Protocol Description

This protocol provides instructions for cell staining with the MultiPro Human Discovery Panel (Cat No G900150). For convenience, this protocol contains steps taken from subsequent 10x Genomics protocols.

Product Description

The Human Discovery Panel (HDP) contains 347 DNA-barcoded antibodies separated into a Cell Surface (purple cap) and Intracellular (white cap) cocktails. Each reaction of the Human Discovery Panel contains enough material to stain up to 2M cells.

This protocol results in the generation of two sequencing libraries: Gene Expression (GEX), and HDP Expression (HEX). Please read the entire protocol before starting.

Oligonucleotide Structure

Human Discovery Panel antibodies are conjugated to **5CFLX** oligonucleotides with the following structure:

CGGAGATGTGTATAAGAGACAG-[15N Barcode]-CCCATATAAGA*A*A

N represents randomly chosen A, T, C, or G.

* Represents Phosphorothioate bonds.

Barcode sequences are available in the HDP **Feature Reference File**, which can be downloaded from the HDP product page.

Workflow and Associated Protocols

1. Prepare single-cell suspension
2. Stain cells with the Human Discovery Panel – Cell Surface cocktail
3. Short cell fixation
4. Stain cells with the Human Discovery Panel – Intracellular cocktail
5. 10x Genomics fixation
6. 10x Genomics Flex
    a. Probe Hybridization
    b. Optional storage*
    c. Single-cell partitioning
    d. Sequencing library prep
    e. Sequencing
    f. Data analysis
    g. Optional storage

This document will guide you through steps 2 to 6 and 7d. For your convenience we've included protocol steps and buffer formulations from the appropriate 10x Genomics protocols below.

* If needed, Proteintech Genomics recommends storing antibody-stained cells post-probe hybridization.

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Staining with the Human Discovery Panel

10x Genomics Demonstrated Protocol for Cell Surface and Intracellular Protein Labeling for GEM-X Flex Gene Expression CG000781 Rev A

10x Genomics Flex Fixation

10x Genomics Fixation of Cells & Nuclei for GEM-X Flex Gene Expression (CG000782 Rev B)

Next Steps

The following documents are required for completing the Flex workflow:

GEM-X Flex Gene Expression Reagent Kit for Multiplex samples with Feature Barcode technology for Protein Expression CG000789 Rev A

Required Reagents and Consumables

Description	Manufacturer	Part Number
MultiPro® Human Discovery Panel	Proteintech Genomics	G900150
MultiPro® Enhanced Blocking Reagent	Proteintech Genomics	G900005-65UL
1.5 mL Protein LoBind tubes	Eppendorf	022431081
Additive C	10x Genomics	PN-2001332
Conc. Fix & Perm Buffer B	10x Genomics	PN-2001301
Conc. Quench Buffer B	10x Genomics	PN-2001300
Protector RNase inhibitor	Roche	3335399001
Tween-20, 10%	Thermo Scientific	28320
NP-40, 10%	Thermo Scientific	85124
Dextran Sulfate Sodium Salt, 8 kDa	Millipore Sigma	RES2029D-A7
Bovine Serum Albumin, Nuclease-free	Millipore Sigma	126609100GM
Paraformaldehyde, 32%	Electron Microscopy Sciences	15714
Human TruStain FcX™	BioLegend	422302
PBS, Ultrapure (10X)	Invitrogen	AM9625
Nuclease-free Water	-	-

Required Equipment

- Centrifuge capable of 14,000 x g, with a rotor suitable for 1.5 mL microcentrifuge tubes.
- Swinging bucket centrifuge with adapter capable of holding 1.5 mL microcentrifuge tubes.
- Tabletop vortex mixer.

Before You Start

Pipetting

- Avoid vacuum aspiration during wash steps to minimize the risk of complete cell loss.
- Use P1000 pipette tips for all aspiration steps. Be careful not to disturb the cell pellet or touch it with the pipette tip. Leaving ~20 µL of residual liquid in each tube at each step is acceptable.
- Handle cell suspensions gently. Avoid vigorous pipetting or mixing that could compromise cell viability or integrity.

