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Improving the Accuracy of Flow Cytometric Assessment of Mitochondrial Membrane Potential in Hematopoietic Stem and Progenitor Cells Through the Inhibition of Efflux Pumps

Claudia Morganti[1,2,3], **Massimo Bonora**[1,2,3], **Keisuke Ito**[1,2,3,4]

[1]Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine

[2]Departments of Cell Biology and Stem Cell Institute, Albert Einstein College of Medicine

[3]Department of Medicine, Albert Einstein College of Medicine

[4]Albert Einstein Cancer Center and Diabetes Research Center, Albert Einstein College of Medicine

Abstract

As cellular metabolism is a key regulator of hematopoietic stem cell (HSC) self-renewal, the various roles played by the mitochondria in hematopoietic homeostasis have been extensively studied by HSC researchers. Mitochondrial activity levels are reflected in their membrane potentials (ΔΨm), which can be measured by cell-permeant cationic dyes such as TMRM (tetramethylrhodamine, methyl ester). The ability of efflux pumps to extrude these dyes from cells can limit their usefulness, however. The resulting measurement bias is particularly critical when assessing HSCs, as xenobiotic transporters exhibit higher levels of expression and activity in HSCs than in differentiated cells. Here, we describe a protocol utilizing Verapamil, an efflux pump inhibitor, to accurately measure ΔΨm across multiple bone marrow populations. The resulting inhibition of pump activity is shown to increase TMRM intensity in hematopoietic stem and progenitor cells (HSPCs), while leaving it relatively unchanged in mature fractions. This highlights the close attention to dye-efflux activity that is required when ΔΨm-dependent dyes are used, and as written and visualized, this protocol can be used to accurately compare either different populations within the bone marrow, or the same population across different experimental models.

Keywords

Biology; Issue 149; mitochondrial membrane potential; hematopoietic stem cell; TMRM; verapamil; flow cytometry; efflux pumps; multi drug resistance

Correspondence to: Keisuke Ito at keisuke.ito@einstein.yu.edu.

Video Link

The video component of this article can be found at https://www.jove.com/video/60057/

Disclosures

The authors have nothing to disclose.

Introduction

Hematopoietic stem cells (HSCs) are self-renewing, multi-potent, and capable of giving rise to all the cells of the blood[1,2]. Cellular metabolism is a key regulator of HSC maintenance, along with transcriptional factors, intrinsic signals and the microenvironment[3,4,5]. The proper control of mitochondrial function and quality is therefore critical to HSC maintenance[6,7].

Mitochondrial membrane potential ($\Delta\Psi m$) is a key parameter in the assessment of mitochondria as it directly reflects their functionality, which derives from the equilibrium of proton pumping activity in the electron transport chain and the proton flow through F_1/F_O ATP synthase. These are both required (depending on gene expression and substrate availability) for the oxygen-dependent phosphorylation of ADP to ATP[8,9]. Taking advantage of the electronegativity of the mitochondrial compartment, various potentiometric dyes have been developed to measure $\Delta\Psi m$. One of them is tetramethylrhodamine methyl ester perchlorate (TMRM), which has been extensively used to measure $\Delta\Psi m$ by flow cytometry in a variety of cells[10], including hematopoietic stem and progenitor cells[11].

Mitochondrial dyes must be used with some caution in HSCs, however, because the high activity of the xenobiotic efflux pumps of these cells can result in dye extrusion[12]. Indeed, the extrusion of mitochondrial dyes such as Rhodamine 123 has allowed researchers to isolate HSCs[13] or identify HSC “side populations” by exploiting the differential extrusion of the dyes Hoechst Blue and Hoechst Red[14,15]. It has also been shown that Fumitremorgin C, a specific blocker of the ATP-binding cassette sub-family G member 2 (ABCG2) transporter, does not affect the staining pattern of MitoTracker in HSPCs[16]. After the publication of these results, multiple studies were performed using mitochondrial dyes in the absence of xenobiotic efflux pump inhibitors, leading to the widespread impression that HSCs have only a small number of mitochondria with low $\Delta\Psi m$[16,17,18].

