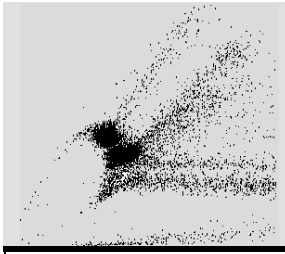


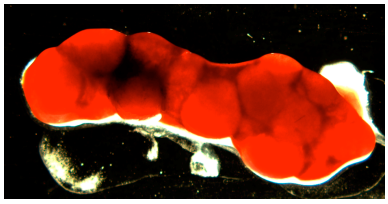
THE GOODELL LABORATORY

Author	Lara Rossi	February, 2009
Title	**Spleen Colony Assay using MSCV-transduced Sca-1$^+$cells**	
Introduction	This protocol represents a variant of the *in vivo* spleen-colony assay originally developed by Till and McCulloch and allows a more sophisticated analysis of hematopoietic progenitors, than traditional *in vitro* colony assays. When hematopoietic cells are transplanted into irradiated recipient mice, they give rise, after 8-13 days, to macroscopic nodules, referred to as spleen colonies, or CFU-S. In particular, when CFU-S are scored at least 11 days after transplantation, they provide an indirect measure of the frequency of pluripotent progenitors originally present in the transplanted cells. The aim of this protocol is to analyze the *in vivo* CFU-S potential of hematopoietic progenitors retrovirally transduced with MSCV virus, capable of driving the enforced expression of a specific experimental gene, associated with the expression of the reporter gene GFP.	
Materials	**5-FU injection of CD45.2 Donor Mice:** 1. 5-FluoroUracil 2. PBS **BM Isolation, Sca-enrichment, and Transduction Sca-1 cells** 3. HBSS+ : Hanks Balanced Salt Solution (Gibco) with 2% Fetal Calf Serum and 10 mM 4. anti-Sca-1 Biotin Antibody 5. anti-Biotin MACS magnetic MicroBeads 6. Red Blood Cell (RBC) Lysis Buffer (1:9 = 0.17 M pH 7.6 TrisCl : NH_4Cl) 7. Culture Medium: StemPro medium (Gibco) Nutrient Supplement (40x), 1X L-Glutamine, 2mM Pen/Strep antibiotics, 1X Thrombopoietin (TPO), 100ng/mL Stem Cell Factor (SCF), 10ng/mL 8. Polybrene, 4ug/mL 9. MSCV- IRES-eGFP (as control) and MSCV-GOI-IRES-eGFP (for your experimental gene) viral suspensions. **Transplant of transduced Sca-1 cells into CD45.1 mice** 10. Serum-free HBSS buffer 11. Insulin needles	

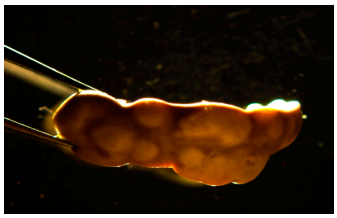
	Analysis of Spleen Colonies (CFU-S assay) at day 13 12. Bouin's Fixative (Sigma-Aldrich, HT10-1-32)	
Protocol		*Notes*
1.	Intraperitoneally inject CD45.2 mice with 5-FU (150mg/Kg).	*Dilute 5-FU 1:5 in PBS (10mg/mL) and inject 300 uL/mouse of 20g of weight.* .
2.	Six days after 5FU injections, proceed to sacrifice the injected mice and harvest BM cells from tibias, femurs, and hips.	
3.	Resuspend BM cells in HBSS+ buffer and stain cells with Biotynilated anti-Sca Antibody for 15 minutes on ice.	
4.	Wash with 10-15 mL HBSS+ buffer and spin cells down (8 min, at 2000 rpm)	
5.	Resuspend BM cells in HBSS+ buffer (at the concentration of 10^7 BM cells in 80 µL buffer) and stain with anti-Biotin MACS MicroBeads for 15 minutes at 4°C.	*Since magnetic MicroBeads tend to settle down, remember to periodically shake the tube during the 15 min – incubation (every 5 minutes).* *We usually obtain about 5 x 10^6 Sca-1 cells/mouse after enrichment*
6.	Add 10-15 mL HBSS+ buffer at the end of the incubation, and spin cells down (8 min, at 2000 rpm)	
7.	Resuspend cells in 500 µL HBSS+ buffer. The sample is now ready for Sca-enrichment on the AutoMacs (recommended enrichment program: PosselD2).	
8.	Take a 10 uL aliquot from the enriched sample and resuspend in 90 µL RBC Lysis buffer (1:10 dilution) and proceed to count cells. In the meantime, add 10-15 mL HBSS+ buffer to the rest of the enriched sample and spin cells down (8 min, @2000 rpm).	
9.	Resuspend Sca-1^+ cells in Culture Medium at the concentration of $1x10^6$/mL.	
10.	Using 24-well plates, plate 500 µL Sca-1^+ - cell suspension / well (500,000 cells/well). Add to each well the appropriate amount of virus and bring the final volume to 1-1.5 mL by adding Culture	*Ensure that the centrifuge you will use for the spin-infection is at RT. If not, it can be*

