FHCRC HVTN Laboratory
Standard Operating Procedure for:
CFSE Proliferation Assay

SOP #: FH-HVTN-A0007 ***Version:*** 3.0

Name: CFSE Proliferation Assay

Effective Date: June 01, 2011

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Approval: **Steve De Rosa,** FHCRC HVTN Co-Investigator

Signature 5/27/2011 Date

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Signature 5/27/11 Date

Revision History:	**Version**	**Description**	**Revised by**	**Revision Date**
	1.0	Initial Version		4/27/10
	2.0	Added EDTA incubation step	Evan Thomas	6/4/10
	3.0	Changed CFSE working solution concentration and α-CD3 reagent. Added EDTA to PFA step.	Evan Thomas	5/26/11

Purpose

This standard operating procedure (**SOP**) describes the procedure of the CFSE Proliferation assay.

Scope

This SOP describes how to perform a CFSE Proliferation assay in the FHCRC HVTN Endpoint Laboratory.

Background

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a compound that passively diffuses into cells. It is colorless and non-fluorescent until the acetate groups are removed by intracellular esterases to yield highly fluorescent carboxyfluorescein succinimidyl ester. The succinimidyl ester groups react with intracellular amines, forming fluorescent conjugates that are well retained. This compound proves useful when determining the ability of T cells to proliferate in response to antigen. Before antigen stimulation, previously frozen PBMC that have been thawed and rested are labeled with CFSE. The cells are then placed in culture with either HIV peptides or AT-2 (aldrithiol-2) inactivated HIV virus for six days. Media only and α-CD3/α-CD28 serve as negative and positive controls, respectively. After cells divide, each daughter cell will have approximately half the level of CFSE as the parent, and the fluorescence will decrease. After 6 days, the cells are stained with a viability dye (AViD) to exclude dead cells, as well as CD3, CD4 and CD8 to define lineage, and collected on an LSR II Flow Cytometer. Proliferation can be reported either as the percent of T cells that are “CFSE low” (defined as the percent of T cells that have lost any level of CFSE labeling) or as “Precursor frequency” (defined as the percent of precursor T cells that have proliferated.)

Authority and Responsibilities

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

Definitions

Term	Definition
BSC	Biological Safety Cabinet
CMV	Cytomegalovirus
D-PBS	Dulbecco's Phosphate Buffered Saline
DMSO	Dimethyl Sulfoxide
FBS	Fetal bovine serum
Guava PCA	Personal Cell Analysis System
PBMC	Peripheral blood mononuclear cells
EDTA	Ethylenediamine-tetraacetic Acid
Hu R10	Complete media, RPMI with 10% Human serum, 1% L-glutamine, 1% penicillin-streptomycin

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 and Solutions

Recommended vendors are listed. Unless otherwise specified, products of equal or better quality than those recommended can be used.

1. RPMI Medium 1640, 500ml +L-Glutamine and +25mM HEPES (Invitrogen, Cat# 22400-89)

1.1 RPMI is ready to use. Store at 4°C.

2. L-Glutamine (100X) (Invitrogen, Cat# 25030-081)

2.1 Dispense into 5ml aliquots.

2.2 Store at -20°C.

3. Penicillin-Streptomycin (Gibco BRL Life Technologies, Cat# 15140-122)

3.1 10,000 Units, Store at -5°C to -20°C.

4. Human Serum AB (GemCell, Cat#100-512)

4.1 Store at -20°C until ready to heat inactivate if necessary.

4.2 Human serum must be heat inactivated and filtered before use. If the current lot is not heat inactivated, the day before heat inactivation, remove serum from the freezer and place at 4°C until completely thawed or overnight.

4.3 To heat inactivate, remove serum directly from 4°C and place into a 56°C water bath. Leave serum in water bath for 54 minutes, mixing gently several times. The serum will take 24 minutes to warm to 56°C from 4°C, then 30 minutes to heat inactivate.

Note: If serum is at a temperature other than 4°C before heat inactivating, place an equivalent blank in the water bath with a calibrated thermometer inside the liquid. This will ensure that the serum reaches the correct temperature before the 30 minute inactivation starts.

4.4 Human serum that goes into the Human R10 will be filtered after being added to the media. For the remaining serum, filter through 0.45μm vacuum filter.

4.5 Human serum can be stored at 4°C for one month.

Note: Human serum has a large amount of fat in it that can clog the filter. More than one filter may be necessary.

5. Human R10

5.1 To one 500ml bottle of RPMI add one 5ml aliquot of L-Glutamine, one 5ml aliquot of Pen Strep and 55ml heat-inactivated Human serum.

