LYMPHOCYTE PROLIFERATION USING SUCCINIMIDYL ESTER OF CARBOXYFLIORESCEIN DIACETATE

1. PRINCIPLE:

The succinimidyl ester of carboxyfluorsecein diacetate [5(6)]- CFSE is the best reagent currently available for the analysis of cellular proliferation. CFSE spontaneously and irreversibly couples to both intracellular and cell surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, CFSE labeling is distributed equally between the daughter cells, which are therefore half as fluorescent as the parents. As a result, halving of cellular fluorescence intensity marks each successive generation in a population of proliferating cells and is readily followed by flow cytometry. The number of divisions, which can be followed, is limited only by the auto fluorescence level of unlabeled cells, and the uniformity in size of the labeled cell population (Hodgkin et al., 1996).

2. SPECIMEN REQUIREMENTS:

2.01 Specimen sources:

- 2.01.1 Heparin anticoagulated peripheral blood
- 2.01.2 ACD anticoagulated peripheral blood
- 2.01.3 EDTA anticoagulated peripheral blood
- 2.01.4 Isolated PBMCs shipped real time or viably frozen for testing

2.02 Volume requirements:

- 2.02.1 Dependent on two factors
  - 2.02.1.1 Patient's absolute lymphocyte count
  - 2.02.1.2 Number of stimulants to be assayed
- 2.02.2 Minimum volume of blood is 1.5-2ml per stimulant.
- 2.02.3 Ideal blood volume is 3ml per stimulant
- 2.02.4 Isolated PBMCs -1 million cells/stimulant

2.03 Whole blood specimens should be transported/shipped at room temperature. Isolated PBMCs should be shipped at 4°C.

2.04 Unacceptable specimen:

- 2.04.1 Clotted specimens
- 2.04.2 Specimens (including isolated PBMCs) that cannot be processed within 30 hours of being drawn.

2.05 Special Handling

Normally, blood samples should be received in the laboratory a short time after they are drawn (1-2 hours).

3. EQUIPMENT AND REAGENTS

3.01 Equipment

3.01.1 Refrigerator - 4°C, 20°C
3.01.2 Sterile Pyrex glass bottles (250-500mL) with screw on caps
3.01.3 Centrifuge – Refrigerated and Room temperature
3.01.4 15ml & 50ml sterile conical tubes
3.01.5 2ml, 5ml, 10ml sterile pipettes
3.01.6 12 x 75 Polystyrene test tubes
3.01.7 Test tube racks
3.01.8 24 well sterile plates
3.01.9 Interval timers
3.01.10 Vortex
3.01.11 Incubator - 37°C with 5% CO^2
3.01.12 Eppendorf Pipettes 1000ul, 200 ul, 10 ul, 5 ul
3.01.13 Disposable sterile pipette tips
3.01.14 Sterile Eppendorf tubes
3.01.15 Ice bucket with lid
3.01.16 Flow Cytometer

3.02 Sterile Reagents:

3.02.1 Stimulants designated by the protocol
3.02.2 Pooled human AB serum (Gemini Bio-Products, Inc.)
3.02.3 RPMI 1640
3.02.4 Hepes (BioWittaker) 1M (Cat# 17-737E)
3.02.5 Pen/Strep antibodies 10,000 Units of Penicillin & 10,000ug of Streptomycin per mL.
3.02.6 Fetal Bovine Serum (FBS) (Sigma)
3.02.7 Dimethyl Sulfoxide (DMSO) (New unopened reagent)
3.02.8 CFSE 25mg vial (Molecular Probes CAT# C1157)
3.02.9 L-Glutamine (Sigma, BioWhittaker) 200mM
3.02.10 Bovine Sera Albumin Faction V (BSA) (Sigma Cat # A-7906)
3.02.11 Phosphate Buffer Saline (PBS) (Sigma, BioWhittaker)
3.02.12 Na azide*
3.02.13 10% Paraformaldehyde**
3.02.14 Monoclonal Antibodies*

*CAUTION: SODIUM AZIDE, under acidic conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be flushed with running water while being discarded to avoid deposits in metal piping in which explosive conditions can develop. If skin or eye contact occurs, wash excessively with water.