Recently, it was demonstrated, however, that Verapamil, a wide spectrum inhibitor of efflux pumps, significantly modifies the staining pattern of the mitochondrial dye MitoTracker Green[19]. This discrepancy is likely due to the fact that Fumitremorgin C is highly selective for Abcg2, while HSCs also express other transporters such as Abcb1a (which is only weakly sensitive to Fumitremorgin C)[19]. We have also reported that other mitochondrial dyes, such as TMRM, Nonyl acridine orange, and Mitotracker Orange (MTO) exhibit the same patterns as Mitotracker Green. More importantly, we have observed that the flow cytometric patterns of HSPCs reflect their $\Delta\Psi m$ in addition to mitochondrial mass[11].

The intake of TMRM dye strictly depends on the negative charge of mitochondria, but the resulting accumulation of dye is in constant balance between its intake and clearance by efflux pumps[20]. The difference in xenobiotic efflux pump expression between HSCs and mature cell populations affects this balance and can lead to biased results. The use of dedicated inhibitors such as Verapamil should be considered in the analysis of $\Delta\Psi m$ by potentiometric dyes. Here we describe a modified protocol for accurate $\Delta\Psi m$ measurement by TMRM-based flow cytometry which corrects for xenobiotic transporter activity through the use of dedicated inhibitors.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Albert Einstein College of Medicine.

1. Preparation of Solutions

1. Staining Buffer (phosphate-buffered saline (PBS) + 2% fetal bovine serum (FBS)): Add 10 mL of FBS in 500 mL of a sterile PBS solution.

   NOTE: This solution can be stored at 4 °C for at least one month in sterile condition. Before starting the following procedures, put an aliquot of this solution (50 mL) on ice.

2. ACK (ammonium-chloride-potassium) lysing buffer: Place an aliquot of ACK lysing buffer (1 mL) on ice before starting the procedure.

   NOTE: To prepare the same non-commercial buffer, dissolve 8.02 g of NH_4Cl, 1 g of $KHCO_3$ and 37.2 mg of Na_2EDTA in 1 L of H_2O. Adjust the pH to 7.2–7.4. Store for up to 6 months at room temperature.

3. Culture medium: Add 50 ng/mL stem cell factor (SCF) and 50 ng/mL thrombopoietin (TPO) to serum-free medium for culture and expansion of hematopoietic cells (see Table of Material for commercial medium recommended).

4. TMRM stock solution: Prepare TMRM (1 μM) solution by dissolving 5 μg of TMRM powder in 10 mL of ethanol. Store this solution at −20 °C protected from the light.

5. Verapamil stock solution: Prepare Verapamil (50 mM) solution by diluting 24 mg of Verapamil in 1 mL of ethanol. Store this solution at −20 °C.

6. FCCP stock solution: Prepare carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) (1 M) solution by dissolving 254 mg in 1 mL of ethanol. Store this solution at −20 °C protected from the light.

2. Bone Marrow Isolation

1. Euthanize the mouse by CO_2 inhalation following institutional guidelines and spray the mice with 70% ethanol.

   NOTE: This step prevents contamination of the cells of interest without compromising experimental results.

2. Using a pair of forceps and sharp scissors, make a small snip in the ventral skin of the mouse and stretch the skin.

3. Extract the femur and tibia while taking care not to dislodge the heads of the femur as they contain a large amount of bone marrow cells. Place the removed bones in a 6-well plate filled with 1.5 mL of staining buffer.

   NOTE: This procedure does not require sterile area.

4. Remove the muscles from the bones and cut the ends of the bones allowing the exit of the bone marrow (Figure 1A). Place the cleaned bones in new wells with 1.5 mL of staining buffer.