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

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 depth

Add a minimum of 10K paired end reads per cell (targeted cell recovery)

Run configuration

Read 1: 28 cycles
Index 1: 10 cycles
Index 2: 10 cycles
Read 2: 90 cycles

Buffer Preparation

20% BSA Stock Solution

Dissolve 4 g of nuclease-free BSA powder in 20 mL of ultrapure water. Filter-sterilize using a 0.2 µm filter and store at 4 °C for up to one week.

1% Dextran Sulfate Stock Solution

Dissolve 0.050 g of dextran sulfate, 8 kDa in 5 mL of nuclease-free water. Store at room temperature for up to 1 month.

Cell Surface (CS) Buffer

Component	[Final]	1 sample +10%	4 samples +10%
BSA, 20%	1%	220	880
10X PBS	1x	440	1760
Nuclease-free water	-	3740	14960
Total	**-**	**4400 µL**	**17600µL**

Intracellular (IC) Buffer

Component	[Final]	1 sample + 10%	4 samples + 10%
RNase inhibitor (200X)	1X	1.1	4.4
Nuclease-free BSA, 20%	7.55%	41.53	166.1
Tween-20, 10%	0.2%	2.2	8.8
NP-40 or Nonidet P40 Substitute, 10%	0.5%	5.5	22
Dextran Sulfate, 8 kDa, 1%	0.025%	2.75	11
Enhanced Blocking Reagent	-	15.95	63.8
Human TruStain FcX™	-	5.5	22
PBS, Ultrapure (10X)	1x	11	44
Nuclease-free Water	-	24.47	97.9
Total		**110 µL**	**440 µL**

Blocking Buffer

Component	[Final]	1 sample +10%	4 samples +10%
CS Buffer	-	47.5	190
Human TruStain FcX	-	7.5	30
Total	**-**	**55 µL**	**220 µL**

Wash Buffer

Component	[Final]	1 sample +10%	4 samples +10%
RNase Inhibitor 40 U/µL	0.2	11	44
Tween-20 10%	0.1%	22	88
Nuclease-free BSA 20%*	1%	110	440
10X PBS	1X	220	880
Nuclease-free water		1837	7348
Total		**2200 µL**	**8800 µL**

*The original 10x Genomics recipe calls for 30% BSA, we've adjusted this to 20% BSA for convenience.

Fixation Buffer B

Component	[Final]	1 sample +10%	4 samples +10%
Conc. Fix & Perm Buffer B Thaw at room temperature. Vortex, check for precipitation, and centrifuge briefly.	1X	110	440
Paraformaldehyde, 32%	4%	137.5	550
Nuclease-free water		852.5	3410
Total		**1100 µL**	**4400 µL**

Quenching Buffer B

Component	[Final]	1 sample +10%	4 samples +10%
Conc. Quench Buffer B Thaw at room temperature. Vortex and centrifuge briefly.	1X	275	1100
Nuclease-free water		1925	7700
Total		**2200 µL**	**8800 µL**

Reconstitution of the Human Discovery Panel

1. Remove the desired number of panel reactions from 4°C storage.

   Reaction (RXN) of HDP

   Critical: All tubes in one reaction pouch are required to stain up to 2 million cells.

2. While keeping the tubes in their pouch, incubate at room temperature for 10 minutes.

3. Remove the cocktail tubes from the pouch and place each tube, with its cap still on, into a 2.0 mL Eppendorf tube.

4. Place the stacked tubes into a centrifuge with a fixed angle rotor.

5. Spin at 1500 *x g* for 30 seconds at room temperature.

6. Carefully remove the cocktail tubes from the Eppendorf tubes and place them into a tube rack.
   Note: Reconstitution of both Cell Surface and Intracellular cocktails can be performed simultaneously.
7. Add 55 µL of **CS Buffer** to the Cell Surface cocktail tube (purple cap)

   Note: Make sure the volume is added BELOW the internal threads of the cocktail tube.

