1.0 PURPOSE

1.1 This procedure describes the process for cell surface staining with fluorescent conjugated antibody reagents and optional amine reactive dye for viability measurement

2.0 SCOPE

2.1 This SOP applies to all ITI Flow Core operators in all locations

3.0 RESPONSIBILITIES

3.1 Cellular Analysis

3.1.1 Reviewing and following the protocol to stain lysed whole blood or other appropriate samples for cell surface with Ab reagents for analysis on flow cytometry

3.2 Qualtity Systems

3.2.1 Reviewing and approving this document.

3.2.2 Notifying initiating department that biennial review is due for an SOP and their associated Form.

4.0 REFERENCES AND RELATED DOCUMENTS

4.1 ITI-00099, RBC lysis with VersaLase lysing buffer

4.2 ITI-00095, Thawing of frozen PBMC

4.3 Batch sample process log sheet by study

4.4 ITI-00067, Individual sample process sheet

5.0 DEFINITIONS

Term	Description
Ab	Antibody
Cocktail	Antibody reagent containing more than1 conjugated antibody
Drop-in	Antibody reagent stored indivually
2xLB	2 times concentrated lysed blood
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
RT	Room temperature
WB	Whole blood

6.0 SAFETY

6.1 Follow all ITI safety policies and procedures. The processing of human whole blood and other blood products are considered a potential biohazard. Treat all human blood as infectious material and process with proper protection. All waste should be disposed in biohazard waste containers. Lab coat, goggles, and gloves should be worn at all times.

7.0 EQUIPMENT AND MATERIALS

7.1 Single channel pipettes (Rainin RL10, 20, 100, 200 and 1000 or equivalent)

7.2 Multi-channel pipettes (Rainin, Cat# L8-20 0.5-20 µl, L8-200 20-200 µl or equivalent)

7.3 Disposable pipette tips (Rainin, Cat# L10, L250 and L1000or equivalent)

7.4 Centrifuge equipped with swinging bucket rotors with appropriate adapters and biohazardous containment covers (Allegra XR-15 or equivalent)

7.5 Vacuum with aspiration line and trap containing bleach in the waste

7.6 8-channel tip (INOTech, Cat# IV-503)

7.7 Sterile serological pipettes (VWR 2, 5, 10, and 25 mL or equivalent)

7.8 Timer (minutes)

7.9 96 well V-bottom plate (VWR, Cat# 62409-104)

7.10 Sterile 50 mL centrifuge tube (VWR, Cat#21008-103) or equivalent

7.11 Solution basins (VWR, Cat# 21007-970)

7.12 IOTest3-Fix (Beckman Coulter, PN IM3515). Stored at 4^{o}C

7.13 PBS (Invitrogen, Ca++ and Mg++ free or equivalent)

7.14 Cell Staining Buffer (PBS contains bovine calf serum and Sodium azide. Biolegend, Cat#420201)

7.15 Amine reactive Live/Dead yellow dye (Invitrogen, Cat#L34959) or alternative color

7.16 Ab-dye conjugates appropriate for the study. Stored at 4^{o}C in the dark

7.17 Gloves (e.g. small, medium and large)

8.0 PROCEDURE

8.1 Cell preparation

8.1.1 Prepare cells for Ab staining following appropriate SOP

- For lysed WB staining follow ITI-00099, RBC lysis with VersaLase lysing buffer to prepare 2xLB
- For fresh PBMC, re-suspend in 3X10^{6} cells/ml in Staining Buffer

- For frozen PBMC follow ITI-00095, thawing frozen PBMC to prepare PBMC suspension

8.2 Ab cell surface staining

8.2.1 Label a 96-well V-bottom plate with sample ID and assay number

8.2.2 Transfer appropriation amount of Ab reagents to each well

8.2.2.1.1 If using ITI Ab reagent kit follow the steps below

8.2.2.1.2 Open cluster tube one column at a time and keep the cluster cap orientation

8.2.2.1.3 Using a multichannel pipette and 0.5-20 µL tips, transfer 20 µL of Ab cocktail to each well

8.2.2.1.4 Using a multichannel and 0.5-20 µL tips transfer 5 µL of each drop in Ab reagent to each well

8.2.3 If using Ab cocktails from other vendor follow the manufacturer's instruction to add Ab to each well

8.2.4 Using a multichannel pipette and 20-200 µL tips add 100 µL of cells at the appropriate concentration to appropriate wells and mix thoroughly by pipetting up and down 3 times.

8.2.5 Cover plate with aluminum foil and incubate the reaction mixture at RT for 15 minutes in the dark

8.3 Washing

Note PBS has to be protein free if follow by amine reactive dye staining

8.3.1 At the end of the incubation, using a multichannel pipette (L12-200) add 100 µL of PBS to all the wells and mix thoroughly

8.3.2 Centrifuge plate at 500 x g for 3 minutes

8.3.3 Dump or aspirate supernatant using vacuum

- For dump, swiftly flip the plate then blot on paper towel to remove access reagents. Care must be taken to prevent cross-well contamination
- For asperating supernatant using vacuum with an 8-channel manifold attached, being careful not to aspirate the cell pellet. This is avoided by placing each aspiration tip along the sidewall of the well and stopping at the junction where the V-bottom begins. This will leave a small amount of supernatant (~10 µL) but will ensure that the cell pellet is not disturbed

8.3.4 Add 200 uL of PBS to all wells

8.3.4.1 Using a multichannel pipette and 20-200 µL tips add 200 µL of PBS to each well

8.3.4.2 Mix well by pipetting up and down 3-5 times

8.3.5 Centrifuge plate at 500 x g for 3 minutes

8.3.6 Dump or aspirate supernatant using vacuum as in steps 8.3.3

8.4 Amine reactive dye staining (optional)

8.4.1 Prepare working concentration of amine reactive dye while doing washing in step 8.3. This has to be prepared fresh and good for 3 hours

8.4.2 Remove one vial of Yellow Live/Dead dye aliquot containing 5ul of Yellow Live/Dead dye (store at -20° C)

8.4.3 Make 1:1000 dilution with PBS to make working dye solution

- PBS must be carrier protein free, such as BSA

8.4.4 Add 200 uL of working dye solution to appropriate wells

8.4.5 Incubate at RT for 30 minutes in the dark

8.4.6 Wash plate one and half times following steps above in the section 8.3 using staining buffer

8.5 Fixing

8.5.1 Make 1:10 dilution of IO-Fix with PBS to make working fixative

8.5.2 Add 250 µl of working fixative to each well

8.5.3 Acquire samples within 2 hours of adding fixative

9.0 RECORD RETENTION

9.1 Compete Study specific Batch Sample Processing forms or individual sample process form and file in study specific data binder and study folder

10.0 APPENDICES

10.1 N/A

REVISION HISTORY					
Superseded Revisions		**Change Control Number**		**Effective Date**	
NA					
Current Revision:	**1.0**	**Change Control Number:**		**Current Effective Date:**	
Section Number	**Description of Changes**			**Justification of Changes**	
All	New ITI document			N/A	