TECHNICAL BULLETIN

FLOW CYTOMETRY METHODS FOR IDENTIFYING MOUSE HEMATOPOIETIC STEM AND PROGENITOR CELLS

Background

The phenotypic characterization of mouse hematopoietic stem and progenitor cells (HSPCs), combined with functional cell-based assays, has greatly improved our understanding of the relationships between hematopoietic stem cells (HSCs), progenitor cells, and mature blood cells. The hematopoietic system is hierarchical in nature, with multipotent long-term HSCs (LT-HSCs) existing at the highest point on a branched hierarchy consisting of more proliferative progenitors further down the tree. While this relatively simple model has recently been challenged,[1-3] the fact remains that primitive HSPC subpopulations are uniquely responsible for generating the billions of mature blood cells that must enter the circulation every day. To regulate these tremendous demands, strict intrinsic and extrinsic mechanisms are in place to control HSC cycling, self-renewal, and differentiation. In order to properly study the complex mechanisms controlling these important cell populations, scientists require methods to identify, isolate, and quantify HSPCs.

Several commonly used methods for phenotyping and purifying mouse HSPCs have relied on the co-expression of c-Kit and Sca1 cell surface proteins, combined with an absence of lineage (Lin) markers that are typically present on mature blood cells such as T and B lymphocytes, NK cells, granulocytes, macrophages/monocytes, and red blood cells.[4] All multipotent hematopoietic cells are encompassed within the $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) fraction of the bone marrow (BM), however, the frequency of true HSCs within this population is only about 10%. Moreover, this phenotype is only appropriate for identifying HSPCs present in steady-state adult BM of certain mouse strains, e.g. C57Bl/6. Additionally, the LSK phenotype is not useful in other mouse strains, e.g. Balb/c, or if HSPCs are activated and proliferating (particularly upon in vitro culture), or from earlier timepoints of mouse embryonic development.[5-7]

The development of the SLAM code of markers, adjudicated by the markers CD150 and CD48, represented a major improvement for further enrichment of the LSK population of mouse HSPCs. The frequency of true HSCs within the $CD150^+CD48^-$ LSK (LSK/SLAM) population is about 40% in normal adult mouse BM.[8] This phenotype also enables improved resolution of HSCs in various experimental contexts and from embryonic sources.[7,9] The ESLAM phenotype ($CD45^+EPCR^+CD150^+CD48^-$) is notable as it does not use the traditional LSK panel. Instead, the SLAM markers are combined with the endothelial cell protein C receptor (EPCR) to increase the purity of isolated HSPCs.[10,11] Inclusion of the CD45 marker in this panel enables the exclusion of contaminating endothelial cells which, along with other cells, express EPCR, and helps standardize gates for flow cytometric-based analyses.[12]

Historically it has been difficult to perform phenotypic analyses of different HSPC subsets due to their low frequency (< 1 in 10,000 cells in BM), absence of specific cell surface markers, and innate functional heterogeneity. The continued evolution of flow cytometers, together with an increasing number of monoclonal antibodies and new fluorochromes to which these can be conjugated, has made methods for studying rare HSPC subsets more readily available.

This technical bulletin describes a general overview of methods for phenotyping LSK, LSK/SLAM, and ESLAM HSPCs from adult C57Bl/6 mouse BM using panels of fluorochrome-conjugated monoclonal antibodies (Tables 1 - 3). Tips for cell preparation and experimental design, including choosing fluorochromes and setting up proper controls, are also provided.

Flow Cytometry Considerations

Several technical aspects must be considered when performing multicolor flow cytometry experiments to identify rare populations of HSPCs.[13]

Choice of Fluorochrome

Given the many different configurations of lasers, band-pass filters, and detectors that exist in modern flow cytometers, it is critical to optimize antibody panels specifically for the user's particular instrument. It is preferable to use fluorochromes with high fluorescence intensities to facilitate the detection of antigens that are expressed at low levels, and conversely to reserve fluorochromes with weaker fluorescence levels to detect markers that are expressed at high levels. Proper titration experiments will help optimize concentrations and combinations of conjugated antibodies to be used together in a phenotyping panel.

The phenotyping panels described in Tables 1 - 3 include a cocktail of Lin markers conjugated to FITC, or an anti-CD45 antibody conjugated to Alexa Fluor® 488. As the relative fluorescence intensities of FITC and Alexa Fluor® 488 are significantly lower than most other fluorochromes, the antigens chosen for detection with these reagents are expressed at high levels. Additionally, although flow cytometry analysis software can automatically compensate overlap between fluorescence channels, it is still

important to minimize spectral overlap when choosing an antibody/fluorochrome combination, as this can impact the ability to detect the population of interest.

