

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

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**SOP #:** FH-HVTN-P0015                      **Version:** 3.0

**Name:** Reagent Titration for the ICS Assay

**Effective Date:** December 06, 2010

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

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

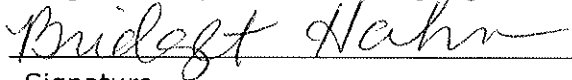


Signature

11-29-10

Date

**Bridget Hahn**, FHCRC HVTN QA/QC Laboratory Manager



Signature

11/29/10

Date

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<b>Revision History:</b>	<b>Version</b>	<b>Description</b>	<b>Revised by</b>	<b>Revision Date</b>
	1.0	Initial Version		6/11/07
	2.0	Updated Info and added steps for surface staining	Nathaniel Chartrand	7/06/09
	3.0	Rewrote the SOP referencing current staining SOPs for the worksheets.	Nathaniel Chartrand and Donald Carter	11/10/10

### 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, of 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 PBMCs for various HVTN projects. The phenotyping panels are designed to examine expression patterns among freshly thawed, unstimulated PBMCs. Sometimes this requires the addition of certain antibodies before permeabilization because FACS Perm 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” staining.

### 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
FH control	A SAC sample with a known CMV response used as a control in Flow assays.
MFI	Median fluorescence intensity
Titer	Amount of antibody to use to properly stain a cell sample.

## Reagents & Solutions

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

1. Specific reagents needed depend on the staining protocol being used. Refer to the appropriate SOP for a reagent list.

## Software

1. FlowJo (TreeStar)
2. Excel (Microsoft)
3. JMP (SAS Institute)

## Specimens

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

## Titration procedure

1. When is titration necessary?
  - 1.1 New antibody to be used.
  - 1.2 New lot of an antibody currently in use.
  - 1.3 New staining conditions for an antibody currently in use.
    - 1.3.1 An antibody with a surface titer may need an intracellular titer, or it may have an intracellular titer using FACS Perm but needs an intracellular titer using BD Perm/wash.
  - 1.4 Extending an expiration date.
    - 1.4.1 An expired antibody can be titrated and this data compared to the original titration done when the antibody arrived. If the data are comparable, the expiration date can be extended by 6 months.
    - 1.4.2 Retesting a current lot due to problems in the assay.

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- 1.4.3 Sometimes antibodies may degrade over time and the staining in the FH control sample over time will reveal this. A fresh titration can be performed to diagnose the problem.
2. Determine ICS protocol to be used.
  - 2.1 Antibodies are used in a variety of staining protocols. Antibody titrations need to be performed in the manner the antibody is going to be used, as variations in the staining protocol can impact the titer.
    - 2.1.1 For example, if the cells are going to be permeabilized and then stained, the antibody should be titrated intracellularly even if it is a surface marker. Thus antibodies can have a surface titer and an intracellular titer; and even the intracellular titer can be broken down into multiple categories depending on the permeabilization reagent used.
  - 2.2 Once the appropriate staining protocol is determined, use that SOP and associated worksheet (along with this titration SOP and attachments) to perform the titration.
3. Determine cells and cell conditions to be used.
  - 3.1 For most titrations, any PBMC may be used.
    - 3.1.1 Specific antibodies may target markers that vary greatly in expression in different individuals or specific cell lines.
  - 3.2 Depending on the marker, the cells may require stimulation before staining. Cytokine markers like IFN $\gamma$  or IL2 are best titrated after stimulation (such as with SEB).
    - 3.2.1 If stimulation is required, use the stimulation cocktail worksheet from the staining SOP selected in step 2 to create the stimulation cocktails.
  - 3.3 Cell preparations:
    - 3.3.1 In general, the various staining SOPs recommend using 1 million PBMC per well. For a titration, far fewer cells are generally needed. For markers with high levels of expression (such as CD3, CD4, or CD8), as few as 100,000 cells would be sufficient (though if cells are available using 500,000 or more is preferable).
    - 3.3.2 Some markers may require more cells if it appears at low frequency.
    - 3.3.3 Titrations do not require the cells to be rested. Using non-rested cells is common for surface markers such as those used in the Phenotyping SOP. For cell stimulations (such as in the ICS SOP) the cells are thawed and rested overnight; however, for purposes of titration this is not necessary and the cells can be stimulated immediately after thaw.
    - 3.3.4 Additionally, the cells can be rested for more than 1 day for use in titrations.
    - 3.3.5 Note that the above are merely guidelines, and the specific antibodies being titrated need to be considered in each case. Some, like Ki67, require 48

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hours incubation with SEB in order to be properly titrated. Consult the lab manager or other trained technician before titrating unfamiliar antibodies.

### 4. Plate layout

- 4.1 See attachment 1 for a sample plate layout. Essentially each row of the plate is a single antibody being titrated in a single set of conditions. The first 9 wells are a serial dilution of the new lot of antibody being titrated, while well 11 is stained with the current lot of the same antibody at the current titer (if one is available).

