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Mapping Enhancer–Gene Interactions with Direct-Capture Targeted Perturb-seq (DC-TAP-seq)

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We use this protocol and it's working

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Abstract

Mapping enhancer-gene (E-G) connections is critical for dissecting the functional impact of noncoding variants in the human genome. Single-cell CRISPRi screens offer a powerful approach to identify these regulatory interactions, but existing methods such as Perturb-seq are limited by high sequencing costs and inefficient guide RNA capture across diverse cell types. Here, we describe a detailed protocol for Direct-Capture Targeted Perturb-seq (DC-TAP-seq) — a cost-effective and scalable approach that integrates TAP-seq¹ with direct capture of Pol III-derived guide RNAs² to sensitively screen thousands of E-G connections across the genome. This protocol describes pooled cloning of gRNAs into an sgOpti-CS vector, high MOI lentiviral transduction, cell preparation for single-cell RNA-seq, and targeted amplification of both transcripts and guide RNAs. DC-TAP-seq enables large-scale mapping of E-G interactions with reduced cost and improved sensitivity, and is broadly applicable for studying E-G regulation in diverse human cell types.

Overview

- 1 The complete end-to-end tool kit for DC-TAP-seq integrates computational design, an optimized experimental protocol, and analysis strategies to identify and quantify the effect sizes of significant E-G regulatory interactions. The design step includes (i) selecting potential enhancer elements to perturb; (ii) designing guide RNAs using the CRISPRDesigner pipeline; (iii) selecting candidate target genes near the perturbation sites; and (iv) designing inner and outer primers for targeted amplification of those genes. In this protocol, we focus specifically on the optimized step-by-step experimental workflow in K562 that follows the upstream computational design.

Materials

- 2 **Guide pool cloning**
 - sgOpti-CS vector
 - BsmBI-v2 (NEB)
 - ClaI (NEB)
 - Ampure XP beads
 - 2x NEBNext Master mix (NEB)
 - PCR1 primers (from here)
 - Fwd_primer_A1 = GTCCTTGCCAATGAACGGGG
 - Rev_primer_E1 = TTCAAGACGGGCGTGA CTGC
 - PCR2 primers
 - GuideAmp Fwd = GGCTTTATATATCTTGTGGAAAGGACGAAACACCG
 - GuideAmp Rev = CTAGCCTTATTTAACTTGCTATGCTGTTTCCAGCTTAGCTCTTAAAC
 - NEB Gibson Assembly 2X Master Mix (NEB)
 - Lucigen Endura ElectroCompetent Cells (Biosearch Technologies)
 - Endo free plasmid midi kit (Qiagen)
 - 2x Q5 Hot Start Master Mix (NEB)
 - PCR primers for sequencing library prep
 - Seq997_cs-A* primer (8 bp index) = AATGATACGGCGACCACCGAGATCTACAC[8bp-index]CGATTTCTTGGCTTTATATATCTTGTG
 - Seq998_cs* primer (4 bp index) = CAAGCAGAAGACGGCATACGAGAT[4bp-index]GCTAGGACCGGCCTTAAAGC
 - Custom sequencing primers:
 - Seq999_hU6_R1 = CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG
 - CS_I1_Updated_Seq_Primer = TATCAACTTGGCCGCTTTAAGGCCGGTCCTAGC

Lentivirus infection and scRNA library preparation

- Polybrene (Sigma)
- psPAX2 (EL1188) (1006 ng/ul)