   NOTE: Muscles and other tissues must be carefully removed to avoid syringe clogging in the next step.

5. Flush out the bone marrow using a 3 mL syringe with a 25 G needle.

   NOTE: Continue to flush the bone marrow until the bone becomes white (Figure 1B).

6. Collect all cells in a 1.5 mL tube, centrifuge for 5 min at 180 × *g* then discard the supernatant (Figure 1C).

7. Resuspend the pellet in 300 μL of ice cold ACK lysing buffer very carefully, put on ice for 1 min and immediately inactive lysis by adding 1 mL of staining buffer. Centrifuge for 5 min at 180 × *g*.

   NOTE: The cells pellet appears white (Figure 1D). The total number of cells isolated is about $2-4 \times 10^7$.

8. Resuspend the pellets in 1 mL of staining buffer and filter using a cell-strainer cap (12×75 mm^2, 5 mL of capacity) with a 35 μm nylon mesh incorporated to obtain mononuclear cells. After blocking, keep the sample on ice.

3. Immunostaining for Detection of HSC

1. Prepare Lineage (Lin) cocktail. In 400 μL of staining buffer, add 4 μL of the following biotinilated antibodies against CD3e, CD4, CD8, B220, CD11b, Gr1, Ter119, CD19, Nk1.1, IgM and Il7Ra to obtain a final dilution 1:100.

2. Prepare fluorophores conjugated-antibodies (Abs). In 400 μL of staining buffer, add 4 μL of the following Abs to obtain a final dilution 1:100. Streptavidin-Pacific Blue, Sca1-PE/Cy7, c-kit-APC/Cy7, CD48-APC, CD150-PerCP/Cy5.5 and CD34-FITC. Keep in ice, protected from light.

3. Centrifuge the sample for 5 min at 180 × *g*, then discard the supernatant.

4. Add 400 μL of Lin cocktail solution to the cells pellet. Add 4 μL of CD135-biotinilated Ab. Vortex quickly to mix and incubate for 30 min in ice.

5. Wash the sample adding 3 mL of staining buffer, spin down for 5 min at 180 × *g* and discard the supernatant.

6. Add 400 μL of Abs solution. Vortex quickly to mix and incubate for 30 min on ice.

7. Wash the samples with 3 mL of staining buffer, spin down for 5 min at 180 × *g* and discard the supernatant.

4. TMRM Staining

1. Prepare TMRM staining solution. Add 2.2 µL of TMRM stock solution and 1.1 µL of Verapamil (2 nM and 50 µM as final concentration, respectively) in 1.1 mL of serum-free medium for culture and expansion of hematopoietic cells (see Table of Material for commercial medium recommended) with TPO and SCF.

   NOTE: This is the most important step of the protocol. Verapamil addition is necessary to block the efflux pumps which are highly expressed in the HSCs and can extrude TMRM.

2. Resuspend the bone marrow in 1 mL of TMRM staining solution, vortex quickly and incubate for 1 h at 37 °C.

   NOTE: TMRM staining must not be washed out. PE-dedicated compensation control is also resuspended in 100 µL of TMRM staining solution, and subjected to incubation for 1 h at 37 °C after quick vortex.

3. Filter the sample using a cell-strainer cap (12 × 75 mm^2, 5 mL of capacity) with a 35 µm nylon mesh incorporated to avoid clogging of the flow cytometer.

   NOTE: TMRM staining increases the possibility of clog formation in the sample. Filtration of the sample just before flow assays is recommended.

4. Add 1 µL of 4',6-diamidino-2-phenylindole (DAPI) to exclude dead cells by flow cytometry.

5. Acquisition by Flow Cytometer

1. Run bone marrow sample and acquire at least 1 × 10^6 events.

2. Set up the gating strategy to identify the different hematopoietic populations (Figure 2).

   1. Display live bone marrow mono-nuclear cells (BM-MNCs), $DAPI^-$ fraction, in plot for Pacific Blue to identify $CD135^-Lin^-$ (Lin^-) and Lin^+ fractions.

