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

Contents

Introduction

For anyone working with **Cytek® 25-Color Immunoprofiling Assay** to prepare and acquire peripheral blood mononuclear cells (PBMCs) on Cytek Aurora cytometer, we recommend the following sample preparation instructions. 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 **Cytek 25-Color IP Assay Template PBMC** 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.*

** 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.*

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Materials

- Frozen PBMCs
- ViaDye Red Fixable Viability Dye, Cytek Biosciences, R7-60008
- 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, 566385
- PBS, pH7.4, Corning 21-040-CM
- Stain Buffer (BSA), BD Biosciences, 554657
- Paraformaldehyde solution 4% in PBS, Santa Cruz Biotechnology, Cat. sc-281692
- 12 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

Thawing PBMCs

1. Pre-warm ~50 mL RPMI (10% FBS, 1% Penicillin) at 37°C for at least 30 minutes
2. Thaw PBMC vial quickly in 37°C water bath until the core is loose
3. Transfer the cells into a 50 mL conical tube
4. Add 1 mL of warm media to the empty cryovial. Set it aside.
5. Drop by drop, slowly add 10 mL of warm media to the cells in the 50 mL conical tube while gently swirling the tube to mix
6. Pour the content of the cryovial from step (4) into the 50 mL conical tube
7. Add additional media to complete the final volume to 20 mL
8. Centrifuge at 300 x *g*, 8 minutes
9. Decant the supernatant and blot on paper towel
10. Gently resuspend the pellet in 2 ml of warm media by pipetting up and down using a serological pipet
11. Repeat steps (7)-(10)
12. Resuspend in proper volume of warm media and count cells
13. Loosen the cap on the 50 mL conical tube, place the cells in the cell culture incubator until ready to use

Preparing ViaDye Red Fixable Viability Dye

1. Completely thaw DMSO
2. Add 100 µL DMSO to the lyophilized ViaDye Red Fixable Viability Dye stock (=1 mM stock solution)
3. Vortex to mix thoroughly
4. Aliquot and freeze at -20°C until use
5. Thaw an aliquot of the stock solution at room temperature, protected from light, before each use.
   ***NOTE**: Do not re-freeze or re-use the viability dye*
6. Dilute the stock solution at 1:500 in PBS (=2 µM working solution)
7. Use the working solution at 5 µL per test

Protocol for Staining PBMCs in Tubes

Plan on using ~400,000 cells for each Single Stain Reference Control (25 fluorescence, 1 Viability and 1 Unstained Control), and ~3 million cells for each Multicolor Sample.

Viability Reference Control

1. Label a 12 x 75 mm tube for Viability Reference Control
2. Add ~400,000 cells to the tube
3. Add PBS to complete the final volume to 3 mL
4. Centrifuge at 400 x *g*, 5 minutes at room temperature
5. Decant supernatant and blot on paper towel
6. Vortex thoroughly
7. Repeat steps (3)-(6) if the volume in step (2) is bigger than 1 mL
8. Add 5 µL of working solution ViaDye Red Fixable Viability Dye to the cell pellet
9. Vortex thoroughly
10. Incubate for 15 minutes at room temperature, protected from light
11. Add 3 mL of Stain Buffer
12. Centrifuge at 400 x *g* , 5 minutes at room temperature
13. Decant supernatant and blot on paper towel
14. Vortex thoroughly
15. Resuspend in 300 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 5 to fix the cells in 1% paraformaldehyde
    NOTE: *If the samples need to be stored at 4ºC for more than 2 hour prior to collecting data, follow the steps in* "Cell Fixation in Tubes" in page 5 *to fix the samples in 1% paraformaldehyde*
16. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

Single Stain Reference Controls

1. Label a 12 x 75 mm tubes for each Single Stain Reference Control
2. Add ~400,000 cells to each tube
   NOTE: *See Table 1 in page 4 for sample type recommendations for each marker.*
3. Add Stain Buffer to complete the final volume to 3 mL
4. Centrifuge at 400 x *g*, 5 minutes at room temperature
5. Decant supernatant and blot on paper towel
6. Vortex thoroughly
7. Repeat steps (3)-(6) if the volume in step (2) is bigger than 1 mL
8. Add 5 µL of appropriate monoclonal antibody to the cell pellet
9. Vortex thoroughly
10. Incubate for 20 minutes at room temperature, protected from light
11. Add 3 mL of Stain Buffer
12. Centrifuge at 400 x *g*, 5 minutes at room temperature
13. Decant and blot on paper towel
14. Vortex thoroughly
15. Resuspend in 300 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 5 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 5 to fix the samples in 1% paraformaldehyde*
16. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

Table 1. Sample Type Recommendations

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

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 ~3 million cells to Multicolor Sample tube
4. Add PBS to complete the final volume to 3 mL
5. Centrifuge at 400 x *g*, 5 minutes at room temperature
6. Decant supernatant and blot on paper towel
7. Vortex thoroughly

8. Repeat steps (4)-(7) if the volume in step (3) is bigger than 1 mL
9. Add 5 µL of working solution ViaDye Red Fixable Viability Dye to the cell pellet
10. Vortex thoroughly
11. Incubate for 15 minutes at room temperature, protected from light
12. Add 3 mL of Stain Buffer
13. Centrifuge at 400 x *g*, 5 minutes at room temperature
14. Decant and blot on paper towel
15. Vortex thoroughly
16. Add 5 µL of **cFluor® BYG710 TCR γδ** and 5 µL of **Brilliant Violet 421™ CCR7**
17. Incubate for 10 minutes at room temperature, protected from light
18. Add the antibody cocktail prepared in step (2)
19. Vortex thoroughly
20. Incubate for 20 minutes at room temperature, protected from light
21. Add 3 mL of Stain Buffer
22. Centrifuge at 400 x *g*, 5 minutes at room temperature
23. Decant supernatant and blot on paper towel
24. Vortex thoroughly
25. Resuspend in 400 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 5 to fix the cells in 1% paraformaldehyde
    NOTE: *If the samples need to be stored at 4°C for more than 2 hours prior to collecting data, follow the steps in "Cell Fixation in Tubes" in page 5 to fix the samples in 1% paraformaldehyde*
26. 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 of fixation.

