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1.0 PURPOSE

- 1.1 This procedure describes process for preparing whole blood samples and washed blood samples with the VersaLyse method for cell surface staining and with 7AAD for viability measurement.

2.0 SCOPE

- 2.1 This WI applies to all ITI Flow Core operators in all locations

3.0 RESPONSIBILITIES


- 3.1 Cellular Analysis
- 3.1.1 Preparing this document
- 3.1.2 Accurately following this WI to stain blood samples for cell surface phenotyping on cytometers
- 3.2 QS
- 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-00056, Assay Plan for specific established ITN and TrialNet studies
- 4.2 ITI-00040, PrepPlus2-QC Use and Maintenance
- 4.3 ITI-00044, PrepPlus2 Panel Definition-Verification
- 4.4 ITI-00046, Whole Blood Washing Method
- 4.5 Beckman Coulter PN IM3648 VersaLyse™ lysing solution package insert
- 4.6 Sample Requisition Form (From ITN Sites)
- 4.7 ITI-00067, Individual Sample Process Sheet
- 4.8 TADA-047-Data Specifications for ITI_BEC v1 7JL.pdf (ITN controlled document)

5.0 DEFINITIONS

Term	Description
Ab	Antibody
HC	Healthy control
Legacy Document	Documents that have been approved and used in prior laboratories that have had an affiliation with ITI.
PBS	Phosphate buffered saline
PID	Patient identification number
PP2	COULTER® PrepPlus2 instrument
QS	Quality system
RBC	Red blood sells

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Term	Description
RT	Room temperature
WBC	White blood cells
WB	Whole blood


6.0 SAFETY

- 6.1 Follow all ITI safety policies and procedures. The processing of human whole blood is 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, RL20, RL100, RL200, RL1000 or equivalents)
- 7.2 Disposable pipette tips (Rainin disposable tips or equivalents)
- 7.3 PrepPlus2 instrument, if following automated setup procedure
- 7.4 Biosafety Hood
- 7.5 Centrifuge capable of 500 x g equipped with swinging bucket rotors, adapters for 12x75 tubes and biohazardous containment covers (Allegra-X15R Centrifuge or equivalent)
- 7.6 Vacuum with aspiration line and trap containing bleach in the waste receptacles
- 7.7 Eppendorf Repeater Plus (VWR, Cat#21516-002) or equivalent
- 7.8 Combitips 2.5 mL, 25 mL, and 50 mL (VWR, Cat# 22-26-740-0; 22-49-613-1; 22-26-770-1) or equivalent
- 7.9 Timer (minutes, hours)
- 7.10 12x75 cm [FC500] polypropylene flow cytometer tubes (VWR, Cat# 60818-430) or equivalent
- 7.11 Sterile 50 mL centrifuge tube (VWR, Cat#21008-103) or equivalent
- 7.12 Gloves (e.g. small, medium and large)
- 7.13 VersaLyse (Beckman Coulter, PN IM3648). Stored at RT
- 7.14 IOTest3-Fix (Beckman Coulter, PN IM3515; contains 8% formaldehyde). Stored at 4°C
- 7.15 PBS Ca⁺⁺ and Mg⁺⁺ free (Invitrogen Cat #14190-136) or equivalent. Stored at RT
- 7.16 7AAD (Beckman Coulter PN A07704). Stored at 4°C
- 7.17 Ab-dye conjugates appropriate for the study. Stored at 4°C, protected from light
- 7.18 Cotton tipped applicators (Pufitan Medical Products Company LLC, Maine, REF#: 806-WC)
- 7.19 Whole Blood (test samples, healthy control, etc.)....