5.2 Filter entire bottle of media through a 0.45μm vacuum filter.

5.3 Human R10 expires 2 weeks from the date it was made.

5.4 Store at 4°C.

6. 1X PBS w/o Ca^{++} and Mg^{++} (Invitrogen, Cat# 14190)

6.1 Store at room temperature.

7. Vybrant CFDA/SE Cell Tracer Kit (Invitrogen, Cat# V12883)

7.1 Kit contains one vial of DMSO and 10 vials of lyophilized CFSE.

7.2 To make CFSE solution, add 90μL DMSO to one vial of CFSE and mix well.

7.3 Any remaining CFSE cannot be frozen and must be discarded.

8. α-CD3 Monoclonal antibody (Clone HIT3a) (BD Biosciences, Cat# 555336)

8.1 Antibody is supplied at 1mg/ml.

8.2 Dilute 1:100 (10μg/ml) in 1X PBS before use.

8.3 Add 3μL/ml as a positive control. The final concentration will be 30ng/ml.

9. α-CD28 Monoclonal antibody, No Azide/Low Endotoxin (BD Biosciences, Cat# 555725)

9.1 Antibody is supplied at 1mg/ml.

9.2 Add 1μL/ml as a positive control. The final concentration will be 1μg/ml.

10. Paraformaldehyde, 10% (Electron Microscopy Sciences, Cat# 15712-S)

10.1 Preparation of the paraformaldehyde working solution referred to as 1% paraformaldehyde

10.1.1 Dilute the 10% paraformaldehyde 1:10 in D-PBS

10.1.2 Paraformaldehyde working solution expires 1 month from date of preparation.

10.1.3 Store paraformaldehyde in a 2-8°C refrigerator.

11. Deep-well plates (Invitrogen, Cat# AB 1068)

11.1 Plates do not come with lids. Remove a lid from a 96-well round bottom plate to use.

12. 96-well Round Bottom Plates (Corning, Cat# 3699)

13. DMSO (Sigma Chemical, Cat# D-2650)

14. Fetal Bovine Serum (FBS) (Gemini Benchmark, Cat# 100-106)

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

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

15. FACS Wash Buffer

15.1 Preparation of FACS Wash Buffer referred to as FACS Wash.

15.1.1 Add 10ml heat inactivated FBS to a 500ml bottle of PBS.

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

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

16. Ethanol, 70%

17. Fluorescently labeled monoclonal antibodies

17.1 Specific reagents and catalog numbers listed in SSP.

17.2 Store at 4°C

18. 0.45μm Vacuum Filters (Millipore, Cat# SCHVU02RE)

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

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

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

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

19.2.2 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.

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

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

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

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

20.2.2 Vortex stock vial of Sphero Rainbow 8 Peak Calibraton Particles.

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

20.2.4 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.

21. AViD Fixable Aqua Dead Cell Stain Kit

21.1 Vendor: Molecular Probes/Invitrogen; Cat #L34957

21.2 Preparation of AViD stock solution referred to as AViD stock

21.2.1 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.

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

21.2.3 Aliquots expire 4 weeks from date of preparation.

21.3 Preparation of AViD working solution

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

21.3.2 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.

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

24.2 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.

24.3 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 -80ºC.

25. Aldrithiol-2 (AT-2) Inactivated HIV-1 Virus and Microvesicle Control

25.1 See the "Study Specific Procedures" for details on AT-2 inactivated HIV-1 virus and microvesicle control specific to the protocol or study being performed.

25.2 AT-2 Inactivated HIV-1 and microvesicle controls are stored in liquid nitrogen. Stock concentrations vary from lot to lot.

25.3 To avoid multiple freeze/thaw cycles, alqiuot AT-2 inactivated virus and microvesicles into 20µl single-use vials.

25.3 Stocks must be diluted to 100µg/ml in PBS before use. The amount of PBS needed will vary from lot to lot. Check vial to ensure appropriate dilution.

