FHCRC HVTN Laboratory
Standard Operating Procedure for:
Reagent Titration for the ICS Assay

SOP #: FH-HVTN-P0015 **Version:** 2.0

Name: Reagent Titration for the ICS Assay

Effective Date: July 06, 2009

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

Approval: **Donald Carter,** FHCRC HVTN Lab Manager

Signature Date 7-6-09

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Signature Date 7/6/09

Revision History:	Version	Description	Revised by	Revision Date
	1.0	Initial Version		6/11/07
	2.0	Updated Info and added steps for surface staining	Nathaniel Chartrand	

Purpose

This standard operating procedure (**SOP**) describes how to titrate new lots of fluorochrome-conjugated antibody reagents and the cell viability marker for use in the intracellular cytokine staining assay.

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 (ICS) is a flow cytometry-based method for enumeration of antigen-specific, cytokine-secreting T cells. This method makes use of monoclonal antibodies specific for cellular markers expressed on the cell surface or inside the cell. Each of the monoclonal antibodies is conjugated (covalently linked) to a fluorescent molecule (a fluorochrome). Each of the fluorochromes has different excitation or emission characteristics so that the expression of each of the different cellular markers can be individually measured. Note that fluorochromes are also referred to as dyes and that fluorochrome-conjugated antibodies, in the context of flow cytometry, are often referred to simply as antibodies. The cell viability marker is not an antibody, but is titrated using the same procedure as for the antibody reagents.

As part of the ICS assay, cells are mixed with these antibody reagents so that they can specifically bind to the cellular markers. This step is referred to as “staining” the cells. In order to determine the amount, or volume, or each antibody reagent that is used for each staining reaction, each reagent must be titrated. For this titration, cells are stained with different amounts of the reagent in order to determine the amount that achieves optimal staining, where the background staining for the antibody is at a low level and there is good separation between the positive and negative cell populations.

Some Antibodies are used for phenotyping PBMC’s for various HVTN projects. The phenotyping panels are designed to examine expression patterns among freshly thawed, unstimulated PBMC’s. Sometimes this requires the addition of certain atibodies before permiabolization because FACSperm can cause some markers to degrade, thus making it unrecognizable to the antibody. This is referred to as “surface staining”. Due to the nature of the antibodies a different titer is sometimes ideal for surface staining than is optimal for intracellular (post perm) staining. This is why it is important to have titers for both “surface” and “intracellular” both some reagents

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
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. Dry ice
6. Microcentrifuge tubes, black
7. Pipet tips
8. Pipets, 5mL, 10mL, 25mL, 50mL
9. Steri-Cup filter unit, Millipore, 0.22μm, Millipore Corp.
10. Desiccators, -20°C, Nalgene
11. 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.

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-5879

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: Sigma Chemical Co.; Cat #ED255

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 -20ºC 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 56ºC for 30 minutes, and store at 4ºC. Initial and date bottle for heat inactivation. If the FBS has already been heat inactivated, it can be thawed and stored at 4ºC. 4ºC 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% Paraformaldahyde

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 4ºC.

18.R10 Media

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.

19.SEB

19.1 Vendor: Sigma Chemical Co.; Cat #S0812

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 7^{th} 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 Calibraton 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

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.

Instrumentation

1. BD LSR II, optionally equipped with High Throughput Sampler (HTS)
2. Guava Cell Analyzer
3. Water bath, 37°C
4. Incubator, 37°C, 5% CO_2

5. Centrifuge
6. Balance
7. BSC
8. Micropipetters
9. Freezer

Specimens

1. Specimens to be analyzed are peripheral blood mononuclear cells (PBMC).

Titration procedure using stimulated PBMC – for markers requiring stimulation for expression

1. Day 1 Thawing

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

1.2 Determine cell number and viability according to Guava Counter SOP FH-HVTN-E0018.

1.3 Centrifuge the cells, 250xG for 10 minutes, decant the supernatant, and gently resuspend the pellet with a 200µl pipetter. Resuspend the cells with R10 at $2x10^6$ cells/ml and place in incubator overnight.

Note: Resting cells is optional. You may proceed with the stimulation or staining immediately after thawing if you wish.

2. Day 2 Stimulation

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 pipetter 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 pipetter. Resuspend the cells with R10 at $5x10^6$ cells/ml and plate (200µl/well) using the sample plate layout (Attachment 1) as a guide.

2.4 Prepare stimulation cocktails for SEB according to the details in the worksheet below (Attachment 2). SEB is only needed for cells to be stained with antibodies with intracellular targets (i.e. cytokines). Titrations targeting surface receptors need no stimulation cocktail (i.e. CD8, CD4 and other lineage markers).

Note: Although not required for the surface antibody reagents and ViViD, stimulated cells can be used for these titrations.

