# Institut für HIV Forschung

**SOP #06-00** (Mai 2016 - BS)

## Surface / Intracellular Cytokine Staining

**Reagents**

| Reagent | Vendor | Catalogue # | Stock Conc. |
|---|---|---|---|
| Facs Tubes | BD Falcon | 352054 | - |
| Anti-CD28/CD49d Antibody | BD Fastimmune | 347690 | - |
| GolgiPlug (Brefeldin A (BFA)) | BD | 555029 | - |
| GolgiStop (Monensin) | BD | 554724 | - |
| Cytofix/Cytoperm Kit | BD | 554722 | - |
| Live/Dead Fixable Aqua Dead Cell Stain Kit | Biolegend | 423102 | - |
| SEB | Sigma | S4881 | 1mg/mL |
| PHA | Fisher | R 30852801 | 250ug/mL |
| PMA | Sigma | 1585 | 1mg/mL |
| Ionomycin | Sigma | 141128 | 1mg/mL |
| RPMI-1640 | Sigma | R0883 | - |
| Pen/Strep | Cellgro | 30-001-Cl | Pen: 5000 IU/mL<br>Strep: 5000ug/mL |
| L-glutamine | Cellgro | 25-002-Cl | 200mM; 29.2mg/mL |
| HEPES (1M; 238.3mg/mL) | Cellgro | 25-060-Cl | 1M |
| FBS, Heat-inactivated | Sigma | F4135 | - |
| PBS | Sigma | D8537 | - |

This protocol is for intracellular cytokine staining of whole PBMC, CTL clones, T-cell specific lines or NK cells. All stimulation procedures will be described for T cell assays and respective comments for other cell types will be indicated if necessary.

**All work should be done in a sterile, laminar flow hood**

### Media:

R10

| Reagent | Stock Concentration | Volume to Add | Final Concentration |
|---|---|---|---|
| RPMI 1640 | - | 500 | - |
| FBS | 100% (v/v) | 55ml | 10% (v/v) |
| Penicillin/Strep | Pen: 5000 IU/mL<br>Strep: 5000ug/mL | 5.5mL<br>5.5mL | Pen: 50 IU/mL<br>Strep: 50ug/mL |
| L-glutamine | 200mM | 5.5mL | 2mM |
| HEPES | 1M | 5.5ml | 10mM |

Staining Buffer (2% FBS/PBS)

| Reagent | Stock Concentration | Volume to Add | Final Concentration |
|---|---|---|---|
| PBS | - | 500 | - |
| FBS | 100% (v/v) | 10ml | 2% (v/v) |

Preparation of the effector cells to be tested:

- ICS works well on cryopreserved cells. For frozen cells, thaw and let them rest for >4 hours in R10 (or rest overnight). This step should reduce background (activation due to freeze/thaw process; apoptotic cells).
- If using fresh cells, you can stain immediately.
- If using cell lines, starve cells of IL-2 the night before the assay (wash and incubate with R10), otherwise background will be too high

## **Protocol**:

1. Re-suspend PBMCs at $10^6$/ml in R10 in FACS tubes
   - *Some people use 96w or 24w plates for the stimulations. Values indicated here are for stimulations in 1ml total volume. If not enough cells, stimulations can also be done at 0.5x10$^6$)*
2. Add 2 µg/ml of peptide to be tested (**e.g. 10 µl of 0.2 mg/ml peptide solution**) to the PBMCs in the flow tubes
   - Unstained control: tube with no fluorescent antibodies added, but treated equally
   - Negative (Unstimulated) control: tube with no peptide/antigen added or PBMCs from a person who does not respond to the peptide (i.e. HIV peptide tested on HIV uninfected individual).
   - Positive control (choose one):

| | Stimulatory Reagent | Stock Concentration | Vol/ml culture |
|---|---|---|---|
| 1 | anti-CD3<br>anti-CD28 | 1mg/ml<br>1mg/ml | 1µl<br>1µl |
| 2 | SEB | 1mg/ml | 5µl |
| 3 | PHA | 250µg/ml | 20µl |
| 4 | PMA<br>Ionomycin | 1mg/ml<br>1mg/ml | 5µl of 1:100 dilution<br>1µl |

   - *Different options exist to stimulate lymphocytes. Most stimulate T cells unspecifically. Superantigens like SEB (Staphylococcus enterotoxin B) work by cross-linking the TCR to MHC class II molecules on APCs (thus it will not work on isolated T cells while PMA/Ionomycin work by activating Protein Kinase C (PKC) via a Ca2+ influx.*
3. Add 1 µl of the combined anti-CD28/anti-CD49d solution (stock is at 1mg/ml; end concentration should be 1µg/ml). Alternatively, add 1 µl of anti-CD28 and 1µl of anti-CD49d if purchased separately.

