

FHCRC HVTN Laboratory
Standard Operating Procedure for:
Intracellular Cytokine Staining (ICS) Assay

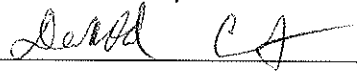
SOP #: FH-HVTN-A0002.GEN **Version:** 8.0

Name: Intracellular Cytokine Staining (ICS) Assay

Effective Date: September 13, 2010

Owner(s): Donald Carter, FHCRC HVTN Lab Manager

Approval: Donald Carter, FHCRC HVTN Lab Manager

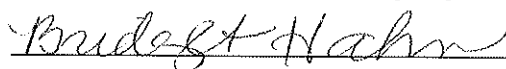


Signature

9-8-10

Date

Bridget Hahn, FHCRC HVTN QA/QC Laboratory Manager



Signature

9/8/10

Date

**Revision
History:**

Version	Description	Revised by	Revision Date
1.0	Initial Version		4/10/07
2.0	Updated the target medians for the new lasers	Donald Carter	5/18/07
3.0	Updated for use with multiple LSRs and changing staining panels	Donald Carter	6/20/08
4.0	Updated pass/fail worksheet	Donald Carter	6/5/09
5.0	Updated retesting form and ICS worksheet.	Donald Carter	12/17/09
6.0	Corrected ICS worksheet and updated Reagents & Solutions	Donald Carter	3/23/10
7.0	Updated retesting forms, FlowJo face page, and fail criteria	Donald Carter	5/11/10
8.0	Added space to record water bath temperature and R10 Benz.	Donald Carter	9/3/10

Purpose

This standard operating procedure (**SOP**) describes how to activate and stain cells for acquisition of intracellular cytokine flow cytometry data. This SOP will be accompanied by a set of “Study Specific Procedures” specific for the protocol or study being performed.

Scope

This SOP applies to intracellular cytokine staining (**ICS**) within the Fred Hutchinson Cancer Research Center (**FHCRC**) HVTN Endpoint Assay Laboratory.

Introduction

Intracellular cytokine staining is a flow cytometry-based method for enumeration of antigen-specific, cytokine-secreting T cells. Peripheral blood mononuclear cells (PBMC) from the subject being tested (typically an HIV-infected or vaccinated individual) are stimulated with HIV peptides in the presence of costimulatory antibodies against CD28 and CD49d. Negative control stimulations with PBMC and costimulatory antibodies but no HIV peptide, and positive control stimulations with superantigens such as staphylococcal enterotoxin B (SEB) are set up in parallel. Stimulations are allowed to proceed for six hours at 37°C in the presence of brefeldin A or monensin, which inhibit protein transport through golgi. Thus any cytokines induced by the HIV peptides in the HIV-specific T cells will be prevented from being secreted and will accumulate within the cells. Responding cells can be further characterized by additional, lineage-specific antibody markers (e.g. CD3 and CD8 antibodies for CD8+ T cells). Once fluorescently labeled, the cells are analyzed using a flow cytometer, which possess lasers and filters to excite specific fluorescent antibodies and measure the released light, permitting enumeration of the responding T cells.

The ICS assay is more informative than the ELISpot assay because it is able to detect multiple cytokines/chemokines and allows precise immunophenotyping using lineage-specific markers to identify the responding cell population(s). In addition, the ICS assay identifies antigen-specific T cells on a single cell level without long-term *in vitro* culture or the addition of exogenous cytokines like IL-2, providing a measured response that more closely mirrors what occurs *in vivo*. Furthermore, the ICS assay does not require the use of fresh cells or radioactive substances, making it a simpler and more transferable technique than other assays, like the chromium release, that have traditionally been used in HIV vaccine trials to measure T cell responses to vaccine antigens.

Comparison of the frequency of antigen-specific T cells before, during, and after an immunization cycle should reflect the relative immunogenicity of the vaccine being evaluated.

SOP for Intracellular Cytokine Staining Assay

Authority and Responsibilities

1. The FHCRC HVTN Laboratory Manager has the authority to establish this SOP.
2. Quality Assurance is responsible for the control of this SOP.
3. The FHCRC HVTN Laboratory Manager is responsible for the implementation of this procedure and for ensuring that all appropriate personnel are trained.
4. FHCRC personnel working on HVTN GLP studies are responsible for reading and understanding this SOP prior to performing the procedures described.

Definitions

Term	Definition
BFA	Brefeldin A
BSC	Biological Safety Cabinet
CEF	Cytomegalovirus, Epstein-Barr Virus, and Influenza peptide pool
CMV	Cytomegalovirus
D-PBS	Dulbecco's Phosphate Buffered Saline
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediamine-tetraacetic Acid
FBS	Fetal bovine serum
Guava PCA	Personal Cell Analysis System
ICS	Intracellular cytokine staining assay
IFN- γ	Interferon gamma
IL-2	Interleukin 2
IL-4	Interleukin 4
PBMC	Peripheral blood mononuclear cells
R10	Complete media, RPMI with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin
SEB	Staphylococcal enterotoxin B
TNF- α	Tumor Necrosis Factor-alpha
ViViD	Violet Live/Dead Stain

Materials

1. Aluminum foil
2. Centrifuge tubes, 50 mL
3. Drierite Absorbants, Fischer Scientific
4. Assay plate, 96 well, U bottom
5. Microcentrifuge tubes
6. Pipet tips
7. Pipets, 5mL, 10mL, 25mL, 50mL
8. Steri-Cup filter unit, Millipore, 0.22µm, Millipore Corp.
9. Desiccators, -20°C, Nalgene
10. FACS tubes, 5mL polystyrene round bottom tubes, 12 X 75mm, BD Falcon, Cat # 352052

Reagents & Solutions

Follow FH-HVTN-S0007, Reagent Preparation and Storage, when preparing, labeling and storing reagents.