   2. Plot Lin^- fraction for APC/Cy7 (c-kit) versus PE/Cy7 (Sca-1) to identify multipotent progenitors (MPP) fraction, as $c\text{-}kit^+$ and $Sca\text{-}1^+$.

   3. Plot MPP fraction for APC (CD48) versus PerCP/Cy5.5 (CD150) to identify HSC fraction, as $CD150^+$ and $CD48^-$.

   4. Display HSC fraction for FITC (CD34) to divide $CD34^-$-HSC and $CD34^+$-HSC.

3. Acquire the TMRM intensity (PE channel) in each population.

4. After acquisition, add to the sample 1 µL of FCCP to obtain the final concentration of 1 mM and incubate at 37 °C for 5 min, then acquire 1 × 10^6 events.

NOTE: FCCP is a mitochondrial uncoupler which is used to dissipate the ΔΨm. It is used as experimental control to confirm that TMRM staining works correctly. After the administration of FCCP, the TMRM intensity should drastically be decreased (Figure 3A).

5. Analyze data normalizing the average intensity of PE of each population by the intensity of PE of all BM-MNCs.

Representative Results

The protocol described above enables the easy isolation of BM-MNCs from a mouse model. Figure 1 summarizes the main steps of the protocol: bone isolation, flushing out of the bone marrow, red blood cell lysis, and antibody staining followed by TMRM staining to measure mitochondrial membrane potential in a specific hematopoietic population.

BM-MNCs contain several cell populations, including HSCs. The antibody cocktails used in this protocol are well-established in the purification of HSCs ($CD34^-$ and $CD34^+$), multipotent progenitor cells (MPPs), Lin^- as well as Lin^+ cells, respectively[21]. The gating strategy for isolating these fractions is shown in Figure 2.

After the identification of populations of interest, TMRM intensity, which should appear as a bright signal, was assessed. TMRM staining in serum-free expansion medium (SFEM) is highly recommended, as TMRM profiles in HSPCs can undergo alteration when staining is performed in PBS +2% FBS (Figure 3A).

Figure 3C shows the average intensity of each population, which is normalized by the intensity of BM-MNCs. HSCs express high activity levels of xenobiotic efflux pumps capable of extruding TMRM dye[20], and indeed, we found TMRM profiles in HSPCs were changed in the presence of Verapamil (Figure 3B,C). Similar results were obtained by other inhibitors such as Cyclosporin H (Figure 3C). Thus, the accurate amount of TMRM loaded in the mitochondria by ΔΨm can be measured after inhibition of the efflux pumps by Verapamil or Cyclosporin H (Figure 3C).

Finally, FCCP can be used to verify the accuracy of TMRM staining. FCCP depolarizes mitochondria, resulting in a reduction in TMRM intensity (Figure 3D). This approach can also be used to determine the background intensity of the staining and/or as a negative control.

Discussion

Mitochondrial membrane potential measurement is a cornerstone of the analysis and assessment of mitochondria, which are critical to the metabolic state of the cell. Here, we describe a protocol for the analysis of ΔΨm by TMRM staining. TMRM is a cell-permeant fluorescent dye which accumulates in active mitochondria due to ΔΨm, and its respective levels remain in equilibrium between the extracellular, cytoplasmic and mitochondrial compartments[10]. This protocol can be adapted for various dyes, including tetramethylrhodamine, ethyl ester (TMRE) and JC-1. Appropriate staining conditions are

critical to achieving accurate results. These include: protection from light exposure, proper incubation time, maintenance of constant temperature (37 °C) and extracellular concentration. When TMRM dye levels have not yet reached perfect equilibrium, unexpected changes in staining intensity can be detected during data acquisition in the flow cytometry process. Therefore the recommended staining period for TMRM is 1 h rather than 30 min (as mentioned in one TMRM manufacturer's protocol). It is also suggested that the sample should be acquired at least two times within 5-min in order to compare the stability of the dye.