2.5 Add 20µl of each stimulation cocktail to appropriate wells (see plate layout Attachment 1).

2.6 Incubate plates undisturbed in an incubator at 37°C, 5% CO_2 for six hours (+/- 15 minutes). Stimulation may go overnight if necessary.

2.7 After incubation, plates can be put into a refrigerator up to 18 hours, or proceed to next step.

2.8 Add 20μl of 20mM EDTA to each well and mix well with a multichannel pipet.

2.9 Incubate plates 10 minutes at room temperature.

2.10 Centrifuge the plates at 750xG for 3 minutes.

2.11 If ViViD or AViD are being titrated, prepare working dilution(s) as described below and record this information in Attachment 5. If neither is being titrated, skip to step 2.12.

Note: The ViViD/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.

2.11.1 In a separate 96 well plate, fill wells A1 to A8 with 50ul PBS. In A9, mix 96ul PBS with 4ul ViViD/AViD.

2.11.2 Remove 50ul from A9 and mix with A8; then remove 50ul from A8 and mix into A7. Repeat this process until well A1, then simply remove 50ul from well A1.

2.11.3 In well A11, add 50ul of the ViViD/AViD lot currently in use, diluted to the appropriate titer in PBS.

2.11.4 After flicking the plate, resuspend the appropriate wells to be stained with 50μl of the ViViD/AViD dilutions. Resuspend the remaining wells containing cells with 50μl PBS.

2.11.5 Incubate at room temperature under foil for 20 minutes.

2.11.6 Add 150μl PBS and centrifuge the plate at 750xG for 3 minutes.

2.12 After centrifugation, flick supernatant from the wells.

2.13 Add 200μl PBS and centrifuge plate at 750xG for 3 minutes.

2.14 After centrifugation, flick supernatant from the wells.

2.15 If a surface titer is desired (pre FACSperm) than resuspend appropriate cells with the desired antibody diluted in the same manner as in step 2.11. Add the current lot of the same antibody at it's appropriate titer to the 11th well of that row to compare results.

Note: If no surface titer is needed than proceed to step 2.16.

2.16 Incubate at room temperature under foil for 20 minutes.

2.17 Add 150μl PBS and centrifuge the plate at 750xG for 3 minutes.

2.18 After centrifugation, flick supernatant from the wells.

2.19 Add 200μl PBS and centrifuge plate at 750xG for 3 minutes.

2.20 After centrifugation, flick supernatant from the wells.

2.21 Resuspend cells in each well with 100μl of 1X BD FACS Lyse Working Solution.

2.22 Incubate the plate at room temperature for 10 minutes.

2.23 Wrap the plate in foil and place in a Low Temperature Freezer (-70 to -80°C) up to 3 weeks.

Note: Freezing the plates at this point is optional. You may proceed straight to day three staining if you wish

3. Day 3 Staining

3.1 Prepare antibody cocktail plate. See the example of a plate layout in Attachment 1.

3.1.1 In a separate 96 well plate, dispense 50ul FACS Wash into wells A1 to A8. Repeat for an additional row of wells for each additional antibody being titrated.

3.1.2 Add solution to wells in column 9 based on what antibody is being titrated, and record this information in Attachment 5:

For antibodies with a titer currently **over** 1uL/test: **60ul FACS Wash and 40ul** of the antibody.

For antibodies with titers currently **under** 1uL/test: **95ul FACS Wash and 5ul** of the antibody.

Note: These numbers are guidelines. You may choose any starting titer you wish based on the regent being tested.

3.1.3 Mix well A9 well and remove 50ul; add to well A8 and mix well. Remove 50ul from well A8 and add to well A7; repeat this process for wells A7 through A1. After mixing 50ul into A1, remove 50ul and discard.

3.1.4 Into well A11, add 50ul of the current lot of antibody in use, diluted to the appropriate titer in FACS wash. This step can be skipped if a new antibody is being titrated that has not been used before or there is none of the current reagent left.

3.1.5 Repeat 3.1.3 and 3.1.4 for any other rows that are being tested. If desired, when titrating many reagents at the same time, all rows may be diluted at the same time with a multichannel pipet. For example, for four reagents, wells A9, B9, C9 and D9 can be mixed at the same time and then 50 ul from each can be transferred to wells A8, B8, C8 and D8, etc.

3.2 Remove the plate from the Low Temperature Freezer (-70 to -80°C) and place in a 37° incubator for ~20 minutes or until all the wells are thawed.

Note: Once the wells are thawed, the processing of the plate should not be delayed. It is recommended that time interval between placing the frozen plate in the incubator and the addition of FACS Wash buffer (as in the next step) should be no longer than 45 minutes.

