STEM CELL LABORATORY (STCL)

DOCUMENT NUMBER: FLOW-GEN-007

DOCUMENT TITLE:

Lyse No Wash Staining for Flow Cytometry

DOCUMENT NOTES:

Document Information

Revision: 11	**Vault:** FLOW-General-rel
Status: Release	**Document Type:** FLOW SOPs

Date Information

Creation Date: 10 Apr 2019	**Release Date:** 06 May 2019
Effective Date: 06 May 2019	**Expiration Date:**

Control Information

Author: MGREESE	**Owner:** MGREESE
Previous Number: FLOW-GEN-007 Rev 10	**Change Number:** STCL-CCR-450

FLOW-GEN-007
LYSE/NO WASH STAINING FOR FLOW CYTOMETRY

1 PURPOSE

1.1 The purpose of this method is to detail how to prepare blood products for analysis by flow cytometry using the lyse/no wash method.

2 INTRODUCTION

2.1 Pre-titered, directly conjugated monoclonal antibodies, are used to stain cells from sources including peripheral blood (PB), fresh and thawed peripheral blood stem cell (PBSC), fresh and thawed umbilical cord blood (UCB), or fresh bone marrow. Red blood cells (rbc) are lysed using a specified lysing buffer with no wash step required. Samples may then be analyzed using a flow cytometer with no less than a 4-color capability.

3 SCOPE AND RESPONSIBILITIES

3.1 This procedure should be used when performing flow cytometry assays using the lyse/no wash staining technique. It is the responsibility of all Stem Cell Laboratory staff using this process to follow the guidelines outlined in this procedure.

4 DEFINITIONS/ACRONYMS

4.1	UCB	Umbilical Cord Blood
4.2	MAB	Monoclonal antibody
4.3	SCE	Stem Cell Enumeration
4.4	BM	Bone Marrow
4.5	PB	Peripheral Blood
4.6	PBSC	Peripheral Blood Stem Cells
4.7	STCL	Stem Cell Laboratory
4.8	BD	Becton Dickinson
4.9	PBS	Phosphate Buffered Saline
4.10	BSA	Bovine Serum Albumin
4.11	MSDS	Material Safety Data Sheet
4.12	NK	Natural Killer

5 MATERIALS

5.1 Monoclonal antibodies, BD Biosciences (Refer to Tables 1-8)

5.2 PBS/ 1% BSA, Gibco BRL

5.3 12 mm x 75mm, 5 ml polystyrene test tubes, Fisher brand or equivalent

5.4 Trucount™ tubes, BD Biosciences

5.5 FACS™ Lysing Solution, BD Biosciences

5.6 Pharm Lyse™ Buffer, BD Biosciences

6 EQUIPMENT

6.1 Vortex mixer

6.2 Adjustable pipettes and tips 10, 20, 100, 1000 microliter (µl), Rainin

6.3 Automated pipette and tips 2000 and 20,000 microliter, Rainin

7 SAFETY

7.1 Review MSDS for monoclonal antibodies used in testing.

7.1.1 Sodium azide warning

7.1.2 Nucleic acid dye warning

7.2 Review MSDS for BD Trucount tubes

7.2.1 Cobalt chloride warning

7.2.2 Silica warning

7.3 Review MSDS for BD FACS Lysing Solution

7.3.1 Diethylene glycol warning

7.3.2 Formaldehyde warning

7.4 Review MSDS for BD Pharm Lyse.

7.5 Wear appropriate personal protective equipment (PPE) when handling any/all potentially infectious blood or body fluid to include, but not limited to, lab coats, gloves, goggles, etc.

8 PROCEDURE

8.1 Fill out the sample and staining information on the flow worksheet and enter the sample information in the specimen log-in book.

8.2 Label tubes as follows:

8.2.1 Applicable products:

8.2.1.1 Label tube 1 of the panel with tube # and the appropriate identifier obtained from the flow worksheet or printed sample label.

8.2.1.2 Remaining tubes should be numbered and labeled with an appropriate sample identifier (patient unique initial or other) or printed label.

