

MultiPro® HashMax™-5 Sample Multiplexing & Sequencing Library Prep Protocol for 10x Genomics Flex Gene Expression

Cat no. G900081

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
Human TruStain™ FcX	BioLegend	422302
2 mL tubes	Eppendorf	022363352
1.5 mL Protein LoBind tubes	Eppendorf	022431081
HashMax-5_suppl primer [2 µM] 5'-GTGACTGGAGTTCAGACGTGTGCTC-3' HPLC purified	Oligo provider	-
Dual Index Kit TT Set A	10x Genomics	1000215

Required Equipment

- Tabletop centrifuge capable of 14,000 x g, with a rotor suitable for 1.5 mL Protein LoBind tubes.
- Swinging bucket centrifuge with adaptor capable of holding 5mL FACS tubes or 1.5 mL Protein LoBind Tubes.
- Tabletop vortex mixer.

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Before You Start

- This protocol is designed to work with the following 10x Genomics assays:
 - Chromium Fixed RNA Profiling Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000673)
 - GEM-X Flex Gene Expression Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture (CG000789)
 - Please make sure you have Fixed RNA Feature Barcode Multiplexing Kit 64 rxns (PN-1000628). The configuration described in 10x Genomics User Guide CG000477 is **NOT** validated with the HashMax™-5 oligo format on MultiPro® HashMax™-5 Cell Hashing antibodies.
- **Time required** - Depending on the number of samples being stained, this protocol is expected to take ~1 hour *after* cell preparation.
- **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.
- **Sequencing Parameters**
 - **Sequencing depth** – 500 paired-end reads per cell (targeted recovery)
 - **Length** -
 - Read 1: 28 cycles
 - Index 1: 10 cycles
 - Index 2: 10 cycles
 - Read 2: 90 cycles

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Buffer Preparation

Prepare a 20% BSA Stock Solution - Dissolve 20 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%
BSA, 20%	1%	175	192.5	770
10X PBS	1x	350	385	1540
Nuclease-free water		2975	3272.5	13090
Total		3500 µL	3850 µL	15400 µL

Prepare HashMax™-5 Staining Solution

NOTE: Steps 1-7 are described for a single MultiPro® HashMax™-5 antibody pool, but should be carried out, in parallel, for all MultiPro® HashMax™-5 antibody pools to be used in the experiment.

1. Clearly label 2 x 1.5 mL Protein LoBind tubes with a unique identifier corresponding to the **HashMax™-5 antibody pool hashcode or sample ID.**
2. Retrieve MultiPro® HashMax™-5 Antibody stock solutions from -20 storage, vortex briefly, and centrifuge at 1500 x g for 30 seconds at RT to ensure the entire volume of antibody is in the bottom of the tube.

Note: The HashMax™-5 antibodies are supplied in 1.4ml Micronics tubes and must be placed in a 2 mL microcentrifuge tube prior to centrifugation.

3. Using a p2 pipettor, transfer 2 µL of the MultiPro® HashMax™-5 Antibody stock solution into one of the pre-labeled 1.5 mL Protein LoBind tubes containing 50 µL of BSA buffer. Return the MultiPro® HashMax™-5 Antibody stock vials to -20 °C
4. Snap shut the tube and vortex for 15 seconds then spin at 1500 x g for 30 seconds at RT.
Critical: Step 4 ensures antibodies are evenly distributed in the HashMax™ staining solution.
5. Spin the tubes containing the **HashMax™ staining solution** at 14,000 x g for 10 minutes at 4°C.

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Critical: Step 5 pellets any protein aggregates that may be present in the cell hashing solution.

6. Being careful NOT to touch the bottom of the tube with your pipette tip, carefully transfer 50 µL of the HashMax™ staining solution to the other pre-labeled 1.5 mL Protein LoBind tube.

Critical: Avoid touching the bottom of the tube with a pipette tip and do not transferring the entire volume, as doing so could result in the transfer of aggregates to the final staining solution.

7. Keep HashMax™ staining solution on ice until ready to use.

Sample Preparation

8. Prepare up to 2×10^6 cells in 1 mL of BSA Buffer in a 1.5 mL Protein LoBind tube.

NOTE: MultiPro® HashMax™-5 Antibodies can be used for various reasons, including but not limited to increasing target cell recovery, reduction of the **Undetected Multiplet Rate**, and enabling users to work with samples with limited cell numbers. Therefore, experimental context may necessitate working with samples with vastly different cell numbers, ranging from as low as 50,000 cells per sample but to several million cells per sample. Although this protocol will not provide specific instructions on how to handle these different cellular inputs, we recommend following some general guidelines.

- Keep staining volumes and antibody concentrations consistent with the approach described above for cellular inputs up to but not exceeding 2×10^6 cells per sample.
- Avoid using vacuum aspiration during cell wash steps, which can lead to complete cell loss.
- Use p1000 tips for aspirations, being careful not to disrupt or touch tips to the cell pellets. It is acceptable to leave ~20ul residual liquid in each tube at each aspiration step.
- Avoid aggressive handling and pipetting of cell suspensions.