As no single specific marker exists for this population, HSPC subsets must be identified by the expression of combinations of markers. Although some high-end flow cytometers can detect up to 18 distinct fluorescence signals, inclusion of many fluorochromes into a phenotyping panel can make experimental design and data analysis difficult. One approach to keep the number of fluorochromes manageable is the use of a common channel to exclude cells that are not of interest, specifically mature blood cells and "late" progenitor cells that express Lin antigens such as CD3, CD11b, CD45R, Gr-1, and Ter119. Adult mouse HSPCs either lack or express only very low levels of Lin markers. Antibodies used to detect these antigens are all conjugated to FITC. In the examples shown in Figures 1 and 2, FITC-conjugated antibodies against a panel of Lin antigens were combined in a single panel to allow detection of the unwanted Lin^+ cells in one fluorescence channel. This frees up the other channels to distinguish HSPC subsets using other, more important markers in the staining panel.[14]

Staining Controls

Two types of controls are required to validate a multicolor flow cytometry panel: (i) "setup" controls, which consist of an unstained sample and single antibody-stained controls, and (ii) "gating" controls, also known as "fluorescence-minus-one" (FMO) controls.[14,15]

Setup controls are used to properly establish various cytometer parameters by facilitating the establishment of photomultiplier tube (PMT) voltage gains and determining the levels of spectral overlap between fluorescent reagents. Unstained cells help set PMT voltage settings and define boundaries to gate out dead cells, but their background fluorescence can differ from cells that have been stained with multiple reagents.[11] Single antibody-stained controls consist of cells stained separately with a single fluorescently labeled antibody. The use of these controls ensures that other reagents in the mix do not adversely affect the fluorescence of the antibody in question.

The use of matching isotype control antibodies, which were historically implemented in multicolor flow cytometry, are now considered to provide little value as they can differ in their background staining from the test reagents they are meant to be compared to.

FMO controls combine all antibodies except for the one of interest. These controls measure the specific fluorescence of a given fluorochrome in the context of other reagents present in the cocktail. They also serve as an important control when setting up gates to analyze target populations. Tables 4, 5, and 6 include FMO controls for the staining panels described in Tables 1, 2, and 3, respectively. Overall, once a phenotyping panel has been confirmed and validated, it may not be necessary to run all FMO and single antibody-stained controls on a routine basis, thus allowing one to conserve cells in small samples and reduce workload.

For both control and test samples, non-specific staining can be reduced by pre-incubating cells with serum (at ~10% v/v concentration) from the same species as the test antibodies and/or using an anti-CD16/32 antibody to block F_c receptor binding.

Compensation

Ideally, cells from the test sample will be used for all of the aforementioned controls. This provides the most accurate context for setting of PMT voltages and interpreting the fluorescence signals observed. However, using cells from precious experimental samples can be difficult when cell numbers are limited, when there are differences in autofluorescence between various cell subsets, or when the target population represents a very small percentage of the bulk population.[13] Compensation beads provide an excellent alternative to the use of cells for setup controls. Commercially available compensation beads bind antibodies with high affinity and thus emit a high fluorescence signal with a low coefficient of variation. To provide an optimal fluorescence signal it is important to titrate the amount of all antibodies.

Table 1. Mouse LSK Phenotyping Panel

CELL SURFACE MARKER	FLUOROCHROME	CLONE	CATALOG #
Lineage cocktail: CD3 CD11b CD45R Gr-1 Ter119	 FITC FITC FITC FITC FITC	 145-2C11 M1/70 RA3-6B2 RB6-8C5 Ter119	 60015FI 60001FI 60019FI 60028FI 60033FI
c-Kit	PE	2B8	60025PE
Sca1	APC	E13-161.7	60032AZ

Table 2. Mouse LSK/SLAM Phenotyping Panel

CELL SURFACE MARKER	FLUOROCHROME	CLONE	CATALOG #
Lineage cocktail: CD3 CD11b CD45R Gr-1 Ter119	 FITC FITC FITC FITC FITC	 145-2C11 M1/70 RA3-6B2 RB6-8C5 Ter119	 60015FI 60001FI 60010FI 60028FI 60033FI
c-Kit	PE	2B8	60025PE
Sca1	Biotin	E13-161.7	60032BT
CD48	APC	HM48-1	60162AZ
CD150	PECy7	TC15-12F12.2	60036CZ