8.0 PROCEDURE

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8.1 Lysis and fix buffer preparation

8.1.1 Make sure stock VersaLyse lysing solution and IOTest3Fix are not expired

8.1.2 Prepare lysis buffer by mixing VersaLyse with IOTest3Fix in 39:1 ratio

8.1.3 Prepare at least 1mL of lysis buffer for every staining tube.

8.1.4 For example prepare 40 mL lysis buffer as follows

8.1.4.1 Label a 50 mL conical tube as Lysis buffer

8.1.4.2 Label a 50 mL combitip as Versalyse

8.1.4.3 Using a repeater pipette and 50 mL combitip add 39 mL VersaLyse to the 50 mL tube

Note: Do not throw away the combitip which will be used later in lysis step

8.1.4.4 Using a RL-1000 pipette add 1 mL IOTest3Fix to the 50 mL tube

8.1.4.5 Mix well by inverting the 50 mL tube 3-5 times

8.1.4.6 Lysis buffer is only good at RT on the day it is made

8.1.5 Prepare 0.1% fix buffer (0.1% formaldehyde) by diluting IOTest3Fix in PBS at 1: 80 dilution.

8.1.6 Prepare at least 0.5 mL 0.1% fix buffer for every staining tube.

8.1.7 For example prepare 20 mL 0.1% fix buffer as following

8.1.7.1 Label a 50 mL conical tube as 0.1% fix

8.1.7.2 Label a 50 mL combitip as PBS

8.1.7.3 Using a repeater pipette and a 50 mL combitip add 19.75 mL PBS to the pre-labeled 50 mL tube

Note: Do not throw away the combitip that will be used later in wash step

8.1.7.4 Using a RL-1000 pipette add 250 µL IOTest3Fix to the 50 mL tube

8.1.7.5 Mix well by inverting the 50 mL tube 3-5 times


8.1.7.6 0.1% fix buffer is only good at RT on the day it is made

8.2 Manual Staining


8.2.1 If blood must be pre-washed, refer to ITI-00046 -Whole Blood Washing Method before proceeding.

8.2.2 All antibody reagents should be protected from light.

8.2.3 Obtain one 12x75 flow cytometry tube for each test per sample and label it with the patient/healthy donor ID, assay name, date and operator initials.

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- 8.2.4 Using a single channel pipette, pipette appropriate volume of antibody reagents into each labeled 12x75 tube. (For volume 5-20 μ L use RL-20, for volume >20 μ L to 100 μ L use RL-100 pipette)
- 8.2.5 Gently invert the WB vacutainer 2-3 times to mix blood before transfer
- 8.2.6 Using an RL-200 pipette, transfer 100 μ l of WB into the appropriate 12x75 tube and mix well by pipetting gently up and down. Be careful not to create blood splatters on the side of the tube. Change tip between tubes.
- 8.2.7 Remove any spattered blood droplets from the sides of the tube using Cotten Tipper Applicator (Puritan). Be careful not to drop cotton fiber in the sample.
- 8.2.8 Incubate for 15 minutes at RT protected from light
- 8.3 Automated staining with PrepPlus 2
 - 8.3.1 Follow ITI-00040 - PrepPlus2-QC Use and Maintenance for operating PP2
 - 8.3.2 Place appropriate reagent racks onto PP2 deck with lids removed
 - 8.3.3 Place a carousel of 12x75 flow cytometry tubes onto PP2 deck
 - 8.3.4 Place the WB sample tubes in rocker on PP2 beginning with position 1.
 - 8.3.5 Choose appropriate PP2 panel (ITI-00044 - PrepPlus2 Panel Definition-Verification) and press RUN.
 - 8.3.6 When complete, select MIX the carousel to mix the test tubes loaded on the carousel
 - 8.3.7 Remove carousel from PP2.
 - 8.3.8 Check each tube on the carousel to make sure the volume is the same and that no blood or Ab reagent is on the side of the tube
 - 8.3.9 If the volume is visibly lower in a tube re-stain manually
 - 8.3.10 If there is blood or Ab on the side of a tube, vortex briefly
 - 8.3.11 Incubate for 15 minutes at RT protected from light
- 8.4 Whole blood lysis and fix
 - 8.4.1 Using an repeater pipette and 50mL combitip from step 8.1.4.3, pipette 1 mL of Lysis Buffer into each 12x75 tube and vortex briefly immediately after adding lysis buffer one tube at a time.
 - 8.4.1.1 Tubes have to be vortexed immediately after adding lysis buffer otherwise lysis may be incomplete.
 - 8.4.1.2 Only vortex briefly for 1-2 seconds, over vortexing may cause incomplete RBC lysis
 - 8.4.2 Incubate the tubes for 15 minutes at RT protected from light
 - 8.4.3 After 15 min of incubation, observe the sample for signs of lysis – properly lysed blood will be translucent and not opaque.
 - 8.4.4 DO NOT proceed if lysis fails and the sample looks opaque. Consult with lab supervisor for next step.


