MultiPro™ Individual Antibody and Cocktail Staining Protocol

Required Reagents and Consumables

Description	Manufacturer	Part Number
PBS, Ultrapure (1X)	Avantor	K812-500ML
PBS, Ultrapure (10X)	Invitrogen	AM9625
Bovine serum albumin, nuclease-free	Millipore Sigma	126609100GM
Enhanced Blocker	Proteintech Genomics	G900005
Protector RNase inhibitor	Roche	3335399001
10% Tween-20	Thermo Scientific	28320
10% NP-40	Thermo Scientific	85124
32% Paraformaldehyde	EMS	15714
Human TruStain™ FcX	BioLegend	422302
5mL FACS tubes with caps, nuclease-free	Stemcell Technologies	38057
2mL tubes	Eppendorf	022363352
1.5mL Protein LoBind tubes	Eppendorf	022431081

Required Equipment

- Tabletop centrifuge capable of reaching 14,000 x *g*, with a rotor suitable for 5 mL FACS tubes.
- Swinging bucket centrifuge with adaptor capable of holding 5mL FACS tubes.
- Tabletop vortex mixer.

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Before You Start

- **Planning your experiment** - Depending on the number of samples being stained, this protocol is expected to take ~3 hours *after* cell preparation. The staining protocol *does not* contain any safe stopping points and users must *immediately* proceed to the "Sample Fixation" step of the "10x Genomics Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling protocol (CG000478 Rev D)" and subsequent "Probe Hybridization" of the "Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture protocol (CG000673 Rev A)", which requires ~2 hours of hands-on time.

- **Note: if you are staining with individual antibodies and *not* using a lyophilized cocktail, skip to the "Sample Preparation" section of this protocol. Make sure to prepare your antibody staining dilution as described in step 13 under the "Intracellular Block & Stain" subsection.**

- **Sample preparation** - A high-quality sample is critical for assay performance. The ideal cell suspension has high viability (> 80%) and minimal cell clumps and debris. We strongly recommend straining cells through a 30µm filter such as Pre-Separation Filters (Miltenyi PN: 130-041-407) to remove cell aggregates. During resuspension and mixing steps, pipette gently and avoid introducing bubbles.

- **RNase Awareness** - To prevent RNA degradation, use nuclease-free reagents and consumables listed in this protocol. Before starting, decontaminate your work surfaces and pipettes using RNase Zap (Invitrogen PN: AM9780).

- **Sequencing Parameters** - MultiPro™ Antibody sequencing libraries require distinct sequencing parameters from 10x Genomics Single Cell Gene Expression Flex sequencing libraries. To sequence Gene Expression and MultiPro™ sequencing libraries in the same run, we recommend using the parameters listed below.

  Critical: Required MultiPro™ antibody library sequencing parameters:

  Read 1: 48 cycles
  Index 1: 10 cycles
  Index 2: 10 cycles
  Read 2: 50 cycles

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Buffer Preparation

Note: this protocol was developed using human Peripheral Blood Mononuclear Cells (PBMCs). Certain samples may require optimization of detergent and fixative concentrations in the buffers listed below.

Prepare a 30% BSA Stock Solution - Dissolve 30 g of nuclease-free BSA powder in 100 mL of ultrapure water. Filter-sterilize using a 0.2 µm filter and store at 4 °C for up to one week.

BSA Buffer

Component	[Final]	1 sample (µL)	1 sample +10%	4 samples +10%
RNase inhibitor (200X)	1X	5.0	5.5	22.0
BSA, 30%	1%	33.3	36.6	146.5
10X PBS	1x	100	110	440
Ultrapure water		861.7	947.9	3791.5
Total		**1000 µL**	**1100 µL**	**4400 µL**

Fixation Buffer

Component	[Final]	1 sample (µl)	1 sample + 10%	4 samples +10%
PFA, 32%	4%	25	27.5	110
10X PBS	1X	20	22	88
Ultrapure water	-	155	170.5	682
Total		**200 µL**	**220 µL**	**880 µL**

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Buffer Preparation (continued)

IC Staining Buffer

Critical: If you are staining using individual antibodies and not a lyophilized cocktail, use the "single sample + 10%" option when preparing your IC Stain Buffer. Individual antibodies will be diluted together in 55 µL of IC Staining buffer.

Component	[Final]	1 sample (µL)	1 sample + 10%	4 samples + 10%
RNase inhibitor (200X)	1x	0.3	0.33	1.3
Tween-20 (10%)	0.2%	1.0	1.1	4.4
NP-40 (10%)	1.0%	5.0	5.5	22.0
BSA (30%)	10%	16.7	18.4	73.5
10X PBS	1x	5.0	5.5	22.0
Ultrapure water	-	22.0	24.2	96.8
Total		**50 µL**	**55 µL**	**220 µL**

IC Blocking Buffer

Component	[Final]	1 sample (µL)	1 sample + 10%	4 samples + 10%
Enhanced Blocker	-	15.5	17.1	68.2
RNase inhibitor (200X)	1x	0.3	0.33	1.3
Tween-20 (10%)	0.2%	1.0	1.1	4.4
NP-40 (10%)	1.0%	5.0	5.5	22.0
BSA (30%)	10%	16.7	18.4	73.5
10X PBS	1x	5.0	5.5	22.0
Ultrapure water	-	6.5	7.2	28.6
Total		**50 µL**	**55 µL**	**220 µL**

Buffer Preparation (continued)

IC Wash Buffer

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Component	[Final]	1 sample (mL)	1 sample + 10%	4 samples+ 10%
RNase Inhibitor (200X)	1X	0.045	0.050	0.198
Tween-20 (10%)	0.2%	0.180	0.198	0.792
NP-40 (10%)	0.1%	0.090	0.099	0.396
BSA (30%)	1%	0.300	0.330	1.320
10X PBS	1X	0.900	0.990	3.960
Ultrapure Water		7.485	8.234	32.934
Total		**9.0 mL**	**9.9 mL**	**39.6 mL**

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Reconstitution of Lyophilized Antibody Cocktail

1. Remove desired number of cocktail tubes from mylar pouch. If additional tubes remain in the provided pouch, place the extra tube(s) and desiccant pack into a 15 mL conical tube store at 4 °C.
2. Without removing the cocktail tube cap, equilibrate antibody cocktail tube to room temperature (RT) for 5 minutes.
3. Insert the cocktail tube into a 2 mL Eppendorf tube and spin at 10,000 x *g* for 30 seconds at RT.