25.4 The final concentration of both the AT-2 inactivated HIV-1 and microvesicle control must be 1µg/ml protein.

26. EDTA, 20mM

26.1 Vendor: Fisher Chemicals, Cat #O2793-500

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

26.2.1 Weigh 744mg of EDTA on a balance.

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

26.2.3 Swirl to dissolve.

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

26.2.5 FACS EDTA expires 6 months from date of preparation.

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

Instrumentation

1. Water Bath, 37°C and 56°C
2. 37° Incubator, 5% CO_2
3. Centrifuge
4. Guava Cell Analyzer
5. BSC
6. Micropipettes
7. BD LSR II, optionally equipped with High Throughput Sampler (HTS)
8. Freezer
9. Microcentrifuge

Specimens

All CFSE 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 One

1.1 Thaw cells according to HVTN SOP# FH-HVTN-P0004. Thaw into Human R10/Benzonase.

1.2 Count cells with Guava according to HVTN SOP# FH-HVTN-E0018

1.3 Resuspend cells at 2M/ml in Human R10. Rest at 37°C, 5% CO2 overnight in 50ml centrifuge tubes.

2. Day 2

2.1 Warm Human R10 and 50ml of 1X PBS in a 37°C water bath, and remove filtered Human serum from refrigerator to warm to room temperature.

2.2 Label a 96-well deep well plate and prepare stimulation cocktails. both defined in the SSP. Add 500μl stimulation cocktails to all negative control, peptide and AT-2 wells, and 750μl to positive control wells. Plates can be held at room temperature while the cells are counted and labeled with CFSE.

2.2.1 For the negative control add DMSO final concentration is 0.5%

2.2.2 For peptide wells, the final concentration is 1μg/ml.

2.2.3 For AT-2 inactivated HIV-1 and microvesicle control, the final concentration of protein is defined in the SSP.

2.2.3 For the positive control well, CD3 final concentration is 30ng/ml and CD28 final concentration is 1μg/ml.

2.3 Centrifuge the cells at 1200 RPM (250xG) for 10 minutes.

2.4 Decant the supernatant from cells, and resuspend in 10 ml Human R10 and determine cell count according to Guava Counter SOP FH-HVTN-E0018.

2.5 Before centrifuging the cells, determine the sample that has at least 6M cells extra to use for compensation. Determine the concentration of the cell suspension (# of cells/resuspension volume) and calculate the amount of suspension needed for 6M cells. Remove this amount and split evenly between the two compensation wells on the 96-well deep well plate (according to SSP.) Add enough Human R10 to each well to bring the final volume to 1ml if needed.

2.6 Centrifuge the cells at 1200 RPM (250xG) for 10 minutes.

2.7 Decant supernatant from cells. Resuspend cell pellet using a P200 micropipette.

2.8 To make CFSE working solution, add 80μL CFSE solution to 40ml warm PBS. Mix well. CFSE working solution must be made fresh daily, right before use.

2.9 Add 3ml room temperature PBS to cells.

2.10 Add 1ml CFSE working solution to cells and **immediately** start a timer for 8 minutes. Gently swirl cells several times during the incubation. Ensure that cells incubate **exactly 8 minutes**. If labeling more than one sample, stagger the time that the CFSE is added by 10 seconds to ensure that no sample goes longer than 8 minutes.

2.11 As soon as the timer goes off, add 4ml room temperature Human serum, swirl gently, and incubate for **exactly** one minute to quench the reaction. Stagger by 10 seconds for multiple samples.

2.12 Immediately after 1 minute incubation, add 12ml Human R10 to the cells and centrifuge at 1200 RPM (250xG) for 10 minutes. The cell pellet should be a bright yellow color.

2.13 Decant supernatant and resuspend cell pellet in Human R10 for a final concentration of 2M/ml.

2.14 Add 500μL cell suspension to all sample wells and 500μL cell suspension (**Note: Positive control wells only get 250μl cell suspension)** into the deep well plate.

2.15 Place plate into the designated CFSE incubator (37°C, 5% CO_2) for 6 days, +/- 5 hours. This incubator should be disturbed as little as possible for the duration of the 6 day incubation.

3. Day 7

3.1 Remove plate from incubator. Add 100μl 20mM EDTA to each well. Incubate for 10 minutes at room temperature, then centrifuge at 2000 RPM (750xG) for 3 minutes. Flick plate.

3.2 Add 100μl PBS to each well and resuspend cell pellets well. Transfer entire volume of each well to a labeled 96 well round-bottom plate. Round-bottom plate layout is defined in the SSP. For the compensation wells, add 600μl PBS to each deep-well and transfer 200μl from the two deep-wells to the labeled compensation wells on the round-bottom plate.

3.4.1 For the CFSE comp, take 50μL of one positive control well and add to the CFSE labeled comp well.

3.5 Centrifuge plate at 2000 RPM (750xG) for 3 minutes. Flick plate.

3.6 Resuspend all sample wells and AViD comp in 50μL AViD solution. Resuspend all other compensation wells in 50μL PBS. Incubate for 20 minutes at room temperature under foil.