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- 8.4.5 Following lysis incubation, centrifuge at 400 x g for 5 minutes at RT with swinging bucket rotors, adapters for 12x75 tubes and biohazardous containment covers
- 8.4.6 Carefully aspirate supernatant from each tube using vacuum, taking care not to disturb the cell pellet.
- 8.4.7 Using a repeater pipette and 50mL combitip from step 8.1.6.3, transfer 3 mL PBS into each tube. The pipetting pressure should mix the cells pellet well with the PBS.
- 8.4.8 Centrifuge again at 400 x g for 5 minutes at RT.
- 8.4.9 Carefully aspirate supernatant using vacuum as before.
- 8.4.10 Add 0.5 mL of 0.1% fix buffer to each tube, mix.
- 8.4.11 Samples are ready for acquiring data on the flow cytometer on the same day
- 8.5 Cell Viability Staining with 7AAD
 - 8.5.1 Gently invert the WB vacutainer 2-3 times to mix blood before transfer
 - 8.5.2 Using an RL-200 pipette, transfer 100µl of WB into the appropriate 12x75 tube
 - 8.5.3 Follow steps 8.4.1 to 8.4.9 to lyse RBC
 - 8.5.4 Re-suspend pellet in remaining volume (approximately 100 µL).
 - 8.5.5 Add 20 µL 7AAD and mix.
 - 8.5.6 Incubate 20 minutes at RT protected from the light.
 - 8.5.7 Add 0.5ml of 0.1% fix buffer to each tube, mix, and acquire sample on the flow cytometer the same day.
- 8.6 Complete ITI-00067-Individual Sample Process Sheet to document deviation and reagents used. Have lab supervisor review and sign the sheet within 24 hr of sample process.

9.0 RECORD RETENTION

- 9.1 The reviewed ITI-00067, Individual Sample Process Sheet will be filed in Trial Specific Data Binder.

REVISION HISTORY					
Superseded Revisions		Change Control Number		Effective Date	
1.0		10-025		13JUL2010	
Current Revision:	1.1	Change Control Number:	10-068	Current Effective Date:	14OCT2010
Section Number	Description of Changes			Justification of Changes	
4.1	Changed title of ITI-00056			Correct an error	

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7.14	Added more detailed information	Make it easier to follow
8.1.5	Added more detailed information	Make it easier to follow
8.1.6	Corrected from lysing buffer to 0.1% fix buffer	Correct an error
8.2.1	Changed ITI-00045-CA-SOP to ITI-00046-CA-WI	Correct an error
8.3	Added more details in staining procedure	Clarify some details to avoid confusion
8.3.1; 8.3.5	Changed format of ITI-##### reference (removed CA-SOP)	Update to current reference format (ITI-#####-Title)
8.4	Deleted 8.4.1.2 and added one tube at a time in 8.4.1	Simplify lysing procedure to one method only
8.5.3	Changed 8.4.10 to 8.4.9	Correct an error
8.6	Changed format of ITI-##### reference (removed CA-SOP)	Update to current reference format (ITI-#####-Title)
9.1	Changed wording and added document reference number (ITI-00067)	Clarify specific form used during processing.