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.

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

Cat no. G900081

Before You Start

- This protocol has been validated to work with the 10x Genomics Chromium GEM-X Single Cell 5' Reagent Kits v3 (CG000734 Rev A)

- **Time required -** Depending on the number of samples being stained, this protocol is expected to take ~1 hour *after* cell preparation. The staining protocol *does not* contain any safe stopping points and users must *immediately* proceed to GEM loading of:

    - 10x Chromium Single Cell 5' Reagent Kits User Guide (v2 - Dual Index) (CG000331)

    - 10x Chromium Single Cell 5' Reagent Kits User Guide (v2 - Dual Index) with Feature Barcoding technology for Cell Surface Protein and Immune Receptor Mapping (CG000330)

    - 10x Genomics Chromium GEM-X Single Cell 5' Reagent Kits v3 (CG000733)

    - 10x Genomics Chromium GEM-X Single Cell 5' Reagent Kits v3 with Feature Barcode technology for Cell Surface Protein (CG000734)

- **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**
    - **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

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 2x10^6 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 step 17-18 to a total of 3 BSA buffer washes.

    NOTE: At this stage cells are ready to be counted and pooled for GEM loading.

10x Genomics Chromium GEM loading and cDNA Amplification

20. Immediately proceed to "GEM generation and Barcoding" in the Chromium GEM-X Single Cell 5' Reagent Kits v3 User Guide.

21. In the Pre-Amplification PCR, spike-in HashMax-5_suppl primer in addition to Feature cDNA Primers

With Feature Barcode Kit

Component	10x Genomics PN	1 sample (µL)
Amp Mix	2000047	50
Feature cDNA primers 4	**2000277**	15
HashMax-5_suppl primer (2 µM)	-	2
Total		**67 µL**

Without Feature Barcode Kit

Component	10x Genomics PN	1 sample (µL)
Amp Mix	2000047	50
cDNA primers	**2000089**	15
HashMax-5_suppl primer (2 µM)	-	2
Total		**67 µL**

Add 67 µL cDNA Amplification Mix to 33 µL Post-GEM-RT Cleanup sample, pipet mix 15x, centrifuge briefly and proceed with cDNA Amplification.

Post cDNA Amplification Cleanup

22. Vortex to resuspend the SPRIselect reagent. Add 60 µL SPRIselect reagent (0.6X) to each sample

23. Thoroughly mix by pipetting 15x. Incubate 5 min at room temperature.

24. Place on the magnet for 3 mins or until the solution clears.

25. Transfer 80 µL supernatant in a new tube strip without disturbing the pellet. Maintain the transferred supernatant at room temperature.
Critical: Do NOT discard the supernatant, as this supernatant contains HashMax and Protein library fragments.)

26. Remove the remaining supernatant from the pellet without disturbing the pellet.
27. DO NOT discard the pellet. Immediately add 200 µL 80% ethanol to the pellet (on magnet) and keep for 30 sec.
28. Aspirate the ethanol without disrupting pellet.
29. Repeat steps 27-28 for a total of 2 washes.
30. Centrifuge briefly and place on the magnet.
31. Remove residual ethanol and air dry pellet for no more than 2 min.
32. Remove from the magnet and add 45.5 µL Buffer EB.
33. Thoroughly mix by pipetting 10-15x and keep at room temperature for ~2 min.
34. Place the tube strip on the magnet for 3 min or until the solution clears.
35. Transfer 45 µL sample to a new tube strip. This sample contains cDNA Amp libraries for VDJ and GEX library generation. This sample is good to be stored at 4°C for 2-3 days or at −20°C for up to 1 month.

Supernatant Cleanup (for HashMax™-5/Protein Library)

36. Vortex to resuspend the SPRIselect reagent.
37. Add 70 µL SPRIselect reagent (2.0X) to 80 µL of the **transferred supernatant** (from step 25) and pipette mix 15x (pipette set to 150 µL).
38. Incubate for 5 min at room temperature.
39. Place on the magnet for 3 min or until the solution clears.
40. Remove the supernatant.
41. Add 200 µL 80% ethanol to the pellet. Wait 30 sec.
42. Remove the ethanol.
43. Repeat steps 41 and 42 for a total of 2 washes.
44. Centrifuge briefly and place on the magnet.
45. Remove any remaining ethanol while the tube strip is on the magnet. Air dry for 2 min.

46. DO NOT exceed 2 min as this will decrease elution efficiency.

47. Remove from the magnet. Add 45.5 µL Buffer EB. Thoroughly mix by pipetting 10-15x.

48. Incubate 2 min at room temperature.

49. Place the tube strip on the magnet for 3 min or until the solution clears.

50. Transfer 45 µL sample to a new tube strip. This is the input sample for HashMax, and Protein Library Sample Indexing PCR

51. Store at 4°C for up to 72 h or at −20°C for up to 4 weeks

HashMax™-5 Sequencing Library Generation

52. In parallel with Sample Index PCR of protein libraries, Transfer ONLY 5 µL Transferred Supernatant Cleanup to a new well of a PCR strip.

53. Add 50 µL Amp Mix (10x Genomics PN-2000047) and 25 µL Buffer EB to the 5 µL sample transferred at last step.

54. Add 20 µL of an individual Dual Index TT Set A well to each sample. (Do not use a well that has been used before, document which well was used) Pipet Mix 5x, centrifuge briefly.

55. Proceed to Sample Indexing PCR with total 16 cycles of amplification. Optimization of PCR cycles may be needed based on the sample types.

HashMax™-5 Library Cleanup

56. Add 60 µL (0.6x) SPRIselect beads, pipet mix 10x and incubate 5 min at room temp
57. pellet beads on magnet for 3 min or until clear.
58. Transfer the supernatant to another tube, add 60 µL (1.2X) SPRIselect to the transferred supernatant, pipet 10x and incubate at room temp for 5 min.
59. pellet beads on magnet for 3 min or until clear.
60. Wash twice with 200 µL of 80% EtOH on magnet.
61. Centrifuge briefly and place on the magnet. Use pipet to remove residual EtOH.
62. Do not air dry, remove tube from magnet, add 35.5 µL buffer EB, resuspend the beads and wait 2 mins.
63. Pellet beads on magnet for 2 min or until clear.
64. Transfer 35 µL eluted DNA to a new tube. HashMax™-5 libraries are ready for Post-Cleanup QC.

Contact Us

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