3.7 Add 150µl PBS to all sample and compensation wells. Centrifuge 2000 RPM (750xG) for 3 minutes and flick plate.

3.8 Resuspend all sample wells in 50µL staining cocktail (see SSP for correct cocktail). Resuspend all compensation wells in 50µL FACS Wash and add appropriate amount of antibody to each well. Incubate 20 minutes at room temperature under foil.

3.9 Add 150µl FACS Wash to all wells.. Centrifuge at 2000 RPM (750xG) for 3 minutes and flick plate.

3.10 Add 200µl FACS Wash to all wells. Centrifuge at 2000 RPM (750xG) for 3 minutes and flick plate.

3.11 Resupend all wells in 200µl 1% paraformaldehyde, then add 20µl 20mM EDTA to each well.

3.12 Wrap the plates in foil and place in refrigerator for up to 18 hours.

3.13 Acquire the data by flow cytometry directly from the plates using the HTS, or manually after transferring cells from the plates to FACS tubes (SOP# HVTN-FH-E0022.)

3.14 After the plates have been collected, check the compensation wells to insure they are acceptable by viewing in FACS Diva software. If there are any problem wells (no staining, not enough cells, wrong stain used, etc.), they will need to be replaced.

3.14.1 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).

3.14.2 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/Negative control wells:** Pass/fail criteria for the positive and negative control wells may be used as indicated in the SSP or determined by the study manager.

5. **Number of CD4 and CD8 T cells collected:** Pass/fail criteria may be used as indicated in the SSP or determined by the study manager.

Data Management

1. Data that have passed review (according to Pass/Fail Criteria), FlowJo analysis, and Labkey analysis (if used) 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 CFSE 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 CFSE Worksheet.

6. LabKey Data: If Lab Key is used, 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 or from data management software (i.e., Excel or JMP) and uploaded to SCHARP.

Attachment 1

CFSE 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 = _____

☐ 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 = _____

☐ 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:_____

Attachment 2

CFSE Proliferation Worksheet

Batch #: _____ 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 Human R10. **Record batch # in Reagent Log Notebook.** Human R10: Lot # _____ Exp. Date: _____	-- N/A--
	2	Prepare Human R10/Benzonase. **Record batch # in reagent log notebook.** R10/Benzonase: Lot#:_____ Exp. Date:_____	-- N/A--
	3	Thaw samples according to SOP FH-HVTN-P0004.	-- N/A--
	4	Add Human R10.	-- 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	Label plate(s) according to plate layout in SSP. Prepare stimulation cocktails as defined in SSP.	-- N/A--
	8	Add 500µl stimulation cocktails (**750µl for positive control)** to appropriate wells.	-- N/A--
	6	Remove cells from incubator. ➡ Time out of incubator:	____:____
	7	Determine cell number and viability using the Guava according to SOP FH-HVTN-E0018. **VIABILITY MUST EXCEED 66% TO PROCEED.**	-- N/A--
	9	For compensation, determine the PBMC sample that has at least 6M extra cells. Calculate concentration, remove 6M cells and add 3M cells each to two wells of a 96-well deep-well plate. Add enough R10 to each well to bring the volume to 1ml if needed. Centrifuge remaining cells.	-- N/A--
	10	Resuspend cell pellet with a P200 pipette. Add 3ml room temperature PBS to each tube. Add 80µl CFSE stock to 40ml warm PBS and mix well. PBS: Lot#:_____ Exp. Date:_____ CFSE Stock: Lot#:_____ Exp. Date:_____	-- N/A--
	11	Add 1ml CFSE mix and **immediately** start a timer for **exactly** 8 minutes. If labeling more than one sample, stagger the timing so that CFSE is added to each sample every 5 seconds. Swirl sample gently several times during incubation at room temperature.	-- N/A--

Initial	Step	Description	Time/Amount
	12	Add 4ml room temperature Human serum to each sample, staggering every 5 seconds for multiple samples. Incubate for one minute at room temperature.	-- N/A--
	13	Add 12ml 37°C Human R10 to each sample. Centrifuge 1200 RPM for 10 minutes.	-- N/A--
	15	Resuspend cell pellet in enough Human R10 to bring the concentration to 2M/ml. Add 500µl cell suspension to all sample wells **except positive control wells which get 250µl**.	-- N/A--
	16	Incubate plates for 6 days, +/- 5 hours at 37°C. ➡ Time in incubator:	____:____

