Sample Preparation (Whole Blood) Guidelines for Cytek® 25-Color Immunoprofiling Assay

Contents

Introduction

For anyone working with the **Cytek® 25-Color Immunoprofiling Assay** to prepare and acquire whole blood cells in Cytek Aurora cytometer, here are Cytek's recommended sample preparation procedures*. These are 3 additional items to make your workflow easier:

1. Import the **Cytek® 25-Color Immunoprofiling Assay Tags** to the fluorescent tag lists in your SpectroFlo® Library section. If you already have existing tags in your library, delete them or overwrite them with the tags in this list.
2. Import experiment template "**Cytek® 25-Color IP Assay Template Whole Blood"** into SpectroFlo®.
3. Refer to **Cytek® 25-Color Immunoprofiling Assay Acquisition Protocol** for a step-by-step guide for sample acquisition and analysis in SpectroFlo®.

** Please note that this kit is designed for research use only and is not for use in diagnostic or therapeutic procedures. Following method has only been tested in blood collected in EDTA tube.*

** For best results, resuspend cells in stain buffer after staining and analyze samples on Aurora within 2 hours post staining. Fixation with 1% paraformaldehyde following the procedure described in this protocol in page 4 can be performed if acquisition needs to be done at a later time, however, be aware of possible changes in the MFI for some antigens as well as quantitative differences compared to fresh samples in the enumeration of some populations.*

Materials

- Whole blood collected in ETDA tubes
- Cytek® 25-Color Immunoprofiling Assay, cFluor® Reagent Kit (18C), Cytek Biosciences, R7-40002
- Immunoprofiling Kit, 7 Color (Brilliant Violet™), BioLegend, 900004160
- Brilliant Stain Buffer Plus, BD Biosciences, 566385Lysing Buffer (RUO), BD Biosciences, 555899
- PBS, pH7.4, Corning 21-040-CM
- Stain Buffer (BSA), BD Biosciences, 554657
- Paraformaldehyde solution 4% in PBS, Santa Cruz Biotechnology, Cat. sc-281692
- UltraComp eBeads™ Plus Compensation Beads, Thermo Fisher Scientific, 01-3333-4112 x 75 mm tubes or 96 well V-bottom deep plates (Corning 3960 or equivalent) and 96 well U-bottom polypropylene plates (Corning 3365 or equivalent)

Sample Preparation

Bulk-lysing Whole Blood

1. Collect whole blood into ETDA tubes*
2. Prepare a fresh working reagent of 1X BD Pharm Lyse Buffer by diluting 1:10 with deionized water.
   NOTE: *Prepare fresh 1X lysis solution on the same day of the experiment.*
3. Transfer 45 mL of room temperature 1X lysis solution into a 50 mL conical tube
4. Transfer 5 mL of well mixed whole blood to the tube containing 45 mL of 1X lysis solution
5. Close and tighten the cap, mix gently by inverting or placing the tube on a tube rocker for 5 minutes
6. Centrifuge at 300 x *g*, for 8 minutes
7. Gently aspirate the supernatant without disturbing the pellet
8. Vortex gently
9. Add 45 mL of room temperature 1X lysis solution to the pellet, mix well
10. Repeat steps (5)-(8)
11. Add 45 mL Stain Buffer, mix well
12. Centrifuge at 300 x *g*, for 8 minutes
13. Gently aspirate the supernatant without disturbing the pellet
14. Vortex gently
15. Repeat steps (11)-(14)
16. Resuspend in **half the volume** of Stain Buffer as original Whole Blood volume
    (ex) If you started with 5 mL of whole blood, resuspend in Stain Buffer to complete the final volume to 2.5 mL

** Please note that only blood collected in EDTA tubes has been tested using this method.*

Protocol for Staining Bulk-lysed Whole Blood in Tubes

Plan on using ~100 µL whole blood* for each Single Stain Reference Control (25 fluorescence and 1 Unstained Control), and ~400 µL whole blood* for each Multicolor Sample*. Viability dye staining is usually not needed for fresh blood samples.

** The recommendations are in volume of original whole blood prior to bulk lysis.*

Single Color Reference Controls

1. Label a 12 x 75 mm tube for each Single Stain Reference Control
2. Add ~50 µL of lysed cells to each Single Stain Reference Control tube
   NOTE: *See Table 1 in page 4 for sample type recommendations for each marker.*
3. Add 5 µL of appropriate monoclonal antibody
4. Vortex thoroughly
5. Incubate for 20 minutes at room temperature, protected from light
6. Add 3 mL of Stain Buffer
7. Centrifuge at 400 x *g*, 5 minutes at room temperature
8. Decant and blot on paper towel
9. Vortex thoroughly
10. Resuspend in 300 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 4 to fix the cells in 1% paraformaldehyde
    NOTE: *If the samples need to be stored at 4ºC for more than 2 hours prior to collecting data, follow the steps in "Cell Fixation in Tubes" in page 4 to fix the samples in 1% paraformaldehyde*
11. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