Table 3. Mouse ESLAM Phenotyping Panel

CELL SURFACE MARKER	FLUOROCHROME	CLONE	CATALOG #
CD45	Alexa Fluor® 488	30-F11	60030AD
CD48	APC	HM48-1	60162AZ
CD150	PECy7	TC15-12F12.2	60036CZ
EPCR	PE	RMEPCR1560	60038PE

Table 4. Staining Matrix for the LSK Phenotyping Panel

	FITC Lin	PE c-Kit	7-AAD	APC Sca1
Unstained	-	-	-	-
Single Antibody-Stained Controls				
FITC	+	-	-	-
PE	-	+	-	-
7-AAD	-	-	+	-
APC	-	-	-	+
FMOs				
FITC-FMO	-	+	+	+
PE-FMO	+	-	+	+
7-AAD-FMO	+	+	-	+
APC-FMO	+	+	+	-
Experimental Samples				
Test Sample	+	+	+	+

Table 5. Staining Matrix for the LSK/SLAM Phenotyping Panel

	FITC Lin	PE c-Kit	7-AAD	APC CD48	PE-Cyanine7 CD150	Biotin Sca1 + SA-BV421
Unstained	-	-	-	-	-	-
Single Antibody-Stained Controls						
FITC	+	-	-	-	-	-
PE	-	+	-	-	-	-
7-AAD	-	-	+	-	-	-
APC	-	-	-	+	-	-
PECy7	-	-	-	-	+	-
BV421	-	-	-	-	-	+
FMOs						
FITC-FMO	-	+	+	+	+	+
PE-FMO	+	-	+	+	+	+
7-AAD-FMO	+	+	-	+	+	+
APC-FMO	+	+	+	-	+	+
PECy7-FMO	+	+	+	+	-	+
BV421-FMO	+	+	+	+	+	-
Experimental Samples						
Test Sample	+	+	+	+	+	+

Table 6. Staining Matrix for the ESLAM Phenotyping Panel

	AF488 CD45	PE EPCR	7-AAD	APC CD48	PECy7 CD150
Unstained	-	-	-	-	-
Single Antibody-Stained Controls					
AF488	+	-	-	-	-
PE	-	+	-	-	-
7-AAD	-	-	+	-	-
APC	-	-	-	+	-
PECy7	-	-	-	-	+
FMOs					
AF488-FMO	-	+	+	+	+
PE-FMO	+	-	+	+	+
7-AAD-FMO	+	+	-	+	+
APC-FMO	+	+	+	-	+
PECy7-FMO	+	+	+	+	-
Experimental Samples					
Test Sample	+	+	+	+	+

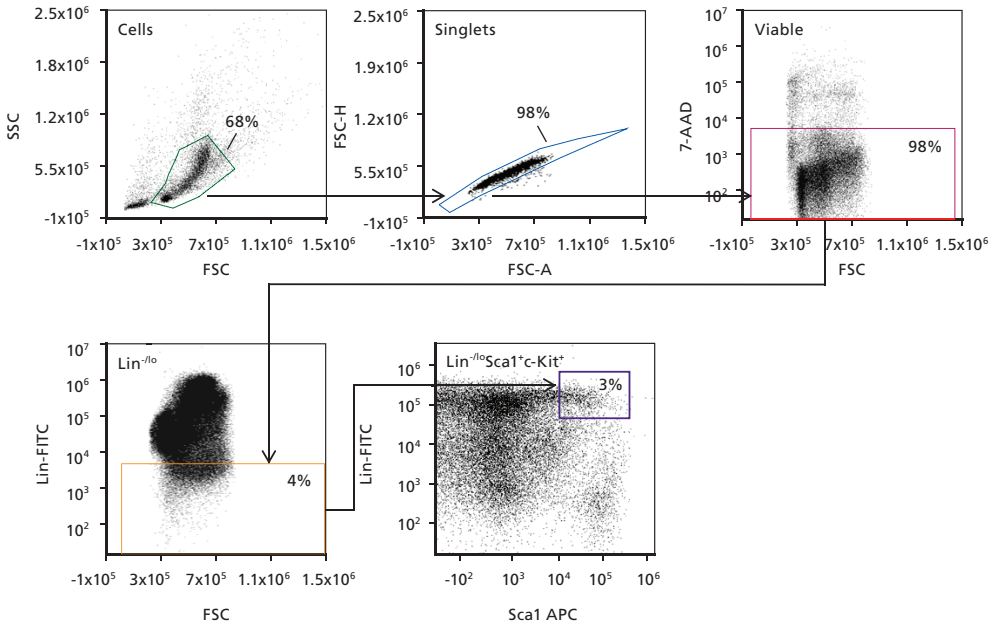
General Protocol for Cell Isolation, Preparation, and Staining