Another critical step of the protocol is the setting up of an efficient color match among antibodies and dye. Hematopoietic populations can be detected by flow cytometry using well-established surface markers[21]. The gating strategy described here (Figure 2) is compatible with TMRM staining, and allows for the measurement of its intensity at different differentiation stages simultaneously. TMRM matches the PE channel, but its intensity is usually much weaker than that of antibodies conjugated with PE (data not shown). As this can affect color compensation, causing unexpected shifts in the spectra of some populations, the sample with TMRM staining should be used as a compensation control for the PE channel.

The major advantage of the current protocol is that it enables the removal of the effect of xenobiotic efflux pumps, whose efficient extrusion of TMRM dye modifies both its equilibrium across the plasma membrane and its mitochondrial accumulation. This is a critical step in the accurate assessment of HSC mitochondrial function by dye absorption. As HSCs express more active efflux pumps than committed cells[19], Verapamil or similar drugs, such as Cyclosporine H, a Ca^{2+}-independent multidrug resistance inhibitor[22] (Figure 3B–C), should be used in the staining procedure for HSCs to more accurately assess levels of mitochondrial activity. Because this critical issue was pointed out only recently[11,19] and is still under discussion[23], the main aim of this report is to provide an easy and detailed protocol which will help to standardize the procedure for obtaining reproducible data and reduce the apparent discrepancies between the results of different research groups. The protocol here described shows in detail each step, including doses, timing and specific media. For instance, TMRM profiles in HSPCs can differ depending on their medium (Figure 3A). A detailed analysis of the mechanisms involved will require further exploration, but since serum-free expansion medium (SFEM) is a well-established method for HSC culture, SFEM rather than PBS is strongly recommended when performing TMRM staining for HSCs. Further, the accurate assessment of mitochondrial membrane potential demonstrated here through the inhibition of efflux pumps highlights possible future applications of Verapamil, as well as other dyes (e.g. MitoTracker, MitoSOX), in the investigation of HSCs.

Importantly, TMRM intensity as shown by flow cytometry may not precisely reflect mitochondrial volume. Flow cytometry measures total fluorescence intensity per cell, but the distribution of mitochondrial dyes to mitochondria depends on $\Delta\Psi m$. It is therefore difficult to discern whether the critical contribution to a TMRM reading derives from $\Delta\Psi m$ or mitochondrial volume[11]. We have confirmed that MTO, one of the dyes most frequently used to measure mitochondrial mass, is affected by both $\Delta\Psi m$ and efflux pump activity. We

have also observed that Verapamil changes the intensity profile of MTO in HSPC populations, but have found no correlation between MTO intensity and mitochondrial volume[11]. Combined measurement of mitochondrial volume and ΔΨm should be used to normalize data and eliminate the effects of membrane potential, and further exploration of mitochondrial content should be carried out using ΔΨm-independent techniques.

Such techniques would include 3D volume analysis based on fluorescent markers (e.g., immunostaining of mitochondrial proteins), electron microscopy[11], and quantitation of mitochondrial/nuclear DNA ratios. The use of mitochondrial dyes (i.e., TMRM) in combination with efflux system inhibitors will doubtless prove of great benefit in the elucidation of mitochondrial biology through flow cytometry.

