# 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]<br>5'-GTGACTGGAGTTCAGACGTGTGCTC-3'<br>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 mL Protein LoBind tubes containing 50 µL of BSA buffer. 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.

   **Critical: Step 5 pellets any protein aggregates that may be present in the HashMax™ staining 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 x $10^6$ 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 2x$10^6$ 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.**

## 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 HashMax™ Staining solution to the Fc Blocked cells, gently pipette mixing 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. Repeat steps 17 -18 for a total of 3 BSA buffer washes.

**NOTE: Completion of Step 19 concludes HashMax™ Staining portion of this protocol.**

20. **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 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 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).

## HashMax™-5 Library Pre-Amplification

21. In the Pre-Amplification PCR step of 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** |

-OR-

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** |

22. 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

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

24. Add 50 µL Amp Mix (10x Genomics PN-2000047/2000103) and 10 µL Nuclease-free water to the 20 µL sample transferred at last step.

25. 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)

26. 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

27. **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 HashMax™-5 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**