8.2.2 Fresh UCB Lymphocyte Subsets:

8.2.2.1 Obtain a test tube rack with at least 6 rows available. This may be the rack used simultaneously for CD34 testing.

8.2.2.2 For each UCB specimen (up to 3 at a time) place two test tubes on a row separated by an empty row.

8.2.2.3 Number the two tubes (3 and 4) and place the ISBT128 bar code label on each tube corresponding to the sample to be tested in those tubes.

8.2.2.4 Assure that the STCL assigned alphabetical letter associated with the UCB sequence is written on each bar code label used.

8.3 Add antibodies as specified in the panel lists included in this procedure for the product type and specific test request.

NOTE: When adding antibodies to BD Trucount tubes, pipette the reagent onto the side of the tube just above the stainless steel retainer.

8.4 Test sample cell concentration must be less than 40 million cells per milliliter (ml) or less than 10 million cells /ml for lymphocyte enumeration using Trucount tubes. Refer to procedure Flow-Gen-023 Specimen Dilution Protocol, for guidelines on diluting specimens. **The residual specimen dilution from the CD34 staining of fresh UCB test samples may be used as the diluted sample for lymphocyte marker staining.** See the tables below for the required sample volume for each test sample. (Vortex on medium speed (see mark on vortex mixer) 2-3 seconds.

NOTE: When using Trucount™ (BD) absolute counting tubes, it is important to use the reverse pipette technique to assure accuracy of measurement when adding test sample. It is necessary to change pipette tips between sample additions and to touch the end of the tip to the inner tube surface approximately 2/3 of the distance from the top of the testing tube while dispensing to the first stop. Reverse pipetting is not required for sample additions to non Trucount test tubes.

8.5 Incubate tubes at room temperatureerature in the dark (e.g. in a drawer) for the time specified in the panel lists below for the testing being performed.

8.6 See the panel list included in this procedure to determine what lysing agent is required for the specific test and the duration of the lyse incubation

8.7 A 1X working solution of the appropriate lysing agent is prepared by adding 1 part of 10x lysing agent and 9 parts of deionized water using a pipette or graduated cylinder depending on the final volume required to complete testing.

8.7.1 The 1X FACS Lysing Solution is usable for 1 month from the date of preparation and is stored at room temperatureerature.

8.7.2 The 1X solution of PHARM Lyse is usable for 2 weeks from the date of preparation and must be stored at the end of the workday at 2-8° C

8.8 Adhesive labels are available for each lysing agent solution preparation. Complete all required information.

10% FACS Lysing Solution or 10% BD Pharm Lyse Solution
LOT #: _____
Made: _____ By: _____
Expires: _____

8.9 Samples lysed with BD FACS lysing solution may be stored at 2-8° C for up to 24 hours after staining. Viable cell assays must be acquired within 1 hour of completion of the red blood cell lyse step g.

8.10 Complete the staining portion of the flow worksheet and take it to the flow cytometer operator or put it in the "to be acquired" tray near the cytometers.

Flow Cytometry Panel List:

NK Cell Selection requirements
Antibody staining panel: Table 1

	FL-1 FITC	FL-2 PE	FL-3	FL-4 APC
Tube 1	*IgG1	IgG2a (10µl)	7AAD(15µl) Cat# 559925	IgG1 (2.5µl) Cat#340754
Tube 2	*CD45	CD14 (10µl)	7AAD(15µl)	none
Tube 3	CD3 (10µl) Cat# 349201	CD56 (10µl) Cat#340724	7AAD(15µl)	CD19 (2.5µl) Cat#340722
Tube 4	CD25 (10µl) Cat#340694	CD3 (2.5µl) Cat#340662	7AAD(15µl)	CD4 (2.5µl) Cat#340672

*** BD Simultest Cat#61611/68026**

- Add antibody and 50µl test sample per tube. **No Trucount tubes used.**
- Vortex gently at speed marked on mixer for 2-3 seconds.
- Incubate ate room temperature in dark 15 minutes.
- Add 450µl of BD Pharm Lyse (1:10 working solution)
- Vortex gently at speed marked on mixer for 2-3 seconds.
- Incubate 15 minutes in dark at room temperature.
- Acquire within 1 hour of completion of staining.