- pMD2.g (EL1189) (1069 ng/ul)
- GFP Clonetester plasmid (1459 ng/μL)
- ENCODE K562 sgOpti-CS pool (113.3 ng/μL x 500 μL =56μg)
- Puromycin
- NucleoSpin-Blood-L-XL-QuickPure kit (Macharey Nagel)
- HindIII-HF (NEB)
- ddPCR primers and probes:
- hALB_F: GCTGTCATCTCTTGTGGGCTGT
- hALB_R: ACTCATGGGAGCTGCTGGTTC
- gRNA_scaffold_F: AGAGCTAAGCTGGAAACAGC
- gRNA_scaffold_R: GTGCCACTTGGCCTTGCTAG
- hALB (M12523.1) Probe: CCTGTCATGCCACACAAATCTCTCC (5'VIC/3'QSY)
- gRNA scaffold Probe: AGGCTAGTCCGTTATCAACTTGGCCG (5'FAM/3'QSY)
- Chromium Next GEM Single Cell 3' GEM Kit v3.1 PN-1000123
- Chromium Next GEM Single Cell 3' Gel Bead Kit v3.1 PN-1000122
- 3' Feature Barcode Kit PN-1000262
- Chromium Next GEM Chip G Single Cell Kit PN-1000120
- Dual Index Kit NT Set A PN-1000242
- KAPA HiFi HotStart ReadyMix PCR Kit (Roche Cat #07958935001)
- DC-TAP seq outer and inner primers
- DC-TAP-seq PCR3 primers
- P5_Truseq-R1_X (10 bp index) =
AATGATACGGCGACCAACCGAGATCTACAC[10bp-index]ACACTCTTTCCCTACACGACGCTCTTCCG
- N7_XXX (8 bp index) =
CAAGCAGAAGACGGCATACGAGAT[8bp-index]GTCTCGTGGGCTCGG

Step 1: Guide pool cloning

3 Restriction digest of plasmid backbone

1. Digest sgOpti-CS plasmid (add enzyme last):

A	B
NEB Buffer 3.1 (10X)	10 μL
20 μg plasmid (450.3 ng/μL)	44.4 μL
BsmBI-v2	4 μL
Water	41.6 μL
Total	100 μL



2. Incubate at 55°C overnight (16 h).
3. Next morning, spike in 4 µL BsmBI-v2 to the tube and continue incubating at 55°C for several more hours. Total 23 h.

4 **0.7X SPRI (Ampure XP) clean up**

1. Pre-warm beads from 4°C to room temperature.
2. Add 70 µL SPRI to the tube, pipette to mix, and incubate for 10 min.
3. Wash with 150 µL 70% ethanol twice.
4. Remove ethanol and air dry for 10 min.
5. Add 89 µL water and heat 37°C for 10 min.
6. Put on magnet and transfer eluate (89 µL) to a new tube.
7. Store at -20°C

5 **PCR1: Amplify Subpools**

N.B: Multiple subpools were ordered in one large oligo pool. So, PCR1 was used to amplify the desired oligo subpool (220228_K562_Random_Screen_Crop) from the large pool.

Preparation of oligo pool solution:

1. Oligo pool from Agilent: 10 pmol of linear, unamplified, pooled oligos. For 114 bp length oligos, 10 pmol = 0.75 µg.
2. Dissolve this in 75 µL water to make 10 ng/µL stock solution.
3. Dilute to make an aliquot of 1 ng/µL pool: 2 µL + 18 µL water.

PCR:

1. Make PCR mix in the following way. Use Fwd_primer_A1 and Rev_primer_E1 primers to dial out the 220228_K562_Random_Screen_Crop oligo subpool.

A	B
2× NEBNext Master Mix	30 µL
Fwd Primer (10 µM)	3 µL
Rev Primer (10 µM)	3 µL
Diluted Oligo Pool (1 ng/µL)	2 µL
Water	22 µL
Total	60 µL

2. Thermocycling

A	B	C
98°C	30 sec	



	A	B	C
	98°C	15 sec	20 cycles
	62°C	15 sec	
	72°C	15 sec	
	72°C	120 sec	
	4°C	Hold	

1X SPRI (Ampure XP) clean up

1. Pre-warm beads from 4°C to room temperature.
2. Add 60 µL SPRI to the tube, pipette to mix, and incubate for 2 min.
3. Wash with 150 µL 70% ethanol twice.
4. Remove ethanol and air dry for 5-10 min.
5. Add 50 µL water and let DNA elute for 2 min.
6. Put on magnet and transfer eluate to a new tube.
7. Run 5 µL cleaned product on a gel. Expected band size = 114-115 bp.
8. Qubit the sample. Concentration = 3.92 ng/ul.
9. Store samples at -20°C. Proceed to PCR2.

