# Individual HSC Proliferation Assay

Ernst Lab 2008

**FACS Buffer:**

HBSS + 2% FBS (0.2µ filtered)

**FACS Buffer + PI:**

HBSS + 2% FBS + Propidium Iodide (1 µg/mL)

**Lineage Mix:**

1 µL lineage mix per 1 million cells (all antibodies = Caltag, 200 µg/mL stocks)

| CD3 | CD8 | CD19 | TER119 | CD4 | GR1 | B220 | IL-7Rα | MAC-1 | Total |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | **14 µL** |

**HSC Expansion Medium:**

Reference: Uchida, Dykstra, Lyons, Leung, and Connie Eaves. *Experimental Hematology* 31 (2003) 1338-1347.

StemSpan SFEM (serum free expansion medium) (Stemcell Technologies)
300 ng/mL murine SCF
20 ng/mL murine IL-11
1 ng/mL human Flt3L

***Filter all media prior to use to remove debris***

**<u>CELL PREPARATION:</u>**

*Perform all steps in sterile conditions and at 4°C*

1. Remove mouse femurs and tibias from hind limbs. Remove any muscle tissue.
2. Crush clean bones using a mortar and pestle in 5 mL FACS Buffer. Filter with 40 µm cell strainer (Becton Dickinson).
3. Rinse bones with additional 2 mL FACS Buffer and filter.
4. Spin cells 4 minutes at 380 x g at 4°C.
5. Resuspend cells in 1 mL 1x RBC Lysis Buffer (eBiosciences). Incubate on ice 5 min.
6. Add 5 mL FACS buffer to stop reaction.
7. Spin cells 4 minutes at 380 x g at 4°C.
8. Resuspend cells in FACS buffer and count.
9. Spin cells 4 minutes at 380 x g at 4°C.
10. Resuspend in 200 µL FACS buffer (staining volume).
11. Stain cells with 1 µL Lineage Mix per $10^6$ cells.
12. Incubate on ice 20 minutes with occasional gentle flicking.
13. Wash Dynal sheep-anti-rat magnetic beads during lineage incubation:
    Dynal Wash:
    i. Mix Dynal beads well by pipetting.
    ii. Pipet appropriate volume beads into FACS tube (100 µL beads per sample… approx $5x10^7$ cells)
    iii. Add 3 mL FACS buffer to wash.
    iv. Place tube with beads in magnet. Wait 2 min.
    v. Aspirate liquid and remove tube from magnet.
    vi. Add back appropriate volume FACS buffer.
14. Wash cells with 5 mL FACS buffer.
15. Spin cells 4 minutes at 380 x g at 4°C.
16. Resuspend cells in 7.5 mL FACS buffer. Add 100 µL washed beads per sample.

17. Incubate cells + beads at 4°C on nutator with gentle rotation for 20 min.
18. Load the 7.5 mL sample into two FACS tubes (~3.25 mL each), and place in magnet.
19. Wait two minutes to deplete, then remove unbound cells (liquid) carefully and transfer to fresh conical tube.
20. Count. Spin cells 4 minutes at 380 x g at 4°C.
21. Resuspend cells in 200 µL FACS buffer.
22. Add 1 µL Caltag Goat-F(ab')2 Anti-Rat **PE-cy5.5** antibody (200 µg/mL). Vortex to mix.
23. Incubate on ice 20 minutes in the dark with occasional flicking.
24. Add 5 mL FACS buffer to wash.
25. Spin cells 4 minutes at 380 x g at 4°C.
26. Resuspend cells in 150 µL FACS buffer.
27. Add 50 µL Rat Ig to block. Incubate 5 minutes on ice.
28. Directly add Sca-1 and c-Kit antibody (all BD Pharmingen) stains to cells + block:
    Following volumes are for ~ $10^7$ cells – if more, scale up.
    **FITC** Rat Anti-Mouse Ly-6A/E (D7) (Sca-1) (0.5 mg/mL stock) 4 uL
    **APC** Anti-Mouse CD117 (c-Kit) (2B8) (0.2 mg/mL stock) 2 uL
    **PE** Anti-Mouse CD48 (BCM1) (HM48-1) (0.2 mg/mL stock) 1.5 uL
29. Incubate on ice 20 minutes in the dark with occasional flicking.
30. Add 5 mL FACS buffer to wash.
31. Spin cells 4 minutes at 380 x g at 4°C.
32. Resuspend in 1 mL FACS Buffer + PI for sorting.

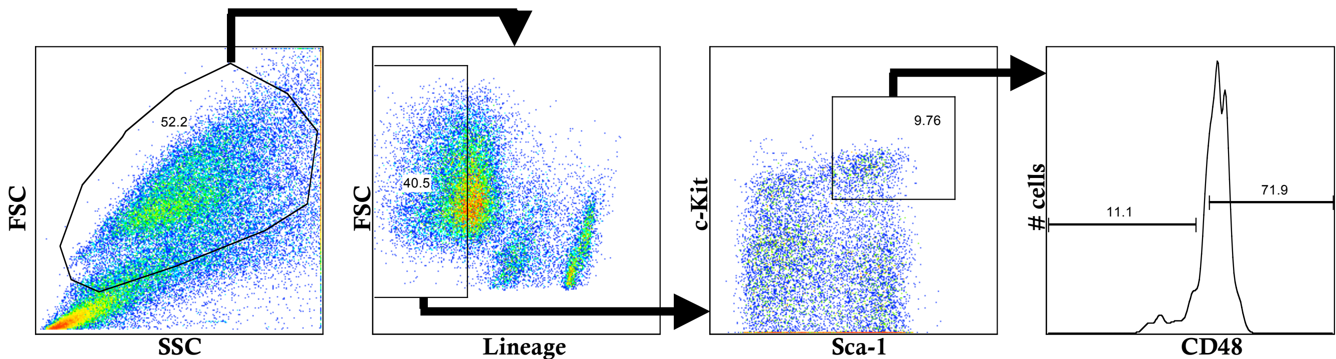
**Staining Controls:**
Use 2 million bone marrow cells from an uninjected control mouse per stain.
Add 1 uL of each antibody except lineage mix (2 µL).
Use 10 ul rat Ig (Sigma) after the goat-anti-rat PEcy5.5 step.
Resuspend in 400 uL either FACS Buffer with or without PI as indicated.