**CAUTION: PARAFORMALDEHYDE is toxic by inhalation and ingestion and is a strong irritant to eyes, skin mucous membrane and respiratory tract. Handle with extreme care and use appropriate protective clothing.

4. REAGENT PREPARATION:

4.01 1% BSA Wash Buffer Solution: To 1 Liter of Phosphate Buffer Saline add 1 gm of Na azide, and 10 gm of BSA. pH 7.2 Test reagent with blood drawn within 24 hours. Spin blood down, remove plasma and replace with wash buffer solution. Mix well by inverting the tube 10 times or place on a rocker for a few minutes. Re-spin the blood and look for lysis. If lysis occurs, remake the solution. Store buffer at 4°C when not in use. Expiration Date: 1 month.

4.02 1% Paraformaldehyde Solution: To 900 ml of Phosphate Buffer Saline add 100 ml of a 10% Para formaldehyde solution. pH to 7.2. Store at 4°C when not in used. Expiration Date: 6 months.

4.03 Suspension of CFSE: To 1 vial of CFSE add 10ml of sterile DMSO (new and unopened bottle) and aliquot the solution into sterile eppendorf tubes (@25-50ul/tube). Always keep this reagent out of the light. Aliquot CFSE in a hood without UV or fluorescent lighting. Store desiccated and in the dark at -20°C. Discard an aliquot after a single use. Frozen CFSE aliquots begins to lose fluorescent intensity after 11 months.

4.04 10% Fetal Bovine Serum in RPMI 1640: Add 10ml of FBS to 90ml of RPMI 1640 containing 1% Hepes, 1% Penn/Strep and 1% L-Glutamine. Store at 4°C. Expiration Date: 1 months.

4.05 1.0% Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS): Add 1.0g of BSA to 100ml of PBS. Filter the reagent using a 2µm filter and store at 4°C. Expiration date: 3 month.

4.06 Working solution of BSA in PBS: Dilute stock solution as needed to prepare a 0.1% to 0.5% working solution. Expiration Date: 1 month

5. PROCEDURE:

Following the ACTG PBMC Consensus Method, isolate peripheral blood mononuclear cells (PBMCs) from heparinized blood. - http://aactg.s-3.com/immmeth.htm You will need $1x10^6$ cells for each stimulant to be assayed. Sterile technique must be used until cells are harvested after stimulation (Section 5.14).

5.01 Harvest cells from the interface of the ficoll layer and wash the cells 2x with RPMI 1640 medium. On the last wash transfer cells to a 15ml conical test tube, obtain an accurate, viable cell count, and then spin at 800xg for 10 min. Remove all of the RPMI medium.

5.02 Resuspend the pellet in 2.5ml of a 0.1% BSA/PBS solution at room temperature. This is to wash out any remaining RPMI and to get the cells prepared for staining.

5.03 Centrifuge cells at 800xg for 10 min. and decant.

5.04 Add 250ul of 0.1% to 0.5% BSA in PBS to the cell pellet and gently resuspend the cells. It is important that cells be well suspended and not aggregated, to ensure uniform labeling with CFSE. *(Please Note: The percentage of BSA added to the PBS can vary from 0.1% to 0.5% depending on the lot of CFSE. Each new lot of CFSE must be tested to determine the best concentration of BSA to use. See Trouble Shooting Section to help determined the percentage of BSA to be used.)*

5.05 Dilute the CFSE stain **immediately** before using it and keep it in the dark. The stain is prepared as follows at room temperature:

5.05.01 Retrieve an aliquot of CFSE from the –20°C freezer. Once a vial is open, do not replace any unused portion of the stain - discard all excess stain.

5.05.02 In the dark, add 4ul of CFSE to 1ml of sterile PBS containing the appropriate concentration of BSA.

5.06 Immediately add 250ul of the diluted CFSE stain to your resuspended cell pellet. You can easily stain 5-25 million cells in this 500ul solution.

5.07 Mix by gently pipetting cells up and down using a sterile transfer pipette and place cells in the 37°C incubator for 10 min. This time is crucial – no longer.

5.08 After incubation, add 5ml of cold 10% FBS in RPMI and incubate on ice for 5 min.

5.09 Wash 3 times with cold 10% FBS/RPMI solution (keep on ice to maintain cold temperature). The centrifuge does not need to be at 4°C. Centrifuge for 5-7 min at 800xg.