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Fc Block

9. Spin cells at 400 x g for 5 minutes at 4 °C.
10. Using a 1 mL pipette tip, carefully remove supernatant and resuspend cells in 45 µL BSA Buffer. Add 7.5 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.

11. Incubate for 10 minutes on ice.

Perform HashMax[™] Staining

12. Add 50 µL of cell hashing solution to the Fc Blocked cells and gently pipette mix 5-10 times.
13. Incubate for 30 minutes on ice.
14. Add 1.4 mL of BSA Buffer and carefully wash cells by gently pipette mixing 5-10 times.
15. Spin cells at 400 x g for 5 minutes at 4 °C.
16. Remove supernatant.

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.

17. Add 1 mL of BSA Buffer and carefully wash cells by gently pipette mixing 5-10 times.
18. Spin cells at 400 x g for 5 minutes at 4 °C.
19. The next steps to be taken depend on the specific goals of each experiment. Here we reference protocols to for some of the most common next steps in a user's experimental workflow:
 - a. If using the MultiPro[®] Human Discovery Panel for subsequent staining:
 - i. For Next GEM chemistry: Immediately Proceed to the [Cell Surface & Intracellular Protein Labeling for Chromium Fixed RNA Profiling \(CG000529 Rev C\)](#)
 - ii. For GEM-X chemistry: Immediately Proceed to the [Cell Surface & Intracellular](#)

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[Protein Labeling for GEM-X Demonstrated Protocol \(CG000781 Rev A\)](#)

- b. If using the MultiPro[®] Immune Profiling cocktail for subsequent staining:
 - i. For Next GEM chemistry: Immediately Proceed to [Cell Surface & Intracellular Protein Labeling for Chromium Fixed RNA Profiling \(CG000529 Rev C\)](#), omit the Cell Surface Protein Labeling section.
 - ii. For GEM-X chemistry: Immediately Proceed to the [Cell Surface & Intracellular Protein Labeling for GEM-X Demonstrated Protocol \(CG000781 Rev A\)](#), omit the Cell Surface Protein Labeling section.
- c. If no additional antibody staining is required:
 - i. For Next GEM chemistry - 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 \(CG000673 Rev B\)](#).
 - ii. For GEM-X chemistry - Immediately Proceed to “Sample Fixation” in the [10x Genomics Fixation of Cells & Nuclei for GEM-X Flex Gene Expression \(CG000782 RevA\)](#). After cell fixation, proceed to [10x Genomics GEM-X Flex Gene Expression Reagent Kits \(CG000789 Rev A\)](#).

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HashMax™-5 Library Pre-Amplification

20. In the “Pre-Amplification PCR” step of the 10x Genomics Flex Library Construction protocol, spike-in the “HashMax-5_suppl primer” in addition to Pre-Amp Primers C as described below, for the appropriate chemistry.

When using Next-GEM Chemistry:

Component	10x Genomics PN	1 sample (μL)
Amp Mix (Next GEM)	2000047	25
Pre-Amp Primers C	2000953	10
HashMax-5_suppl primer (2 μM)	-	2
Total		37 μL

When using GEM-X Chemistry:

Component	10x Genomics PN	1 sample (μL)
Amp Mix C (GEM-X)	2001311	25
Pre-Amp Primers C	2000953	10
HashMax-5_suppl primer (2 μM)	-	2
Total		37 μL

21. Add 37 μL preamplification Mix to aqueous sample recovered from the Post-GEM Incubation – Recovery step, cap firmly, invert 8x, centrifuge briefly and proceed with Pre-Amplification PCR.

HashMax™-5 Sequencing Library Generation

22. In parallel with Sample Index PCR of protein library, transfer ONLY 20 μL pre-amplified DNA to a new well of a PCR strip

23. Add 50 μL Amp Mix (10x Genomics PN-2000047/2000103) and 10 μL Nuclease-free water to

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the 20 µL sample transferred in step 22.

24. Add 20 µL of an individual Dual Index TT Set A well to each sample. Pipet Mix 5x, centrifuge briefly.

Critical: Do not use a well that has been used before, document which well was used)

25. Proceed to Sample Indexing PCR with the following cycle numbers:

Target Cells	Total Cycles
< 4,000	20
4,000-7,000	19-20
7,000-12,000	18-19
12,000-25,000	17-18
25,000-50,000	16-17
50,000-128,000	15-16

HashMax™-5 Library cleanup

26. **NOTE:** HashMax™-5 libraries have a similar length as protein libraries and cleanup of both libraries can be performed in parallel using the same parameters. For isolating cell hashing library, perform 0.6X SPRIselect clean up the same way as protein library cleanup described in the Protein library - Post Sample index PCR Size Selection -SPRIselect section of 10x Genomics User Guide.

Contact Us

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