Protocol 2: Multimodal single-cell screening using GuEST-List without phenotypic enrichment

This protocol assumes you have human cells in culture infected with a CRISPRi GuEST-List library that have undergone puromycin selection for an appropriate number of days (cell line dependent) and, if necessary, live-dead selection using a magnetic separation method (eg, Miltenyi Biotec 130-090-101) or similar. Cells should be >90% live before beginning the protocol– if not, include a fixation-compatible live/dead stain. This protocol is written assuming 1-2 million cells as input, but can be scaled up or down as needed.

I) Preparation of buffers

1 M Glycine

- Dissolve 750.7 mg glycine powder in 10 mL molecular grade water.
- Store at room temperature for 1 year

3% BSA in PBS

- Dissolve 15 g of BSA powder in 500 mL PBS with gentle agitation.
- Filter through a 0.22 uM membrane and store at 4 °C for 1 year

MACS Buffer (1x PBS, 0.5% BSA, 2 mM EDTA)

- Combine 2 mL 500 mM EDTA and 5 mL 10% BSA in PBS with 493 mL 1x PBS.
- Filter through a 0.22 uM membrane and store at 4 °C for 1 year

16% Formaldehyde

- Combine 219 uL 36.5% Formaldehyde with 281 uL PBS. Use within 1 hour.

Wash Buffer #1

- Prepare fresh, supplement with DTT immediately before use

Reagent	Final Concentration	Volume to prepare 10 mL
1M Tris-HCl (pH 7.5)	10 mM	100 uL
5M NaCl	10 mM	20 uL
1M MgCl2	3 mM	34 uL
10% BSA	1%	1 mL
0.1M DTT	1 mM	100 uL
Molecular Grade Water	-	8.5 mL

Permeabilization Buffer

- Prepare fresh on ice, supplement with RNAse inhibitor immediately before use

Reagent	Volume to prepare 1 mL
Wash Buffer #1	940 uL
10% NP-40	10 uL
NxGEN RNAse Inhibitor	50 uL

Wash Buffer #2

- Prepare fresh on ice, supplement with DTT immediately before use

Reagent	Volume to prepare 10 mL
PBS with 3% BSA	9.8 mL
10% Tween	100 uL
0.1 M DTT	100 uL

Blocking Buffer

- Prepare fresh on ice, supplement with DTT and RNAse inhibitor immediately before use

Reagent	Volume to prepare 1 mL
PBS + 3% BSA	905 uL
20 mg/mL Random ssDNA	50 uL
NxGEN RNAse Inhibitor	25 uL
Fc Block	10 uL
0.1 M DTT	10 uL

II) Protocol

Part 1: Permeabilization and Fixation

1. Harvest 1-2 million cells. Centrifuge 300 g for 5 minutes at room temperature
    a. Use a swing bucket centrifuge for all spin steps to minimize cell loss
2. Aspirate supernatant and resuspend pellet in 475 µL PBS
3. Filter cell solution through a 40 µm cell strainer into a fresh 1.5 mL tube
4. Add 30 µL 16% formaldehyde and pipette to mix.
5. Leave at room temperature for 10 minutes. Swirl/invert occasionally.
6. Quench fixation by the addition of 68.5 µL of 1 M glycine
7. Fill tube with ice cold PBS.
8. Centrifuge for 5 minutes at 400 g at 4 °C
9. Remove supernatant, add 1 mL ice-cold PBS, and repeat spin down.
10. Discard supernatant, resuspend pellet in 100 µL permeabilization buffer.
11. Incubate on ice for 5 minutes.
12. Add 900 µL Wash Buffer #1
13. Centrifuge for 5 minutes at 500 g at 4 °C

Part 2: Intracellular staining

14. Resuspend cell pellet in 100 µL of blocking buffer.
15. Spike in additional 1 µg of hashing antibody to each blocking solution.
16. Incubate with rotation for 30 minutes at room temperature
17. While blocking proceeds, combine oligo-antibody pool with single-stranded binding protein (SSB), such that there is 8 ug of SSB / 1 ug antibody. Add molecular grade water to bring volume up to 180 uL, then add 20 uL 10x NEB Buffer 4 and pipette mix thoroughly for a final reaction volume of 200 uL. See sample recipe below.