3.3 Add 100µl of FACS Wash Buffer to each well.

3.4 Centrifuge the plate at 750xG for 3 minutes.

3.5 Flick supernatant from the wells.

3.6 Resuspend the cells in the wells with 200µl of FACS Perm II Working Solution.

3.7 Incubate the plate 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).

3.8 Centrifuge the plate at 750xG for 3 minutes.

3.9 Flick supernatant from the wells.

3.10 Add 200µl of FACS Wash Buffer to each well and resuspend cells.

3.11 Centrifuge the plate 750xG for 3 minutes.

3.12 Flick supernatant from the wells.

3.13 Add 200µl of FACS Wash Buffer to each well and resuspend cells.

3.14 Centrifuge the plate at 750xG for 3 minutes.

3.15 Flick supernatant from the wells.

3.16 Resuspend wells with 50ul of the appropriate antibody dilutions from the prepared antibody cocktail plate. Resuspend wells not receiving antibody with 50ul FACS Wash.

3.17 Incubate at room temperature, in the dark for 30 minutes (acceptable range is 25 to 35 minutes).

3.18 After the completion of incubation, add 150µl of FACS Wash Buffer to all wells, and resuspend cells.

3.19 Centrifuge the plate at 750xG for 3 minutes.

3.20 Flick supernatant from the wells.

3.21 Add 200µl of FACS Wash Buffer to each well

3.22 Centrifuge the plate at 750xG for 3 minutes.

3.23 Flick supernatant from the wells.

3.24 Resuspend the cells with 150ul of 1% Paraformaldehyde Working solution.

3.25 Wrap the plate in foil and place in refrigerator for up to 18 hours.

3.26 Acquire the data by flow cytometry directly from the plate using the HTS, or manually after transferring cells from the plate to FACS tubes.

Titration procedure using unstimulated PBMC – for markers that need no stimulation (such as CD3, CD4, or CD8)

1. When to use this procedure:

1.1 This procedure may be used for titrating any antibodies that do not require stimulation, such as: CD4, CD3, CD8, Granzyme B, and Perforin; as well as for titrating ViViD or AViD. If you are uncertain if an antibody may be titrated with this procedure ask the lab manager. These reagents may also be titrated using stimulated cells as described above. This is often convenient if anti-cytokine antibodies also need to be titrated.

1.2 Reagents whose targets require stimulation (such as IL2, TNFα, IL4, and IFNγ) may NOT be titrated using this procedure.

2. Day 1 Thawing

2.1 Thaw cryopreserved PBMC according to SOP FH-HVTN-P0004. After counting the cells, do not resuspend them at $2x10^6$ cells/ml; instead, carry on with step 2.2.

2.2 Resuspend the cells with R10 at $5x10^6$ cells/ml and plate (200µl/well) using the sample plate layout (Attachment 1) as a guide.

2.3 Centrifuge the plate at 750xG for 3 minutes.

2.4 If ViViD is being titrated, prepare ViViD working dilution(s) as described below, and record this information in Attachment 5. If ViViD is not being titrated, skip to step 2.5.

Note: The ViViD is 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.

2.4.1 In a separate 96 well plate, fill wells A1 to A8 with 50ul PBS. In A9, mix 96ul PBS with 4ul ViViD.

2.4.2 Remove 50ul from A9 and mix with A8; then remove 50ul from A8 and mix into A7. Repeat this process until well A1, then simply remove 50ul from well A1.

2.4.3 In well A11, add 50ul of the ViViD lot currently in use, diluted to the appropriate titer in PBS.

2.4.3 After flicking the plate, resuspend the appropriate wells to be stained with 50µl of the ViViD dilutions. Resuspend the remaining wells containing cells with 50µl PBS.

2.4.4 Incubate at room temperature under foil for 20 minutes.

2.4.5 Add 150µl PBS and centrifuge the plate at 750xG for 3 minutes.

2.5 If a surface titer is desired (pre FACSperm) than resuspend appropriate cells with the desired antibody diluted in the same manner as in step 2.11. Add the current lot of the same antibody at it's appropriate titer to the 11^{th} well of that row to compare results.

Note: If no surface titer is needed than proceed to step 2.9.

2.5.1 Incubate at room temperature under foil for 20 minutes.

2.5.2 Add 150µl PBS and centrifuge the plate at 750xG for 3 minutes.

2.6 After centrifugation, flick supernatant from the wells.

2.7 Add 200µl PBS and centrifuge plate at 750xG for 3 minutes.

2.8 After centrifugation, flick supernatant from the wells.

2.9 Add 200µl PBS and centrifuge plate at 750xG for 3 minutes.

2.10 After centrifugation, flick supernatant from the wells.

2.11 Resuspend cells in each well with 100µl of 1X BD FACS Lyse Working Solution.

2.12 Incubate the plate at room temperature for 10 minutes.

2.13 Add 100µl of FACS Wash Buffer to each well.

2.14 Centrifuge the plate at 750xG for 3 minutes.

2.15 Flick supernatant from the wells.

2.16 Resuspend the cells in the wells with 200µl of FACS Perm II Working Solution.

2.17 Incubate the plate 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).

2.18 Prepare antibody cocktail plate.

2.18.1 In a separate 96 well plate, dispense 50ul FACS Wash into wells A1 to A8. Repeat for an additional row of wells for each additional antibody being titrated.

