Flow Cytometry Immune Activation SOP

Purpose

This SOP standardizes the procedure for measuring immune activation of T cells using flow cytometry in ACTG Immunology Laboratories.

Materials

1. 12x75mm flow tubes
2. RPMI 1640
3. Flow Buffer
    a. RPMI 1640 + 1% BSA
4. BD CompBead, Anti-mouse Ig (Catalog #552843)
5. Molecular Probes ArC Amine Reactive Compensation Bead Kit (Catalog #A10346)
6. 0.5% Formaldehyde
7. Staining Antibodies (for CD3, CD4, CD8, HLA-DR, and CD38)
8. Live/Dead Fixable Aqua Dead Cell Stain Kit for 405 Excitation (Molecular Probes, Catalog #L34957)
9. Spherotech 7-peak beads (Fisher, catalog RCP-30-5A-7)

1. Sample Requirement
    a. Blood should be collected using heparin or EDTA as an anticoagulant.
    b. All testing will be done on frozen samples.
        i. Whole blood should be processed within 6 hours and cryopreserved at clinical site to avoid being shipped.
        ii. The samples are to be cryopreserved using the ACTG Cryopreservation SOP (Cross-Network PBMC Processing SOP. HANC-LAB-P0001v4).
        iii. Once samples are frozen, they will then be shipped to BRI. BRI will send batched samples to ISL for flow cytometry analysis.
            1. One ISL will be responsible for all flow cytometry analysis for a particular study protocol. Samples are not to be split between sites.
2. Validation
    a. Reagent Validation
        i. All antibodies should be titrated prior to thawing of study samples.
            1. Should use the same lot of antibody for each study. However, if a new lot is used it must be titrated as well.
        ii. HLA-DR and CD38 FM2 (fluorescence minus two) tube should be run only during initial study setup on HIV+ donors. It is not necessary to run FM2 tubes daily with patient samples.
            1. Set the gates based on the negative population in the FM2 plot and these gates should be carried over for use with study samples.
        iii. A control sample will be run weekly, at minimum, for the duration of the study.
    b. Instrument Validation
        i. Instrument should be standardized daily to maximize instrument performance and minimize interassay variability.
            1. If using a BD machine, CST must be run daily to ensure cytometer performance.
        ii. When setting up the experiment for the first time, choose your voltages to minimize bleed over between fluorochromes and then run Spherotech 7-peak beads to establish *baseline* MFI value for each fluorochrome.
            1. Draw a gate around the 7-peak beads in a FSC/SSC plot.
            2. In histogram view, draw a gate around the peak for each fluorochrome. This should be gated off the prior bead gate.
            3. Use the statistics view to determine the MFIs.
            4. Record these values on a worksheet (example shown in Appendix B).
3. Thawing Samples
    a. All labs must participate in a cross lab validation of thawing that will be established by the IQA.
    b. Thaw using the IQA SOP.
    c. Wash the cells 2 times with 10mL RPMI to remove FBS. Proceed with staining the cells.
4. Staining
    a. Stain for viability with Molecular Probes Live/Dead® Fixable Aqua Dead Cell Stain Kit.
        i. Resuspend cells at $1x10^6$/mL in RPMI in 12x75mm flow tube. Stain at a concentration of 1µL per 1mL of sample.
        ii. Also stain Live/Dead compensation tube at this time by placing 3µL of stain and 3 drops of positive ArC Amine beads in a flow tube.
        iii. Incubate at room temperature for 15-30 minutes in the dark. Protect samples from light from this step forward.
    b. Wash cells once with 2mL RPMI to remove Live/Dead stain. After wash decant liquid. Do not add additional volume to the tube.

i. Add 3 drops of negative ArC Amine beads to the Live/Dead compensation tube and fix in 0.5% formaldehyde.

c. Use the following antibodies as the core immune activation panel.