1. BD FACS Lyse Solution, 10x

- 1.1 Vendor: BD Biosciences; Cat #349202
- 1.2 Preparation of the FACSLyse working solution referred to as 1X FACSLyse
 - 1.2.1 Dilute the 10X BD FACS Lyse 1:10 in deionized water.
 - 1.2.2 BD FACS Lyse working solution expires 1 month from date of preparation.
 - 1.2.3 Store FACS Lyse at room temperature.

2. BD FACS Perm II, 10X

- 2.1 Vendor: BD Biosciences; Cat #340973
- 2.2 Preparation of the FACSPerm working solution referred to as 1X FACSPerm
 - 2.2.1 Dilute the 10X BD FACS Perm II 1:10 in deionized water.
 - 2.2.2 BD FACS Perm II working solution expires 1 month from date of preparation.
 - 2.2.3 Store FACS Perm II at room temperature.

3. BFA

- 3.1 Vendor: Sigma Chemical Co.; Cat #B-7651
- 3.2 Preparation of BFA stock solution.
 - 3.2.1 Add DMSO directly to a vial of Brefeldin A (1ml DMSO per 5mg BFA).
 - 3.2.2 Cap the vial and shake to dissolve.

SOP for Intracellular Cytokine Staining Assay

3.2.3 BFA stock expires 1 year from the date of preparation.

3.2.4 Dispense aliquots into microcentrifuge tubes and freeze in -20°C freezer.

3.3 Preparation of BFA working solution.

3.3.1 Dilute stock 1:10 by mixing BFA stock solution with D-PBS.

3.3.2 The diluted BFA working solution may be stored in a 2-8° C refrigerator for up to a week.

4. CD28/49d

4.1 Vendor: BD Biosciences; Cat #347690

5. Deionized water

6. DMSO

6.1 Vendor: Sigma Chemical Co.; Cat #D-2650

7. Dulbecco's Phosphate-Buffered Saline solution w/o Ca⁺⁺ and Mg⁺⁺

7.1 Vendor: Gibco BRL Life Technologies; Cat # 14190-144

8. FACS Wash Buffer

8.1 Preparation of FACS Wash Buffer referred to FACS wash.

8.1.1 Add 10mL heat inactivated FBS to a 500mL bottle of PBS.

8.1.2 FACS Wash Buffer expires 2 weeks from date of preparation.

8.1.3 Store FACS Wash Buffer in a 2-8° C refrigerator.

9. EDTA, 20mM

9.1 Vendor: Fisher Chemicals, Cat #O2793-500

9.2 Preparation of the EDTA working solution referred to as EDTA solution

9.2.1 Weigh 744mg of EDTA on a balance.

9.2.2 Add the EDTA to a bottle containing 100mL D-PBS.

9.2.3 Swirl to dissolve.

9.2.4 Bring to pH 7.2-7.4 with 1M NaOH.

9.2.5 FACS EDTA expires 6 months from date of preparation.

9.2.6 Store EDTA in a 2-8° C refrigerator.

10. Ethanol, 70%

11. Fetal Bovine Serum (FBS)

11.1 Vendor: Gemini Benchmark; Catalog #100-106

11.2 FBS should be stored at -20°C until thawed for use.

11.3 In most cases, FBS lots purchased for use in HVTN assays will have been heat inactivated in a batch by the vendor. This will be indicated on the bottle. If the FBS has not already been heat inactivated, heat inactivate at 56°C for 30 minutes, and store at 4°C. Initial and date bottle for heat inactivation. If the FBS has already been heat inactivated, it can be thawed and stored at 4°C.

SOP for Intracellular Cytokine Staining Assay

4°C storage of heat-inactivated FBS is not recommended for longer than one month.

- 11.4 FBS lots must be pre-screened for intracellular cytokine staining (ICS) assay for low background activity as well as the capacity to optimally support antigen-specific (e.g. CEF pool) responses. A single lot of FBS must be used for all ICS assays performed in conjunction with a given vaccine trial.

12. Fluorescently labeled monoclonal antibodies

- 12.1 Store at 4°C.

13. Guava ViaCount

- 13.1 Vendor: Guava Technologies; Cat #4000-0041

14. L-glutamine

- 14.1 Vendor: Gibco BRL Life Technologies; Cat #25030-081
- 14.2 200mM L-glutamine, 100x concentration, store at -20°C.

15. Paraformaldehyde, 10 %

- 15.1 Vendor: Electron Microscopy Sciences; Cat #15712-S
- 15.2 Preparation of the paraformaldehyde working solution referred to as 1% Paraformaldehyde
 - 15.2.1 Dilute the 10% Paraformaldehyde 1:10 in D-PBS.
 - 15.2.2 Paraformaldehyde working solution expires 1 month from date of preparation.
 - 15.2.3 Store Paraformaldehyde in a 2-8° C refrigerator.

16. Penicillin-Streptomycin

- 16.1 Vendor: Gibco BRL Life Technologies; Cat #15140-122
- 16.2 10,000 Units, Store at -5°C to -20°C.

17. RPMI 1640 with 25mM HEPES buffer and L-glutamine

- 17.1 Vendor: Gibco BRL Life Technologies; Cat # 22400-089
- 17.2 Store at 4°C.

18. R10 Media and R10/Benz

- 18.1 Preparation of culture media referred to as **R10**
 - 18.1.1 Into 500ml of RPMI 1640 with 25mM HEPES buffer and L-glutamine add 55ml of FBS, 5ml of L-glutamine, 5ml of Penicillin-Streptomycin.
 - 18.1.2 R10 can be stored for 2 weeks at 4°C.
- 18.2 Preparation of thawing media, **R10/Benz** (R10 supplemented with benzonase at 50U/ml)
 - 18.2.1 For 25U/μl Benzonase: Into 565ml of **warmed** R10 add 1.13ml of benzonase (or 20μl/10ml if less media is needed)

SOP for Intracellular Cytokine Staining Assay

18.2.2 For 250U/μl Benzonase: Into 565ml of R10 add 0.113ml of benzonase (or 2μl/10ml if less media is needed)

18.2.3 R10/Benz must be prepared fresh each time it is used. Filter with a 0.22μm filter (Millipore filter from Fisher, cat# 5CGVUOIRE, or equivalent).

