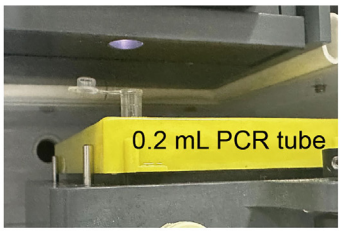
10. Start the sorter (BD FACS Aria III) for a standard quality control setup. Set the sorter with a 70 μm nozzle, a sorting speed of 20,000–30,000 events per second, sheath pressure at 70 pounds per square inch (psi), and drop drive frequency (ddf) set to 89 kHz, with a threshold rate maintained at 1/5–1/3 of ddf. Sort precision mode set to Purity Precision Mode.
11. Transfer 1 mL cell suspension to a 5 mL Polystyrene tube and filter the cells right before sorting. Turn on Sample Agitation to 100 g to prevent cell clumping. Use the sample temperature command to set the temperature inside the sample injection chamber at 4°C.
12. Use a 200 μL PCR tube as a collection tube during sorting. Coating the collection tube with FBS or BSA before adding the cell isolation buffer is recommended. Each collection tube can acquire up to 5×10^5 cells in 30 μL collection buffer (Figures 2 and 3).

A

BD FACS Aria III setup parameters	
Nozzle	70 μm
Sorting mode	Purity
Sheath Pressure	70 psi
Amplitude	60 volts
Frequency	89 kHz
Drop1	150
Gap (upper limit)	7 (14)
Maintain event rate	around 20,000-30,000 events/s

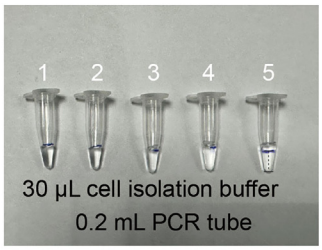
Parameters of the sorter (BD FACS Aria III)

B



Set collection 0.2 mL PCR tube

C



HSC collecting 0.2 mL PCR tubes

Figure 3. Schema for cell sorting

(A) The parameters used for cell sorting (BD FACS Aria III) were shown.

(B) A 200 μL PCR tube containing 30 μL of cell isolation buffer is used as a collection tube for cell sorting.

(C) The collection tubes were numbered from 1 to 5 and received 100, 500, 1,000, 5,000, and 10,000 HSCs, respectively.

⚠ CRITICAL: It usually takes 1–1.5 h to start and setup the sorters. Cell viability is critical for successful cell sorting; therefore, it is essential to sort the stained cells immediately. EPPENDORF PCR cooler 0.2 mL rack (Catalog no. 022510525) is recommended for holding the PCR tube and keeping the sample cool. Sorting 1.5 × 10^8 bone marrow cells from one 3–4-month-old mouse takes approximately 1.5 h, and the frequency of HSCs is around 0.015%. Typically, (2–2.5) × 10^4 HSCs are collected from one 3–4-month-old mouse. Always keep cells on ice or on a cooling rack during sorting. To maintain a high cell yield rate, it is crucial to aliquot the cell suspensions, filter them right before sorting, and turn on the sample agitation mode. If the sample agitation mode is unavailable for the sorter, the cells must be resuspended manually.

Post sort analysis

Timing: 10 min

13. The isolated HSCs were reanalyzed by Attune NxT (Thermo Fisher Scientific). The purity of sorted HSCs is typically over 90% (Figure 4).

Low-input cells western blotting

Timing: ~ 20 h

This step describes a western blot protocol suitable for low numbers of HSCs (100–10,000 cells) (Figures 5 and 6). Typically, 10,000 cells are used for one analysis, but highly abundant proteins can be detected with as few as 500 HSCs.