| | | | | | |
|---|---|---|---|---|---|
| 1 | **Unstained** | | | | **NO PI** |
| 2 | **CD44- FITC** | | | | **NO PI** |
| 3 | **B220- APC** | | | | **NO PI** |
| 4 | **FcR- PE** | | | | **NO PI** |
| 5 | **Lin- PEcy5.5** | | | | **NO PI** |
| 6 | **c-Kit-APC** | **Sca-1-FITC** | **CD48-PE** | **-** | **+PI** |
| 7 | **-** | **+** | **+** | **Lin-PEcy5.5** | **+PI** |
| 8 | **+** | **-** | **+** | **+** | **+PI** |
| 9 | **+** | **+** | **-** | **+** | **+PI** |
| 10 | **+** | **+** | **+** | **+** | **+PI** |

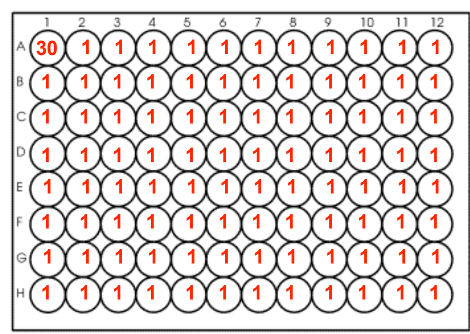
**<u>SORTING (we use an Aria):</u>**

1. Gate Lineage$^{neg/lo}$/c-Kit$^{+}$/Sca-1$^{+}$/CD48$^{-}$ cells as depicted below and sort into coated collection tubes containing ~ 300 µL FACS buffer supplemented with high serum (~20 % filtered FBS).

2. Resort single Lineage$^{neg/lo}$/c-Kit$^{+}$/Sca-1$^{+}$/CD48$^{-}$ cells into individual U-bottom wells of TC Microwell 96U W/Lid Nunclon Δ SI plates containing 100 μL HSC Expansion Medium. Optimal flow rate was found to be the minimum setting, or 1. This corresponds to something between a few to 50 cells per second.
3. Sort 10-50 cells into one of the 96 wells for visual confirmation of focal plane when scoring plates later. See below for example plate.
4. After sort, spin plates 4 minutes at 380 x g at 4°C.
5. Incubate plates at 37°C with 5% $CO_2$ for 1 hour.
6. Visually inspect each well to confirm that they contain no more that 1 cell/well (except for the focus control well).
7. Incubate plates at 37°C with 5% $CO_2$ and score divisions as desired for up to 5 days.

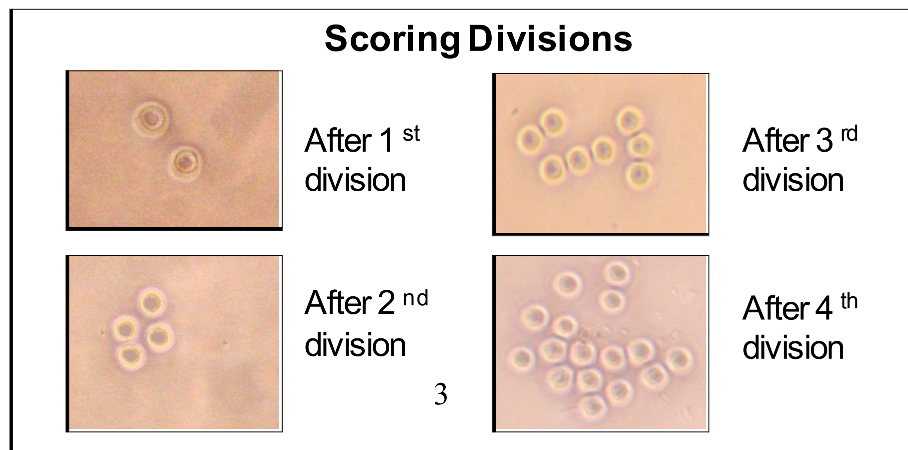



Example Plate Sorting Setup (red numbers indicate # cells sorted into each well):




**SCORING DIVISIONS:**

1. Use well with 10-100 cells to focus on the correct plane.
2. Count # of cells in each well.



**Notes:**

- Typically, ~$10^4$ Lineage$^{neg/lo}$/c-Kit$^+$/Sca-1$^+$/CD48$^-$ cells can be sorted from a single wild type B6 mouse aged 6-12 weeks.
- Nunc 96 well U-bottom plates were selected after comparisons to other brands because they had the best optical clarity for scoring cell growth.
- It is very important to filter the medium to avoid particulate in the wells.
- The one-hour incubation period prior to visual confirmation of the wells was implemented because the cells tend to be very small right after sorting and grow in size after a short incubation, facilitating easier identification in the wells.