Sample Preparation Guidelines for Cytek® cFluor® TBMNK Kit, 8 Color

Cytek cFluor® TBMNK Kit contains 8 single-color antibody conjugates that are optimized, in terms of fluorochrome/specificity pairing and titer, for a flow cytometer with violet, blue and red lasers. The kit allows for the identification of lymphocytes; total T cells, helper T cells, cytotoxic T cells, NK-Like T cells, B cells, NK cells, and total monocytes; nonclassical and classical monocytes in human peripheral blood mononuclear cells and in whole blood.

Materials needed

- Cytek® cFluor® TBMNK 8 Color, Cytek Biosciences, Cat. R7-40001
- ViaDye™ Red Fixable Viability Dye, Cytek Biosciences, Cat. R7-60008
- Human peripheral blood mononuclear cells (PBMCs) or whole blood
- Falcon™ 5 ml polystyrene Round-Bottom Tube, 12 x 75 mm, Cat. 352008, or equivalent
- Corning™ 96 well polypropylene Round Bottom microplates, Cat. 3365, or equivalent
- PBS, 1X Corning™, Cat. 21-040-CM, or equivalent
- BD Pharmingen™ Stain Buffer (BSA), Cat. 554657, or equivalent
- BD FACS™ Lysing Solution, Cat. 349202, or equivalent
- 4% paraformaldehyde in PBS, Santa Cruz Biotechnology, Cat: sc-281692, or equivalent

Thawing PBMCs

For this kit, plan on using 3 x 10^6 to 6 x 10^6 cells for Reference Controls and for the Multicolor Sample.

1. Pre-warm ~50 mL RPMI (supplemented with 10% FBS, 1% Penicillin/Streptomycin) 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 contents 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 200 x *g*, 8 minutes
9. Decant the supernatant and blot on paper towel or remove the supernatant by aspiration
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 for a final cell concentration of 2.5 x 10^6 cells/ml and verify by counting
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. Allow the DMSO to thaw completely
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 2 x 10^5 cells for each Reference Control sample (8 fluorescence Reference Controls, one viability Reference Control and 1 Unstained Control) and 1 x 10^6 cells for each Multicolor sample.

Viability Reference Control

1. Label a 12 mm x 75 mm tube for the Viability Reference Control
2. Add 2 x 10^5 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 or remove the supernatant by aspiration
6. Vortex thoroughly
7. Repeat steps (3)-(6) if the volume in step (2) is greater 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 or remove the supernatant by aspiration
14. Vortex thoroughly
15. Resuspend in 150 µL Stain Buffer
16. Acquire at medium flow rate within 2 hours post staining if cells are not fixed
    ***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" to fix the samples in 1% paraformaldehyde*

Single Stain Reference Controls

1. Label a 12 x 75 mm tube for each Single Stain Reference Control
2. Add 2 x 10^5 cells to each tube
   ***NOTE:** See Table 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 or remove the supernatant by aspiration
6. Vortex thoroughly
7. Repeat steps (3)-(6) if the volume in step (2) is greater 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 supernatant and blot on paper towel or remove the supernatant by aspiration
14. Vortex thoroughly
15. Resuspend in 150 µL Stain Buffer
16. Acquire at medium flow rate within 2 hours post staining if cells are not fixed
    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" to fix the samples in 1% paraformaldehyde.*

Table 1. Sample Type Recommendations for optimal Reference Controls

Laser	Target	Fluorochrome	Recommended Sample Type
Violet	CD3	cFluor® V420	Cells or Beads
	CD14	cFluor® V450	Cells or Beads
	CD45	cFluor® V547	Cells Only
Blue	CD8	cFluor® B515	Cells or Beads
	CD19	cFluor® BYG710	Cells or Beads
Red	CD16	cFluor® R668	Cells Only
	CD56	cFluor® R720	Cells or Beads
	CD4	cFluor® R780	Cells or Beads