6 PCR2: Add Gibson Arms to Subpool

1. Make PCR mix in the following way. Use PCR2 primers GuideAmp-Fwd and GuideAmp-Rev.

	A	B
	2× NEBNext Master Mix	30 µL
	Fwd primer (10 µM)	3 µL
	Rev primer (10 µM)	3 µL
	PCR1 product (7 ng)	1.79 µL
	Water	22.21 µL
	Total	60 µL

2. Thermocycling:

	A	B	C
	98°C	30 sec	



	A	B	C
	98° C	15 sec	3 cycles
	62° C	15 sec	
	72° C	15 sec	
	98° C	10 sec	9 cycles
	72° C	15 sec	
	72° C	5 sec	
	72° C	120 sec	
	4°C	Hold	

Ampure XP clean up

1. Add 90 µL SPRI (1.5X) to the tube, pipette to mix, and incubate for 2 min.
2. Wash with 150 µL 70% ethanol twice.
3. Remove ethanol and air dry for 5-10 min.
4. Add 30 µL water and let DNA elute for 2 min.
5. Put on magnet and transfer eluate to a new tube.
6. Run 5 µL cleaned product on a gel. Expected band size = 103 bp.
7. Qubit the sample. Concentration = 7.45 ng/ul.

7 Gibson Assembly

1. Make Gibson mix in the following way

	A	B	C
	Reagents	Pool	Backbone only control
	NEB Gibson Assembly 2X Master Mix	22.7 µL	18.6 µL
	Digested sgOpti- CS backbone (45.9 ng/µL)	13.3 µL	10.9 µL
	PCR2 product (7.45 ng/µL)	9.4 µL	0



	A	B	C
	water	0	7.7 μ L
	Total	45.4 μ L	37.2 μ L

2. Incubate at 50°C for 1 hr in the thermocycler.

Purify assembled plasmid with 0.7X SPRI (Ampure XP)

1. Pre-warm beads from 4°C to room temperature.
2. Add 0.7x SPRI (e.g. 31.8 μ l for the sample tube), pipette to mix, and incubate for 1 min.
3. Wash with 150 μ L 70% ethanol x 3.
4. Remove ethanol and air dry for 10 min.
5. Add 15 μ L water and heat 37°C for 10 min.
6. Put on magnet and transfer eluate to a new tube.
7. Store at -20°C.

8 Electroporation

1. Electroporate 12 μ L into Lucigen Endura Competent Cells. Use cleaned Gibson product into Lucigen Endura ElectroCompetent Cells using the BioRad Xcell Electroporator with the settings 10 μ F, 600 Ohms, 1800 volts, exponential wave.
2. Transfer to 1 mL LB recovery media (no antibiotic) and grow for 1 hour at 30°C.

9 Expand bacteria and estimate transformed colonies

1. Take 10 μ L (1% of total) to estimate the number of transformed colonies by plating a serial dilution of transformation mixture (Do this for both the backbone-only control, and the desired pool).
 - Dilute into 90 μ L LB in a 96-well plate. Continue for 6 dilutions (i.e. 1:1K, 1:10K, 1:100K, 1:1M, 1:10M, 1:100M from electroporation).
 - Streak 10 μ L from each dilution on a Carb plate.
 - Grow overnight.
 - Quantify coverage by counting a dilution with distinguishable colonies and multiplying by that dilution value (e.g. 20 colonies in the 1:100K dilution means the full electroporation has 2M colonies. In general, aiming for >1000-fold coverage of the library).
 - Quantify background by counting a dilution of the backbone-only-control with distinguishable colonies and multiplying by that dilution value (e.g. 15 colonies in the 1:10K dilution means the full electroporation has 150K empty colonies).
2. From the rest of the 1 mL culture (in recovery media), inoculate a 50mL LB+Carb for midiprep, and grow for 18h at 30°C.

