Protocol 1: VIPseq Phenotypic Enrichment and Library Preparation for Intracellular Screens

This protocol assumes you have human cells in culture infected with the genome-wide 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 10-20 million cells as input, but can be scaled up or down as needed.

I) Preparation of buffers

Staining Buffer (3% BSA in 1x 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

Sorting and Collection Buffer (1x PBS, 1% BSA)

- Combine and 10 mL 10% BSA in PBS with 490 mL 1x PBS.
- Filter through a 0.22 uM membrane and store at 4 °C for 1 year

4% Paraformaldehyde

- Combine 10 mL 20% Paraformaldehyde with 40 mL PBS. Use within 1 day.

70% Ethanol

- Combine 35 mL 100% etOH with 15 mL H_2O
- Place in freezer for permeabilization >1hr before needed

1X PBS-T

- Pipette 500 µL of Tween-20 into 500 mL of PBS

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 40 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 40 mL 4% Paraformaldehyde
3. Leave undisturbed at room temperature for 1 hour.
4. Centrifuge 850 x g for 5 mins.
5. Remove supernatant, resuspend in 40 mL PBS-T.
6. Centrifuge 850 x g for 5 mins.
7. Repeat Steps 5 and 6.
8. Add 40 mL ice-cold 70% Ethanol.
9. Incubate overnight at 4° C.
10. Remove from 4° C and centrifuge 850 x g for 5 minutes.
11. Remove supernatant, resuspend in 40 mL PBS-T.
12. Centrifuge 850 x g for 5 mins.
13. Repeat Steps 11 and 12.

Part 2: Intracellular staining

14. Resuspend cell pellet in 8 mL of blocking buffer.
15. Incubate with rotation for 30 minutes at room temperature.
16. After blocking of the cells, centrifuge at 850 x g for 5 minutes at 4 °C and resuspend in 8 mL PBS-T.
17. Remove supernatant and repeat wash step.
18. After final wash step, resuspend pellet directly in 4 mL of staining solution with 40 µL of vimentin-AF647 antibody.

    Note: Choose a volume of fluorophore-conjugated antibody appropriate for detecting target screen phenotype. You can follow guidance from the antibody manufacturer as a starting point, but we recommend running a small pilot experiment ahead to optimize the staining protocol for your particular screen. Can also prepare additional stained controls here as appropriate for your FACS experiment.

19. Incubate on a tube rotator at room temperature for 1 hour, protected from light.

Part 3: Phenotypic enrichment via fluorescence-activated cytometric sorting (FACS)

20. After incubation has completed, spin down at 850 x g for 5 minutes at 4 °C and remove supernatant.
21. Resuspend in 8 mL PBS-T. Count cells and repeat spindown.
22. Repeat wash step.

23. Calculate volume of sorting and collection buffer to resuspend cells in to achieve a concentration ideal for sorting (instrument dependent, for our Sony SH800 we target 5-10 million cells/mL).

Reagent	For 10 mL supplemented buffer
15 BSA in 1x PBS Buffer	10 mL
RNAse Inhibitor	33.3 µL

24. Resuspend pellet in a volume of supplemented sorting buffer prepared in the previous step.
25. Prepare 2 x 1.5 mL sample collection tubes with 500 uL supplemented sorting buffer each.
    Adjust this if you are planning to sort a smaller or larger number of fractions.
    a. May need to prepare additional sample collection tubes as sort proceeds– aim to have cells sitting at room temperature for no more than 1 hour. After that, prepare more collection tubes with freshly supplemented sorting buffer.
26. Perform FACS to isolate cells exhibiting the phenotype targeted by your screen. Aim to recover a minimum of 100,000 cells per fraction.
    a. 10x Genomics recommends 300k cells as input into each hybridization reaction, with a minimum of 25k and a maximum of 500k.
27. *OPTIONALLY* - at this step, if desired, hashing (to compare multiple sorted flow populations from a single experiment, for example), and or staining with oligonucleotide-conjugated antibodies to capture protein data along with the gene-expression modality can be performed. If so, from this point follow Protocol 2 beginning at Part 2.

Part 4: 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 5: 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 6: 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
	72 °C	1 min

1 cycle	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.