Marker	Clone
CD3	UCHT1
CD4	RPA-T4
CD8	SK1
HLA-DR	G46-6
CD38	HIT2

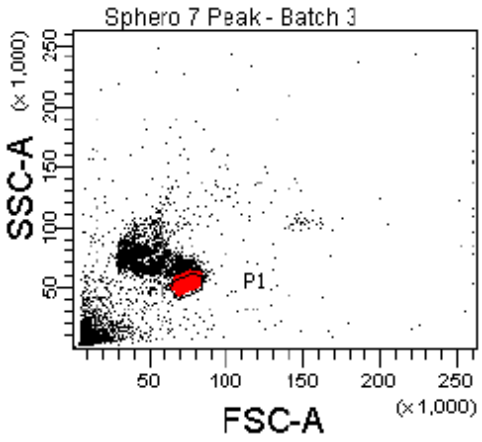
i. Prepare antibody cocktail for appropriate number of samples + 1 extra. Using Flow Buffer (RPMI + 1% BSA) bring the volume up to 100µL/test in the cocktail tube.
ii. Prepare compensation tubes at this time using BD CompBeads.
1. Add one test worth of antibody to the specific compensation tube. Add 1 drop each of the positive and negative CompBeads.
iii. Add 100µL of the antibody cocktail to each sample tube and vortex. Incubate at room temperature for 30 minutes in the dark.

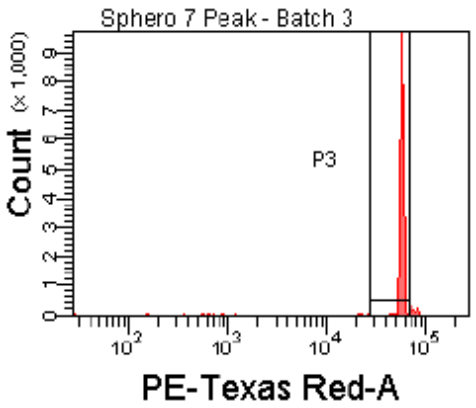
d. Wash the cells with 2mL Flow Buffer.
e. Fix cells in 0.5% formaldehyde and run on a flow cytometer within 48 hours of fixation.

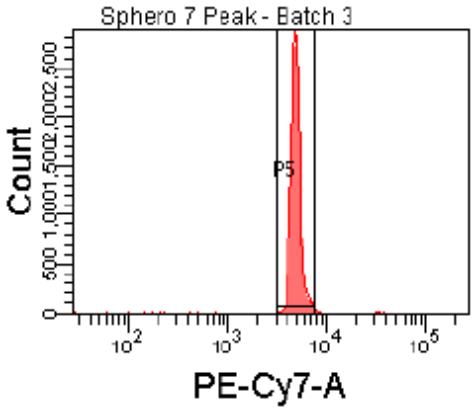
5. Sample Collection
a. Ensure proper filters are in place for selected fluorochromes.
b. Spherotech 7-peak beads should be run first.
i. Prepare Spherotech 7-peak beads by putting 3 drops of beads in 2mL PBS. Protect the beads from light and store at 2-8 °C. The beads are good for one week.
ii. Run at an event rate of 150-350 events/second. Adjust PMT voltages to match the baseline MFI values acquired at study set up (see section 2.b.ii).
iii. Record at least 25,000 events and track the daily MFI values on a worksheet.
c. Run compensation tubes and collect 25,000 events per tube.
d. Run sample and aim to collect $1x10^6$ events. This will not always be possible due to poor sample quality and in these cases collect all events.
i. Be sure to collect FSC-A, FSC-H, and SSC-A parameters for samples.