	Medium.	*brought at RT by letting the centrifuge spin at 4,000rpm for 15-20 min at RT.*
11.	Add Polybrene to each well to a final concentration of 4µg/mL.	
12.	Spin-infection: centrifuge plates at 1,100 rpm for 2 hrs. The centrifuge must be at Room Temperature.	*Need plate adaptor*
13.	During the 2hrs-spin-infection, perform the first cycle of irradiation on CD45.1 recipient mice.	
14.	At the end of the spin-infection, remove the plates from the centrifuge and incubate cells for 1 hr at 37°C, 5%CO_2.	
15.	Harvest cells from each well and count cells.	
16.	Add 5-10 mL plain HBSS buffer to the sample and spin cells down (8 min, @2000 rpm).	
17.	Resuspend cells at the concentration of 35,000cells/200uL in plain HBSS buffer.	
18.	Perform the second cycle of irradiation. Load needles with 200 µL cell suspension, and proceed to transplant CD45.1 mice with the transduced Sca-1^+ cells (35,000 cells/mouse).	
19.	13 days after transplantation, sacrifice recipient mice and proceed to analyze their spleen for the presence of macroscopic colonies.	*CFU-S colonies could also be analyzed at earlier time points (e.g. 8 days after transplantation), though their origin (and, therefore, biological significance is different from day 13 CFU-S). Please consult reference for details.*
20.	In order to make colonies more evident, fix the spleens in Bouin's Fixative for 10-30 minutes and count colonies under a dissecting microscope. We recommend using this procedure in order to obtain an accurate count of CFU-S.	*After fixation in Bouin's Fixative, the splenic stroma will shrink, making CFU-S easier to score.*
Alternative 20.	According to the purpose of your experiment, it may be important for you to know which colonies, within the same spleen, are GFP+ and which are GFP-. If this is the case, do not fix the spleens with Bouin's fixative, but proceed to dissect every single colony from each spleen. The colony can be then	*This analysis is more time-consuming, but it will give you an indication of what the proportion is between transduced and*

	dissociated into a single-cell suspension and analyzed on the flow cytometer for the presence of GFP+ cells. We recommend mixing the cell suspension derived from each colony with a fixed amount of carrier cells (for instance, use splenocytes stained with B220-APC antibody).	*untransduced colonies within a given spleen. However, be aware that the total numenr of CFU-S scored on an unfixed spleen may significantly diverge from the ones scored after fixation.*



CFU-Ss before fixation



CFU-Ss after fixation with Bouins' fixative

References.

1.Siminovitch L, McCulloch EA, Till JE. The distribution of colony-forming cells among spleen colonies. *J Cell Physiol* 1963. Dec;62:327-36.

2. Magli MC, Iscove NN, and Odartchenko N. Transient nature of early hematopoietic spleen colonies. *Nature* 1982. 295:527-529.