18.2.4 Place the R10/Benz in a 37°C, 5% CO₂ incubator until use to maintain temperature.

18.2.5 R10/Benz expires the same day as it was prepared.

19. SEB

19.1 Vendor: Sigma Chemical Co.; Cat #S4881

19.2 Preparation of culture media referred to as SEB working solution

19.2.1 Add D-PBS directly to a new vial of SEB (2ml D-PBS per 1mg SEB).

19.2.2 SEB expires 1 year from date of preparation.

19.2.3 Store vial in a 2-8° C refrigerator.

20. Sphero 7th Peak Fluorescent Particles, 3.8 μm, 2 mL

20.1 Vendor: Spherotech, Cat#RCP-30-5A-7

20.2 Preparation of Ultra Rainbow beads working dilution referred to as 1X Rainbow Beads:

20.2.1 Add 1 mL 1x PBS to a 5 mL FACS Tube.

20.2.2 Vortex stock vial of Sphero 7th Peak Rainbow Fluorescent Particles.

20.2.3 Add 3 drops of the stock beads to the PBS in the FACS Tube.

20.2.4 Label tube with "7th Peak Beads", the lot number and size of beads, your initials and the expiration date (1 week from preparation date.)

20.2.5 Store at room temperature in minimal light.

21. Sphero Rainbow Calibration Particles (8 Peaks), 3.0 μm, 5 mL

21.1 Vendor: Spherotech, Cat# RCP-30-5A

21.2 Preparation of working dilution referred to as 8x Rainbow beads:

21.2.1 Add 1 mL 1x PBS to a 5 mL FACS Tube.

21.2.2 Vortex stock vial of Sphero Rainbow 8 Peak Calibration Particles.

21.2.3 Add 3 drops of the stock beads to the PBS in the FACS tube.

21.2.4 Label tube with "Rainbow 8x", the lot number and size of beads, your initials and the expiration date (1 week from preparation date.)

21.2.5 Store at room temperature in minimal light.

22. ViViD Fixable Violet Dead Cell Stain Kit

22.1 Vendor: Molecular Probes/Invitrogen; Cat #L34955

22.2 Preparation of ViViD stock solution referred to as ViViD stock

SOP for Intracellular Cytokine Staining Assay

22.2.1 Add 50µl of anhydrous DMSO (supplied with kit) directly to a vial of the ViViD stain provided in the kit and resuspend contents of vial.

22.2.2 Prepare aliquots in microcentrifuge tubes, for single use, and freeze in dessicator at -20°C.

22.2.3 Aliquots expire 4 weeks from date of preparation.

22.3 Preparation of ViViD working solution

22.3.1 Thaw a vial of the ViViD stock by leaving at room temperature until thawed.

22.3.2 Dilute in PBS immediately before use (dilution ratio dependent on titration results for that lot of ViViD). Mix the working solution well.

23. AViD Fixable Aqua Dead Cell Stain Kit

23.1 Vendor: Molecular Probes/Invitrogen; Cat #L34957

23.2 Preparation of AViD stock solution referred to as AViD stock

23.2.1 Add 50µl of anhydrous DMSO (supplied with kit) directly to a vial of the AViD stain provided in the kit and resuspend contents of vial.

23.2.2 Prepare aliquots in microcentrifuge tubes, for single use, and freeze in dessicator at -20°C.

23.2.3 Aliquots expire 4 weeks from date of preparation.

23.3 Preparation of AViD working solution

23.3.1 Thaw a vial of the AViD stock by leaving at room temperature until thawed.

23.3.2 Dilute in PBS immediately before use (dilution ratio dependent on titration results for that lot of AViD). Mix the working solution well.

24. Peptides

24.1 See the "Study Specific Procedures" for details on peptides specific to the protocol or study being performed.

24.2 HIV-1 (15-mer peptides), CMV peptides, and other peptides provided individually or in pools (≥70% purity).

24.3 Pools of peptides are reconstituted and stored in aliquots at -80°C. Peptides should be only thawed once and then may be stored at 4°C for up to 7 days. Record date thawed on the peptide vial.

24.4 CMV pp65 peptide pool – A panel of 138 15-mer CMV pp65 peptides recognized by CD8+ and CD4+ T cells. Working stocks at 40 µg/ml of each peptide have been prepared, aliquoted, and stored at -80°C.

Instrumentation

1. BD FACSCalibur flow cytometer, optionally equipped with High Throughput Sampler (HTS)
2. BD LSR II, optionally equipped with High Throughput Sampler (HTS)
3. Guava Cell Analyzer
4. Water bath, 37°C
5. Incubator, 37°C, 5% CO₂
6. Centrifuge
7. Balance
8. BSC
9. Micropipetters
10. Freezer

Specimens

All ICS assays will be conducted on peripheral blood mononuclear cells (PBMC) cryopreserved using the established SOP for collection processing and cryopreservation of PBMC.

Procedure

1. Day 1 Thawing

- 1.1 Thaw cryopreserved PBMC according to SOP FH-HVTN-P0004.

Note: When 2 vials of the same participant/visit need to be thawed, it is acceptable to thaw more than 2 vials at one time. The maximum number of vials can be 4 for the case when 2 vials of each of 2 participant/visits need to be thawed.