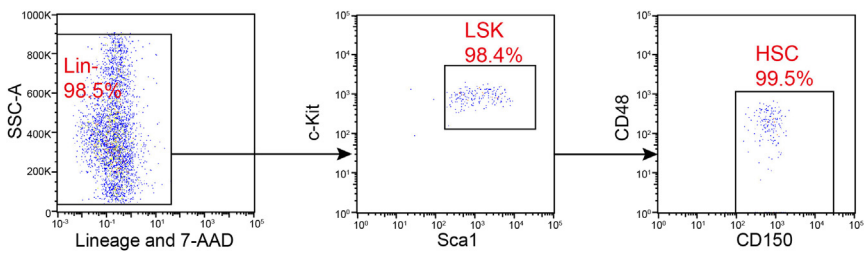


Figure 4. Post-sort analysis for isolated HSCs

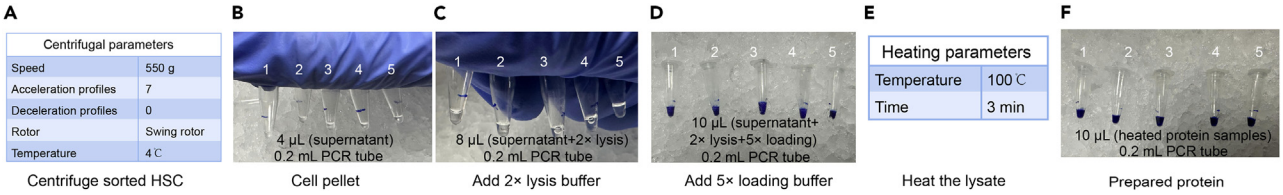


Figure 5. Schema for protein extraction

(A) The parameters of centrifugation for HSC preparation after sorting.

(B) HSC pellets in a 200 μL PCR tube with 4 μL leftover collection buffer.

(C) Add 4 μL of 2× lysis buffer to each collection tube.

(D) Add 2 μL of 5× loading buffer to each collection tube.

(E) Heat the protein samples at 100°C for 3 min.

(F) The collection tubes with heated protein samples from 100, 500, 1,000, 5,000, and 10,000 HSCs were numbered 1 to 5, respectively.

⚠ CRITICAL: Equal cell numbers are used for normalization.

14. Sample preparation and western blot.
    a. Insert the PCR tube with sorted 10,000 HSCs in a PCR tube holder and spin down the cells at 550 g in a swinging bucket rotor for 5 min at 4°C without a break.
    b. Gently remove the supernatant with a 10 μL pipette until ~ 4 μL cell suspension left.
    c. Add 4 μL of 2× lysis buffer and leave the 8 μL 1× lysis buffer on ice for 10 min 10,000 cells were lysed with 8 μL 1× lysis buffer.
    d. Add 2 μL 5× loading buffer into the 8 μL cell lysate, and heat the lysate on a heat block at 100°C for 3 min.

Pause point: The samples can be immediately used for western blot or stored at -80°C freezer after snap-freezing in liquid nitrogen.

    e. Modify the teeth of a Mini-PROTEAN 10-well 1.0 mm comb by cutting the width from 5 mm to 1.5 mm (Figure 6). Each well can accommodate up to 10 μL of cell lysate for loading.

⚠ CRITICAL: This is a critical step to increase the sensitivity of western blot for cells in small number. The 1.5 mm comb is a homemade creation in the lab.

    f. After electrophoresis, transfer the protein bands from the gel to a polyvinylidene difluoride (PVDF) membrane with standard protocol.

15. Antibody staining and detection.
    a. Incubate with primary antibodies (1:1000 in Primary antibody dilution buffer, Cat#P0023A, Beyotime) on a shaker at 4°C for 12–16 h.

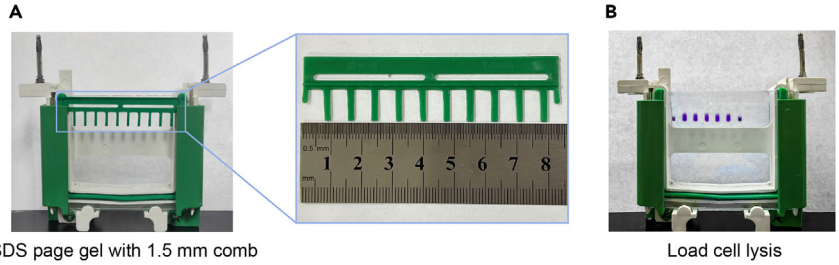


Figure 6. Schema for protein loading

(A) Homemade 1.5 mm width comb.