2.18.2 Add 60ul FACS Wash to the 9th well of each row being tested; mix well with 40ul of the appropriate antibody for that row.

2.18.3 Mix well A9 well and remove 50ul; add to well A8 and mix well. Remove 50ul from well A8 and add to well A7; repeat this process for wells A7 through A1. After mixing 50ul into A1, remove 50ul and discard.

2.18.4 Into well A11, add 50ul of the current lot of antibody in use, diluted to the appropriate titer in FACS wash. This step can be skipped if a new antibody is being titrated that has not been used before.

2.18.5 Repeat 2.14.3 and 2.14.4 for any other rows that are being tested. If desired, when titrating many reagents at the same time, all rows may be diluted at the same time with a multichannel pipet. For example, for four reagents, wells A9, B9, C9 and D9 can be mixed at the same time and then 50 ul from each can be transferred to wells A8, B8, C8 and D8, etc.

2.19 Centrifuge the plate at 750xG for 3 minutes.

2.20 Flick supernatant from the wells.

2.21 Add 200µl of FACS Wash Buffer to each well and resuspend cells.

2.22 Centrifuge the plate 750xG for 3 minutes.

2.23 Flick supernatant from the wells.

2.24 Add 200µl of FACS Wash Buffer to each well and resuspend cells.

2.25 Centrifuge the plate at 750xG for 3 minutes.

2.26 Flick supernatant from the wells.

2.27 Resuspend wells with 50ul of the appropriate antibody dilutions from the prepared antibody cocktail plate. Resuspend wells not receiving antibody with 50ul FACS Wash.

2.28 Incubate at room temperature, in the dark for 30 minutes (acceptable range is 25 to 35 minutes).

2.29 After the completion of incubation, add 150µl of FACS Wash Buffer to all wells, and resuspend cells.

2.30 Centrifuge the plate at 750xG for 3 minutes.

2.31 Flick supernatant from the wells.

2.32 Add 200µl of FACS Wash Buffer to each well

2.33 Centrifuge the plate at 750xG for 3 minutes.

2.34 Flick supernatant from the wells.

2.35 Resuspend the cells with 150ul of 1% Paraformaldehyde Working solution.

2.36 Wrap the plate in foil and place in refrigerator for up to 18 hours.

2.37 Acquire the data by flow cytometry directly from the plate using the HTS, or manually after transferring cells from the plate to FACS tubes.

Modifying the FACS Diva titration template

1. Open the titration template by going to Experiment and selecting new experiment. Select Titration Template and click Ok.
2. Rename the experiment using the naming convention in the titration worksheet.
3. Delete wells that are not used in this titration, if any. To do this, highlight the rows that are not used in the titration plate, right click, then select delete wells.
4. Under Experiment, select Experiment Layout.

    4.1 There are three tabs in the Experiment Layout window: Layout, Keywords, and Acquisition

    4.2 Layout Tab

        4.2.1 Find the column for the first row of the plate that corresponds to the reagent being tested (i.e. for CD4 FITC, the FITC column). Label all 12 wells in that column with the antibody being tested (CD4, CD8, Etc.) or ViViD.

        4.2.2 Repeat 4.2.1 for all other titration rows on the plate.

    4.3 Keywords Tab

        4.3.1 In the Antibody keyword column, put the name of the antibody or ViViD stain being titrated in the corresponding wells.

    4.4 Acquisition Tab: no changes required.

    4.5 Click Ok.

Analysis of Titration Data

1. Add samples into a new FlowJo workspace.
2. Put the titration samples into separate groups for each antibody.
3. Add a new keyword “titer” to the samples
4. Click on the empty “titer” box at the first antibody dilution and choose workspace and with the option key held down select “create value series”
5. In the pop up window you will see the following