2. Day 2 Activation

- 2.1 Remove cells from incubator. Fill tubes to 20mL with R10 (if not already at 20mL). Centrifuge the cells, 250xG for 10 minutes. Decant the supernatant. Gently resuspend the pellet with a 200µl pipettor and resuspend in 5-10ml R10.
- 2.2 Determine cell number and viability according to Guava Counter SOP FH-HVTN-E0018.
- 2.3 Centrifuge the cells, 250xG for 10 minutes, decant the supernatant, and gently resuspend the pellet with a 200µl pipettor. Resuspend the cells with R10 at 5×10^6 cells/ml and plate (200µl/well) according to the plate layout (See the Plate Layout section of the study specific Study Plan). For the compensation wells, cells from any donor can be used, any remaining cells from different donors can be pooled, or cells from another experiment can be used.

Note: If fewer than the desired number of cells are recovered, then the cells may be resuspended at less than 5×10^6 cells/ml to allow for 200ul of cell

SOP for Intracellular Cytokine Staining Assay

suspension to be distributed to each well, to a minimum of 2.5×10^6 cells/ml. If there are still not enough cells, refer to the "Study Specific Procedures" or contact the study manager.

- 2.4 Thaw assay specific peptide pool aliquots and BFA by leaving the vials at room temperature up to one hour on the bench top or in the BSC.
 - 2.5 Prepare stimulation cocktails for DMSO (negative control), SEB (positive control), and peptide pools according to the details in the "Study Specific Procedures."
 - 2.6 Add 20µl of each stimulation cocktail to appropriate wells.
 - 2.7 Incubate plates undisturbed in an incubator at 37°C, 5% CO₂ for six hours (+/- 15 minutes).
 - 2.8 After incubation, plates can be put into a refrigerator up to 18 hours, or proceed to next step.
 - 2.9 Add 20µl of 20mM EDTA to each well and mix well with a multichannel pipette.
 - 2.10 Incubate plates for 10 minutes at room temperature.
 - 2.11 Centrifuge the plates at 750xG for 3 minutes.
 - 2.12 After centrifugation, flick supernatant from the wells.
 - 2.13 Prepare viability dye working dilution(s) as described in the worksheet.
 - 2.13.1 Depending on the protocol, ViViD or AViD may be used as the viability dye. Which to use will be indicated in the SSP for that protocol.
- Note:** ViViD and AViD are extremely labile once thawed and diluted with PBS for the working dilution. Therefore, do not thaw and prepare the working dilution until immediately before use.
- 2.14 After flicking the plates, resuspend the appropriate wells to be stained with 50µl of the viability dye working solution. Also resuspend the cells in the viability dye compensation well with 50µl of the ViViD working solution. Resuspend the remaining wells containing cells with 50µl PBS.
 - 2.14.1 In some cases, cells may be tested in duplicate with a peptide pool and then pooled at this step. Refer to the "Study Specific Procedures" which will contain instructions if this is the case. This only applies to certain peptide pools and only if so instructed in the "Study Specific Procedures." It does NOT apply to the negative controls (receiving DMSO), which are generally tested in duplicate.
 - 2.15 Incubate at room temperature under foil for 20 minutes.
 - 2.16 Add 150µl PBS and centrifuge the plates at 750xG for 3 minutes.
 - 2.17 After centrifugation, flick supernatant from the wells.
 - 2.17.1 If there is a surface stain, prepare surface antibody cocktail according to the "Study Specific Procedures", using the appropriate titer for each lot of antibody. If there is no surface stain, proceed to step 2.18.
 - 2.17.2 Add 50µl antibody solution to the appropriate wells and resuspend the cells. Resuspend compensation wells with 50ul FACS wash, and then add individual antibodies to the compensation wells.

SOP for Intracellular Cytokine Staining Assay

- 2.17.3 Incubate at room temperature, in the dark for 20 minutes (acceptable range is 17 to 23 minutes).
- 2.17.4 Add 150µl PBS and centrifuge the plates at 750xG for 3 minutes. After centrifugation, flick supernatant from the wells.
- 2.17.5 If there is no secondary antibody, proceed to step 2.18.
- 2.17.6 Add 200µl PBS and centrifuge plates at 750xG for 3 minutes. After centrifugation, flick supernatant from the wells.
- 2.17.7 Resuspend test wells in 50µl secondary antibody cocktail. Resuspend all remaining wells in 50µl PBS. Add secondary antibodies to appropriate compensation wells.
- 2.17.8 Incubate at room temperature, in the dark for 20 minutes (acceptable range is 17 to 23 minutes).
- 2.17.9 Add 150µl PBS and centrifuge the plates at 750xG for 3 minutes. After centrifugation, flick supernatant from the wells.
- 2.18 Add 200µl PBS and centrifuge plates at 750xG for 3 minutes.
- 2.19 After centrifugation, flick supernatant from the wells.
- 2.20 Resuspend cells in each well with 100µl of 1X BD FACS Lyse Working Solution.
- 2.21 Incubate the plates in the dark at room temperature for 10 minutes.
- 2.22 Wrap the plates in foil and place in a Low Temperature Freezer (-70 to -80°C) for up to 3 weeks.

3. Day 3 Staining

- 3.1 Remove the plates from the Low Temperature Freezer (-70 to -80°C) and place in a 37°C incubator for ~20 minutes or until all the wells are thawed.
Note: Once the wells are thawed, the processing of the plates should not be delayed. It is recommended that time interval between placing the frozen plates in the incubator and the addition of FACS Wash buffer (as in the next step) should be no longer than 45 minutes.
- 3.2 Add 100µl of FACS Wash Buffer to each well.
- 3.3 Centrifuge the plates at 750xG for 3 minutes.
- 3.4 Flick supernatant from the wells.
- 3.5 Resuspend the cells in the wells with 200µl of FACS Perm II Working Solution.
- 3.6 Incubate the plates for 10 minutes in the dark at room temperature.
Note: Cells should not be exposed to the FACS Perm solution for more than 10 minutes. Start the timer as FACS Perm is added to the first well (instead of starting the timer after FACS Perm has been added to all the wells).
- 3.7 Centrifuge the plates at 750xG for 3 minutes.
- 3.8 Flick supernatant from the wells.
- 3.9 Add 200µl of FACS Wash Buffer to each well and resuspend cells.
- 3.10 Centrifuge the plates 750xG for 3 minutes.
- 3.11 Flick supernatant from the wells.