(B) Protein sample loading.

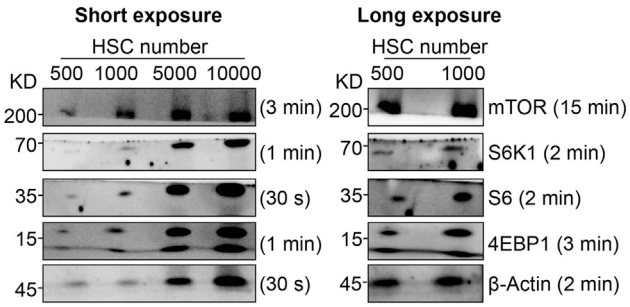


Figure 7. Western Blot for HSCs

The western blots with indicated cell numbers of HSCs for proteins as indicated. The exposure time for each protein band was indicated in the brackets.

b. Wash the membrane 3 times and incubate the membrane with HRP-conjugated secondary antibodies for 1 h at 25°C–30°C.
c. Use Western Chemiluminescent HRP Substrate with high sensitivity (WBKLS0050, Millipore) and Ultra-high sensitivity chemiluminescence imaging system(Bio-Rad ChemiDoc Touch) for western blot detection.

EXPECTED OUTCOMES

This protocol typically yields approximately 20,000 HSCs from individual 3–4-month-old mouse, which is sufficient for running 2–3 western blots to detect 4–5 antibodies, depending on the molecular weight and the antibody sensitivity of the targeted proteins. To detect multiple antibodies in the same protein-transferred PVDF membrane, the membrane can be cut based on molecular weight (Figure 7). The membrane can be used to detect low-abundance proteins first and then stripe the membrane to detect another more abundant protein. For one replicate experiment, 1–3 mice are used, depending on the number and sensitivity of the antibodies being detected. For three independent experiments, 3–9 mice are typically used.

LIMITATIONS

High-speed and high-performance sorting instruments are essential for long-term sorting. It is important to note that the HSC purity in this experiment is approximately 90%, and performing a double sort for further enrichment of purity is challenging due to additional cell loss. Moreover, the combs and SDS-page gels used were homemade and not commercially available. It is difficult to measure the low protein concentration; therefore, the cell numbers were used to normalize protein loading in the western blot analysis.

TROUBLESHOOTING

Problem 1

The cell viability is low (step 2a, 2b).

Potential solution

Use crush instead of grind method to extract bone marrow cells from minced bones and maintain all cell isolation steps on ice to preserve sample integrity. Perform cell sorting immediately after staining and ensure to keep the collection tube cool during the process.

Problem 2

Sort nozzle clogs during cell sorting (step 7).

Potential solution

To prevent leftover bone fragments or cell aggregation during sorting, carefully remove bone fragments before staining and filter the cell suspensions immediately prior to sorting. Avoid cell settlement during sorting by utilizing Sample Agitation or manually shaking the tube.

Problem 3

Cell loss during sample preparation (step 14b).

Potential solution

To prevent cell loss during sample preparation, use a centrifuge with a swinging bucket rotor and do not stop the rotor during the process. Hold the tube firmly and carefully remove the supernatant using a 10 μL pipette.

Problem 4

Difficulties in loading samples into western blot gels (step 14e).

Potential solution

As a small comb is used, traditional loading tips may encounter difficulties in loading samples and lead to sample loss. The QSP thin-loading tips (010-Q, Thermo Fisher Scientific) are recommended.

Problem 5

Low abundance of the target protein (step 15c).

Potential solution

For low abundant protein, we recommend increasing the number of cells for each western blot analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Meng Zhao (zhaom38@mail.sysu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

Conceptualization, M.Z. and C.L.; methodology, C.L. and Y.L.; investigation, C.L., J.G., Y.L., X.T., Y.Z., W.L., Huixuan Tian, Huiqi Tian, and Y.Y.; writing – review and editing, M.Z., C.L., and J.G.; funding acquisition, M.Z.; supervision, C.L. and M.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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