Reagent	Concentration	Volume
Oligo-Antibody Pool	0.5 ug/uL	40 uL
SSB	5 ug/uL	32 uL
Water	-	108 uL
10x NEB Buffer 4	10x	20 uL

Note: SSB concentration may vary across lots. Oligo-antibody pool concentration and volume will depend on experiment.

18. Incubate SSB-oligo-antibody pool mix at 37 °C for 30 minutes.
19. After incubation of the oligo-antibody pool, add 100 uL of 3x PBS + 9% BSA for a final concentration of 3% BSA in 1x PBS.

20. After blocking of the cells, centrifuge at 600 g for 5 minutes at 4 °C and resuspend in 500 uL wash buffer #2.
21. Remove supernatant and repeat wash step.
22. While final centrifugation step occurs, prepare intracellular staining mix

Reagent	Volume
Antibody + SSB in 3% BSA in PBS (Step 18)	300 uL
0.1 M DTT	7.5 uL
NxGen RNAse Inhibitor	18.75 uL
3% BSA in PBS	up to 500 uL

23. After final wash step, resuspend each pellet directly in 1/3rd of the staining solution and combine into a single tube.
24. Incubate on a tube rotator at room temperature for 1 hour, protected from light.
25. After incubation has completed, spin down at 500 g for 5 minutes at 4 °C and remove supernatant.
26. Resuspend in 1 mL wash buffer #2. Count cells and repeat spindown.
27. Repeat wash step.

Part 3: Modified 10x Flex Workflow for Secondary Fixation, Hybridization, GEM Generation and Pre-amplification

28. Follow 10x Genomics Demonstrated Protocol for Fixation of Cells & Nuclei for GEM-X Flex Gene Expression (CG000782) as described.
29. Follow 10x GEM-X Flex Gene Expression Protocol for Singleplex Samples (CG000786) through the end of step 4 (GEM Recovery and Pre-Amplification) with the following modifications.
    a. After step 1.1.g, spike in 2.5 uL of 40 nM GDO-BC Probe Set Working Stock, prepared as per 10x recommendations (CG000621). The final resulting hybridization mix will be as follows:

Reagent	Volume for 1x reaction
Hyb Buffer B (PN 2001312)	35 uL
Enhancer (PN 2000482)	5 uL
Human WTA Probes (PN 2001249-20001274)	10 uL
GDO-BC Probe Set Working Stock	2.5 uL

b. At step 3.1.b, if hashing was performed, can increase the concentration of cell solution input into GEM reaction to raise target cell recovery (ie, “super-loading”, as described in Stoeckius et al., 2018; see that publication for more detail)
c. At step 4.2.a, adjust pre-amplification mix as follows:

Reagent	Volume for 1x reaction
Amp Mix C (PN 2001311)	25 uL
Pre-Amp Primers B (PN 2000529)	10 uL
0.2 uM ADT Additive Primer	1 uL
0.2 uM GDO Additive Primer	1 uL
0.2 uM HTO Additive Primer	1 uL

30. After completing Step 4 of the 10x protocol, you will have 100 uL preamplification material that will be used as input into separate library preparations for each modality

Part 4: Gene Expression Library Preparation

31. Prepare the gene expression PCR reaction mixture on ice as described below. Pipette mix thoroughly

Reagent	Volume for 1x reaction
Amp Mix C (PN 2001311)	50 uL
Preamplification Material (step 4.3.o)	20 uL
Nuclease-free Water	25 uL
10 uM Universal Sample Index Forward Primer	2.5 uL
10 uM GEX Sample Index Reverse Primer	2.5 uL

32. Perform the PCR using the reaction conditions described below

Lid Temp: 105 °C		Reaction Volume: 100 uL
1 cycle	98 °C	45 s
6-11 cycles, use guidelines described in step 5.1.b of 10x protocol	98 °C	20 s
	54 °C	30 s

	72 °C	20 s
1 cycle	72 °C	1 min
	4 °C	∞

33. Run out 2 uL reaction on a 2% E-gel to confirm formation of desired product. If band is very faint, perform 2 additional cycles of PCR. Repeat as necessary.
34. Perform a 1.0x SPRI clean-up and elute the gene expression library in 41 uL Buffer EB.