8. Add 55 µL of **IC Buffer** to the Intracellular cocktail tube (white cap)

   Critical: If your pouch contains TWO tubes with white caps add 27.5 µL of IC Buffer to each.
   Note: Make sure the volume is added BELOW the internal threads of the cocktail tube.

9. Securely cap the tubes and vortex for 10 seconds.

10. Place each cocktail tube into an Eppendorf tube.

11. Incubate the cocktail at room temperature for 5 minutes.

12. Place the stacked tubes into a centrifuge with a fixed angle rotor.

13. Spin at 10,000 *x g* for 30 seconds at room temperature.

14. Remove the stacked tubes carefully from the centrifuge and place them into a tube rack.

15. Set the temperature of the centrifuge to 4˚C

16. Using a P200 pipette with an **extended length pipette tip**, carefully transfer the contents of each cocktail tube to a new 1.5 mL Eppendorf tube.

    Critical: For processing multiple samples, you can pool multiple CS (purple) or IC (white) tubes into a single CS or IC tube for convenience.

17. Place each Eppendorf tube containing the reconstituted CS and IC pools into a centrifuge with a fixed angle rotor that has been chilled to 4˚C.

18. Spin at 14,000 *x g* for 10 minutes at 4˚C.

19. Label two new 1.5 mL Eppendorf tube **CS** and **IC**.

20. Without touching the bottom of the Eppendorf tubes, transfer 52 µL of the contents of the **CS** and **IC** tubes to the newly labeled Eppendorf tubes.

    Critical: This step removes protein aggregates that may be present.

21. Keep **CS** and **IC** tubes on ice until use.

Stain Cells with Human Discovery Panel (HDP)

22. Prepare up to 2 x 10^6 cells in 1 mL of **CS Buffer** in a 1.5 mL Protein LoBind tube.

Fc Block

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

24. Using a 1 mL pipette tip, carefully 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.

25. Resuspend cells in 50 µL of **Blocking Buffer** and gently pipette mix.

26. Incubate on ice for 10 minutes.

Cell Surface Stain

27. Without removing blocking solution, add 50 µL of **CS (purple cap)** to the Fc Blocked cells and gently pipette mix 5-10 times.

28. Incubate on ice for 30 minutes

Wash

29. Add 1.4 mL of **CS Buffer** and gently pipette mixing 5-10 times
    - Add 700µL of **CS Buffer** to each sample and gently mix the cells by pipette 5X.
    - Add an additional 700µL of **CS Buffer** and gently mix the cells by pipette 5X.

30. Centrifuge cells at 400 x *g* for 5 minutes at 4°C.

31. Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet. Up to 30 µL supernatant may be left behind if working with < 3 x 10^5 cells.

32. Add 1.5 mL of CS Buffer and gently pipette mix 5 - 10 times.
    - Add 750 µL of **Sample Buffer** to each sample and gently mix the cells 5X.
    - Add an additional 750 µL of **Sample Buffer** and gently mix the cells 5X.

33. Centrifuge cells at 400 x *g* for 5 minutes at 4°C.

34. 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. Ensure no more than 20µL of volume is left behind. If you are working with <500k cells, 30µL of volume can be left behind.

Cell Fixation

35. Add 0.5 mL room temperature **Fixation Buffer B** to the and pipette mix 5x.

36. Incubate for 20 minutes at room temperature (20°C).

    Critical: DO NOT agitate or mix the sample during incubation.

37. Add 0.5 mL room temperature **Additive C** to the sample in Fixation Buffer B and pipette mix 5x.

38. Centrifuge at 2,000 *x g* for 5 minutes at room temperature.

39. Remove the supernatant without disturbing the pellet.

    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. Ensure no more than 20µL of volume is left behind. If you are working with <500k cells, 30µL of volume can be left behind.

