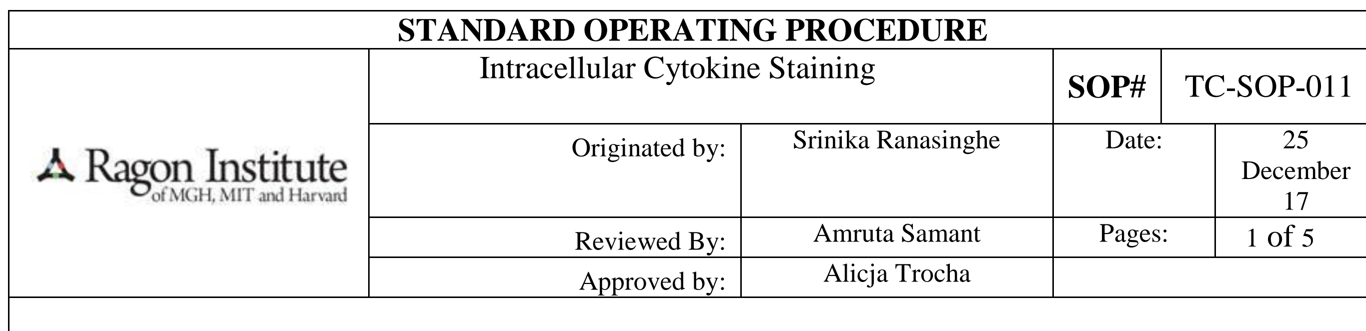

STANDARD OPERATING PROCEDURE				
Ragon Institute of MGH, MIT and Harvard	Intracellular Cytokine Staining		SOP#	TC-SOP-011
	Originated by:	Srinika Ranasinghe	Date:	25 December 17
	Reviewed By:	Amruta Samant	Pages:	1 of 5
	Approved by:	Alicja Trocha		

I. PURPOSE

The purpose of this procedure is to establish and outline the requirements for attire and personal protective equipment (PPE) for Ragon Institute laboratories.

II. SCOPE

This procedure applies to all employees, students, contractors and visitors that enter and/or work in the Ragon Institute laboratories.

III. RESPONSIBILITIES

A. The Ragon Institute **Director and all Laboratory Principal Investigators** are responsible for the overall implementation of this procedure and ensuring compliance.
B. **Environmental Health & Safety (EH&S)** is responsible for periodically reviewing the application and maintenance of this procedure, and initiating any updates to this procedure.
C. **All employees, students, contractors and visitors** are required to follow this procedure. Non-compliance with this procedure will result in the assignment of a corrective action plan.
D. **The Partners Institutional Biosafety Committee, Ragon Laboratory Managers and/or EH&S** are responsible for outlining additional PPE beyond what is stated in Section IV. (Procedure) if warranted through a regulatory requirement or industry best-practice.

IV. PROCEDURE

Reagent	Vendor	Catalogue #
Facs Tubes	BD Falcon	352054
Anti-CD28/CD49d Antibody	BD Fastimmune	347690
Brefeldin A (BFA)	Sigma-Aldrich	B7651
Golgistop (Monensin)	BD	554724
Fix Perm A	Invitrogen	GAS001S100
Fix Perm B	Invitrogen	GAS002S100
Live/Dead Fixable Violet Dead Cell Stain Kit	Invitrogen	L34955

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.

Preparation of the effector cells to be tested:

- ICS works well on Cryopreserved cells. For frozen cells, thaw and let them rest for 2-4 hours in R10 (or rest overnight). This step should reduce background (activation due to freeze/thaw process; apoptotic cells).
- If using fresh cells, use 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