10 Plasmid preparation and QC

1. Take 4 mL out of culture and spin down for miniprep.
2. For the rest, do Midiprep with Qiagen EndoFree Midi Kit. Can freeze pellets for up to one week at -20°C or one month at -80°C.

**3. Digest the plasmid to check insertion (Add enzyme last)**

A	B
NEB Buffer 3.1 (10X)	2 μ L
500 ng plasmid	1 μ L
BsmBI-v2	0.5 μ L
Water	16.5 μ L
Total	20 μ L

4. Incubate at 55°C incubation for >1 h (incubated overnight). Verify on gel that no undigested vector exists.

11 Library preparation for sequencing

1. Make PCR mix. Use Fwd primer Seq997_cs-A* and Rev primer Seq998_cs* .

A	B
2× Q5 Hot Start Master Mix	25 μ L
Fwd primer (10 μ M)	2.5 μ L
Rev primer (10 μ M)	2.5 μ L
Diluted plasmid pool (1 ng/ μ L)	1 μ L
Water	19 μ L
Total	50 μ L

2. Thermocycling

A	B	C
98° C	30 sec	
98° C	15 sec	4 cycles
64° C	15 sec	
72° C	16 sec	



	A	B	C
	98° C	15 sec	16 cycles
	72° C	5 sec	
	72° C	15 sec	
	72° C	120 sec	
	4°C	Hold	

Purification

1. Clean with 1X SPRI beads, elute in 40µL H₂O.
2. Clean again with 1X SPRI beads, elute in 20µL H₂O.
3. Measure library concentration with Qubit. Concentration = 7.16 ng/ul .
4. Check on 2% gel or a Tapestation. Expected PCR product = 215 bp.
5. The plasmid pool was named ENCODE K562 sgOpti-CS pool.

12 Sequencing

Use custom primers Seq999_hU6_R1 (Read 1) and CS_I1_Updated_Seq_Primer (Index 1)
Sequencing strategy (MiSeq):

- Read 1: 25 nucleotides (reads the guide spacer)
- Index 1: 4 nucleotides
- Index 2: 8 nucleotides

Step 2. Lentivirus generation with guide pool**13 Day 0. Plate HEK293Ts**

Seeded 6-well plate (550K cells per well) with HEK293Ts in DMEM + 10% FBS (no P/S) making sure cells are at optimal concentration (70-90%).

Day 1. Transfect Cells

1. Confirm cells are 70-90% confluent.
2. (~1 hour before starting) Warm Opti-MEM (OMEM) and XtremeGENE to RT.
3. Remove media from cells and add 1.8 mL fresh media.
4. Briefly vortex XtremeGENE.
5. Combine OMEM and XtremeGENE as per the table below. Add XtremeGENE directly into OMEM (not against the side of the tube). Mix well by flicking. Incubate for 2-5 min RT.
6. In a separate tube prepare DNA mixes (psPAX2, pMD2.g, and transfer plasmid) according to the table below. For the transfer plasmid, use the ENCODE K562 sgOpti-



CS pool (113.3 ng/ul) for 5 wells or GFP clonetester plasmid (1459 ng/ul) for 1 well of a 6-well plate.

	A	B
	Reagent	Per well of a 6-well plate
	Media volume	2 mL
	OMEM	192 μ L
	XtremeGENE	5.8 μ L
	psPAX2	900 ng
	pMD2.g	360 ng
	Transfer plasmid	1200 ng

7. Add the OMEM/XtremeGENE mix to the respective tubes with the plasmid DNA. Flick to

mix. DO NOT VORTEX.

8. Incubate 15 min at RT. Spin briefly.

9. Add 198 μ L transfection complex slowly and dropwise to cells.

10. Rock plate back-and-forth and side-to-side to mix. Do not swirl.

11. Return plate to incubator.

Day 2. Check cells

In 24 h, verify the GFP samples are fluorescent.

Day 3. Harvest virus

1. Pool each library and mix well.

2. Filter virus through syringe filter into 15 ml tubes.

3. Measure titer as below.

4. Aliquot virus into labeled and dated cryotubes. Virus to be used within the next 2 weeks should be stored at 4°C. Virus to be used beyond a week or two should be stored at -80°C. Aliquot whenever possible since multiple freeze/thaw affects viral titer.