5.10 Resuspend cells in complete media. (RPMI, 10% AB Sera, 1% L-Glutamine, 1% Hepes buffer, 1% of Pen/Strep antibiotics). 1ml of media for each million cells. You do not need to recount cells.

5.11 Plate 1ml of cells for each stimulus in a 24 well plate.

The following are examples of various stimulants that could be used. The concentration of each stimulant is usually determined by what has been optimal in a lymphocyte proliferation assay.

- CMV - 1:40 dilution
- CMV Control – 1:40 dilution
- PHA – 5ug/ml
- Vβ3 antibody – 3ul/ml of the BD/PharMigen antibody
- CD3 antibody – 3ul of stock (Stock solution is a 1:15 dilution of the BD/PharMigen antibody)
- HIV-MN – 2.5ul of stock (1:400 dilution)
- HIV-MN control – 2.5ul of stock (1:400 dilution)
- Tetanus – 1LFU/ml to 2LFU/ml
- Vaccine - Lot dependent
- Media – 10% Pooled Human Serum

5.12 Incubate at 37° in 5% CO^2 from 4 to 7 days depending on the stimulant. The length of incubation time should be stated in the Protocol Immunology Information Sheet for each study.

5.13 After incubation, harvest the stimulated cells into appropriate labeled 12 x 75 test tubes and centrifuged for 3 min at 800xg.

5.14 Harvest tubes in the dark, as light will quench the CFSE stain. Proceed as much as possible in the dark with the cells.

5.15 Surface stain each stimulated cell with 20ul of CD8 PerCP and 10ul of CD4 PE for 3 color Flow Cytometry. The choice of fluorochromes to be used should be stated in the Protocol Immunology Information Sheet for each study. Example histograms of 4 color CFSE Flow Cytometry will be shown at the end of this procedure and again this will be protocol specific.

5.16 Incubate at room temperature for 10 min in the dark.

5.17 Add 2ml of the wash buffer solution, centrifuge the cells at 800xg for 2 min and decant the supernatant.

5.18 Add 200ul wash buffer and 200ul of the 1% Paraformaldehyde solution.

5.19 Refrigerate tubes in the dark until ready to run on the Flow Cytometer. Fixed cells are stable for analysis for up to 48 hours.

6. ACQUISTION OF CELL ON THE FLOW CYTOMETER.

6.01 Create a dot plot of forward scatter (FSC) on the x-axis versus side scatter (SSC) on the y-axis. Place a gate (R1) around the lymphocyte population. Cells that are proliferating will become larger with more granules. Adjust the gate to include these cells.

6.02 Create two, single parameter histogram plots with CD4 or CD8 on the x-axis. Anchor gate around cells that are CD4 bright (R2) and CD8 bright and dim (R3).

6.03 For 3-color analysis, a second histogram plot needs to be created for both CD4 and CD8 with CFSE (FITC) on the x-axis. The 1st histogram plot will have the logic of R1 and R2. The 2nd histogram plot will have R1 and R3.

6.04 For 4-color analysis, a dot plot needs to be created with CFSE on the x-axis and PE on the y-axis for both CD4 and CD8. The 1st dot plot will have the logic of R1 and R2. The 2cd dot plot will have R1 and R3.

6.05 Cells stained with CFSE have very bright fluorescence. As the cells proliferate the cell population moves from right to left – from bright to dim. Daughter cells have half the fluorescent intensity of the parent cell. The placement of the M1 cursor extends from the y-axis up to the very bright CFSE stained cells.

6.06 PMT voltages may have to be adjusted downward because of the brightness of the CFSE stain. Once the PMT range is determined for your lot of CFSE, it will pretty