SOP for Intracellular Cytokine Staining Assay

- 3.12 Add 200µl of FACS Wash Buffer to each well and resuspend cells.
- 3.13 Centrifuge the plates at 750xG for 3 minutes.
- 3.14 Flick supernatant from the wells.
- 3.15 Prepare antibody cocktails according to the "Study Specific Procedures", using the appropriate titer for each particular lot of antibody.
- 3.16 Add 50µl antibody solution to the appropriate wells and resuspend the cells.
- 3.17 Resuspend compensation wells with 50ul FACS wash, and then add individual antibodies to the compensation wells.
- 3.18 Incubate at room temperature, in the dark for 30 minutes (acceptable range is 25 to 35 minutes).
- 3.19 After the completion of incubation, add 150µl of FACS Wash Buffer to all wells.
- 3.20 Centrifuge the plates at 750xG for 3 minutes.
- 3.21 Flick supernatant from the wells.
- 3.22 Add 200µl of FACS Wash Buffer to each well
- 3.23 Centrifuge the plates at 750xG for 3 minutes.
- 3.24 Flick supernatant from the wells.
- 3.25 Resuspend the cells with 150ul of 1% Paraformaldehyde Working solution.
- 3.26 Add at least 150ul of FACS wash, 1% Paraformaldehyde, or PBS to empty plates wells as directed by the protocol-specific plate layout.
- 3.27 Wrap the plates in foil and place in refrigerator for up to 18 hours.
- 3.28 Acquire the data by flow cytometry directly from the plates using the HTS, or manually after transferring cells from the plates to FACS tubes.
- 3.29 After the plates have been collected, check the compensation wells to insure they are acceptable. If there are any problem wells (no staining, not enough cells, wrong stain used, etc.), they will need to be replaced.
 - 3.29.1 If comp wells need to be replaced, use leftover comp cells from a previous experiment if at all possible. Those leftover cells may be transferred from the old plate into the plate being collected in the same position as the failed comp well (if the incorrect stain was used, rinse the well out twice with 200ul PBS or FACS wash using a pipette first).
 - 3.29.2 Once the replacement cells have been added to the plate, put it on the HTS and select the well to be collected in FACS Diva. When collecting a well where the program already has data, it will prompt you that the data will be over-written. Be certain to only re-collect the problem well(s) and that the machine does not continue on and over-write other data it has collected.

Pass/Fail Criteria

1. **Viability:** On day 2, the viability must be at least 66% to proceed with the experiment. If the day 2 viability is less than 66%, the sample must be repeated. If a second thaw also results in a day 2 viability of less than 66%, the sample will not be tested.
2. **Cell number:** On day 2, there may not be enough cells to perform the assay. The minimum number of cells needed will be indicated in the Study Specific Plan, and is subject to the discretion of the study manager. If it is determined that there are too few cells to perform the assay, the sample will not be plated and must be repeated. If a second thaw also results in too few cells for testing on day 2, the sample will not be tested.
3. **Positive control wells:** Each specimen tested will have a SEB control for CD4⁺ and CD8⁺ T cells. If the SEB control is less than 1.2% for either the CD4⁺ or CD8⁺ T cells, the sample for that specimen at that time point must be re-tested. The percentage response used for this is the combined response for IFN- γ and IL-2. This is the sum of the percentages for IFN- γ ⁺IL-2⁺, IFN- γ ⁺IL-2⁻ and IFN- γ ⁻IL-2⁺ populations. Any additional cytokines included in the assay are not used to determine this percentage. If the re-tested results are below 1.2%, then the sample data from the second collection will be used. This data will be flagged identifying the low SEB response.
4. **Negative control wells:** If the average cytokine response for the negative control wells is above 0.1% for either the CD4⁺ or CD8⁺ T cells, then the sample must be re-tested. The same criteria as in the positive control wells are used for determining this response. If the re-tested results are above 0.1% then the sample results data from the second collection will be used. The data will be flagged identifying the high background response.
5. **Number of CD4 and CD8 T cells collected:** If the number of CD4 or CD8 cells, as counted in the CD4 or CD8 gates determined by the software analysis of the data after collection is less than 5,000 for any of the wells for a particular sample, then the data for that well is unreliable and will be filtered out, and the sample may need to be re-tested. For CMV and SEB-stimulated wells, low CD4 or CD8 cell numbers are not criteria for re-testing. If only one of the 2 negative control (DMSO) replicates has a CD4 or CD8 count below 5,000, the other replicate may be used by itself for data analysis. However, if both negative control wells fail for low CD4 or CD8 counts, then the same will need to be retested. Antigen stimulated wells with low cell counts will not be analyzed, and the sample will be re-tested at the discretion of the study manager. If the sample is retested, the retest results are taken as the final data. If the re-tested results include wells with fewer than 5,000 CD4 or CD8 cells, those wells will be filtered from the final analysis.