Multicolor Sample

1. Label a 12 x 75 mm tube for each Multicolor sample
2. Prepare the antibody cocktail in a 1.5 mL tube. For one Multicolor sample, add 5 µL of all antibodies included in the kit.
   NOTE: *Prepare one extra test for the multicolor cocktail to account for any reagent loss in the process (ex. make multicolor cocktail for 6 tests if you have 5 multicolor samples to stain). Take 40 µL of the cocktail per multicolor sample and discard any leftover if not used within 10 days of cocktailing.*
3. Add 1 x10^6 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 or remove the supernatant by aspiration
7. Vortex thoroughly
8. Repeat steps (4)-(7) if the volume in step (3) is greater than 1mL
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 supernatant and blot on paper towel or remove the supernatant by aspiration
15. Vortex thoroughly
16. Add 40 µL/test of the antibody cocktail prepared in step (2).

17. Vortex thoroughly
18. Incubate for 20 minutes at room temperature, protected from light
19. Add 3 mL of Stain Buffer
20. Centrifuge at 400 x *g*, 5 minutes at room temperature
21. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
22. Vortex thoroughly
    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" to fix the samples in 1% paraformaldehyde*
23. Resuspend in 300 µL Stain Buffer
24. 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. Pellet the cells by centrifugation at 400 x g, 5 minutes at room temperature, if the cells are in suspension
3. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
4. Add 300 µL of 1% paraformaldehyde to the cell pellet
5. Vortex thoroughly
6. Incubate for 20 minutes at room temperature, protected from light
7. Add 3 mL of Stain Buffer
8. Centrifuge at 400 x *g*, 5 minutes at room temperature
9. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
10. Vortex thoroughly
11. Resuspend in 150 µL Stain Buffer for Single Stain Reference Controls and 300 µL for Multicolor Samples
12. Store at 4°C and acquire within 24 hours post fixation

Protocol for Staining PBMCs in 96 well Plates

Plan on using 2 x 10^5 cells for each Reference Control sample (8 fluorescence Reference Controls, one viability Reference Control and 1 Unstained Control) and 1 x 10^6 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, if required.

Viability Reference Control

1. Using a 96 deep well V-bottom plate, add 2 x 10^5 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 or remove the supernatant by aspiration
5. Resuspend well by pipetting up and down
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 or remove the supernatant by aspiration
13. Resuspend in 200 µL Stain Buffer by pipetting up and down
14. Transfer the sample to 96 well U-bottom polypropylene plate, if required
15. Acquire at medium flow rate within 2 hours post staining if cells are not fixed
    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 Plates" to fix the samples in 1% paraformaldehyde*

Single Stain Reference Control

1. Using a 96 deep well V-bottom plate, Add 2 x 10^5 cells to each Single Stain Reference Control well
   NOTE: *See Table 1 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 supernatant and blot on paper towel or remove the supernatant by aspiration
5. Resuspend well by pipetting up and down
6. Repeat steps (2)-(5) if the volume in step (1) is greater 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 supernatant and blot on paper towel or remove the supernatant by aspiration
13. Resuspend in 200 µL Stain Buffer by pipetting up and down
14. Transfer the sample to 96 well U-bottom polypropylene plate, if required
15. Acquire at medium flow rate within 2 hours post staining if cells are not fixed
    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 Plates" to fix the samples in 1% paraformaldehyde*

Multicolor Sample

1. Prepare antibody cocktail in a 1.5 mL tube. For one Multicolor sample, add 5 µL of all the antibodies included in the kit.
   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 40 µL of the cocktail per multicolor sample and discard any leftover if not used within 10 days of cocktailing.*
2. Using a 96 deep well V-bottom plate, add 1 x 10^6 cells to Multicolor Sample wells
3. Add PBS to complete the final volume to 2 mL
4. Centrifuge at 400 x *g*, 5 minutes at room temperature
5. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
6. Resuspend well by pipetting up and down
7. Repeat steps (3)-(6) if the volume in step (2) is greater than 1 mL
8. Resuspend well by pipetting up and down