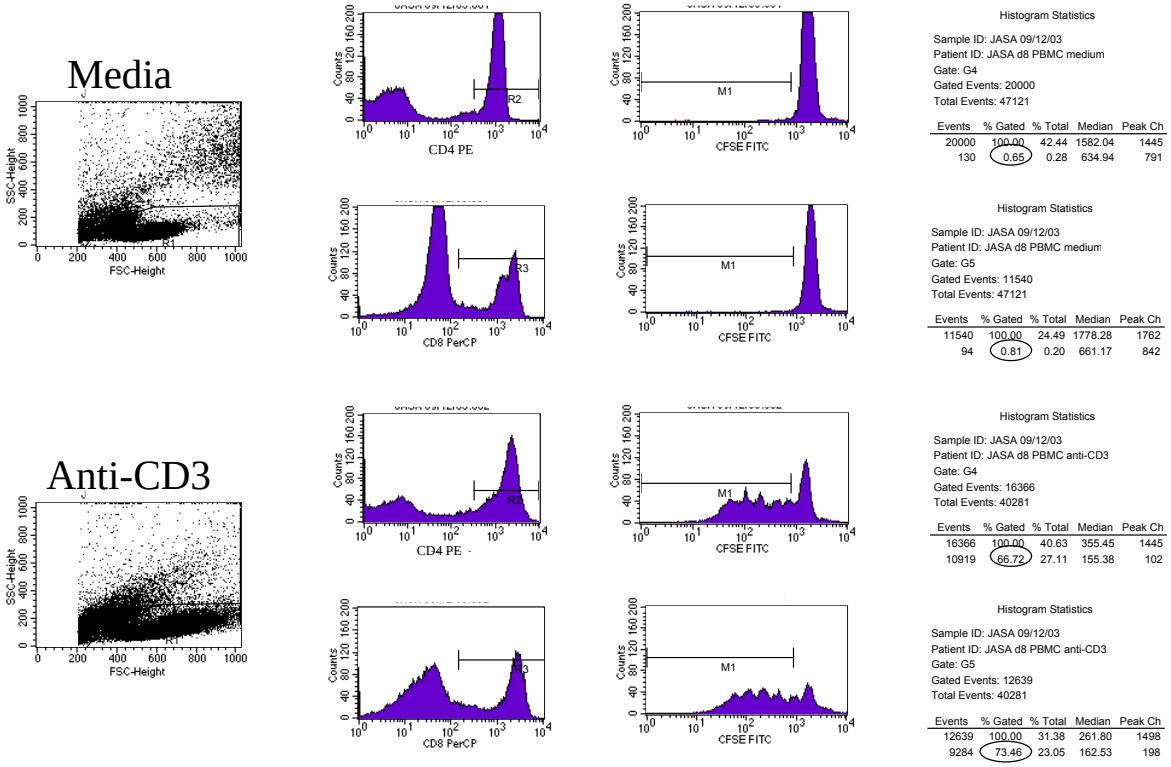
much remain the same unless you have exposed the cells to excessive light while processing.

6.07 Below are examples of histograms using different stimulants and examples of both 3 and 4 color Flow Cytometry. Please note the location of the M1 marker position used in the 3-color flow.

6.08 For both 3 and 4-color Flow Cytometry, at least 20,000 gated lymphocytes should be acquired.

Three Color Flow

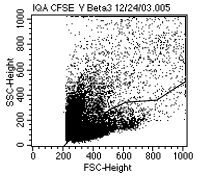
The stimulants used in the following proliferation assay were media (negative control) and anti-CD3 (positive control). The cells were incubated for 7 days and then stained with CFSE (FITC)/CD4 (PE)/CD8 (PerCP). The circled results would be reported in a protocol specific excel spreadsheet.

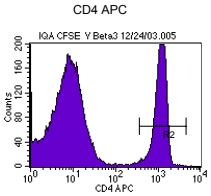


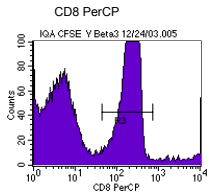
Four Color Flow Cytometer

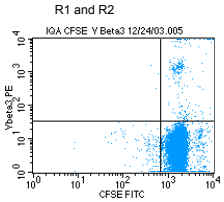
The stimulants used in the following proliferation assay were media (negative control) and anti-Vβ3 (positive control). The cells were incubated for 7 days and then stained with CFSE (FITC)/Vβ3 (PE)/CD8 (PerCP)/CD4 (APC). The percent gated quadstat results would be reported via a protocol specific excel spreadsheet.