CD34 Selection requirements
Antibody staining panel: Table 2

	FITC-FL-1	PE-FL-2	FL-3	APC- FL-4
1	* IgG1	IgG2 10µl	7-AAD 15µl Cat# 559925	IgG1 2.5µl Cat#340754
2	* CD45	CD14 10µl	7-AAD 15µl	None
3	CD3 10µl Cat# 349201	CD56 10µl Cat#340724	7-AAD 15µl	CD19 2.5µl Cat#340722
4	CD25 10µl	CD3 2.5µl Cat#340662	7-AAD 15µl	CD4 2.5µl Cat#340672
5	None	CD3 2.5µl	7-AAD 15µl	CD8 2.5µl Cat#340659
Add tube(s)	Use appropriate CD34 method for product type.			

* BD Simultest Cat#61611/68026

- Add antibody and 50µl of test sample to tubes 1-5.
- Vortex gently at speed marked on mixer for 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark
- Add 450µl of Pharm lyse (1:10 working solution).
- Vortex gently at speed marked on mixer 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark.
- Acquire within 1 hour of completion of staining process.

For Adult Allo donor PBSC, Allo donor Bone Marrow, or Umbilical Cord Blood Allogeneic donor infusion products
Antibody staining panel: Table 3

	FL-1 FITC	FL-2 PE	FL-3 PerCP	FL-4 APC
Tube 1	*BD SCE tube			None
Tube 2	**CD3	CD8	CD45	CD4 (10µl)

*BD SCE KIT Cat# 344563 Tube 1 See SOP FLOW-GEN-040 for staining requirements
**BD Multitest reagents cat#340499

- Add antibody and 50µl of test sample to tube.
- Vortex gently at speed marked on mixer for 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark
- Add 450µl of FACS Lysing solution (1:10 working solution).
- Vortex gently at speed marked on mixer 2-3 seconds.
- Incubate 15 minutes at room temperatureerature in the dark.
- Acquire within 24 hours of completion of staining process.

Fresh cord blood requirements

Table 4: Lymphocyte subset staining panel

	FL-1 FITC	FL-2 PE	FL-3 PerCP	FL-4 APC
Tube 1	**CD3	CD16+56	CD45	CD19 (10µl)
Tube 2	**CD3	CD8	CD45	CD4 (10µl)

**BD Multitest reagents cat#340500/340499
Lysing agent required: BD FACS Lysing Solution (1:10 working solution)

- Add antibody and 50µl of test sample to tubes 1-2.
- Vortex gently at speed marked on mixer for 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark.
- Add 450µl of BD FACS Lysing solution (1:10 working solution)Vortex gently at speed marked on mixer 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark.
- Store at 2-8 degrees C if not acquired right away.
- Acquire within 24 hours of completion of staining process.

Donor lymphocyte infusion (DLI) requirements

	FL-1 FITC	FL-2 PE	FL-3 PerCP	FL-4 APC
Tube 1	**CD3	CD8	CD45	CD4 (10µl)
***	**CD3	CD16+56	CD45	CD19 (10µl)

**BD Multitest reagents cat# 340499

***Add for QC only when needed to help define lymphocyte gate (i.e. CTL019 study)
Lysing agent required: BD FACS Lysing Solution (1:10 working solution)

- Add antibody and 50µl of test sample to tube. (Use BD Trucount tube if single platform method is required).
- Vortex gently at speed marked on mixer for 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark.
- Add 450µl of BD FACS Lysing solution (1:10 working solution)
- Vortex gently at speed marked on mixer 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark.

- Store at 2-8 degrees C if not acquired right away.
- Acquire within 24 hours of completion of staining process.