Lenti-X GoStix

1. Download Lenti-X GoStix App on your smartphone. Scan (or enter) the lot number of the Lenti-X GoStix Plus strip. Enter your sample information. The below steps can also be followed on the app itself.

2. To spot check titer on Lenti-X GoStix Plus strip:

- Take 20 μ L media of one virus well and add onto lentiX titer strip.
- Add 80 μ L of lentiX chase buffer.



- c. Incubate for 10 min at RT.
- d. Scan the GoStix cassette with Lenti-X GoStix App. This will give you a quantitative titer.
- e. Qualitatively, 2 equally bright bands indicate a great virus (very roughly 1M infectious particles per mL). 1 band indicates very little or no virus (<100K infectious particles /mL).

Day 4. Additional harvest (optional)

Filter virus through syringe filter into labeled and date the tube and store accordingly.

Step 3: High MOI lentiviral infection of K562 cells**14 Infection and selection**

N.B. 1M BFP+ K562 cells (top 70% of BFP distribution) were sorted beforehand and passed for a few rounds prior to infection.

Day 1. Infection

1. Thaw virus on ice for a few hours.
2. Warm centrifuge to 37°C (this is done faster by setting the temperature and spinning at max speed).
3. Prepare cell (total cells needed 100M).
4. Add 8 µg/mL polybrene to the cells to help infection efficiency. Total volume needed: 120ml media + 96µl polybrene (stock = 10µg/µl).
5. Spin down 100M cells.
6. Resuspend at 1M/ml with 100ml polybrene media.
7. Aliquot the below volumes into two tubes: No Virus, MOI10 (1.5ml), MOI5 (3ml), MOI2 (7.5ml), MOI1 (37.5ml).
8. Add 300µl or 600µl of virus into each pair of tubes.
9. Plate 1 ml into each well of a 12-well plate. N.B. The number of final cells to be used for infection depends on the number of guides, coverage and the desired MOI. In our experiment we were targeting a ~1000x coverage for MOI 5 for a pool of ~15,000 guides.
10. Wrap the plate in a giant kimwipe and place in the specialized plate holders (with the airtight lids) in the centrifuge. This step is to protect you if anything happens during the spin. You don't want virus leaking everywhere.
11. Spin the cells + virus at 1200g for 40 minutes at 37°C.
12. Transfer the plate to an incubator and let the cells recover overnight.

Day 2. Puromycin Selection

Add puromycin 24 hrs after the spinfection. Because the media volumes will be different due to adding higher volumes of virus, we did spin+resuspended the cells instead of spiking in the puromycin.

1. Prepare 1 µg/mL puromycin media: 240 ml media + 24 µl puro (10 mg/mL).
2. Spin the cells and resuspend in 1 ml puromycin media.

Day 3-5. Continuing Puromycin selection:

Check the cells visually for death % and keep incubating cells in puromycin media.

Day 6. Moved to low puromycin media (0.3µg/ml puromycin)

Day 8-15. Keep on passing the cells in low puromycin media.

Check MOI with digital droplet PCR (ddPCR)

gDNA purification/quantification:

Follow manufacturer's instructions for preparing buffers. Preheat Elution Buffer BE to 70°C in incubator or water bath before experiment. Thaw Proteinase K.

