INTRACELLULAR Ki67 STAINING

1. PRINCIPLE

Ki67 is a nuclear antigen associated with cell proliferation and is present throughout the active cell cycle (G_1, S, G2 and M phases) but absent in resting cells (G_0). The increased intracellular Ki67 expression in HIV-1 infected subject has been taken to indicate an increase in T cell turnover.

2. SPECIMEN REQUIREMENT

2.01 K_3EDTA (purple top) vacutainer is the preferred anticoagulant used in immunophenotyping.

2.02 Sodium heparin (green top) vacutainer is an acceptable alternative.

2.03 One ml of whole blood is required and ideally taken from blood drawn for a Three Color Advanced Flow Panel. If isolated cells are used (i.e.: Peripheral Blood Mononuclear Cells, lymph node aspirate cells, etc.), it is highly recommended that at least 1-2 X 10^6 cells be used to initiate the assay.

2.04 Specimens will be kept at room temperature (18-22ºC) and must be processed within 24 hours of specimen draw. Please Note: 24 hours is the longest time to process samples. Intracellular staining works best on fresh samples (under 6 hours).

2.05 Specimens must be labeled with Patient Identification number (PID), date, time of draw and protocol number.

2.06 Criteria for specimen rejection based on suboptimal specimen conditions (e.g. clotting, hemolysis) are described in NIAID guidelines for flow cytometric immunophenotyping, http://www.wiley.com/legacy/products/subject/life/cytometry/guide.html (1).

3. REAGENTS

3.01 Monoclonal antibody panel will be specified by the protocol. (Ki67 FITC manufacturers – BD Biosciences Cat # 36624K, Beckman-Immunotech Cat # IM0606)
- 3.01.1 Store reagents at 2-8ºC to assure proper performance. Avoid exposing reagents to light. Discard reagents after expiration date.
- 3.01.2 Fluorochrome designations for monoclonal antibody combinations will be protocol-specified and must be followed.
- 3.01.3 Monoclonal antibody combinations will be obtained from a protocol-specified vendor.

3.02 Dulbecco's phosphate buffered saline (dPBS, e.g. Sigma, #5652). Store unused buffer at 2-8ºC.

3.03 FACS™ Lysing Solution (BD Biosciences Cat#349202)

3.04 FACS™ Permeabilizing Solution (BD Bioscience Cat#340457)

* 3.05 Sodium Azide
3.06 Bleach
3.07 BSA (Bovine Serum Albumin) or FBS (Fetal Bovine Serum)
**3.08 2% methanol-free Paraformaldehyde in dPBS or 2% methanol-free formaldehyde in dPBS.
3.09 If the protocol calls for the isolation of PBMCs from whole blood to be stain with Ki67 (i.e. 48 hour cultured cells), refer to the Specimen Processing Guide on the web at: http://aactg.s-3.com/pub/download/SpecimenProcessingGuide.pdf for additional reagents needed.

*CAUTION: SODIUM AZIDE, when 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. If swallowed, seek medical advice immediately.

**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. EQUIPMENT

4.01 Class 2 Biosafety cabinet
4.02 Centrifuge
4.03 Refrigerator - 4°C
4.04 12 x 75 mm polystyrene tubes (Falcon #352008)
4.05 Micropipettes capable of dispensing 20 μL, 100 μL, and 500 μL
4.06 Disposable pipette tips
4.07 Disposable transfer pipettes
4.08 Vortex
4.09 Interval timers
4.10 Disposable gloves
4.11 Lab coat
4.12 Safety glasses
4.13 Flow cytometer capable of 3-color detection

5. REAGENT PREPARATION

5.01 FACS Lysing Solution:
Mix 100 mL of BD FACS Lyse Solution and 900 mL of Reagent Grade water. Store at Room Temperature. Expiration Date: 1 month

5.02 FACS Permeabilizing Solution:
Mix 1 mL of FACS Permeabilizing Solution and 9 mL of Reagent Grade water. (Make only the amount you are going to use. FACS Permeabilizing Solution should be prepared fresh). Keep at Room Temperature until used.

5.03 1% BSA Wash Buffer Solution:
Combine 1 Liter of dPBS with 1 g of Na Azide and 10 g of BSA. Mix and pH to 7.2. Store at 4°C when not in use. Expiration date: 3 months. A 2% FBS can be used as an alternative.