Multicolor Sample

1. Label a 12 x 75 mm tube for each Multicolor sample
2. Prepare antibody cocktail in a 1.5 mL tube. For one Multicolor sample, first add 10 µL of Brilliant Stain Buffer Plus, then 5 µL of 23 mAbs one by one, **except for cFluor® BYG710 TCR γδ and** Brilliant Violet 421™ **CCR7**.
   NOTE: *cFluor® BYG710 TCR γδ and* Brilliant Violet 421™ *CCR7 need to be added separately.* ***DO NOT*** *add these antibodies in the cocktail mix.*
   NOTE: *Brilliant Stain Buffer must be added first to the cocktail.*
   NOTE: *Prepare one extra test for the multicolor cocktail to take in account for any reagent loss in the process (ex. make multicolor cocktail for 6 tests if you have 5 multicolor samples to stain). Take 125 µL of the cocktail per multicolor sample and discard any leftover. Make antibody cocktails fresh each time before use and* ***DO NOT*** *re-use pre-made cocktails.*
3. Add 200 µL of RBC lysed cells to Multicolor Sample tube
4. Add 5 µL of **cFluor® BYG710 TCR γδ** and 5 µL of **Brilliant Violet 421™ CCR7**
5. Vortex thoroughly
6. Incubate for 10 minutes at room temperature, protected from light
7. Add the antibody cocktail prepared in step (2)
8. Vortex thoroughly
9. Incubate for 20 minutes at room temperature, protected from light
10. Add 3 mL of Stain Buffer
11. Centrifuge at 400 x g, 5 minutes at room temperature
12. Decant supernatant and blot on paper towel
13. Vortex thoroughly
14. Resuspend in 300 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 4 to fix the cells in 1% paraformaldehyde
    NOTE: *If the samples need to be stored at 4ºC for more than 2 hours prior to collecting data, follow the steps in "Cell Fixation in Tubes"* on page 4 *to fix the samples in 1% paraformaldehyde*

15. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

Cell Fixation in Tubes

If the samples need to be stored at 4°C for more than 2 hours prior to collecting data, follow these steps to fix the samples in 1% paraformaldehyde and acquire within 24 hours post fixation.

1. Dilute 4% paraformaldehyde in PBS to make 1% paraformaldehyde solution
2. Add 300 µL of 1% paraformaldehyde to cell pellet.
3. Vortex thoroughly.
4. Incubate for 20 minutes at room temperature, protected from light
5. Add 3 mL of Stain Buffer
6. Centrifuge at 400 x g, 5 minutes at room temperature
7. Decant and blot on paper towel
8. Vortex thoroughly
9. Resuspend in 300 µL Stain Buffer for Single Stain Reference Controls and 400 µL for Multicolor Samples
10. Store at 4°C and acquire within 24 hours post fixation

Table 1. Sample Type Recommendations for Single Color Reference Controls

Laser	Target	Fluorochrome	Recommended Sample Type
Violet	CCR7	Brilliant Violet 421™	Cells or Beads
	CD45RA	cFluor® V450	Cells or Beads
	IgM	Brilliant Violet 510™	Cells or Beads
	CD20	cFluor® V547	Cells Only
	CD3	Brilliant Violet 570™	Cells Only
	CD28	Brilliant Violet 650™	Cells Only
	CD38	Brilliant Violet 711™	Cells Only
	CD56	Brilliant Violet 750™	Cells or Beads
	PD-1	Brilliant Violet 785™	Cells or Beads
Blue	CD141	cFluor® B515	Cells or Beads
	CD8	cFluor® B532	Cells or Beads
	CD14	cFluor® B548	Cells or Beads
	HLA-DR	cFluor® B690*	Cells Only
Yellow/Green	CD25	cFluor® BYG575*	Cells or Beads
	CD4	cFluor® YG584	Cells or Beads
	CD16	cFluor® BYG610	Cells or Beads
	IgD	cFluor® BYG667*	Cells Only
	TCRγδ	cFluor® BYG710	Cells or Beads
	CD11c	cFluor® BYG781*	Cells or Beads
Red	CD127	cFluor® R659*	Cells or Beads
	CD1c	cFluor® R668	Cells or Beads
	CD19	cFluor® R685	Cells Only
	CD123	cFluor® R720	Cells or Beads
	CD45	cFluor® R780*	Cells or Beads
	CD27	cFluor® R840	Cells or Beads

cFluor® V547, cFluor® B515, cFluor® B532, cFluor® R668 and cFluor® R720 are equivalent to CF®405L, CF®488A, CF®503, CF®647, and CF®700 respectively, manufactured and provided by Biotium, Inc. under an Agreement between Biotium and Cytek (LICENSEE). The manufacture, use, sale, offer for sale, or import of the product is covered by one or more of the patents or pending applications owned or licensed by Biotium. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. No right under any other patent claim, no right to perform any patented method, and no right to perform commercial services of any kind, including without limitation reporting the results of purchaser's activities for a fee or other commercial consideration, is conveyed expressly, by implication, or by estoppel.

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*Fluor conjugated antibody manufactured and supplied by BioLegend Inc.

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