

# UNIVERSITY OF ROCHESTER MEDICAL CENTER

Human Immunology Center Core Laboratory

David H. Smith Center for Vaccine Biology and Immunology

Aab Institute of Biomedical Sciences

STANDARD OPERATING PROCEDURE

NUMBER HIC-1-0016

Date: 6-September-2007

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**Approved: Sally Quataert**

## **Title:**

Intracellular Staining of human lymphoid cells for FoxP3 Expression

## **Purpose and Scope:**

This procedure describes a micro-method for FoxP3 staining of human single cell suspensions for analysis using the BD LSR II fluorescent antibody cell sorter. This method is used by the Human Immunology Core Laboratory in exploratory clinical research studies to analyze human clinical specimens for immune status intracellular and cell surface characterization.

## **Principle:**

Single cells or peripheral blood mononuclear cells are stained with cell surface reactive antibodies conjugated to fluorochrome in a v-bottom microtiter plate with the cell pellet and 10 to 15  $\mu$ L of Ab-fluorochrome(s) solution and then the cells are fixed/permeabilized and stained for FoxP3. Antibody fluorochrome conjugates with up to eleven or 18 different fluorescent light emissions at different wavelengths may be used to detect cell surface markers simultaneously on human cells with the BD LSR II flow cytometer. Single cell suspensions or peripheral blood mononuclear cells are prepared from human specimens and stained with either direct antibody (Ab) fluorochrome conjugates or antibody-biotin conjugates followed by streptavidin-fluorochrome conjugates or Molecular Probes, Zenon technology kits. The cells are fixed and permeabilized prior to intracellular staining with an anti-FoxP3 antibody fluorochrome conjugate. Control cell suspensions or antibody capture beads are individually stained with a single Ab fluorochrome conjugate of each antibody fluorochrome used in the multiple stain reactions to compensate for the spill over in the emission spectrums for each fluorochrome. This allows the instrument to calculate and subtract the appropriate overlap to yield the specific signal intensity for each fluorochrome. After surface staining and intracellular staining of the cells is completed the cells are resuspended in staining buffer. The cells must be analyzed by flow cytometry the same day.

## **References:**

Current Protocols in Immunology (2001) 5.3.1-5.3.24 John Wiley & Sons, Inc.

Peter Schenkle et al. Evaluation of a Novel Mononuclear Cell Isolation Procedure for Serological HLA Typing. Clin. Diag. Lab Imm. Nov. 1998 p.808-813.

Current protocols in Immunology (1996) 7.1.1-7.1.3 John Wiley & Sons, Inc.

Recommendations for Prevention of HIV Transmission in Health-Care Settings: Universal Blood and Body Fluid Precautions Guideline CDC 1987

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CDC Biosafety in Microbiological and Biomedical Laboratories 4<sup>th</sup> edition U.S.H&HS, Public Health Service

Product information document for FoxP3 from eBioscience

HIC-1-0004: Micro-immunofluorescence Staining for Multichromatic FACS Analysis with Human Single Cell Suspensions.

## Safety:

Personnel will adhere to safe work processes outlined in U.S. Public Health Universal Precautions Guidelines for use of human blood and body fluids and follow biosafety level 2 (BSL2) practices.

## Reagents and Material:

FACSFlow Sheath Fluid buffer Cat. # 340398 or equivalent

Staining buffer: 1X Hank's balanced salt solution (HBSS) with 1% W/V BSA

FoxP3 eBioScience, Cat. # 53-4776

BD Allegra X-12 centrifuge with SX4750 swinging bucket rotor and inserts with seals or equivalent

Biological Safety Cabinet, Class II

Household bleach (5% sodium hypochlorite)

Absorbent towels

Sterile serological pipets 10, 5, 1 mL size

Pipet aide for serological pipets

Sterile pipet tips

Digital variable single channel air displacement pipets 5-50  $\mu$ L, and 30-300  $\mu$ L

Waste pan

10% bleach solution

variable speed vortex mixer

sterile 15 mL & 50 mL conical polypropylene tube

Ice bath for microtiter plates

10X Permeabilization buffer, eBioScience Cat# 008333-56

Fixation/Permeabilization Concentrate, eBioScience Cat# 005123-43

Fixation/Permeabilization Diluent, eBioScience Cat# 00522356

Normal Rat Serum, eBioscience, Cat. #24-5555-93 or equivalent

**Reagent Preparation:** Fixation and Permeabilization reagents are prepared fresh.

Permeabilization Buffer: Dilute 1:10 in diWater based on the volume needed for staining. (ie. 1mL of 10X permeabilization buffer + 9mL of diWater).

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Fixation/Permeabilization buffer: Dilute 1 part Fixation/Permeabilization concentrate into 3 parts Fixation/Permeabilization diluent (250 $\mu$ L Fixation/Permeabilization concentrate+750 $\mu$ L Fixation/Permeabilization Diluent)

Blocking Buffer: Prepare 2% normal rat serum in permeabilization buffer by adding 2  $\mu$ L of normal rat serum to 0.1 mL of permeabilization buffer.

*Note: anti-FoxP3 antibody fluorochrome conjugate will bind to the bangs beads for use in compensation.*

### Intracellular Staining of cells for FoxP3 FACS analysis

1. Follow SOP HIC-1-0004 for surface staining of cells. Prepare fixation/permeabilization buffer and permeabilization buffer during wash steps. After completion of last wash step continue to step 2 below.
2. Add 100  $\mu$ L of fixation/permeabilization buffer to appropriate wells. Incubate for 30-60 minutes at 4<sup>0</sup> C. **(Do not incubate for longer than 60 minutes)**
3. Wash by adding 100  $\mu$ L permeabilization buffer at 4<sup>0</sup> C to each well. Centrifuging the plate for 6 minutes at 4<sup>0</sup> C at 400 X g. Working in a biological safety cabinet, decant supernatant into a waste container containing 10% bleach solution and gauze (never decant directly into fluid) followed by blotting the last drop on absorbent toweling before inverting upright. Repeat wash one time using 200  $\mu$ L permeabilization buffer.
4. Add 10  $\mu$ L of NRS blocking solution to appropriate wells. Incubate for 15 minutes at 4<sup>0</sup> C in the dark.
5. Wash the microtiter wells once as in step 3 using 200  $\mu$ L permeabilization buffer.
6. Add 10 $\mu$ L of diluted FoxP3 antibody (80 $\mu$ L of HBSS/1%BSA + 20 $\mu$ L of FoxP3 Antibody fluorochrome). Incubate for 30 minutes at 4<sup>0</sup> C in the dark.
7. Wash 2X as in step 5.
8. Resuspend in 200  $\mu$ L of staining buffer (HBSS/1%BSA) and leave in microtiter plate for reading using the High Throughput Autosampler System on the LSR II or transferred to either large volume microwell tubes or to 12 X 75 mm tubes for manual reading. Samples must be

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analyzed on the BD LSR II FACS immediately after the staining is complete. If using 12 X75 mm tubes, add additional FACSFlow buffer to make @ 0.5 mL final volume immediately before reading on LSR II. Samples from microtiter wells may be transferred to microwell tubes that will fit into a 12 X 75 mm size tube for reading on the LSR II.

### Data Analysis and Interpretation:

Data is collected and auto-compensated using FACS Diva software on the LSR II. The FCS files are transferred onto the HIC server into individual accounts and a back up data disc is burned from the BD LSR II computer D drive. Files are analyzed using Flowjo software program.

### Revision History

Version	Change	Impact	Justification	Change Date:
HIC-1-1004	new			09-06-2007