5.04 2% paraformadehyde or 2% formaldehyde:
A suggested source of this fixative is a 10% Formalin Neutral Buffered Solution from Sigma (# HT50-1-1) that is diluted 1:2 with dPBS to obtain a working concentration of 2% formaldehyde. Another source is a 10% Formaldehyde Solution from PolySciences (#0418), which must be diluted 1:5 with dPBS to obtain a working concentration of 2% formaldehyde. Use of methanol-free 2% (w/v) Paraformaldehyde (Sigma, #P-6148) in a buffered salt solution, pH 7.4 – 7.6, is also acceptable. Store in an amber glass container or wrap container in aluminum foil to avoid exposure to light. Diluted 2% formaldehyde may be stored up to 1 week at 2 – 8 ^{o}C, while stock solutions of formaldehyde should be stored no longer than one year. All formaldehyde solutions are highly toxic and carcinogenic.

6. INSTRUMENTATION

6.01 Each laboratory will have a comprehensive quality assurance program that will assure proper instrument operation, calibration stability and personnel training.

7. PROCEDURE FOR WHOLE BLOOD

7.01 Place 1 mL of blood into a 17 x 75 test tube and wash 2 times with 1% BSA Wash Buffer (Wash Buffer). Centrifuge for 3-5 minutes at 300 g between each wash. (Blood from 3 color Advanced Flow may be used if the protocol elects to do both assays).

7.02 Aspirate the supernate and resuspend blood with Wash Buffer to its original volume (1mL).

7.03 Add 10-20 μL of the following antibodies to four 17 x 75 test tubes:

Test Tube #	Antibodies Added
1	CD4(PE)/CD3(PerCP)
2	CD4(PE)/CD3(PerCP)
3	CD8(PE)/CD3(PerCP)
4	CD8(PE)/CD3(PerCP)

Please Note: This antibody combination may change depending on the protocol. Regardless, use the manufacturer's recommended amount of antibody for cell surface staining.

7.04 Add a 100 μL of washed blood to each tube. (If the patient is known to have a very low White Blood Count, it may be necessary to add 200 μL of washed blood to each tube).

7.05 Vortex gently for 3 seconds and incubate in the dark for 15 minutes at room temperature.

7.06 Add 2 mL of 1X FACS Lysing Solution

7.07 Vortex gently for 5 seconds and incubate for 10 minutes in the dark at room temperature.

7.08 Centrifuge at 300 g for 5 minutes at room temperature. Aspirate the supernatant trying to remove as much lysing solution without disturbing the pellet.

7.09 Add 500 μL of freshly diluted 1X FACS Permeabilzing Solution to the pellet of each tube.

7.10 Resuspend cells by gently flicking the tube or with a disposable plastic transfer pipette. (Vortexing can be too harsh on the cells.) Incubate in the dark for 15 minutes.

7.11 Add wash buffer directly to the four test tubes and centrifuge at 300 g for 5 minutes.

7.12 Aspirate supernatant leaving as little liquid as possible and add 5 uL of IgG FITC to tubes #1 and #3. Add 10 to 20 uL of Ki67 FITC to tubes #2 and #4. The amount of antibody added is dependent on the manufacturer used.

7.13 Gently mix and incubate cells for 30 minutes in the dark at room temperature.

7.14 Add wash buffer directly to each of the four tubes, centrifuge at 300g for 5 minutes and aspirate supernatant.

7.15 Add 300 uL of fixative (2% paraformadehyde or 2% formaldehyde) and keep in the dark at 4ºC until ready to run on a Flow Cytometer. Cells can be store at 4ºC up to 48 hours.

Please Note: If the protocol calls for PBMCs to be stained, you do not need to FACS Lyse (Step 7.06). After surface staining 0.5 to 1 x 10^6 PBMCs as outline in 7.03-7.05, wash out the surface staining antibodies 1 time with 2 ml of wash buffer, centrifuge, aspirate and proceed to step 7.09.

8. FLOW CYTOMETER SETUP

8.01 The compensation settings should have been set up for the Advanced Flow Panel. However if that panel is not being run, follow the compensation instructions in the Consensus Protocol for Advanced Panel Immunophenotyping on the web **http://aactg.s-3.com/immmeth.htm**.

8.02 Create a dot plot using a linear Forward Scatter (X axis) versus a linear Side Scatter (Y axis) histogram and gate on the lymphocytes. This is the same set up as the Advance Flow panel analysis.