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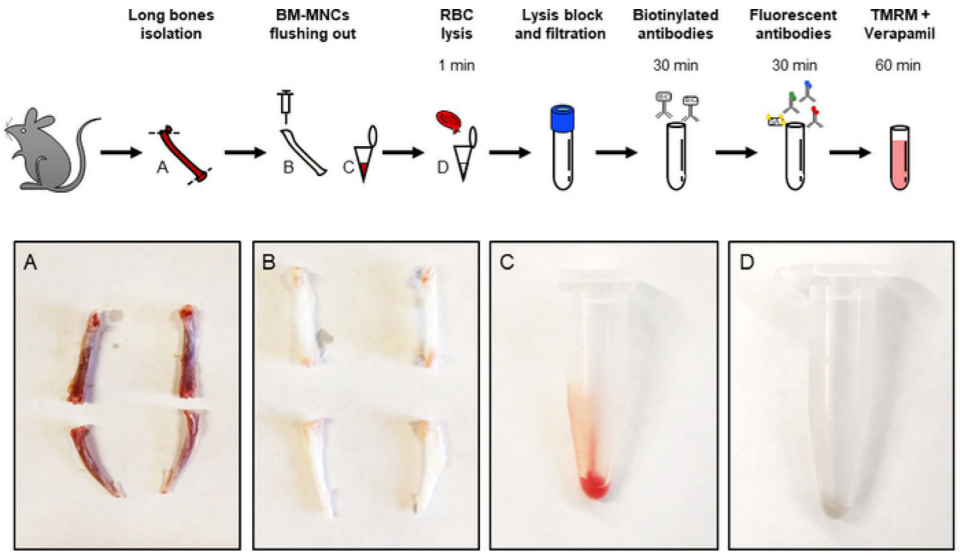

Figure 1: Protocol flowchart.

Graphical summary of the procedure to isolate and stain BM-MNCs to determine ΔΨm. Critical steps are highlighted by picture inserts (**A-D**). Femurs and tibias from adult C57BL/6 mice were isolated and their ends are removed (**A**). Long bones as in A after flush out (**B**). Isolated BM-MNCs before (**C**) and after (**D**) ACK lysis.

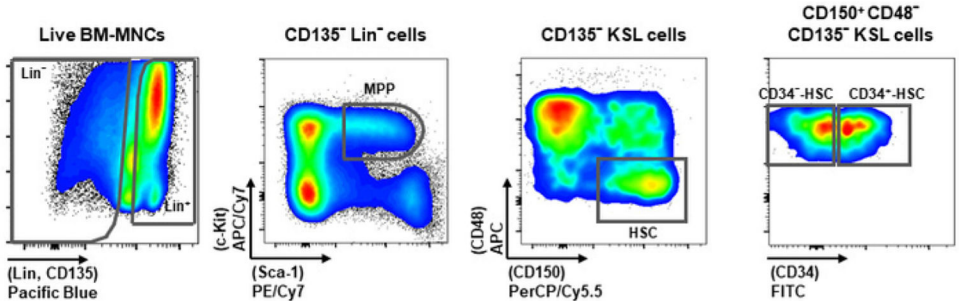

Figure 2: Gating setup.

Schematic representation of gating strategy to identify the different hematopoietic populations, including $CD34^{-}$-HSC and $CD34^{+}$-HSC, MPP, Lin^{-} and Lin^{+} cells. The panels were modified from Bonora, M. et al.[11].