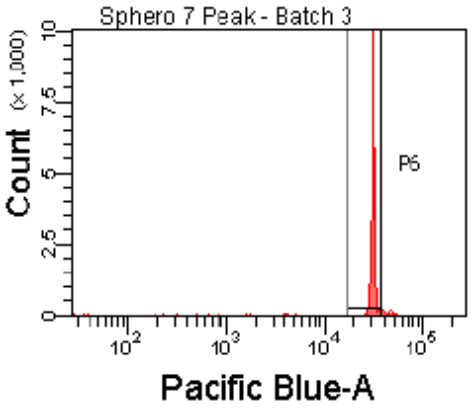
6. Analysis
a. All analysis for a given study should be performed by one technician using FlowJo, FACS Diva, or other appropriate software. This will reduce variability in gating strategies.
b. Only samples with viability >50% will be reported. Viability is to be determined by the CD3+ lymphocyte and singlet-gated subset. See gating strategy in Appendix C.
c. Due to the standardization of the machine with Spherotech beads daily, the fluorescence gates should not change from day to day. The scatter gates may change slightly.
d. Use the gating strategy in Appendix C to analyze T lymphocytes.
e. The following parameters, at minimum, must be reported for all activation studies
i. CD3+ CD4+ CD38+HLA-DR- (report as % of CD3+CD4+).
ii. CD3+ CD4+ CD38+HLA-DR+ (report as % of CD3+CD4+).
iii. CD3+ CD4+ CD38- HLA-DR+ (report as % of CD3+CD4+).
iv. CD3+ CD8+ CD38+HLA-DR- (report as % of CD3+CD8+).
v. CD3+ CD8+ CD38+ HLA-DR+ (report as % of CD3+CD8+).
vi. CD3+ CD8+ CD38- HLA-DR+ (report as % of CD3+CD8+).

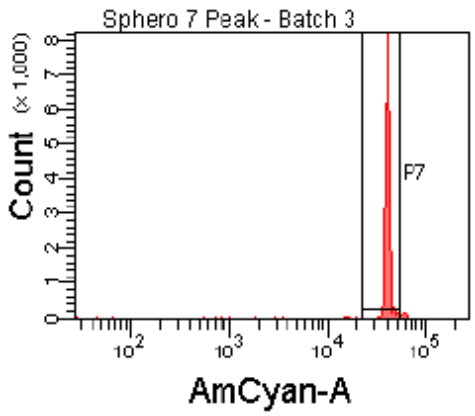
Appendix A: Spherotech 7-Peak Bead Acquisition

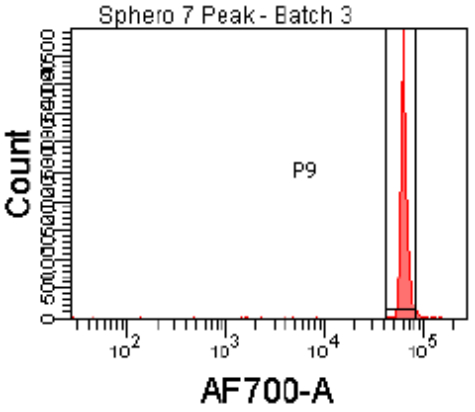


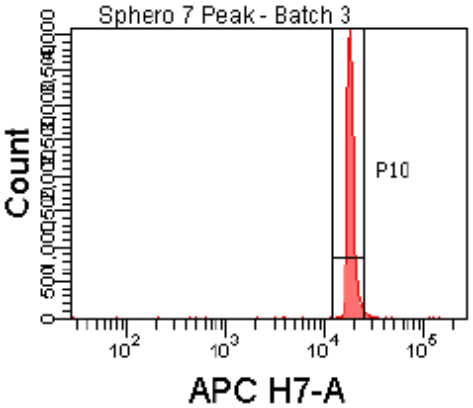












Experiment Name: 7 Peak Beads
Record Date: Feb 24, 2012 11:00:26 AM

Population	PE-Texas R... Median	PE-Cy7-A Median	Pacific Blue... Median	AmCyan-A Median	AF700-A Median	APC H7-A Median
All Events	57,250	4,902	31,606	40,867	62,389	18,529
P1	57,117	4,856	31,527	40,742	62,076	18,412
P3	57,003	4,819	31,453	40,623	61,800	18,313
P5	57,106	4,851	31,521	40,730	62,044	18,401
P6	57,056	4,834	31,479	40,663	61,891	18,346
P7	57,080	4,844	31,502	40,703	61,984	18,380
P9	57,082	4,845	31,505	40,706	61,986	18,381
P10	57,092	4,848	31,511	40,717	62,011	18,391

Appendix B: Example of Spherotech 7-Peak Bead Worksheet for MFIs

Date	PE-Tx Red	PE-Cy7	PB	AmCyan	AF700	APC-H7	Tech
Baseline	**57,003**	**4,851**	**31,479**	**40,703**	**61,986**	**18,391**	**JP**
2/24/2012							

Appendix C: Gating Strategy