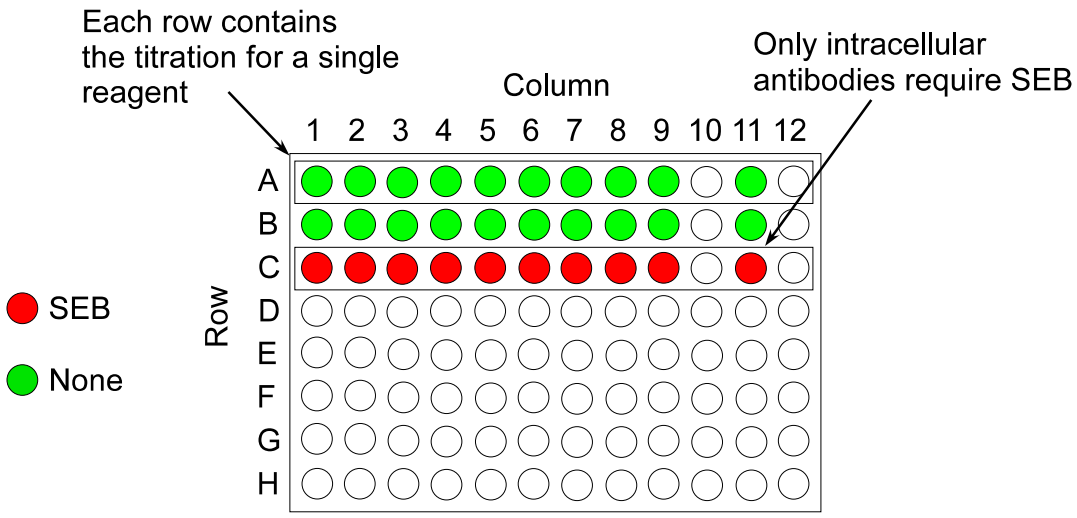
   Starting Value: This is the starting titer of the reagent

   Increment: 0

   Multiplier: 0.5 (since we are using a 1:2 dilution factor)
6. Click ok and the program will automatically assign the appropriate titer to all your samples within the group

   NOTE: This function will only work if your samples are sorted by dilution from highest to lowest. Example 9,8,7,6 etc.
7. Within each group, assign a singlet and lymphocyte gate to one sample.
8. For the cells within the lymphocyte gate, set the axes to FSC on the X axis and set the Y axis to the channel for the antibody being titrated.
9. Apply the gates to the entire group.
10. Gate on the cells that are positive for the antibody being titrated separately for each dilution of the titration, and add a median statistic to this population.
11. Create a layout of the lymphocyte gate for all 10 samples, and save a printout in the Titration binder.
12. Repeat steps 3 to 7 for each titration performed.
13. Export the median data for all samples to JMP (or other suitable software such as excel). Assign an antibody volume to each row indicating the volume of antibody added to each well. Since the experiment uses 2-fold dilutions, a formula can be used to generate the antibody volume corresponding to each dilution. Graph the median vs. antibody volume for each antibody titrated, and save a printout for each antibody in the titration binder with the appropriate FlowJo printout.
14. The lab manager (or other experienced individual) can determine what titer is appropriate for each antibody. The titer chosen is a compromise between several factors: a median fluorescence near the saturation point is desirable, as is optimal separation between the positive and negative populations, and the negative population should remain low. A summary of the antibodies titrated, their lot numbers, and the titer to be used should be included in the titration binder (see Attachment 4).
15. If there is no acceptable titer, the particular lot of reagent titrated may be unusable in the ICS assay. If this is the case, the company that provided the reagent will be contacted, and arrangements made for a replacement to be sent out. This new lot will need to be similarly titrated, and can only be used if an acceptable titer is determined.

Attachment 1
ICS Assay Sample Plate Layout for Antibody Titration



This is only a sample plate layout; the actual titration plates may differ from this example. SEB only needs to be added to those wells being tested with reagents requiring stimulated cells (such as PE IL2, Ax700 TNFα, APC IL4, or PeCy7 IFNγ); in the above example, row C. After staining is complete, add 150ul PBS or FACS Wash to all wells in columns 10 and 12 that are in a row being tested.

Attachment 2
ICS Assay Worksheet for titration using stimulated PBMC for anti-cytokine reagents

Date: _____

Reagent Lot and Batch Numbers

Reagent	Lot or Batch # /Expiration	Initials
R10		
R10/Benz		
PBS		
EDTA solution		
FACSwash		
1X FACSLyse		
1X FACSPerm		
1% Paraformaldehyde		
ViViD stock (current lot)		
ViViD stock (lot being titrated)		
BFA Working Solution		
7th Peak beads, 3.8uM		
8x Ultra-rainbow beads, 3.0uM		

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.**	-- N/A--
	2	Prepare R10/Benzonase. **Record batch # in reagent log notebook.**	-- N/A--
	3	Thaw samples according to SOP FH-HVTN-P0004.	-- 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:	 ____:____

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 to the recipes listed below.	-- N/A--

Stimulation Cocktails:

Stim condition	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
SEB	0.5mg/ml (1:50)	5*x=___	25*x=___	50*x=___	170*x=____	250*x=____	

For SEB, multiply each volume by x, where x = number of reagents requiring stimulated cells being titrated (such as PE IL2, Ax700 TNFα, APC IL4, or PE-Cy7 IFNγ).