8.03 A minimum of 10,000 lymphocytes should be acquired.

8.04 Create a histogram plot, with CD3 Cy-Chrome on the X-axis versus Counts on the Y-axis. Place an anchor marker on the bright CD3 positive cells.

8.05 Analyze the Ki67/CD4 or KI67/CD8 cells with an anchor on the CD3 positive cells. (R1 and R2 on a BD Flow Cytometry. AB on a Coulter Flow Cytometry.)

8.06 Create a dot plot containing quadrant markers. On the X-axis place the FITC stained antibodies (IgG or Ki67) and on the Y-axis place the PE stained antibodies (CD4 or CD8).

8.07 Using the IgG FITC isotype as a guideline to set your quadrant markers, report out the 4 quadrant statistics for the CD4 and CD8 cells.

9. TROUBLE SHOOTING

An unusual increase in debris seen on the histograms could be caused by too vigorously mixing cells, especially by vortexing cells, after the FACS lysing step. These cells tend to be fragile at this point, and prone to destruction if treated too harshly.

A high intracellular fluorescence background staining with the IgG Fitc isotype may be cause by the whole blood being exposed to too high or too low temperatures. The WBC cells in this assay are even more sensitive to temperature than in the Three Color Advanced Flow Panel analysis. Also if the time from blood draw is greater than 24 hours, the cells tend to bind the IgG antibodies nonspecifically. Staining the blood as soon as possible decreases a high fluorescence background.

A patient with a low WBC count may require an increased volume of blood to be used at the initial step. An additional 100 μl of washed blood may be added into each tube. An increased amount of surface staining antibody is not usually required, but gentle vortexing midway through that incubation period is advised.

10. QUALITY ASSURANCE ISSUES

10.01 Perform daily instrument quality control, 3-color compensation and set-up procedures. Laboratories will ensure quality of monoclonal antibody reagents by periodic testing on a blood specimen obtained from a normal donor or on stabilized, control cells.

10.02 A normal donor may show less expression of Ki67 on unstimulated lymphocytes than a HIV-1 infected individual. With stimulated lymphocytes (PHA, PWM etc.) the expression of this antigen is much higher and could be used to test the quality of the antibody if there is any question of its reactivity.

10.03 Please Note: Participation in the bimonthly proficiency testing from the NIAID Flow Cytometry Quality Assessment Program is required. This testing includes the Advance Flow Cytometry Panel with results being reported to the Immunology Quality Assurance (IQA) Program.

11. REFERENCES

1. Calvelli, T., Denny, T.N., Paxton, H., Gelman, R., and Kagan, J. Guideline for flow cytometric immunophenotyping: A report from the National Institute of Allergy and Infectious Diseases, Division of AIDS. Cytometry 14:702-715, 1993.

2. Nicholson, J., Kidd, P., Mandy, F., Livnat, D., and Kagan, J. Three-color supplement to the NIAID/DAIDS guideline for flow cytometric immunophentoyping. Cytometry (Communications in Clinical Cytometry) 26:227-230, 1996.

3. Fleury S., Rizzardi G.P., Chapuis A., Tambussi G., Knabenhans C., Simeoni E., Meuwly J.Y., Corpataux J.M., Lazzarin A., Miedema F., Pantaleo G. Long-term kinetics of T cell production in HIV-infected subjects treated with highly active antiretroviral therapy. Pro Natl Acad Sci USA 2000 May. 9;97(10):5393-8

4. Kaur, A., Hale, C.L., Ramanujan, S., and Johnson, R.P. Massive Proliferation of CD8+ T Lymphocytes and Natural Killer (NK) Cells during Acute SIV Infection in Rhesus Macaques. 7th Conference on Retroviruses and Opportunistic Infections San Francisco, Calf. Jan. 2000. Abstract 575.

12. PROTOCOL VERSIONS EFFECTIVE DATE

Version 1.0 effective date: October, 2000

13. AUTHORS

Version 1.0 Laboratory Technologists Subcommittee of the ACTG Site and Data Management Group

Procedure: Intracellular Ki67 Staining

Prepared by: ACTG Laboratory Technologist Committee

Preparation Date: 19 October 2001

Date Implemented into the Laboratory: _____

Updated on:

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Reviewed by: Date:

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