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 supernatant and blot on paper towel or remove the supernatant by aspiration
15. Resuspend well by pipetting up and down
16. Add 40 µl /test of the antibody cocktail prepared in step (1)
17. Mix well by pipetting up and down
18. Incubate for 20 minutes at room temperature, protected from light
19. Add Stain Buffer to complete the final volume to 2 mL
20. Centrifuge at 400 x *g*, 5 minutes at room temperature
21. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
22. Resuspend in 200 µL Stain Buffer by pipetting up and down
23. Transfer the sample to 96 well U-bottom polypropylene plate, if required
24. Acquire at medium flow rate within 2 hours post staining if cells are not fixed

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 Plates" to fix the samples in 1% paraformaldehyde*

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. Pellet the cells by centrifugation at 400 x g, 5 minutes at room temperature, if the cells are in suspension
3. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
4. Add 300 µL of 1% paraformaldehyde to cell pellet in each well.
5. Mix well by pipetting up and down
6. Incubate for 20 minutes at room temperature, protected from light
7. Add Stain Buffer to complete the final volume to 2 mL
8. Centrifuge at 400 x *g*, 5 minutes at room temperature
9. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
10. Resuspend in 200 µL Stain Buffer
11. Transfer the sample to 96 well U-bottom polypropylene plate, if required
12. Store at 4°C and acquire within 24 hours post fixation

Protocol for Staining in Whole Blood

Plan to use 100 µl of whole blood for each Reference Control and for each Multicolor Sample.

Single Stain Reference Controls

1. Label a 12 x 75 mm tube for each Single Stain Reference Control
2. Add 100 µl of whole blood to each tube

NOTE: *See Table for sample type recommendations for each marker.*

3. Add 5 µL of appropriate monoclonal antibody to the cell pellet
4. Vortex thoroughly
5. Incubate for 20 minutes at room temperature, protected from light
6. Add 2 mL of 1X FACS Lysing Solution, vortex well for 10 seconds
7. Incubate the samples in the dark for 10 minutes, at room temperature
8. Centrifuge at 400 x *g*, 5 minutes at room temperature
9. Decant and blot on paper towel or remove the supernatant by aspiration
10. Wash with 3 ml of Stain Buffer
11. Centrifuge at 400 x *g*, 5 minutes at room temperature
12. Decant and blot on paper towel or remove the supernatant by aspiration
13. Resuspend in 300 µL Stain Buffer
14. Acquire at medium flow rate within 2 hours post staining

NOTE: *The samples can be stored at 4°C for up to 24 hours post-staining.*

Table 1. Sample Type Recommendations for Optimal Reference Controls

Laser	Target	Fluorochrome	Recommended Sample Type
Violet	CD3	cFluor® V420	Cells or Beads
	CD14	cFluor® V450	Cells or Beads
	CD45	cFluor® V547	Cells Only
Blue	CD8	cFluor® B515	Cells or Beads
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Red	CD16	cFluor® R668	Cells Only
	CD56	cFluor® R720	Cells or Beads
	CD4	cFluor® R780	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, 5 µL of all the antibodies included in the kit.

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 40 µL of the cocktail per multicolor sample and discard any leftover if not used within 10 days of cocktailing.*

3. Add 100 µl of whole blood to the Multicolor Sample tube
4. Add 40 µL/test of the antibody cocktail prepared in step (2)
5. Vortex thoroughly
6. Incubate for 20 minutes at room temperature, protected from light

7. Add 2 mL of 1X FACS Lysing Solution, vortex well for 10 seconds
8. Incubate the samples in the dark for 10 minutes, at room temperature
9. Centrifuge at 400 x *g*, 5 minutes at room temperature
10. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
11. Wash with 3 mL of Stain Buffer
12. Centrifuge at 400 x *g*, 5 minutes at room temperature
13. Decant supernatant and blot on paper towel or remove the supernatant by aspiration
14. Resuspend in 300 µL Stain Buffer
15. Acquire at medium flow rate within 2 hours post staining

NOTE: *The samples can be stored at 4°C for up to 24 hours post-staining.*

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" to fix the samples in 1% paraformaldehyde*

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