*Final concentrations after mixing stimulation cocktails with cells:
DMSO = 1%; CD28/49d = 1 ug/ml; BfA = 10ug/ml; SEB = 1ug/ml
Calculations are based on 4 wells for DMSO and 12.5 wells for SEB per antibody to be titrated.

Initial	Step	Description	Time/Amount
	10	Plate samples and add 20 ul of stimulation cocktails to the appropriate wells.	-- 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.	Time in: ____:____ Time out: ____:____
	14	Centrifuge plate and flick supernatant.	

Initial	Step	Description	Time/Amount
	15	Skip this step if not titrating ViViD. Prepare ViViD serial dilution **immediately** before use. In a separate 96 well plate, fill wells A1 to A8 with 50ul PBS. In A9, mix 96ul PBS with 4ul ViViD. Remove 50ul from A9 and mix with A8; then remove 50ul from A8 and mix into A7. Repeat this process until well A1, then simply remove 50ul from well A1. Record the manufacturer's lot number and expiration date in the appropriate row on the table in step 21. In well A11, add 50ul of the ViViD lot currently in use, diluted to the appropriate titer in PBS. ViViD Volume (in μL): PBS Volume (in mL):	 _____ _____
	16	Skip this step if not titrating ViViD Resuspend the appropriate sample wells with the appropriate dilution of ViViD solution from your ViViD plate. Resuspend the remaining wells containing cells with 50 ul of PBS. Incubate for 20 minutes at RT.	Time in: ____:____ Time out: ____:____
	17	Skip this step if not titrating ViViD Add 150 ul PBS and centrifuge plate. Flick supernatant.	-- N/A--
	18	Add 200ul PBS and centrifuge plate.	-- N/A--

	19	Prepare **surface** antibody cocktail plate: Dispense 50ul FACS Wash into wells A1 to A8. Repeat for an additional row of wells for each additional antibody being titrated. Add solution to wells in column 9 based on what antibody is being titrated: For FITC CD4, PE IL2, TR-PE CD3, PerCP-Cy5.5 CD8, Ax700 TNFα, PE CD8, and APC CD3: 60ul FACS Wash and 40ul of the antibody. For APC IL4: 90ul FACS Wash and 10ul of the antibody. For PE-Cy7 IFNγ: 95ul FACS Wash and 5ul of the antibody. Mix well A9 well and remove 50ul; add to well A8 and mix well. Remove 50ul from well A8 and add to well A7; repeat this process for wells A7 through A1. After mixing 50ul into A1, remove 50ul and discard. In well A11, add 50ul of the current lot of antibody in use, diluted to the appropriate titer in FACS wash. This step can be skipped if a new antibody is being titrated that has not been used before. Repeat for any other rows that are being tested. If desired, all rows may be diluted at once with a multichannel pipet. **If no surface stain proceed to step 24**	-- N/A--
	20	Resuspend cells in 50uL of surface cocktail and incubate for 20 minutes at RT covered in foil	-- N/A--
	21	Add 150uL of FACS wash and centrifuge plate.	-- N/A--

	22	Flick supernatant and wash again with 200uL of FACS wash.	-- N/A--
	23	Centrifuge plate	-- N/A--
	24	Flick supernatant, resuspend with 100ul of 1x FACSlyse solution and incubate for 10 minutes at RT. Place in freezer or continue with rest of staining.	-- N/A--
	25	Store at –70°C. ➡ Time placed in freezer:	____:____

Day 3 Day 3 Date: _____/_____/_____

Initial	Step	Description	Time/Amount
	26	Record name and lot of antibodies to be titrated, as well as the row it is being titrated in, on the Table for Antibody Plate (Attachment 5).	-- N/A--
	27	Prepare **intracellular** antibody cocktail plate: Dispense 50ul FACS Wash into wells A1 to A8. Repeat for an additional row of wells for each additional antibody being titrated. Add solution to wells in column 9 based on what antibody is being titrated: For FITC CD4, PE IL2, TR-PE CD3, PerCP-Cy5.5 CD8, Ax700 TNFα, PE CD8, and APC CD3: 60ul FACS Wash and 40ul of the antibody. For APC IL4: 90ul FACS Wash and 10ul of the antibody. For PE-Cy7 IFNγ: 95ul FACS Wash and 5ul of the antibody. Mix well A9 well and remove 50ul; add to well A8 and mix well. Remove 50ul from well A8 and add to well A7; repeat this process for wells A7 through A1. After mixing 50ul into A1, remove 50ul and discard. In well A11, add 50ul of the current lot of antibody in use, diluted to the appropriate titer in FACS wash. This step can be skipped if a new antibody is being titrated that has not been used before. Repeat for any other rows that are being tested. If desired, all rows may be diluted at once with a multichannel pipet. **If no Intracellular stain is needed proceed to step 25**	
	28	Remove plates from freezer, thaw at 37°C and add 100ul of FACS Wash buffer to each well.	-- N/A--