1. Prepare 1M K562 cells in 200 µl PBS in 1.5 mL tubes. Use 2 samples of uninfected K562 Ci cells for control.
2. Add 25 µl **Proteinase K**.
3. Add 200 µl **Buffer B3** to the samples and vortex vigorously (10-20s). Note: Vigorous mixing is important to obtain high yield and purity of DNA.
4. Incubate samples at 70°C for 10-15 min.
5. Add **210 µL ethanol** (96 – 100%) to each sample and vortex again.
6. For each preparation, take one NucleoSpin Blood Column placed in a collection Tube and load the sample.
7. Bind DNA: Centrifuge 1 min at 11,000g. If the samples are not drawn through the matrix completely, repeat the centrifugation at higher g-force (< 15,000g).
8. Discard Collection Tube with flow-through.
9. 1st wash: Place the NucleoSpin Blood Column into a new Collection Tube (2 mL) and add 500 µl **Buffer BW**. Centrifuge 1 min at 11,000g and discard Collection Tube with the flow-through.
10. 2nd wash: Place the NucleoSpin Blood Column into a new Collection Tube (2 mL) and add 600 µl **Buffer B5**. Centrifuge 1 min at 11,000g. Discard flow-through and reuse collection Tube.
11. Place the NucleoSpin Blood Column back into the Collection Tube and centrifuge 1 min at 11,000g. Residual ethanol is removed during this step.
12. Place the NucleoSpin Blood Column in a 1.5 mL microcentrifuge tube and add 100 µL preheated **Buffer BE** (70°C). Dispense buffer directly onto the silica membrane.
13. Incubate at room temperature for 1 min. Centrifuge 1 min at 11,000g.
14. Quantify samples using Qubit dsDNA HS assay (Quantitation Range 0.1 - 120 ng).
15. Dilute gDNA to 500 ng / 44 µl = 11.36 ng/µl concentration.

Nucleic acid digestion:

Digest purified gDNA (500 ng) with HindIIIHF (NEB) enzyme with 10–20 units in a total reaction mixture of 50 µl at 37°C for 1 hr. HindIIIHF stock is 20,000 units/ml.

Make a master mix on ice.



A	B
gDNA (11.36 ng/μl)	44 μL (500 ng)
Water	0
10X rCutSmart Buffer	5 μL
HindIIIHF	1 μL (0.4 U/μL)
Total	20 μL

1. Aliquot 6 μl master mix into strip tubes and 44 μl of each gDNA.
2. Incubate at 37°C for 1 hr.
3. Heat kill at 80°C for 20 min.
4. Add 150 μL water to dilute gDNA samples 1:20 to obtain concentrations of 2.5 ng/μL.

Multiplex ddPCR:

1. Make 50 μl primer mix at a final concentration of 22.4 μM. Combine 11.2 μl of each primer (100 μM stock) + 5.2 μl H₂O. Use primers hALB_F, hALB_R, gRNA_scaffold_F, gRNA_scaffold_R.
2. Make 32 μl TaqMan probe mix at a final concentration of 6.25 μM. Combine 2 μl of both probes (100 μM stock) + 28 μl H₂O. Wrap the tube in aluminum foil. Use probes hALB (M12523.1), gRNA scaffold Probe.
3. Thaw and equilibrate reaction components to RT. Vortex ddPCR Supermix thoroughly to ensure homogeneity, as a concentration gradient may form during -20°C storage. Centrifuge briefly.
4. Prepare ddPCR master mix:

A	B
Primers (22.4 μM)	1 μL
TaqMan Probes (6.25 μM)	1 μL
gDNA (2.5 ng/μl)	10 μL (25 ng)
2X ddPCR Supermix	12.5 μL
Water	0.5 μL
Total	25 μL

Aliquot 15 μl master mix in strip tubes and add 10 μl gDNA. Mix by vortexing in short pulses, and centrifuge briefly.

**Droplet generation on QX100:**

1. Transfer 20 µl of samples into the sample wells using multi-channel.
2. Put 700 µl Droplet Generation Oil for Probes into a reservoir. Using a multichannel, dispense 70 µl oil in the bottom row of the cartridge. Use 50% glycerol for empty wells as the chip will not run dry.
3. Hook the gasket over the cartridge holder using the holes on both sides.
4. Place the cartridge holder into the QX100 droplet generator and initiate droplet generation.

PCR amplification

1. Pipet 40 µl of the contents of the top wells (the droplets) into a 96-well plate slowly
2. Cover the 96-well plate with a tip box lid and seal the plate.
3. Transfer the sealed 96-well plate to a thermal cycler.
4. Perform PCR within 30 min after completing droplet generation using a UNO96 thermal cycler with the following cycling conditions (ramp rate should be 2°C at every step).