To isolate, prepare, and stain BM cells for flow cytometric analysis of HSPCs, follow the general protocol below:

1. Isolate cells from mouse femora and tibiae by flushing bones with 1 - 3 mL phosphate-buffered saline (PBS) (without Mg^{2+} and Ca^{2+}) supplemented with 5 mM EDTA plus 1% fetal calf serum. Flushing may be done using a 21 - 26G needle attached to a 1 - 10 mL syringe.
2. Generate a single-cell suspension by gently triturating the cells through the needle until large clumps are no longer present. Note: The number of harvested BM cells can be increased by including other bones such as the iliac crest or the spinal column. Crushing the bones using a mortar and pestle has also been shown to significantly improve the recovery of LSK cells.[14]
3. Lyse red blood cells (RBCs) by adding cold ammonium chloride solution and incubating BM cells on ice for 10 minutes.
4. Following RBC lysis, top up the sample with an appropriate volume of PBS or similar wash buffer, and centrifuge at 300 x *g* for 5 minutes at room temperature (RT; 15 - 25°C). Resuspend the cell pellet in PBS and filter through a 70 µm filter to remove aggregated cell clumps and debris.
5. Count the cells in the filtered suspension and dilute to 1 x 10^7 cells/mL in the same medium. The expected recovery of nucleated cells from both tibiae and femora (i.e. 4 long bones) of a normal male C57Bl/6 mouse is approximately 4 - 5 x 10^7 cells.
6. To reduce non-specific binding of fluorescent antibodies, block F_c receptors by incubating cells with anti-CD16/32 (clone 2.4G2) antibody and 10% rat serum for 10 minutes on ice.
7. For single antibody-stained controls, aliquot 3 x 10^4 cells per tube and stain with optimized concentrations of individual antibodies in a total volume of ~100 µL.
8. In parallel, set up FMOs and test samples by aliquoting 2 - 3 x 10^6 cells per tube and stain with optimized concentrations of antibodies.
9. Incubate cells for 20 minutes on ice, in the dark.
10. Wash cells in an appropriate volume of PBS and centrifuge at 300 x *g* for 5 minutes at RT, then aspirate the supernatant.
11. Resuspend cells in 100 µL PBS.
12. Just prior to acquisition, add 1 µg/mL 7-AAD to all samples, except unstained, single antibody-stained, and 7-AAD-FMO controls. Addition of 7-AAD to separate unstained and single antibody-stained controls should be considered if there are many dead or apoptotic cells that can cause increased autofluorescence or non-specific staining. Note: This general protocol uses 7-AAD to detect non-viable cells, but other viability dyes such as propidium iodide (PI) or amine-reactive dyes may also be used.
13. If using compensation beads, stain controls according to the manufacturer's directions. To provide an optimal fluorescence signal, it is important to titrate the antibody so that the highest staining index is achieved.
14. Typically 1 x 10^4 events are acquired for unstained and single antibody-stained controls. The minimum number of events to be aquired from multiply-stained samples can vary, however, since the frequency of the various HSPC subpopulations within the BM is typically very low, it is helpful to acquire a large number of events (as many as a million or more may be necessary for small HSPC subsets).