- CD28 and CD49d are co-stimulatory molecules on the T cell surface (and also on other cell types, such as granulocytes (CD28, CD49), B cells, NK cells, Monocytes etc (CD49d)) that reinforce the TCR-MHC signal and increase cytokine release. The natural ligands for CD28 are CD80 (alternative name B7.1) and CD86 (B7.2) which are expressed on activated dendritic cells and for CD49d (alternative name VLA-4) VCAM-1 a cell adhesion molecule. Thus adding these antibodies simulates the co-stimulatory signal of T cell adhesion to an activated antigen presenting cells. *For NK cell assays these antibodies are not needed!*
- Remember to add anti-CD28/Cd49d to all tubes to be tested, including the negative control(s)!
- These costimulatory molecules can increase background. If background is a problem, try using only CD28.

5. Incubate the cells at 37˚ C for 30 minutes before adding 1µl of 1mg/ml Brefeldin A (BFA) and 1µl of 1mg/ml Golgi-Stop (Monensin) to each tube (including controls)!
    - BFA and Monensin block the transport of generated cytokines from the Golgi to the granules, thus holds back cytokines that would otherwise be synthesized and then released upon stimulation of the effector cells. Thus forgetting to add these will render your experiment useless when doing ICS!
    - This is harmful to the cells, these reagents should not be added until the last 5 hours of the experiment.
6. Incubate for a total of 5-6 hours at 37˚ C. (Some investigators incubate overnight/14h. In that case, the amounts of BFA/GolgiStop should be reduced to avoid cell death/apoptosis)
7. Post-stimulation, wash cells once with 2mL PBS at 350xg, 5 minutes.
8. Check that your cells have pelleted at the bottom of your FACS tubes carefully. Your positive controls and stimulated samples may be more dispersed on the bottom and may not clump as well as the unstimulated cells. This is normal and they will clump together better after each washing step.
9. You may choose either to decant your samples into the liquid waste or aspirate the supernatant off. Be consistent between experiments especially when titrating antibodies!

**THE REST OF THE PROTOCOL IS DONE IN THE DARK (i.e. turn light in the hood off to avoid activation/bleaching of the fluorochromes).**

10. Create a master mix for the Zombie dye by creating a 1:100 dilution. We use 1ul Zombie viability dye in 99ul PBS per sample (so for 9 tubes make a master mix by adding 9ul Zombie to 891ul PBS and don't forget to vortex!!).
11. Pipette 100ul master mix from step 10 to each tube
12. Incubate for 20-30 minutes at RT in the dark
13. Wash with 2ml cold staining buffer.
14. Discard supernatant and add surface antibodies (i.e. immunophenotype antibodies such as anti-CD3, CD4, CD8, CD16, CD14, CD19, CD56 etc, surface receptors such as CXCR4, CCR5, CCR7, etc) to the cell pellet using a

master mix (normally at a final volume of 100ul per tube). Briefly vortex tubes after adding master mix to each tube.

   a. Titrating antibodies is a great way to save reagent and $€. Manufacturers typically recommend using more antibody per test than is needed. Titrate your antibodies individually by performing single staining serial dilutions and comparing MFI of your positive populations with the MFI of your negative populations.

15. Incubate cells with surface antibodies at 4°C for 10-20 minutes in the dark.
16. Wash once with 2ml of staining buffer and spin at 350xg, 5 minutes at room temperature and decant/aspirate supernatant
    a. If you are only staining surface antibodies then go to Step 24. If you want to fix your cells and perform intracellular staining (ICS) then continue to Step 17.
17. Add 100 µl of CytoFix/CytoPerm solution to each sample, vortex shortly and then incubate for 20 minutes at 4°C in the dark
18. Create enough 1X Permeabilization buffer to wash your samples (2ml per tube) and to create enough master mix for ICS (normally about 1-2ml extra). 1X Perm Buffer is made by diluting 10X Perm Solution with $ddH_2O$
19. Wash with approx 2ml 1X Permeabilization Buffer at 500xg, 5 minutes at room temperature and decant/aspirate supernatant
    a. Fixation of cells causes the cells to slightly expand and become a fairly rigid ball. This stretching causes the cell to become less dense and thus spinning the cells harder is necessary to ensure all of them are pelleted
20. Create a master mix with your ICS antibodies in 1X Permeabilization buffer. Add 100ul of your master mix to each tube and vortex briefly.
    a. Permeabilization is a reversible process so it is incredibly important that ICS is done in the presence of some kind of permeabilization reagent so that the antibodies can enter the cell (the 1X Permeabilization buffer or a homemade one can be substituted using a reagent such as saponin). For transcription factor staining, a much harsher fix/perm set of buffers must be used. Foxp3 Staining Kit (eBioscience # 00-5523-00) is a good kit for intra-nuclear staining.
21. Incubate at 4˚C for 30 minutes
22. During ICS incubation, prepare compensation tubes for each antibody you use (including the viability dye). Please refer to the Compensation Beads Protocol
23. Wash ICS samples with 2ml of staining buffer at 500xg, 5 minutes room temperature.
24. Discard supernatant and resuspend in 150µl PBS. You are now ready to go to the flow cytometer!
    a. Fixed cells can remain in 4˚C overnight as long as they are protected from light with aluminum foil. This is not encouraged and flow should be done the same day whenever possible as many tandem dyes will begin breaking down. Compensation tubes should **always** be made the same day as flow analysis. Flow **must** be done on the same day as staining for unfixed cells.