   Critical: **Step 3 ensures that all lyophilized material is at the bottom of the tube prior to reconstitution. Steps 4 to 7 ensure the reconstituted cocktail is at the correct concentration.**
4. Carefully reconstitute the lyophilized cocktail by pipetting 55 µL of IC Staining Buffer down the side of the tube.
5. Re-cap the cocktail tube and vortex for 15 seconds and spin at 10,000 x *g* for 30 seconds at RT.
6. Incubate for 5 minutes at RT.
7. Vortex the tube for 15 seconds and spin at 10,000 x *g* for 30 seconds at RT.
8. Transfer the entire volume of the reconstituted cocktail (~60 µL) into a 1.5 mL Protein LoBind Eppendorf tube and spin at 14,000 x *g* for 10 minutes at 4 °C.

   Critical: **Step 8 removes any protein aggregates that may be present in the reconstituted cocktail.**
9. Being careful not to touch the bottom of the tube with your pipette tip, carefully transfer 50µL of the cocktail to a new 1.5 mL Protein LoBind tube.

   Critical: **Touching the bottom of the tube with a pipette tip or transferring the entire volume could result in the transfer of aggregates to the final staining solution.**
10. Keep reconstituted cocktail on ice until ready to use.

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Sample Preparation

1. Prepare 1 x 10^6 cells in 1 mL of BSA Buffer in a 5 ml FACS tube.

   Critical: **Staining more than 1 x 10^6 cells per cocktail *tube* may adversely affect performance.**

Fc Block

2. Spin cells at 400 x *g* for 5 minutes at 4 °C.

   Critical: **For step 2, use a swinging bucket rotor centrifuge. Use of a fixed angle rotor can result in smearing of the cell pellet, which may negatively impact cell recovery.**

3. Using a 1 mL pipette tip, carefully remove supernatant and resuspend cells in 95µL BSA Buffer. Add 5 µL Human TruStain™ FcX and *gently* pipette mix.

   Critical: **Centrifugation at low-speed results in loose pellets that can be easily disturbed and aspirated during washing steps. Avoid disruption of the cell pellet as much as possible.**

4. Incubate for 10 minutes on ice.

5. Bring up to 1 mL with BSA Buffer.

Cell Fixation

6. Spin Fc blocked cell suspension at 400 x *g* for 5 minutes at 4 °C.

7. Being careful not to disturb or aspirate from the cell pellet, remove supernatant. Set a timer for 15 minutes, then add 200 µL of Fixation Buffer without resuspending the pellet. Immediately start the 15-minute timer and then gently pipette mix *10 times*.

   Critical: **Carefully follow the sequence of Step 7 to avoid over fixation of cells.**

8. Incubate for 15 minutes at room temperature (RT).

9. Bring up to 1 mL with 1X PBS.

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Intracellular Block & Stain

10. Spin fixed cell suspension at 850 x *g* for 5 minutes at 4 °C.

11. Being careful not to disturb or aspirate from the cell pellet, remove supernatant, and resuspend in 50 µL of IC Block buffer.

12. Incubate on ice for 10 minutes.

13. If using a lyophilized cocktail, add all 50 µL of reconstituted antibody cocktail and gently pipette mix *10 times*.

    Critical: **If not using a lyophilized cocktail, combine antibody staining volume with enough IC Staining Buffer so that the final volume is 50 µL. Add 50 µL of the diluted antibody to the cells in 50 µL of IC Block Buffer.**

14. Incubate for 30 minutes on ice.

15. Wash 1: Add 900 µL of IC Wash Buffer to sample. Wash cells by gently pipetting 10 times then add 2 mL of IC Wash Buffer.

16. Spin at 850 x *g* for 5 minutes at 4 °C.

17. Wash 2: Remove supernatant and resuspend in 1 mL of IC Wash Buffer. Wash cells by gently pipetting 10 times then add 2 mL of IC Wash Buffer.

18. Spin at 850 x *g* for 5 minutes at 4 °C.

19. Wash 3: Remove supernatant and resuspend in 1 mL of IC Wash Buffer. Wash cells by gently pipetting 10 times then add 2 mL of IC Wash Buffer.

20. Immediately proceed to “Sample Fixation” in the 10x Genomics Fixation of Cells & Nuclei for Chromium Fixed RNA Profiling protocol (CG000478 Rev D). After cell fixation using the 10X Genomics protocol, proceed to “Probe Hybridization” in the Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture protocol (CG000673 Rev A).

MultiPro™ Individual Antibody and Cocktail Staining Protocol

Intracellular Block & Stain (continued)

21. Post probe hybridization, we recommend the "Individual Wash Workflow" on page 94 of the “Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture protocol (CG000673 Rev A)”_protocol on day 2 of the 10x Genomics Flex assay. Washing the samples individually reduces cell clumping.

Contact Us

Questions or concerns? Please contact us at **genomics.support@ptglab.com**