# 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 Blocking Reagent | Proteintech Genomics | G900005 |
| Protector RNase inhibitor | Roche | 3335399001 |
| Tween-20, 10% | Thermo Scientific | 28320 |
| NP-40, 10% | Thermo Scientific | 85124 |
| Paraformaldehyde, 32% | 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 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.

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## Before You Start

- **Planning your experiment -** Some experiments will require a "Stain-Fix-Stain" approach where live cells are stained with antibodies in between Fc Block and Cell Fixation. If your experiment requires a pre-fixation antibody stain, ensure that you prepare enough BSA Buffer by following the BSA Buffer recipe for experiments requiring a Pre-Fixation Stain.

- **Time required -** Depending on the number of samples being stained, this protocol is expected to take ~3-4 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.

- **Staining with single antibodies -** 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 23 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).

- **Centrifuge selection** – All steps requiring centrifugation of a cell suspension should be performed using 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.

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## Before You Start, *continued*

- **Sequencing Parameters**

  For Cell Ranger v8.0 or newer
  - Read 1: 28 cycles
  - Index 1: 10 cycles
  - Index 2: 10 cycles
  - Read 2: 90 cycles

  For Cell Ranger v7.2
  - Read 1: 48 cycles
  - Index 1: 10 cycles
  - Index 2: 10 cycles
  - Read 2: 50 cycles

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## 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 for experiments NOT requiring a Pre-Fixation Stain

| **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** |

### BSA Buffer for experiments requiring a Pre-Fixation Stain

| **Component** | [Final] | 1 sample (µL) | 1 sample +10% | 4 samples +10% |
|---|---|---|---|---|
| RNase inhibitor (200X) | 1X | 19.5 | 21.5 | 85.8 |
| BSA, 30% | 1% | 130 | 143 | 572 |
| 10X PBS | 1x | 390 | 429 | 1716 |
| Ultrapure water | | 3360.5 | 3696.6 | 14786.2 |
| **Total** | | **3900 µL** | **4290 µL** | **17160 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** |

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## 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 Blocking Reagent | - | 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** |

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## IC Wash Buffer

| 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** |

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## 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 and 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 the appropriate amount of Staining Buffer down the side of the tube.
   - If using a lyophilized cocktail for pre-fixation staining – 105 µL of BSA Buffer
   - If using a lyophilized cocktail for post-fixation staining – 55 µL of IC Staining Buffer

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 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 pellets 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 the appropriate volume of reconstituted cocktail to a new 1.5 mL Protein LoBind tube:
   - If using a lyophilized cocktail for pre-fixation staining – 100 µL of BSA Buffer

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- If using a lyophilized cocktail for post-fixation staining – 50 µL of IC Staining Buffer

**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.

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## Sample Preparation

1. Prepare 1-2 x $10^6$ cells in 1 mL of BSA Buffer in a 5 ml FACS tube.

   **Critical: Staining more than 2 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.
3. Using a 1 mL pipette tip, carefully remove supernatant and resuspend cells in 95 µL 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.
6. Spin Fc blocked cells at 400 x *g* for 5 minutes at 4 °C.

## Pre-Fixation Stain (optional)

**Critical: If omitting Pre-Fixation Staining, skip to step 14.**

7. If using single antibodies instead of a lyophilized cocktail - Prepare antibody staining mix by combining antibodies and BSA Buffer to make a total of 100 µL.
8. Remove supernatant from cells and resuspend pellet in 100 µL of BSA Buffer + Antibodies, gently pipette mix.
9. Incubate for 30 minutes on ice.
10. Add 1.4 mL of BSA Buffer, wash cells by gently pipette mixing 10 times.
11. Spin cells at 400 x *g* for 5 minutes at 4 °C.
12. Remove supernatant

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13. Add 1.4 mL of BSA Buffer, wash cells by gently pipette mixing 10 times.

14. Spin Pre-Fixation-Stained cells at 400 x *g* for 5 minutes at 4 °C.

    **Critical:** If omitting intracellular staining, remove supernatant and proceed directly to step 28, 10X Genomics sample fixation.

## Cell Fixation

15. Being careful not to disturb or aspirate from the cell pellet, remove supernatant.

    **Critical:** **Remove as much buffer as possible prior to fixation without disturbing the cell pellet to ensure proper cell fixation, however leaving ~15 µL of residual buffer will not impact assay performance.**

16. 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 16 to avoid over fixation of cells.

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

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

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

20. Being careful not to disturb or aspirate from the cell pellet, remove supernatant.

    **Critical:** Remove as much **Fixation Buffer as possible prior to resuspending cells in IC Block Buffer, however leaving ~15 µL of residual buffer will not impact assay performance.**

## Intracellular Block & Stain

21. Resuspend cell pellet in 50 µL of IC Block buffer.

22. Incubate on ice for 10 minutes.

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23. 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 pool to the cells in 50 µL of IC Block Buffer.**

24. Incubate for 30 minutes on ice.

25. 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.

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

27. 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.

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

29. 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.

30. 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).

31. 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)" on protocol on day 2 of the protocol. This aids in reducing cell clumping.

## Contact Us

# MultiPro™ Individual Antibody and Cocktail Staining Protocol

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