Data Management

1. Data that have passed review (according to Pass/Fail Criteria), FlowJo analysis, and Labkey analysis will move forward to data management.
2. A conformance check will be performed by the lab manager (using Attachment 6). The following steps will be completed and documented on Attachment 6 prior to data transmission to SCHARP:
 3. Record the FCS file folder name for one set of plates. This name is unique for each batch of samples.
 4. The original ICS worksheets will be checked for completeness and accuracy.
5. Data printout: After FlowJo analysis is completed, the data will be printed out and stored in a labeled binder. The lab manager will then review the printouts to ensure that the gating is appropriate and QC the data for anomalies. Any deviations should be noted on the Face page for FlowJo Analysis (Attachment 5) (if affecting assay criteria) and/or the ICS Worksheet.
6. LabKey Data: The lab manager will check that the PTID data is accurate and has been properly joined to the FCS file data. Then, the lab manager will check the pass/fail criteria using a LabKey query. Any samples that fail criteria must be checked against the FlowJo printout for accuracy. If not previously filled out, the ICS Sample Retesting Form (Attachment 1) can be filled out by the lab manager at this time.
7. Data ready for SCHARP: After review of the data, the lab manager or designee will work to resolve any issues with documentation or assay acceptance criteria. When the lab manager is satisfied with the quality of the data, it can be exported from LabKey and uploaded to SCHARP.

SOP for Intracellular Cytokine Staining Assay

Attachment 1 ICS Sample Retesting Form

PTID: _____ Visit: _____

Name of LSR collection file: _____ Sample order on plate: _____

Reason for repeating (explain in comments for each fail):

- ☐ % Viability: Day 1 viability = _____ Day 2 viability = _____
- ☐ Cell Number: Day 2 cell count = _____
- ☐ Negative control: CD4+ = _____ CD8+ = _____
- ☐ Positive control: CD4+ = _____ CD8+ = _____
- ☐ # of T cells collected (describe in comments the stimulations that failed for CD4/CD8)
- ☐ Other (describe in comments below)

Comments: _____

Pass/Fail Analysis Performed By/Date: _____

Sample Tracking: CD4 = _____ CD8 = _____ Both = _____

☐ **Final Data?**

Entered into protocol tracking file By/Date: _____

Retest Date: _____ **LSR collection file:** _____ **Sample order:** _____

Result of repeat: ☐ Pass all criteria

- ☐ % Viability: Day 1 viability = _____ Day 2 viability = _____
- ☐ Cell Number: Day 2 cell count = _____
- ☐ Negative control: CD4+ = _____ CD8+ = _____
- ☐ Positive control: CD4+ = _____ CD8+ = _____
- ☐ # of T cells collected (describe in comments the stimulations that failed for CD4/CD8)
- ☐ Other (describe in comments below)

Comments _____

Pass/Fail Analysis Reviewed By/Date: _____

Sample Tracking: CD4 = _____ CD8 = _____ Both = _____

☐ **Final Data?**

Entered into protocol tracking file By/Date: _____

SOP for Intracellular Cytokine Staining Assay

Attachment 1 (page 2)

Empty Vector Sample Retesting Form

Only use page 2 for Empty Vector repeats

PTID: _____ Visit: _____

Name of LSR collection file: _____ Sample order on plate: _____

Reason for repeating (explain in comments for each fail):

- ☐ % Viability: Day 1 viability = _____ Day 2 viability = _____
- ☐ Cell Number: Day 2 cell count = _____
- ☐ Negative control: CD4+ = _____ CD8+ = _____
- ☐ Positive control: CD4+ = _____ CD8+ = _____
- ☐ # of T cells collected (describe in comments the stimulations that failed for CD4/CD8)
- ☐ Other (describe in comments below)

Comments: _____

Pass/Fail Analysis Performed By/Date: _____

Sample Tracking: CD4 = _____ CD8 = _____ Both = _____

☐ Final Data?

Entered into protocol tracking file By/Date: _____

Retest Date: _____ LSR collection file: _____ Sample order: _____

Result of repeat: ☐ Pass all criteria

- ☐ % Viability: Day 1 viability = _____ Day 2 viability = _____
- ☐ Cell Number: Day 2 cell count = _____
- ☐ Negative control: CD4+ = _____ CD8+ = _____
- ☐ Positive control: CD4+ = _____ CD8+ = _____
- ☐ # of T cells collected (describe in comments the stimulations that failed for CD4/CD8)
- ☐ Other (describe in comments below)

Comments _____

Pass/Fail Analysis Reviewed By/Date: _____

Sample Tracking: CD4 = _____ CD8 = _____ Both = _____

☐ Final Data?

Entered into protocol tracking file By/Date: _____

SOP for Intracellular Cytokine Staining Assay

Attachment 2 ICS Assay Worksheet

Batch #: _____

Date: _____

Reviewed By/Date: _____

NOTE: All deviations should be recorded on a Deviation Report Form. (FH-HVTN-Q0011).

Day 1 **Day 1 Date:** / /

Initial	Step	Description	Time/Amount
	1	Prepare R10. Record batch # in reagent log notebook. R10: Lot#: _____ Exp. Date: _____	-- N/A--
	2	Prepare R10/Benzonase. Record batch # in reagent log notebook. R10/Benzonase: Lot#: _____ Exp. Date: _____	-- N/A--
	3	Thaw samples according to SOP FH-HVTN-P0004. <u>Waterbath(s) Equipment #</u> <u>Temperature</u> _____ °C _____ °C _____ °C _____ °C	-- N/A--
	4	Add R10/Benzonase.	-- N/A--
	5	Determine cell number and viability using the Guava according to SOP FH-HVTN-E0018.	-- N/A--
	6	Begin overnight incubation.  Time in incubator: _____	_____ : _____

SOP for Intracellular Cytokine Staining Assay

Day 2

Day 2 Date / /

Initial	Step	Description	Time/Amount
	7	Remove cells from incubator. ➡ Time out of incubator:	 :
	8	Determine cell number and viability using the Guava according to SOP FH-HVTN-E0018. VIABILITY MUST EXCEED 66 % TO PROCEED.	-- N/A--
	9	Label plate(s) according to plate layout and prepare stimulation cocktails according the ICS Stimulation Cocktail Worksheet. PBS: Lot# _____ Exp. Date: _____ BFA working solution: Lot# _____ Exp. Date: _____	-- N/A--
	10	Plate samples and add 20 ul of stimulation cocktails to the appropriate wells. Note that the SEB cocktail may also be added to some compensation wells (see plate layout). No stimulation cocktail is added to the column of wells including the other compensation wells and the unstained cells.	-- N/A--
	11	Incubate plates for 6 hours at 37 deg (+/- 15 minutes). ➡ Time in incubator:	 :
	12	Remove plates from incubator. ➡ Time removed from incubator:	 :
	13	Add 20ul EDTA solution, mix and incubate 10 minutes at RT. EDTA solution: Lot# _____ Exp. Date: _____	Time in: : Time out: :