Initial	Step	Description	Time/Amount
	29	Centrifuge the plates, resuspend cells in 200ul of FACS Perm II solution and incubate for 10 minutes at RT.	Time in: ____:____ Time out: ____:____
	30	Centrifuge the plates and wash twice with 200 ul FACS Wash buffer.	-- N/A--
	31	After flicking supernatant, resuspend all sample wells with 50ul of the appropriate antibody dilution from the antibody cocktail plate. Any wells not receiving an antibody dilution should be resuspended in 50ul FACS Wash.	Time in: ____:____ Time out: ____:____
	32	Add 150ul of FACSWash buffer and centrifuge the plate.	-- N/A--
	33	Wash plate once with FACS Wash Buffer.	-- N/A--
	34	Resuspend wells with 150 ul of 1% Paraformaldehyde working solution and store in refrigerator until FACS collection.  Time staining completed:	____:____

FACS Collection Date: _____/_____/_____

Initial	Step	Description	Time/Amount
	35	Refer to the LSR SOP (FH-HVTN-E0022). Time FACS collection begun:	____:____
	36	Create QC folder	
		Record QC folder name (follow convention, e.g., **060412-Titration-QC**):	_____
	37	Run alignment beads and adjust PMT voltages to match target medians.	-- N/A--
	38	Check CV's: Are CV's within tolerance limits:	YES/NO
	38.1	If the CVs are outside the tolerance limits, inform the study manager or other qualified supervisor who will determine if the experiment can proceed.	-- N/A--

LSRII alignment using Sphero 7th Peak Rainbow beads, 3.8uM

Laser	Channel	Target Median	Accept-able 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)		<10%			
	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%			

Initial	Step	Description	Time/Amount
	39	Copy instrument settings.	-- N/A--
	40	Perform cleaning procedure on HTS.	-- N/A--
	41	Create a new experiment from the protocol-specific template and paste the instrument settings. ➡ Record folder name (follow convention as noted above for QC file, e.g., **060412-Titration**):	_____
	42	Collect plates according to the LSRII equipment SOP (FH-HVTN -E0022).	
	43	Export the experiment folder and the QC folder ➡ Time collection completed:	____:____
	44	Perform HTS and instrument cleaning procedures as per the LSRII equipment SOP (FH-HVTN-E0022).	

Comments _____

Attachment 3
ICS Assay Worksheet for Titration procedure using unstimulated PBMC

Date: _____

Reagent Lot and Batch Numbers

Reagent	Lot or Batch # /Expiration	Initials
R10		
R10/Benz		
PBS		
FACSwash		
1X FACSLyse		
1X FACSPerm		
1% Paraformaldehyde		
ViViD stock (current lot)		
ViViD stock (lot being titrated)		
7th Peak beads, 3.8uM		
8x Ultra-rainbow beads, 3.0uM		

Reviewed By/Date:_____

Reagent Titration for the ICS Assay

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.**	-- N/A--
	2	Prepare R10/Benzonase. **Record batch # in reagent log notebook.**	-- N/A--
	3	Thaw samples according to SOP FH-HVTN-P0004.	-- 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	Label plate(s) according to plate layout and plate samples.	-- N/A--
	7	Centrifuge plate and flick supernatant.	
	8	Skip this step if not titrating ViViD Prepare ViViD serial dilution **immediately** before use. In a separate 96 well plate, fill wells A1 to A9 with 50ul PBS. In A10, mix 96ul PBS with 4ul ViViD. Remove 50ul from A10 and mix with A9; then remove 50ul from A9 and mix into A8. Repeat this process until well A1, then simply remove 50ul from well A1.	Lot Number of ViViD: _____
	9	Skip this step if not titrating ViViD Resuspend the appropriate sample wells with the appropriate dilution of ViViD solution from your ViViD plate. Resuspend the remaining wells containing cells with 50 ul of PBS. Incubate for 20 minutes at RT.	Time in: ____:____ Time out: ____:____
	10	Skip this step if not titrating ViViD Add 150 ul PBS and centrifuge plate. Flick supernatant.	-- N/A--