1. Dilute 4% paraformaldehyde in PBS to make 1% paraformaldehyde solution
2. Add 300 µL of 1% paraformaldehyde to the 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

Protocol for Staining PBMCs in 96 well Plates

Plan on using ~400,000 cells for each Single Stain Reference Control (25 fluorescence, 1 Viability and 1 Unstained Control), and ~3 million cells for each Multicolor Sample. Prepare separate plates for Single Stain Reference Controls and Multicolor Samples.

Use a 96 deep well V-bottom plate (polystyrene or polypropylene) to prepare the cells and transfer the final sample to a 96 well U-bottom plate (polypropylene) for acquisition.

Viability Reference Control

1. Using a 96 deep well V-bottom plate, add ~400,000 cells to Viability Reference Control well
2. Add PBS to complete the final volume to 2 mL

3. Centrifuge at 400 x *g*, 5 minutes at room temperature
4. Decant supernatant and blot on paper towel
5. Vortex thoroughly
6. Repeat steps (2)-(5) if the volume in step (1) is bigger than 1 mL
7. Add 5 µL of working solution ViaDye Red Fixable Viability Dye to the cell pellet
8. Mix well by pipetting up and down
9. Incubate for 15 minutes at room temperature, protected from light
10. Add Stain buffer to complete the final volume to 2 mL
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 200 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 7 to fix the cells in 1% paraformaldehyde
    NOTE: *If the samples need to be stored at 4°C for more than 1 hour prior to collecting data, follow the steps in "Cell Fixation in Tubes" in page 7 to fix the samples in 1% paraformaldehyde*
15. Transfer the sample to 96 well U-bottom polypropylene plate
16. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

Single Stain Reference Control

1. Using a 96 deep well V-bottom plate, Add ~400,000 cells to each Single Stain Reference Control well
    NOTE: *See Table 1 in page 4 for sample type recommendations for each marker.*
2. Add Stain Buffer to complete the final volume to 2 mL
3. Centrifuge at 400 x *g*, 5 minutes at room temperature
4. Decant and blot on paper towel
5. Vortex thoroughly
6. Repeat steps (2)-(5) if the volume in step (1) is bigger than 1 mL
7. Add 5 µL of Mab to the cell pellet in each well
8. Mix well by pipetting up and down
9. Incubate for 20 minutes at room temperature, protected from light
10. Add Stain Buffer to complete the final volume to 2 mL per well
11. Centrifuge at 400 x *g*, 5 minutes at room temperature
12. Decant and blot on paper towel
13. Vortex thoroughly
14. Resuspend in 200 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 7 to fix the cells in 1% paraformaldehyde
    NOTE: *If the samples need to be stored at 4°C for more than 2 hours prior to collecting data, follow the steps in "Cell Fixation in Tubes" in page 7 to fix the samples in 1% paraformaldehyde*
15. Transfer the sample to 96 well U-bottom polypropylene plate
16. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

Multicolor Sample

1. 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.*
2. Using a 96 deep well V-bottom plate, add ~3 million cells to Multicolor Sample well

3. Add PBS to complete the final volume to 2 mL
4. Centrifuge at 400 x *g*, 5 minutes at room temperature
5. Decant and blot on paper towel
6. Vortex thoroughly
7. Repeat steps (3)-(6) if the volume in step (2) is bigger than 1 mL
8. Vortex thoroughly
9. Add 5 µL of working solution ViaDye Red Fixable Viability Dye to the cell pellet
10. Mix well by pipetting up and down
11. Incubate for 15 minutes at room temperature, protected from light
12. Add Stain Buffer to complete the final volume to 2 mL
13. Centrifuge at 400 x *g*, 5 minutes at room temperature
14. Decant and blot on paper towel
15. Vortex thoroughly
16. Add 5 µL of **cFluor® BYG710 TCR γδ** and 5 µL of **Brilliant Violet 421™ CCR7**
17. Mix well by pipetting up and down
18. Incubate for 10 minutes at room temperature, protected from light
19. Add the antibody cocktail prepared in step (1)
20. Mix well by pipetting up and down
21. Incubate for 20 minutes at room temperature, protected from light
22. Add Stain Buffer to complete the final volume to 2 mL
23. Centrifuge at 400 x *g*, 5 minutes at room temperature
24. Decant and blot on paper towel
25. Vortex thoroughly
26. Resuspend in 200 µL Stain Buffer or go to step (1) in "Cell Fixation in Tubes" in page 7 to fix the cells in 1% paraformaldehyde
    NOTE: *If the samples need to be stored at 4°C for more than 2 hours prior to collecting data, follow the steps in "Cell Fixation in Tubes" in page 7 to fix the samples in 1% paraformaldehyde*
27. Transfer the sample to 96 well U-bottom polypropylene plate
28. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

Cell Fixation in Plates

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 in each well.
3. Mix well by pipetting up and down
4. Incubate for 20 minutes at room temperature, protected from light
5. Add Stain Buffer to complete the final volume to 2 mL
6. Centrifuge at 400 x *g*, 5 minutes at room temperature
7. Decant and blot on paper towel
8. Vortex thoroughly
9. Resuspend in 200 µL Stain Buffer
10. Transfer the sample to 96 well U-bottom polypropylene plate
11. Store at 4°C and acquire within 24 hours post fixation

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