Day 7 Day 7 Date: _____/_____/_____

Initial	Step	Description	Time / Amount
	17	Prepare antibody cocktail as described in the CFSE Antibody Cocktail Worksheet following these steps:	-- N/A--
	17.1	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--
	17.2	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--
	17.3	Calculate the final stain volume. Multiply the number of tests x 55ul. Record this in the table.	-- N/A--
	17.4	Calculate the amount of PBS to add to the antibody cocktail by subtracting the total antibody volume from the final stain volume. Record this in the table.	-- N/A--
	17.5	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. If more than 1.5ml, split evenly into microcentrifuge tubes. Microcentrifuge at 14,000RPM (1100 G) for 5 minutes to remove aggregates. After centrifugation, pipette supernatant and be careful to not disturb pellet.	-- N/A--
	18	Remove plates from incubator.	-- N/A--
		Time removed from incubator:	____:____
	19	Add 100µl EDTA solution, mix and incubate 10 minutes at RT, then centrifuge plate and flick supernatants. EDTA solution: Lot#_____ Exp. Date:_____	Time in: ____:____ Time out: ____:____

Initial	Step	Description	Time/Amount
	20	Resuspend each well (except two comp wells) in 100µl PBS and transfer entire volume to a labeled 96-well round-bottom plate (see SSP). Resuspend comp wells in 600µl PBS and distribute volume to comp wells on the 96-well round bottom plate. Centrifuge at 2000 RPM (750XG) for 3 minutes.	-- N/A--
	20	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. **AViD** Lot#_____ Exp. Date:_____ Dilution (in ul/mL PBS): _____	-- N/A--
	21	After plate is done centrifuging, flick supernatants. Reusupend all sample wells and AViD comp in 50µl AViD solution. Incubate in the dark for 20 minutes at room temperature.	Time in: ____:____ Time out: ____:____
	22	Add 150µl PBS to all wells. Centrifuge 2000 RPM for 3 minutes. Flick supernatant.	-- N/A--
	23	Resuspend all sample wells in 50µl surface stain cocktail. Reusupend all compensation wells in 50µl PBS and add appropriate antibody to each. Incubate 20 minutes in the dark at room temperature.	Time in: ____:____ Time out: ____:____
	24	Add 150µl FACS Wash to all wells. Centrifuge and flick plate. (1st wash)	-- N/A--
	25	Add 200µl FACS Wash to all wells . Centrifuge and flick. (2nd wash)	-- N/A--
	26	Resuspend wells with **200µl** of 1% Paraformaldehyde working solution, **then add** 20µl EDTA solution and store in refrigerator until FACS collection. 1% Paraformaldehyde: Lot#_____ Exp. Date:_____ ➧ Time staining completed:	____:____

FACS Collection Date: _____/_____/_____

Initial	Step	Description	Time/Amount
	27	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):**	_____
	28	Run alignment beads and adjust PMT voltages to match target medians as appropriate for each machine. 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--
	29	Check CV's: Are CV's within tolerance limits?	YES/NO
	30	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--

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. Refer to SSP for list of channels to be used.

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)		<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%			

Initial	Step	Description	Time/Amount
	31	Perform plate clean on HTS (this may also be done prior to running the bead QC step).	-- N/A--
	32	Copy instrument settings.	-- N/A--
	33	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):	_____
	34	Paste in instrument settings from QC folder.	-- N/A--
	35	Collect plates according to the LSRII equipment SOP (FH-HVTN-E0022). ➧ Time FACS collection begun:	____:____
	36	After collection is completed, check all compensation wells to insure they are acceptable by viewing each in the Diva software. 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--
	37	Export the experiment folder and the QC folder to the Data Export folder on the desktop. **Note: Export as "Experiment" not as FCS files.**	Time collection completed ___:____
	38	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--
	39	Perform HTS and instrument cleaning procedures as per the LSRII equipment SOP (FH-HVTN-E0022).	-- N/A--

Comments _____

Attachment 3

CFSE 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)
=_____

				For comp wells		For cocktail	
Antibody	**Lot#**	**Exp date**	**Titre (μl)**	**Comp well (μl)**	**By**	**Volume (μl) to add to mix**	**By**
CD3 APC							
CD4 ECD							
CD8 PE							
			Total Ab Volume (ul):				N/A
			FACS Wash Volume (ul):				
			Total stain cocktail volume (ul) = #tests x 55				N/A

Attachment 4

Face Page for FlowJo Analysis

FlowJo analysis for protocol:_____

Use a separate face page for each separate experiment

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.)

_____

_____

_____

_____

_____

_____

_____

Data Reviewed By/Date:_____

Attachment 5

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**