Initial	Step	Description	Time/Amount
	11	Prepare **surface** antibody cocktail plate: Dispense 50ul FACS Wash into wells A1 to A8. Repeat for an additional row of wells for each additional antibody being titrated. Add solution to wells in column 9 based on what antibody is being titrated: For FITC CD4, PE IL2, TR-PE CD3, PerCP-Cy5.5 CD8, Ax700 TNFα, PE CD8, and APC CD3: 60ul FACS Wash and 40ul of the antibody. For APC IL4: 90ul FACS Wash and 10ul of the antibody. For PE-Cy7 IFNγ: 95ul FACS Wash and 5ul of the antibody. Mix well A9 well and remove 50ul; add to well A8 and mix well. Remove 50ul from well A8 and add to well A7; repeat this process for wells A7 through A1. After mixing 50ul into A1, remove 50ul and discard. In well A11, add 50ul of the current lot of antibody in use, diluted to the appropriate titer in FACS wash. This step can be skipped if a new antibody is being titrated that has not been used before. Repeat for any other rows that are being tested. If desired, all rows may be diluted at once with a multichannel pipet. **If no surface stain proceed to step 16**	-- N/A--
	12	Resuspend cells in 50uL of surface cocktail and incubate for 20 minutes at RT covered in foil	
	13	Add 150uL of FACS wash and centrifuge plate.	
	14	Flick supernatant and wash again with 200uL of FACS wash.	

	15	centrifuge plate	
	16	Flick supernatant, resuspend with 100ul of 1x FACSlyse solution and incubate for 10 minutes at RT.	Time in: ____:____ Time out: ____:____
	17	Record name and lot of antibodies to be titrated, as well as the row it is being titrated in, on the Table for Antibody Plate (Attachment 5).	-- N/A--
	18	Add 100ul of FACS Wash buffer to each well.	-- N/A--
	19	Centrifuge the plates, resuspend cells in 200ul of FACS Perm II solution and incubate for 10 minutes at RT.	Time in: ____:____ Time out: ____:____
	20	Prepare **Intracellular** antibody cocktail plate: Dispense 50ul FACS Wash into wells A1 to A9. Repeat for an additional row of wells for each additional antibody being titrated. Add 60ul FACS Wash to the 10th well of each row being tested; mix well with 40ul of the appropriate antibody for that row. Mix well A10 well and remove 50ul; add to well A9 and mix well. Remove 50ul from well A9 and add to well A8; repeat this process for wells A8 through A1. After mixing 50ul into A1, remove 50ul and discard. Repeat for any other rows that are being tested. If desired, all rows may be diluted in this way at once with a multichannel pipet. **If no Intracellular stain is needed proceed to step 23**	
	21	Centrifuge the plates and wash twice with 200 ul FACS Wash buffer.	-- N/A--

Initial	Step	Description	Time/Amount
	22	After flicking supernatant, resuspend all sample wells with 50ul of the appropriate antibody dilution from the antibody cocktail plate. Any wells not receiving an antibody dilution should be resuspended in 50ul FACS Wash.	Time in: ____:____ Time out: ____:____
	23	Add 150ul of FACSWash buffer and centrifuge the plate.	-- N/A--
	24	Wash plate once with FACS Wash Buffer.	-- N/A--
	25	Resuspend wells with 150 ul of 1% Paraformaldehyde working solution and store in refrigerator until FACS collection. ➡ Time staining completed:	____:____

FACS Collection Date: _____/_____/_____

Initial	Step	Description	Time/Amount
	26	Refer to the LSR SOP (FH-HVTN-E0022). Time FACS collection begun:	____:____
	27	Create QC folder	
		➡Record QC folder name (follow convention, e.g., **060412-Titration-QC**):	_____
	28	Run alignment beads and adjust PMT voltages to match target medians.	-- N/A--
	29	Check CV's: Are CV's within tolerance limits:	YES/NO
	29.1	If the CVs are outside the tolerance limits, inform the study manager or other qualified supervisor who will determine if the experiment can proceed.	-- N/A--

LSRII alignment using Sphero 1x Ultra-rainbow beads, 3.8uM

Laser	Channel	Target Median	Accept-able 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)		<10%			
	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%			

Initial	Step	Description	Time/Amount
	30	Copy instrument settings.	-- N/A--
	31	Perform cleaning procedure on HTS.	-- N/A--
	32	Create a new experiment from the titration template and paste the instrument settings. ➡ Record folder name (follow convention as noted above for QC file, e.g., **060412-Titration**):	_____
	33	Collect plates according to the LSRII equipment SOP (FH-HVTN -E0022).	
	34	Export the experiment folder and the QC folder ➡ Time collection completed:	____:____
	35	Perform HTS and instrument cleaning procedures as per the LSRII equipment SOP (FH-HVTN-E0022).	

Comments _____

Attachment 4

Summary of Titration Analysis

Antibody	Lot#	Exp date	Appropriate Titer

Titration Analysis Reviewed By/Date:

_____

Attachment 5
Table for Antibody Plate

Row on Plate	Reagent Name	Reagent to be Titrated				Current Reagent		
		Lot#	Exp Date	Amount of reagent added to well 9	Amount FACS Wash or PBS added to well 9	Lot #	Exp Date	Titer (ul)
A								
B								
C								
D								
E								
F								
G								
H								