Gating

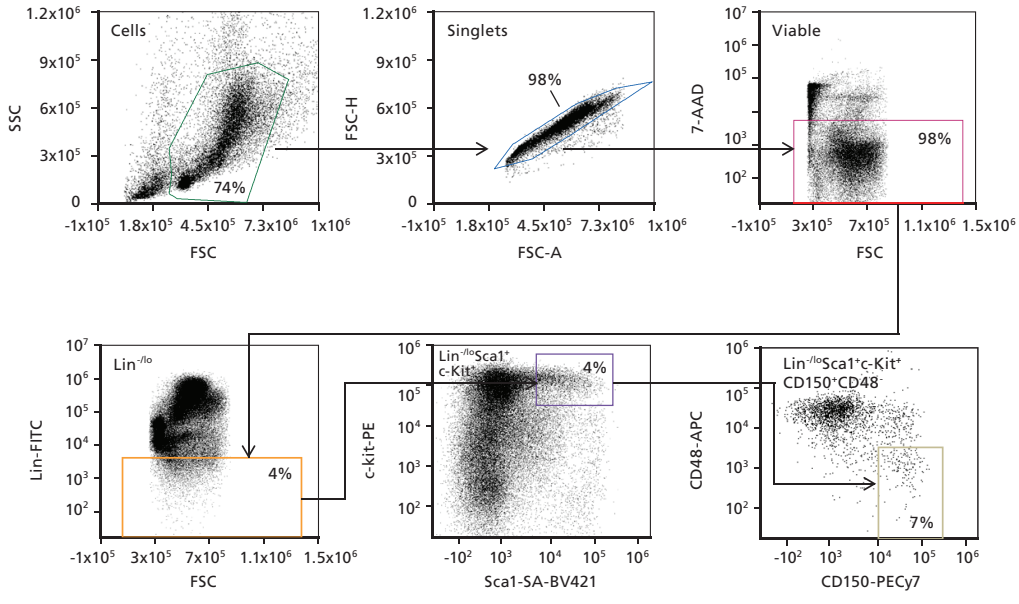
Several gating strategies can be employed to eliminate unwanted events prior to phenotyping HSPCs. Knowing the forward scatter (FSC) and side scatter (SSC) characteristics of your cells of interest can aid in proper gating. HSPCs are relatively uniform in size and lack intracellular complexity. As shown in Figures 1 - 3, the use of a tight FSC or SSC gate allows for the inclusion of small, non-granular cells that contain HSPCs while excluding dead cells, debris, and myeloid cells that are large and have lobular nuclei and/or a granular cytoplasm. FSC-area (FSC-A) and -height (FSC-H) parameters are also used to exclude cell doublets or triplets, which may be interpreted as cells co-expressing multiple markers. SSC signals can also be used to discriminate singlet events. Dead cells typically exhibit a greater autofluorescence than live cells and can non-specifically bind antibodies, thus appearing as false positives in the sample. Their exclusion improves the detection of live cells that express antigens of interest but at lower levels of fluorescence. After such pre-gating steps have been implemented, the frequency of LSK and LSK/SLAM HSCs can be determined by gating out Lin^+ cells, followed by the analysis of cells expressing c-Kit, Sca1 (Figure 1), and SLAM markers (Figure 2). To identify ESLAM HSCs, follow the steps to pre-gate as above, and then set gates to identify $CD45^+EPCR^+$ cells followed by the analysis of the $CD150^+CD48^-$ population (Figure 3).



Population	# of Events	% of Gated Cells	% of All Cells
No gate	1022592	N/A	100
Cells	699595	68	66
Singlets	687887	98	67
Viable	677325	98	66
Lin-/lo	28401	4	3
Lin-/loSca1+c-Kit+	977	3	0.1

Figure 1. Gating Strategy to Phenotype Mouse LSK HSCs

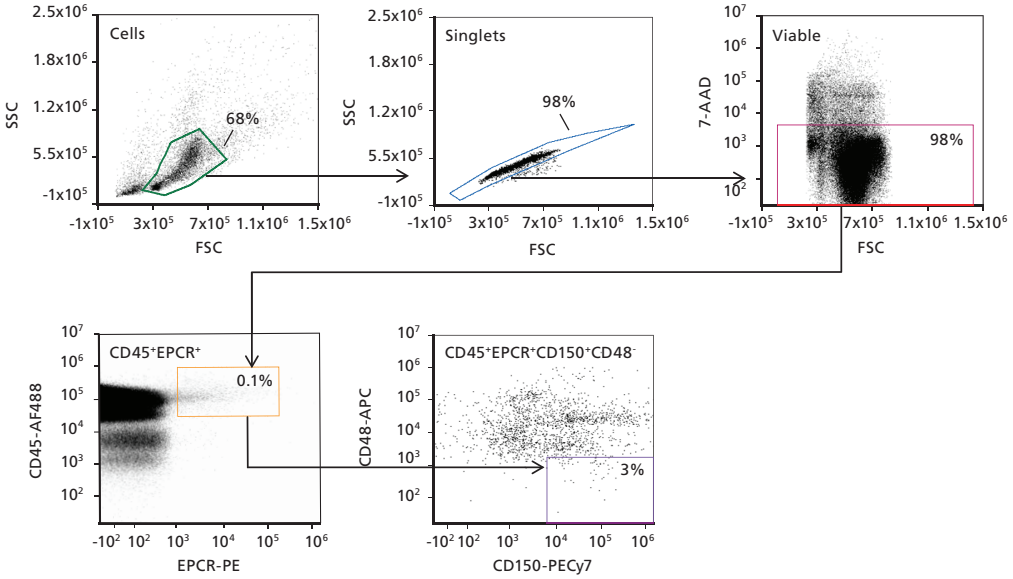
Adult C57Bl/6 BM cells were stained with a FITC lineage cocktail, PE c-Kit, and APC Sca1 antibodies. Primitive cells are initially refined using a light scatter gate followed by the exclusion of doublets and dead cells. The LSK population is identified by first excluding Lin$^+$ cells followed by gating on the Sca1$^+$c-Kit$^+$ population. Population subset analysis of the number and percent of cells in gated regions and within the entire population, is shown.