SOP for Intracellular Cytokine Staining Assay

Initial	Step	Description	Time/Amount
	14	Centrifuge plates and flick supernatant.	-- N/A--
	15	Prepare viability dye working dilution immediately before use. The viability dye is diluted in PBS at a ratio determined by the titration for the current lot. ViViD or AViD (circle one to use as listed in SSP and on the antibody worksheet): Lot# _____ Exp. Date: _____ Dilution (in ul/mL PBS): _____	-- N/A--
	16.1	NOTE: Perform this step only if there are samples tested against Env peptide pools in duplicate. If not, skip to step 16.2 For the duplicate wells stimulated with PTE Env 1, 2, and 3, combine the duplicates by first resuspending the left column (first replicates) with 50ul of the viability dye working solution and then transferring these cells to the second column (second replicates), and resuspending the second replicate wells. Note that ONLY the Env replicates are pooled, and NOT the DMSO replicates.	-- N/A--
	16.2	Resuspend the remaining sample wells with 50ul of viability dye working solution. This does not include the compensation wells. Only resuspend the viability dye compensation well with 50ul of this working solution. Resuspend the remaining wells containing cells with 50ul of PBS. Incubate for 20 minutes at RT in the dark.	Time in: _____ Time out: _____
	17	Add 150ul PBS and centrifuge plates. Flick supernatant. If no surface stain, proceed to step 19	-- N/A--
	18.1	If there is a surface stain: Resuspend test wells in 50µl surface stain cocktail. Resuspend all compensation wells in 50µl PBS. Add surface stain antibodies to appropriate compensation wells. Incubate at RT under foil 20 minutes.	Time in: _____ Time out: _____
	18.2	Add 150ul PBS and centrifuge plates. Flick supernatant. Add 200µl PBS and repeat.	-- N/A--

SOP for Intracellular Cytokine Staining Assay

Initial	Step	Description	Time/Amount
	18.3	If there is no secondary antibody, skip to step 20 Resuspend test wells in 50µl secondary antibody cocktail. Resuspend all remaining wells in 50µl PBS. Add secondary antibodies to appropriate compensation wells. Incubate 20 minutes RT under foil.	Time in: _____ Time out: _____
	18.4	Add 150ul PBS and centrifuge plates. Flick supernatant.	-- N/A--
	19	Add 200ul PBS and centrifuge plates. Flick supernatant.	-- N/A--
	20	Resuspend with 100ul of 1x FACS lyse solution and incubate for 10 minutes at RT in the dark. FACS Lyse: Lot# _____ Exp. Date: _____	-- N/A--
	21	Store at –80°C.  Time placed in freezer: _____	_____

SOP for Intracellular Cytokine Staining Assay

Day 3 **Day 3 Date:** / /

Initial	Step	Description	Time/Amount
	22	Prepare antibody cocktail as described in the ICS Antibody Cocktail Worksheet following these steps:	-- N/A--
	23.1	Calculate the number of stain tests required. The number of tests is calculated as the number of vaccine samples x number of stimulation conditions. Record this in the table.	-- N/A--
	23.2	Calculate the amount of each antibody to add to mix by multiplying the titer by the number of tests. Write these antibody volumes in the right column in the table and total these values.	-- N/A--
	23.3	Calculate the final stain volume. Multiply the number of tests x 55ul. Record this in the table.	-- N/A--
	23.4	Calculate the amount of FACS wash to add to the antibody cocktail by subtracting the total antibody volume from the final stain volume. Record this in the table.	-- N/A--
	23.5	Prepare the antibody cocktail by adding the FACS wash and the volumes of each antibody in the last column in the table. Initial the table after the reagents are added. Gently mix the cocktail after the last reagent is added. Centrifuge at 1100 G for 5 minutes.	-- N/A--
	24	Remove plates from freezer, thaw at 37°C and add 100ul of FACS Wash buffer to each well. FACS Wash: Lot# _____ Exp. Date: _____	-- N/A--
	25	Centrifuge the plates, resuspend cells in 200ul of FACS Perm II solution and incubate for 10 minutes at RT in the dark. FACS Perm II: Lot# _____ Exp. Date: _____	Time in: _____ : _____ Time out: _____ : _____

SOP for Intracellular Cytokine Staining Assay

Initial	Step	Description	Time/Amount
	26	Centrifuge the plates and wash twice with 200ul FACS Wash buffer.	-- N/A--
	27	After flicking supernatant, resuspend the sample wells with 50ul of the antibody cocktail. Resuspend the compensation wells with 50ul of FACS wash and then add each compensation antibody. Gently mix the compensation wells after addition of antibody. Incubate for 30 min (+/- 5 min) at RT in the dark.	Time in: _____: _____: Time out: _____: _____:
	28	Add 150ul of FACS Wash buffer and centrifuge the plates.	-- N/A--
	29	Wash plates once with 200ul FACS Wash Buffer.	-- N/A--
	30	Resuspend wells with 150ul of 1% Paraformaldehyde working solution and store in refrigerator until FACS collection. 1% Paraformaldehyde: Lot# _____ Exp. Date: _____ ➡ Time staining completed:	_____: _____:

SOP for Intracellular Cytokine Staining Assay

FACS Collection Date: ____/____/____

Initial	Step	Description	Time/Amount
	31	Create QC folder by opening the QC template:	-- N/A--
		➡ Record QC folder name (follow convention, e.g., #####-X-000-QC: ##### is a placeholder for the batch number, X is a placeholder for the machine designation [such as L or M], 000 is a placeholder for the protocol number, QC indicates this is the QC folder): _____	
	32	Run alignment beads and adjust PMT voltages to match target medians as appropriate for each machine and record results in Attachment 7 LSR QC. Channels not used in an experiment do not have to have their voltages adjusted, but there is no harm in doing so. Those channels may also be deleted from the QC file if desired. 7th Peak Rainbow Beads: Lot# _____ Exp. Date: _____ 8x Ultra-Rainbow Beads: Lot# _____ Exp. Date: _____	-- N/A--
	33	Check CV's: Are CV's within tolerance limits:	YES/NO
	34	If the CVs are outside the tolerance limits, inform the study manager or other qualified supervisor who will determine if the experiment can proceed. Comments: _____	-- N/A--
	35	Staple Attachment 7 LSR QC sheet to the back of the worksheet.	-- N/A--

SOP for Intracellular Cytokine Staining Assay

Initial	Step	Description	Time/Amount
	36	Perform plate clean on HTS (this may also be done prior to running the bead QC step).	-- N/A--
	37	Copy instrument settings.	-- N/A--
	38	Create a new experiment from the protocol-specific template. ➡ Record folder name (follow convention as noted above for QC file, e.g., ####-L-000. If desired, this can be followed by a description of the assay, such as ###-L-000-prof for proficiency samples):	
	39	Paste in instrument settings from QC folder.	-- N/A--
	40	Collect plates according to the LSRII equipment SOP (FH-HVTN-E0022). ➡ Time FACS collection begun:	:
	41	After collection is completed, check all compensation wells to insure they are acceptable. If any have a problem (no staining, wrong stain, or not enough events), those comp wells should be re-collected using leftover comp cells from a previous day, if possible. If not, explain in the comments section.	-- N/A--
	42	Export the experiment folder and the QC folder to the Data Export folder on the desktop. ➡ Time collection completed:	:
	43	Copy the experiment folder and the QC folder from the Data Export folder into a folder based on the date of collection: Sluf50\\Vaccine\FACS_Data_for_ICS\[year]\[month]\[day] Where the year month and day are given as: [year] = 4 digit year [month] = 2 digit month [day] = 2 digit day Once that is done, delete the data from the Data Export folder.	-- N/A--
	44	Perform HTS and instrument cleaning procedures as per the LSRII equipment SOP (FH-HVTN-E0022).	-- N/A--

Comments _____

SOP for Intracellular Cytokine Staining Assay

Attachment 3

ICS Stimulation Cocktail Worksheet (See Study Specific Procedures)

Stimulation Cocktails:

Stim condition	Peptide Lot #	Stock conc. (Dilution in mix)	Volume peptide or DMSO (uL)	Volume CD28/49 use at 1:10 (uL)	Volume of BfA use at 1:5 (uL)	Volume of PBS (uL)	Final volume (uL)	Performed by

SOP for Intracellular Cytokine Staining Assay

Attachment 4

ICS Antibody Cocktail Worksheet (See Study Specific Procedures)

Preparation of antibody cocktail

Number of tests required calculated as:

Number of PBMC samples _____ x (# of stim conditions) + 4 (for FH Ctrl wells)
= _____

Antibody	Lot#	Exp date	Titre (µl)	For comp wells		For cocktail	
				Comp well (µl)	By	Volume (µl) to add to mix	By
				Total Ab Volume (ul):			N/A
				FACS Wash Volume (ul):			
				Total stain cocktail volume (ul) = #tests x 55			N/A

SOP for Intracellular Cytokine Staining Assay

Attachment 5

Face Page for FlowJo Analysis

FlowJo analysis for protocol:_____

Date of FlowJo analysis:_____ Performed by:_____

Name of folder containing FCS files_____

Make a note here if the folder seems to be named inappropriately (i.e., any errors):

Note below any deviations as noted during the FlowJo analysis (e.g., incorrect keywords, missing samples, populations not falling within template gates, etc.)

List unreliable data, if any:_____

Data reviewed by/date:_____

SOP for Intracellular Cytokine Staining Assay

Attachment 6

Lab Manager Data Review

Enter FCS file folder name	Initial and date each column below after review:				
	ICS worksheet	FlowJo printout (check gating and QC/criteria)	Labkey data (check PTIDs and repeats)	Export data to SCHARP	Reviewed SCHARP's Exported Data

SOP for Intracellular Cytokine Staining Assay

Attachment 7: LSR QC

LSR name:_____ **Date:**_____

Batch:_____ **Protocol:**_____

Note: Channels not used in an experiment do not have to have their voltages adjusted (you may just enter N/A), but there is no harm in doing so. Those channels may also be deleted from the QC file if desired.

Laser	Channel	Target Median	Acceptable CV	After Adjustment		By
				PMT Voltage	CV	
Blue	Forward Scatter		N/A		N/A	
	Side Scatter		N/A		N/A	
	FITC		<7%			
	PerCP Cy55 Blue		<10%			
Red	APC-Cy7 (for collection of APC Alx750)		<12%			
	Alexa 680 (for collection of Alexa 700)		<12%			
	APC		<12%			
Green	PE-Cy7		<10%			
	PE-Cy5.5 (for collection of PerCP-Cy5.5)		<8%			
	PE Cy5		<10%			
	PE-TR (also referred to as ECD)		<8%			
	PE green laser (also referred to as PE)		<8%			
Violet	Am Cyan (for collection of AViD)		<10%			
	Pacific Blue (for collection of ViViD)		<20%			
	Qdot 565		<10%			
	Qdot 585		<10%			
	Qdot 605		<10%			
	Qdot 655		<10%			
	Qdot 705		<10%			
	Qdot 800		<10%			