Part 5: Guide-Barcode Library Preparation

35. Set up a guide-barcode enrichment PCR reaction on ice according to the following recipe.

Reagent	Volume for 1x reaction
Amp Mix C (PN 2001311)	50 uL
Preamplification Material (step 4.3.o)	20 uL
Nuclease-free Water	25 uL
10 uM Universal Sample Index Forward Primer	2.5 uL
10 uM GDO Enrichment Reverse Primer	2.5 uL

36. Perform the enrichment PCR using the reaction conditions described below.

Lid Temp: 105 °C		Reaction Volume: 100 uL
1 cycle	98 °C	45 s
9 cycles	98 °C	20 s
	63 °C	30 s
	72 °C	20 s
1 cycle	72 °C	1 min
	4 °C	∞

37. Clean up the reaction with a 1.8x SPRI and elute into 30 uL Buffer EB.
38. Set up a guide-barcode sample indexing PCR reaction on ice according to the following recipe.

Reagent	Volume for 1x reaction
2x KAPA Mix	50 uL
Guide-enriched material (from previous step)	30 uL
Nuclease-free Water	15 uL
10 uM Universal Sample Index Forward Primer	2.5 uL
10 uM GDO Sample Index Reverse Primer	2.5 uL

39. Perform the sample indexing PCR using the reaction conditions described below.

Lid Temp: 105 °C		Reaction Volume: 100 uL
1 cycle	95 °C	3 min
8 cycles	95 °C	20 s
	60 °C	30 s
	72 °C	20 s
1 cycle	72 °C	5 min
	4 °C	∞

40. Run out 2 uL reaction on a 2% E-gel to confirm formation of desired product. If band is very faint, perform 2 additional cycles of PCR. Repeat as necessary.
41. Clean up the reaction with a 1x SPRI clean-up. Elute into 20 uL Buffer EB.

Part 6: ADT and HTO Library Preparation

42. Set up one ADT sample indexing PCR reaction and one HTO sample indexing PCR reaction on ice according to the following recipe.

Reagent	Volume for 1x reaction
2x KAPA Mix	50 uL
Preamplification Material (step 4.3.o)	20 uL
Nuclease-free Water	25 uL
10 uM Universal Sample Index Forward Primer	2.5 uL

10 uM ADT **OR** HTO Sample Index Reverse Primer	2.5 uL

43. Perform the sample indexing PCR using the reaction conditions described below.

Lid Temp: 105 °C		Reaction Volume: 100 uL
1 cycle	95 °C	3 min
12 cycles	95 °C	20 s
	60 °C	30 s
	72 °C	20 s
1 cycle	72 °C	5 min
	4 °C	∞

44. Run out 2 uL reaction on a 2% E-gel to confirm formation of desired product. If band is very faint, perform 2 additional cycles of PCR. Repeat as necessary.
45. Clean up the reaction with a 1.6x SPRI clean-up. Elute into 20 uL Buffer EB.

References

1. Blair, J. D. *et al.* Phospho-seq: integrated, multi-modal profiling of intracellular protein dynamics in single cells. *Nat Commun* **16**, 1346 (2025).
2. 10x Genomics. “Fixation of Cells & Nuclei for GEM-X Flex Gene Expression.” CG000782, Rev C. Demonstrated Protocol.
3. 10x Genomics. “GEM-X Flex Gene Expression Reagent Kits For Singleplexed Samples.” CG000786, Rev B. User Guide.
4. 10x Genomics. “Custom Probe Design for Visium Spatial Gene Expression and Chromium Single Cell Gene Expression Flex.” CG000621, Rev C. Technical Note.
5. Stoeckius, M. *et al.* Cell Hashing with barcoded antibodies enables multiplexing and doublet detection for single cell genomics. *Genome Biology* **19**, 224 (2018).