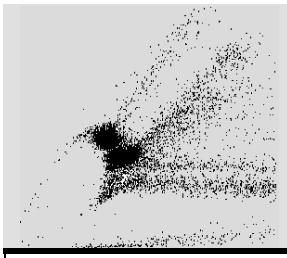

THE GOODELL LABORATORY

Author	Nathan Boles	Feb.5, 2009
Title	**Hematopoietic Progenitor Staining**	
Introduction	This protocol describes the analysis or isolation of the early hematopoietic progenitors.	
Materials	HBSS+ : Hanks Balanced Salt Solution (from Gibco) with 2% Fetal Calf Serum and 10 mM DMEM+ Hoechst 33342 *LT-HSC, ST-HSC, MPP antibody cocktail* Antibody — Concentration Lineage- Fitc — 1:100 Gr-1 Mac-1 Ter-119 CD4 CD8 B220 Sca-1 – Pe-Cy7 — 1:100 cKit – APC-AF750 — 1:100 Flk2/Flt3 – PE — 1:50 CD34 – AF647 — 1:50 *CMP* Antibody — Concentration Lineage – biotin — 1:100 Gr-1 Ter-119 CD4 CD8 CD3 B220 CD19 Il7ra – Pe-Cy7 — 1:50 Sca-1 – Fitc — 1:100 cKit – Pe — 1:100	

	CD34 – APC 1:50 CD16/32 – AF750 1:50 *CLP* Antibody — Concentration Lineage – biotin 1:100 Gr-1 Mac-1 Ter-119 CD4 CD8 CD3 B220 Il7ra – Pe-Cy7 1:50 Sca-1 – Fitc 1:100 cKit – Pe 1:100	
Protocol		*Notes*
1.	Prewarm DMEM+ (see below) while preparing the bone marrow.	*Ensure that a water bath is at precisely 37° C (check this with a thermometer!).*
2.	Using mice 8-12 weeks of age, prepare bone marrow from femurs and tibias and resuspend in HBSS+	
3.	Aliquot bone marrow into two equal portions	*We find an average of 5 x 10^7 nucleated cells per C57Bl/6 mouse. This number varies from strain to strain.*
4	Set cell concentration to 1x10^6 cells/mL using 37º C DMEM+ for one aliquot (hereafter to be referred to as SP aliquot). Place other aliquot in 4°C fridge (hereafter to be referred to as Prog aliquot).	…
5	Add 200X Hoechst solution to the SP aliquot in the 37º C DMEM+	
6	Incubate the SP aliquot at 37°C for 90 minutes exactly with occasional shaking.	*Use this time to eat lunch.*
7	Set a fraction of Prog aliquot to the side for staining controls, then split the remainder into two equal fractions (hereafter referred to as Prog1 and Prog2)	*For controls 5 million cells is sufficent.*

8	With 45 minutes left go ahead and make the 3 antibody cocktails outlined in the Materials section. Make enough to bring the final concentration of cells to $1x10^8$ cells/mL during staining for each aliquot. After you complete the cocktails go ahead and label all the tubes you will need for the rest of your experiment.	*Be extra careful when making the cocktails because a mistake here can give wacky results. Also make 10% more of each cocktail than you need to ensure an adequate amount.*
9	Take a small fraction of your SP aliquot to use as SP controls at the end of the 90 minutes.	
10	Spin down SP aliquot for 8 min. at 2000 rpm at 4°C, and resuspend in cold Hanks+ to a volume of $1x10^8$ cells/mL and keep on ice throughout the rest of the protocol.	
11	Now add the appropriate amount of antibodies to their correct aliquots (LT-HSC, ST-HSC, MPP cocktail to the SP aliquot. CMP cocktail to Prog1. CLP to Prog2)	
12	Incubate for thirty minutes on ice.	
13	Add enough Hank's+ to each control fraction to have 100µL for each color (including negative control).	
14	Stain controls for each color for both setups using Gr-1 or B220. However for the following antibodies use the actual antibody at a concentration of 1:50: Il7ra – Pe-Cy7, CD34 – AF647, CD16/32 – AF750, Flk2/Flt3 – Pe. (Stain for at least 10 minutes)	
15	Prepare a PI solution by using the lab stock of PI (1:100concentration) and Hank's+. Make enough to resuspend each of your samples in 500µL.	
16	Prepare a secondary staining solution. Put strepavidin-Pac Blue into Hank's+ at a concentration of 1:50. Make enough to stain all of your Prog1 and Prog2 samples at a cell concentration of $1x10^8$ cell/mL.	
17	Wash all samples and controls with 20x Hank's+ and spin down for 8 min. at 2000 rpm at 4°C.	
18	Resuspend SP aliquots and controls in 300-500µL of PI solution, keep on ice.	

19	Incubate for 15 minutes	
20	Wash all Prog1 and Prog2 samples with 20x Hank's+ and spin down for 8 min. at 2000 rpm at 4°C.	
21	Resuspend Prog1 and Prog2 in 300-500µL of PI solution, keep on ice.	
22	Go and analyze.	*Colors are appropriate for a 4-laser LSRII, however if using another machine verify color scheme.*

References.