Population	# of Events	% of Gated Cells	% of All Cells
No gate	2000000	N/A	100
Cells	1475601	74	74
Singlets	1442238	98	72
Viable	1412164	98	71
Lin-/lo	57071	4	3
Lin-/loSca1+c-Kit+	2454	4	0.1
Lin-/loSca1+c-Kit+CD150+CD48-	180	7	0.01

Figure 2. Gating Strategy to Phenotype Mouse LSK/SLAM HSCs

Adult mouse C57Bl/6 BM cells were stained with a FITC lineage cocktail, PE c-Kit, Biotin Sca1, APC CD48, and PECy7 CD150 antibodies. Binding of the Biotin Sca1 antibody was revealed by secondary staining with streptavidin conjugated to BV421 (SA-BV421). The hematopoietic progenitor population was initially refined using a light scatter gate followed by discrimination of doublets and dead cells. Lin$^+$ cells were excluded, followed by gating on the Sca1$^+$c-Kit$^+$(LSK) population. CD150$^+$CD48$^-$ cells within the LSK population were identified and quantitated in the final gating step. Population subset analysis of the number and percent of cells in gated regions and within the entire population, is shown.



Population	# of Events	% of Gated Cells	% of All Cells
No gate	2000000	N/A	100
Cells	1358778	68	68
Singlets	1337125	98	67
Viable	1310053	98	66
$CD45^+EPCR^+$	1856	0.1	0.1
$CD45^+EPCR^+CD150^+CD48^-$	51	3	0.002

Figure 3. Gating Strategy to Phenotype Mouse ESLAM HSCs

Adult mouse C57Bl/6 BM cells were stained with Alexa Fluor® 488 CD45, APC CD48, PECy7 CD150, and PE EPCR antibodies. The hematopoietic progenitor cell population was initially refined using a light scatter gate followed by discrimination of doublets and dead cells. The population was then gated on $CD45^+EPCR^+$, followed by gating on $CD150^+CD48^-$ cells. Population subset analysis of the number and percent of cells in gated regions and within the entire population, is shown.

Summary

Studies of hematopoiesis, and HSC biology in particular, are greatly facilitated by the use of highly purified populations of primitive cells whose phenotype has been very well defined by the work of many investigators over the past 30 years. The procedures described herein highlight the relative ease of interrogating the phenotype of mouse HSCs by flow cytometry using three independent antibody staining panels described in the literature. Additional pre-enrichment procedures, not described herein but employed before antibody staining and sorting, can be used to increase the frequency of HSPCs. This approach can reduce fluorescence-activated cell sorting (FACS) time and facilitate more reliable detection (and isolation) of rare subsets under certain experimental conditions. $Lin^{-/lo}$ cells can be pre-enriched using the EasySep™ Mouse Hematopoietic Progenitor Cell Isolation Kit (Catalog #19856) for this purpose. By combining the correct antigen-fluorochrome combinations with the preparation of proper controls, flow cytometry is a powerful tool for studying mouse hematopoiesis.

Recommended Products

PRODUCT	CATALOG #
Anti-Mouse CD117 Antibody, Clone 2B8	60025PE
Anti-Mouse Sca-1 Antibody, Clone E13-161.7	60032AZ 60032BT
Anti-Mouse CD48 (SLAMF2) Antibody, Clone HM48-1	60162AZ
Anti-Mouse CD150 Antibody, Clone TC15-12F12.2	60036CZ
Anti-Mouse CD45 Antibody, Clone 30-F11	60030AD
Anti-Mouse EPCR Antibody, Clone RMEPCR1560 (1560)	60038PE

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