1. Re-suspend PBMCs at 10^6/ml in R10 in Flow tubes
   - *Some people use 96w or 24w plates for the stimulations. Values indicated here are for a 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
   - Negative control= tube with no peptide added or PBMCs from a person who does not respond to the peptide (i.e. HIV peptide tested on HIV uninfected individual).
   - Positive control= Use either 10 µl SEB (1 mg/ml) or 20 µl PHA per tube for T cells or 1µl PMA/0.5µl Ionomycin for NK cells (NB: Although SEB stimulation is frequently 5-6hrs, it is recommended that PHA and PMA/Ionomycin positive controls are only stimulated for 2hours max to prevent cell death).
   - *Different options exist to stimulate lymphocytes. Most stimulate T cells unspecifically as superantigens like SEB by cross-linking the TCR (Staphylococcus enterotoxin B), T cell specific mitogens like PHA (phytohaemagglutinin), or Protein Kinase C (PKC) activation/Ca2+ influx via PMA/Ionomycin.*
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).

5. Incubate the cells at 37˚ C for 15minutes before adding 6µl of 1mg/ml Brefeldin A (BFA) and 0.5µl 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!
    - It may be more accurate to add 5 µl of Golgi-Stop (Monensin) after a 1:10 dilution instead of neat Golgistop.
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. After the incubation, cells can either be stained directly (but then also will need to be aquired on the flow cytometer immediately) or alternatively be transfered to a 4°C fridge overnight. Make sure to close lids tightly. The cold will stop/slow down cytokine production by putting the cells in a hibernating state.
8. Post-stimulation, wash cells once in PBS at 1700 rpm x 5 minutes if you use a viability dye (which we highly recommend) to reduce unspecific binding of the dye to proteins in the solution (ie FCS in the media).

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).

9. Add 1ul viability dye in 1ml PBS per tube and incubate for 20 minutes at RT in the dark
10. Wash with Flow buffer (PBS/FCS1%)
11. Discard supernatant and add surface antibodies (i.e. immunophenotype antibodies such as anti-CD3, CD4, CD8, CD16, CD14, CD19, CD56 etc) to the cell pellet (usually 150-200µl remain in the flow tube after decanting the supernatant) using a master mix and then mix cell suspension well using P200 pipet
    a. There are several 'standard Flow panels' in place at the Ragon Institute to assess polyfunctionality or cell subset function (eg memory T cells). Refer to the respective PIs if you need suggestions for panels
12. Incubate cells with surface antibodies at RT for 15 minutes in the dark. (Alternatively, incubation can also be done at 4˚C for 30 minutes, which should reduce unspecific binding of antibodies and thus should be done if background is an issue).
13. Wash once with PBS/1%FCS at 1700 rpm x 5 minutes at RT and decant/aspirate supernatant
14. Add 100 µl of Perm A Fixation Buffer to each sample, resuspend with P200 pipette (or vortex shortly), and then incubate for 20 minutes
15. Wash with approx 2ml PBS/1%FCS at 1700 rpm x 5 minutes at RT
16. Resuspend cell pellets with 100 µl of Perm B Fix/Permeabilization Buffer and immediately add intracellular antibodies directly to cells and Perm B. Resuspend with a P200 pipette (or vortex shortly).
    a. Alternatively the BD Fix/Perm System can be used: for that add 250 µl of Cytofix/Cytoperm™to each sample, vortex shortly, incubate for 15 min wash cells with 1ml of 1X Perm/Wash™ solution (dilute 1:10 with milliQ H_2O), dump supernatant and add intracellular antibodies to pellet
17. Incubate at RT for 30 minutes (or 4˚C for 40 minutes)
18. During ICS incubation prepare compensation tubes for each antibody you use (including the viability dye) and prepare a tube with rainbow beads (mid-range). Add one drop of compensation beads to each tube and then the respective antibody at the same amount as used for the cell staining.

For viability dyes from invitrogen use the Arc-reactive beads with 1ul of undiluted viability dye and prepare a separate tube with unstained Arc beads.

19. Wash with PBS/1%FCS (or 1X Perm/Wash™ solution (1 ml/wash) if you use the BD system)
20. Spin down, discard supernatatnt and resuspend in 200µl FACS buffer. You are now ready to go to the flow cytometer!