### 5. Titration staining

- 5.1 Titrations generally are performed using a single antibody at a time. In some cases, the antibody being titrated may have poor staining, and a second antibody may help to visualize the population of interest. The second antibody is used at a constant titer.
- 5.2 The steps below detail the methodology for setting up the dilutions for staining for ViViD, AViD, and antibody stains. In all cases, specific antibodies may have slightly different requirements and these steps should be seen as guidelines only. Record the dilution information as well as antibody and lot number in Attachment 2.
- 5.3 If ViViD or AViD are being titrated, prepare working dilution(s) as described below and record this information in Attachment 2.  
**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.
  - 5.3.1 In a separate 96 well plate, fill wells A1 to A8 with 50ul PBS. In A9, mix 96ul PBS with 4ul ViViD/AViD.
  - 5.3.2 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 and discard from well A1.
  - 5.3.3 In well A11, add 50ul of the ViViD/AViD lot currently in use, diluted to the appropriate titer in PBS.
  - 5.3.4 Proceed with processing the cells for running on the LSR as described in the staining SOP being used.
- 5.4 For titrating antibodies, prepare an antibody cocktail plate.

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- 5.4.1 In a separate 96 well plate, dispense 50ul buffer (FACS Wash or BD Perm/Wash or other reagent depending on the SOP used) into wells A1 to A8. Repeat for an additional row of wells for each additional antibody being titrated.
- 5.4.2 Add solution to wells in column 9 based on what antibody is being titrated, and record this information in Attachment 2.
- 5.4.3 For antibodies with a titer currently over 1uL/test: 60ul buffer and 40ul of the antibody.
- 5.4.4 For antibodies with titers currently under 1uL/test: 95ul buffer and 5ul of the antibody.

**Note:** These numbers are guidelines. Different starting titers may be chosen depending on the desired titration range for the reagent being tested.

- 5.4.5 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.
- 5.4.6 Dilute the current lot of antibody in use to the appropriate titer in buffer, and add 50ul into well A11. This step can be skipped if a new antibody is being titrated that has not been used before or if there is none of the current reagent left.
- 5.4.7 Repeat 5.4.2 to 5.4.6 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..
- 5.4.8 Proceed with processing the cells for running on the LSR as described in the staining SOP being used.

## 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/AViD.

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- 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, enter the name of the antibody or ViViD/AViD 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 titer keyword values and the median data for the positive population of 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. If the titer keyword was not created in FlowJo, 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

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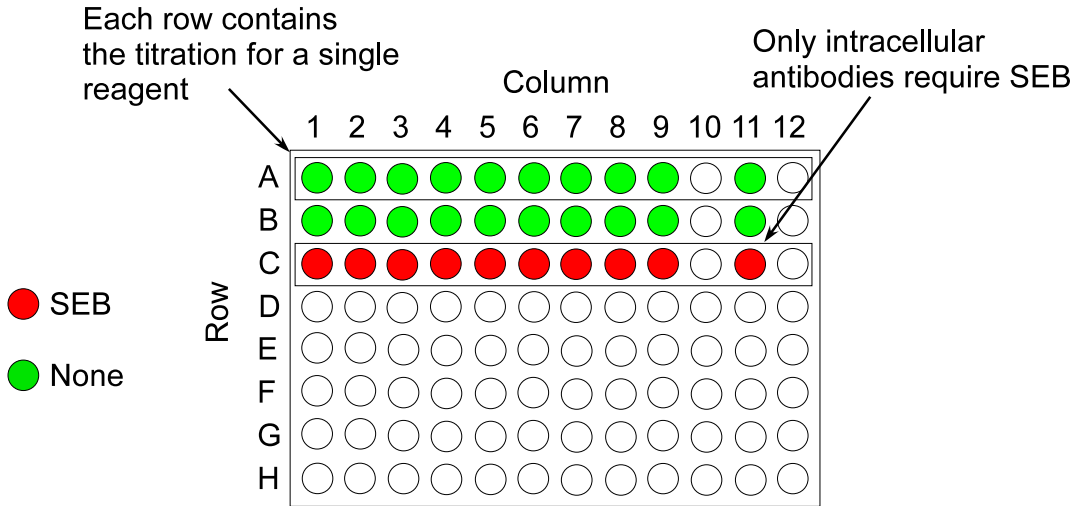
factors: a median fluorescence near the saturation point is desirable, as is optimal separation between the positive and negative populations, and the negative population should remain low. A summary of the antibodies titrated, their lot numbers, and the titer to be used should be included in the titration binder (see Attachment 3).

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

### Sample Plate Layout for Titrations



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 TNF $\alpha$ , APC IL4, or PeCy7 IFN $\gamma$ ); 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.

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**Attachment 2**  
**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 (uI)
A								
B								
C								
D								
E								
F								
G								
H								

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**Attachment 3**  
**Summary of Titration Analysis**

Antibody	Lot#	Exp date	Appropriate Titer				
			Surface	Intracellular with FACS Perm	Intracellular With BD Perm/wash	Other staining conditions	
						Description	Titer

Titration Analysis Reviewed By/Date:

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