Peripheral blood immune reconstitution requirements
Antibody staining panel: Table 6

Tube #	*1 10µl	*2 10µl	3 10µl	4	5	6	7	*8
	Multitest Lymphocytes cat#340500	Multitest T-subsets cat#340499	Multitest RTE Cat#340977	T-reg	CTL	Ctrl	DC ctrl	DC subsets
FL-1	CD3	CD3	CD45RA	CD25-**9µl** Cat#340694	CD57-**7µl** Cat#340706	IgG1-**2µl** Cat#340755	Lin-**9µl** Cat#340546	Lin-**9µl**
FL-2	CD16+56	CD8	CD62L	CD62L-**5µl** Cat#341012	CD28-**2µl** 348047	IgG2a-**1µl** Cat#340766	IgG1-**1µl** Cat#349043	CD123-**4µl** Cat#340545
FL-3	CD45	CD45	CD3	CD3-**7µl** Cat#340663	CD8-**7µl** Cat#341049	CD3-**7µl** Cat#340663	HLA-DR-**7µl** Cat#347364	HLA-DR-**7µl** Cat#347364
FL-4	CD19	CD4	CD4	CD4-**1µl** Cat#340672	HLA-DR-**1µl** Cat#340691	IgG2a **5µl^** Cat# 340757	IgG2a-**5µl^** Cat# 340757	CD11c-**2µl** Cat#340544

* TrucountTube-BD ^ Make a 1:10 dilution using 1µl antibody per 9µl PBS or multiples thereof. Add 5µl of this dilution to the staining tube. Volumes are color coded.
Lysing agent required: BD FACS Lysing Solution (1:10 working solution)

- Add antibody and 50µl of test sample to tube.
- Vortex gently at speed marked on mixer for 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark.
- Add 450µl of BD FACS Lysing solution (1:10 working solution)
- Vortex gently at speed marked on mixer 2-3 seconds.
- Incubate 15 minutes at room temperature in the dark.
- Store at 2-8 degrees C if not acquired right away.
- Acquire within 24 hours of completion of staining process.

9 RELATED DOCUMENTS/FORMS

9.1 FLOW-GEN-012 (FRM 5) Stem Cell Laboratory Flow Cytometry Worksheet

9.2 FLOW-GEN-023 Specimen Dilution Protocol

9.3 FLOW-GEN-040 Using the BD Stem Cell Enumeration Kit with the BD FACSCalibur or FACSCanto II Flow Cytometer Systems to Assay Viable CD34+ Cells in Hematopoietic Transplant Products and Mobilized Peripheral Blood

9.4 FLOW-GEN-007 JA1 Immune Reconstitution Alternate Performance Assessment Process

9.5 FLOW-GEN-007 JA2 Car-T-Cell Protocols

10 REFERENCES

10.1 Becton Dickinson Multitest Reagent, product insert.

10.2 Paul Szabolcs; Kyung-Duk Park; Melissa Reese; Luciana Marti; Gloria Broadwater; Joanne Kurtzberg, Absolute values of dendritic cell subsets in bone marrow, cord blood, and peripheral blood enumerated by a novel method Stem Cells 2003;21(3):296-303.

11 REVISION HISTORY

Revision No.	Author	Description of Change(s)
11	M. Reese	Added Job Aids 1 and 2 to Related Docs section

Signature Manifest

Document Number: FLOW-GEN-007 **Revision:** 11

Title: Lyse No Wash Staining for Flow Cytometry

All dates and times are in Eastern Time.

FLOW-GEN-007 Lyse No Wash Staining for Flow Cytometry

Author

Name/Signature	Title	Date	Meaning/Reason
Melissa Reese (MGREESE)		12 Apr 2019, 02:13:23 PM	Approved

Management

Name/Signature	Title	Date	Meaning/Reason
Barbara Waters-Pick (WATE02)		16 Apr 2019, 01:48:40 PM	Approved

Medical Director

Name/Signature	Title	Date	Meaning/Reason
Joanne Kurtzberg (KURTZ001)		16 Apr 2019, 04:25:34 PM	Approved

Quality

Name/Signature	Title	Date	Meaning/Reason
Bing Shen (BS76) Lisa Eddinger (LE42) Taylor Orr (TSO4) Richard Bryant (RB232)		17 Apr 2019, 07:36:34 AM	Approved

Document Release

Name/Signature	Title	Date	Meaning/Reason
Sandy Mulligan (MULLI026)		22 Apr 2019, 07:36:15 PM	Approved