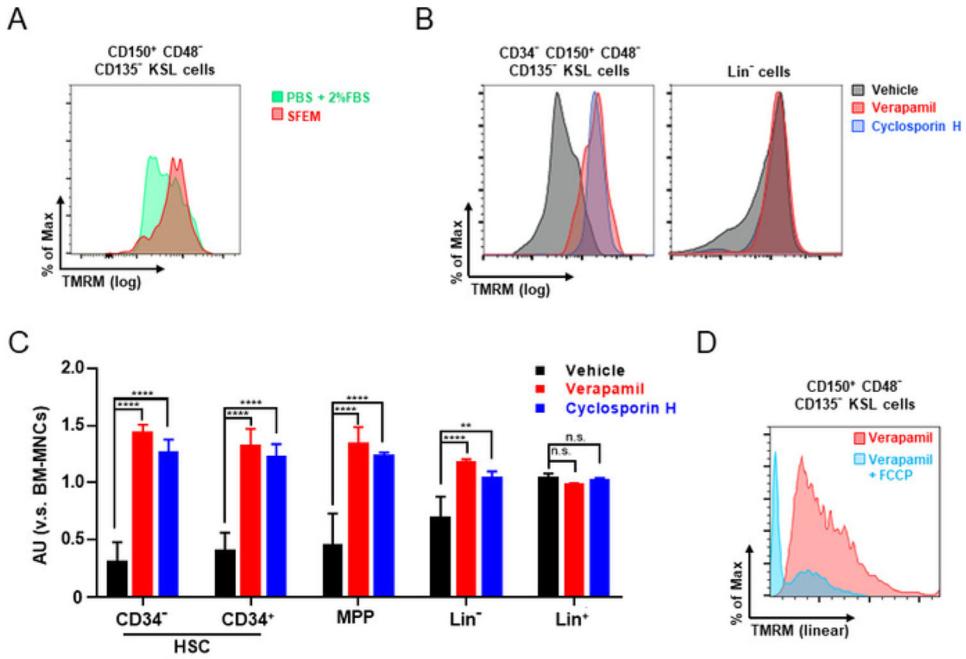

Figure 3: Flow cytometry analysis of mitochondrial membrane potential.

(**A**) Representative distribution of ΔΨm in HSCs stained with TMRM in PBS+2%FBS (green) or in serum-free expansion medium (SFEM) (red). (**B, C**) Representative distribution of ΔΨm in $CD34^{-}$-HSC and Lin^{-} cells (**B**) and quantification of ΔΨm in $CD34^{-}$-HSC, $CD34^{+}$-HSC, MPP, Lin^{-} and Lin^{+} cells (**C**) stained with TMRM in presence or absence of efflux pump inhibitors. TMRM intensity of each population was normalized by the TMRM intensity of own BM-MNCs (modified from Bonora, M. et al.[11]). (**D**) Representative histogram of TMRM intensity distribution in HSCs before (pink) and after (light blue) FCCP addition.

Materials

Name	Company	Catalog Number	Comments
ACK lysing buffer	Life Technologies	A1049201	
B220-biotin	BD Bioscience	553086	
CD3e-biotin	Life Technologies	13-0031-85	
CD4-biotin	Fischer Scientific	BDB553782	
CD8-biotin	Life Technologies	13-0081-85	
CD11 b-biotin	BD Bioscience	553309	
CD19-biotin	BD Bioscience	553784	
CD34-FITC	eBioscience	11-0341-85	
CD48-APC	eBioscience	17-0481-82	
CD135-biotin	eBioscience	13-1351-82	
CD150-PerCP/Cy5.5	Biolegend	115922	
c-kit-APC/Cy7	Biolegend	105826	
Cyclosporin H	Millipore Sigma	SML1575-1MG	
DAPI solution (1mg/mL)	Life Technologies	62248	
Fetal Bovine Serum (FBS)	Denville	FB5001-H	
FCCP	Millipore Sigma	C2920-10MG	
Grl-biotin	Biolegend	108404	
IgM-biotin	Life Technologies	13-5790-85	
Il7Rα-biotin	eBioscience	13-1271-85	
Nk1.1-biotin	Fischer Scientific	BDB553163	
Phosphate buffered saline (PBS)	Life Technologies	10010023	
Sca-1-PE/Cy7	eBioscience	25-5981-81	
SCF murine	PEPROTECH	250-03-10UG	
StemSpan SFEM medium	STEMCELL technologies	9605	
Streptavidin-Pacific Blue	eBioscience	48-4317-82	
Ter119-biotin	Fischer Scientific	BDB553672	
TMRM	Millipore Sigma	T5428-25MG	
TPO	PEPROTECH	315-14-10UG	
Verapamil hydrochloride	Millipore Sigma	V4629-1G	