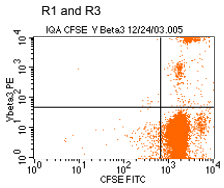
Media











Quadrant Statistics

File: IQA CFSE V Beta3 12/24/03.005
Log Data Units: Linear Values
Sample ID: CFSE VBeta3 Media
Gated Events: 14950
Total Events: 73610

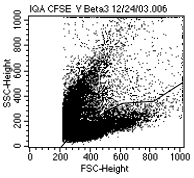
Quad	Events	% Gated	% Total	X Mean
UL	3	0.02	0.00	516.35
UR	273	1.83	0.37	2827.11
LL	153	1.02	0.21	410.25
LR	14521	97.13	19.73	1757.73

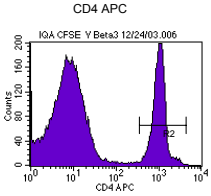
Quadrant Statistics

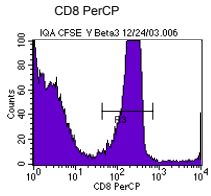
File: IQA CFSE V Beta3 12/24/03.005
Log Data Units: Linear Values
Sample ID: CFSE VBeta3 Media
Gated Events: 17808
Total Events: 73610

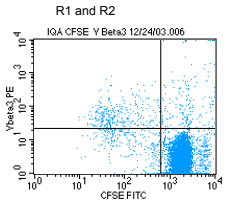
Quad	Events	% Gated	% Total	X Mean
UL	14	0.08	0.02	370.16
UR	565	3.17	0.77	2795.42
LL	224	1.26	0.30	417.05
LR	17005	95.49	23.10	1783.19

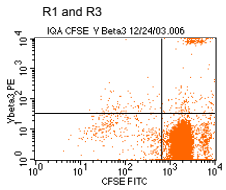
VBeta 3











Quadrant Statistics

File: IQA CFSE V Beta3 12/24/03.006
Log Data Units: Linear Values
Sample ID: CFSE VBeta3 Stimulated
Gated Events: 14415
Total Events: 75328

Quad	Events	% Gated	% Total	X Mean
UL	334	2.32	0.44	118.30
UR	199	1.38	0.26	3493.18
LL	272	1.89	0.36	250.10
LR	13610	94.42	18.07	2059.81

Quadrant Statistics

File: IQA CFSE V Beta3 12/24/03.006
Log Data Units: Linear Values
Sample ID: CFSE VBeta3 Stimulated
Gated Events: 17573
Total Events: 75328

Quad	Events	% Gated	% Total	X Mean
UL	82	0.47	0.11	149.83
UR	290	1.65	0.38	3627.41
LL	380	2.16	0.50	238.74
LR	16821	95.72	22.33	2087.28

6. TROUBLESHOOTING

6.01 BSA Concentration: The concentration of protein (BSA) included in the staining step improves the viability of cells. This is particularly important when using cells sensitive to being driven into apoptosis. If there is a viability problem, it may be necessary to titrate the CFSE stain to determine a level at which cell survival is obtained, while maintaining sufficient CFSE fluorescence intensity.

6.02 CFSE resuspension in DMSO: A new bottle of DMSO is necessary when dissolving a vial of CFSE. A bottle of DMSO older than 6 months will greatly affect the cells ability to proliferate.

6.03 Very heavy staining often results in suboptimal cell viability, as well as making compensation between detecting channels difficult or impossible.

6.04 Do not add CFSE directly to your cell suspension. It can be detrimental to the cells. Always dilute the CFSE first before adding it to the cells.

7. REFERENCES

Hodgkins, P.D., Lee, J.H., and Lyons, A.B. 1996. B cell differentiation and isotype switching is related to division cycle number. J. Exp. Med. 184:277-281.

Lyons, A.B. and Parish, C.R. 1994. Determination of lymphocyte division by flow cytometry. J. Immunol. Methods 171:131-137.

Lyons, A.B. and Doherty, K.V. 1998. Flow cytometry analysis of cell division by dye dilution. Current Protocols in Cytometry 9.11.1-9.11.9.

Molecular Probes, Inc. 1996-2002 webmaster@probes.com Assays for cell enumeration, cell proliferation and cell cycle. 15.4.

Procedure: Dye Dilution (CFSE) Proliferation

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 01 June 2004

Date Implemented into the Laboratory: _____

Updated on:

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Reviewed by: Date:

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Supersedes Archived Protocol: No Previous ACTG Protocol