40. Add 1 mL chilled Quenching Buffer B to the sample pellet and pipette mix 5x and keep on ice.

Intracellular Block

41. Centrifuge at 2,000 *x g* for 5 minutes at 4°C. Remove the supernatant without disturbing the pellet.

42. Resuspend pellet in 50 µL IC Buffer

43. Incubate for 10 mins at 4°C.

Intracellular Staining

44. Without removing blocking solution, add 50 µL reconstituted Intracellular (IC) Cocktail to the cells and gently pipette mix 10x.

45. Incubate for 30 minutes at 4°C.

Intracellular Wash

46. Add 1 mL chilled Intracellular **Wash Buffer** to the labeled cells. Gently pipette mix.

47. Centrifuge cells at 2,000 *x g* for 5 minutes at 4°C.

48. Remove the supernatant without touching the bottom of the tube to avoid dislodging the pellet. Up to 30 µL supernatant may be left behind if working with $<3 \times 10^5$ cells.

49. Repeat 46-48 one more time for a total of 2 washes.

Post Stain Fixation

50. Add 0.5 mL room temperature **Fixation Buffer B** to the sample pellet and pipette mix 5x.

51. Incubate for 1 hour at room temperature (20°C) or for 16-24 hours at 4°C. If planning to store fixed samples, a 16–24-hour fixation at 4°C. is recommended.

    Critical: DO NOT agitate or mix the sample during incubation.

52. Add 0.5 mL room temperature **Additive C** to the sample in Fixation Buffer B and pipette mix 5x

53. Centrifuge at 2000 *x g* for 5 minutes at room temperature.

54. Add 1 mL* chilled Quenching Buffer B to the sample pellet and pipette mix 5x and keep on ice.

    *If cell numbers are expected to be < 2 x 10^5, resuspend sample pellet in 200 µL Quenching Buffer B. After counting, add an additional 800 µL Quenching Buffer B before proceeding to GEM-X Flex Gene Expression protocols or storage.

55. Determine cell concentration of the fixed sample using an automated cell counter or hemocytometer.

56. Proceed immediately to the appropriate GEM-X Flex Gene Expression protocols or store the sample as directed in the protocols below:

    GEM-X Flex Gene Expression Reagent Kit for Multiplex samples with Feature Barcode technology for Protein Expression CG000789 Rev A

Optional Storage

Fixed cell or nuclei suspensions can be stored at 4°C for up to 1 week or at -80°C for up to 12 months after resuspending in appropriate reagents.

Short-term Storage at 4°C

a. Thaw Enhancer (10x Genomics PN-2000482) for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

   DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.

b. Add 0.1 volume pre-warmed Enhancer to fixed sample in Quenching Buffer B.

   For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer B. Pipette mix.

   Alternatively, to conserve the Enhancer volume, centrifuge cells at 850 rcf, remove 500 µl Quenching Buffer B, and add 50 µl Enhancer to the sample.

c. Store sample at 4°C for up to 1 week.

Long-term Storage at −80°C

a. Thaw Enhancer (10x Genomics PN-2000482) for 10 min at 65°C. Vortex and centrifuge briefly. Keep warm and verify no precipitate before use.

   DO NOT keep the thawed reagent on ice, or the solution will precipitate. Once thawed, Enhancer can be kept at 42°C for up to 10 min. If not used within 10 min and precipitation
   is observed, reheat at 65°C to ensure enhancer is fully dissolved again before use.

b. Add 0.1 volume pre-warmed Enhancer to fixed sample in Quenching Buffer B.

   For example, add 100 µl Enhancer to 1,000 µl fixed sample in Quenching Buffer Pipette mix.

   Alternatively, to conserve the Enhancer volume, centrifuge cells at 850 rcf, remove 500 µl Quenching Buffer B, and add 50 µl Enhancer to the sample.

c. Add 50% glycerol for a final concentration of 10%.

   For example: add 275 µl 50% glycerol to 1,100 µl fixed sample in Quenching Buffer B and Enhancer. Pipette mix.

d. Store at −80°C for up to 12 months.

   If planning to store the fixed samples, it is strongly recommended to perform a 16-24 h fixation at 4°C during the fixation step and store the fixed samples at −80°C for best results.

Contact Us

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