1. Challen G, Boles NC, Lin KY, Goodell MA. Mouse Hematopoietic Stem Cell Identification And Analysis. Cytometry A. 2009 Jan;75(1):14-24.

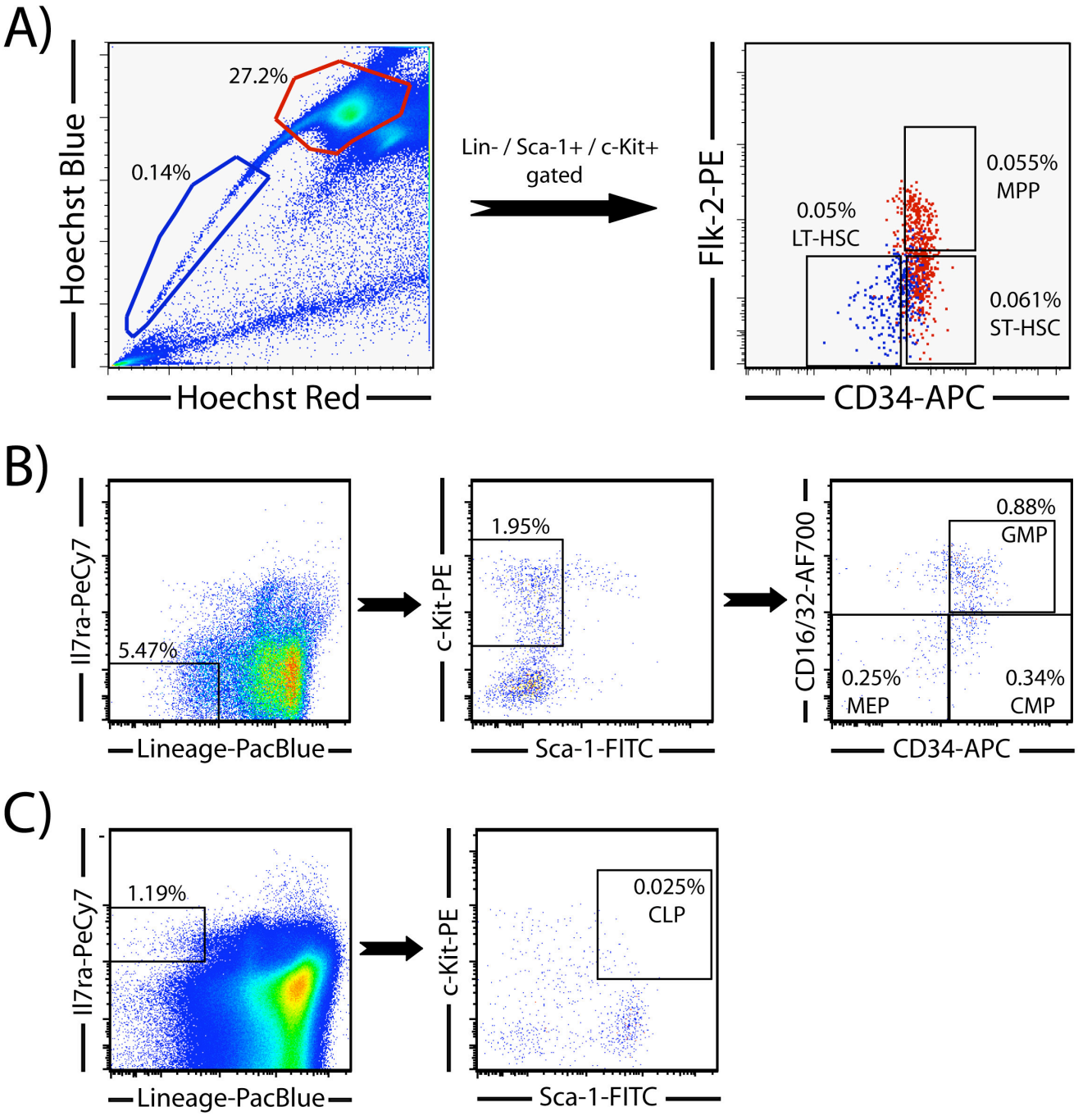

Figure1. Separation of hematopoietic progenitor populations by flow cytometry. (A) LT-HSC, ST-HSC, and MPP gating scheme. SP cells are gated to KLS as described for Figure 1, and shown here displayed in red on a CD34 / Flk-2 plot; the SPKLS are negative for both of these markers (the Hoechst-stained cells here have been previously magnetically enriched for Sca-1 to increase the overall proportion to 0.14%). The non-SP population, also shown gated on the Hoechst plot, is also taken through a KLS selection (not shown), then displayed for CD34 and Flk2. The KLS-Flk2+CD34+ cells are multi-potential progenitors (MPP), and the Flk2-CD34+ cells are considered short-term (ST) HSC. Thus, all three of these populations can be readily sorted from one sample. (B) Gating scheme for the common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs). (C) Gating scheme for the common lymphoid progenitor (CLP).