	A	B	C
	95°C	Hold	
	95°C	10 min	
	94°C	30 sec	40 cycles
	60°C	60 sec	
	98°C	10 min	
	4°C	Hold	

5. After thermal cycling, store the plate at 4°C overnight in the dark.
6. Next day, secure the PCR plate containing the droplets in the plate reader holder of QX200 droplet reader (Bio-Rad).
7. Analyze the data in the QuantSoft software. Use Reference Copies = 3 (for triploid genome of K562).

Step 4. Single-cell RNA library preparation**15 Part 0 - Prepare cells**

1. Treat MOI5 cells with 1 µg/ml dox for 48 hrs in 0.3 µg/ml puromycin media in the following way:
 - MOI 5: 10M / 25 ml in T75 flask (1.16×10^6 cells/ml (97%)).
2. On the day of harvest:
 - Check BFP for CRISPRi expression and scatter plot distribution for cell viability using flow cytometry. Take pictures of cells with and without BFP.



- Freeze 1M cells / vial x 5 vials or use fresh cells downstream.

Part 1 - Harvest cells

1. Prepare 1.5M MOI5 cells in a 15 ml tube.
2. For frozen cells, thaw 1 vial of MOI5 cells and add to 5 ml media in a 15 ml tube. For the fresh sample we directly used the cells from culture.
3. Centrifuge cells at 200g for 3 min at RT.
4. Remove supernatant without disrupting the cell pellet.
5. Using a wide-bore pipette tip, add 1 ml PBS with 0.04% BSA to the tube. Gently pipette mix 5 times to resuspend the cell pellet.
6. Centrifuge cells at 200g for 3 min.
7. Remove supernatant without disrupting the cell pellet.
8. Add 1 ml of PBS with 0.04% BSA to MOI5 cells to achieve cell concentration of 700-1200 cells/ μ l. Gently pipette mix 10 – 15 times or until the cells are completely suspended.
9. Use a 70 μ m Flowmi™ Tip Strainer to remove any remaining cell debris and large clumps.
10. For fresh cells, wash them like the frozen cells in PBS with 0.04% BSA.
11. Determine the final cell concentration. 900-975 cell/ μ l.
12. Place the cells on ice and immediately proceed to 10x Genomics Single Cell Protocol.

Part 2 - 10X loading and library preparation

Part 2.1. GEM Generation & Barcoding

Follow step 1 of the **10X Genomics protocol**. Load ~16,500 cells onto each lane.

Part 2.2. Post GEM RT Cleanup

Follow only step 2.1 from the 10X protocol. The final volume of the purified material will be 35 μ l.

Part 2.3. TAP-seq PCRs

PCR1 (all-in-one)

1. Thaw outer primers, which have already been pooled.
2. Make a 10 μ M dilution of Partial Read 1 primer (Truseq_Partial-R1_Fw).
3. Make a 40 μ M dilution of Partial Read 1N primer (Partial_Read_1N).
4. Make the mastermix for PCR1 in the following way (on ice):

A	B
Sample RT Cleanup	35 μ L
10 μ M Partial Read 1	4 μ L
100 μ M ENCODE outer primer mix	2.5 μ L
40 μ M Partial Read 1N	1 μ L



A	B
100 μ M Partial TSO	1.2 μ L
KAPA HiFi	50 μ L
Water	6.3 μ L
Total	100 μ L

5. Add 65 μ L mastermix into each of the 35 μ L RT Cleanup from Step 2.

6. Run the PCR1 program below.

A	B	C
95°C	3 min	
98°C	20 sec	12 cycles
67°C	60 sec	
72°C	60 sec	
72°C	5 min	
4°C	Hold	

SPRI cleanup

1. Add 65 μ L SPRI beads (0.65x) to the reaction and pipette mix 15x (pipette set to 150 μ L).
2. Incubate at room temperature for 5 min.
3. Place the tube on a 10x magnet (high) until the solution clears. Transfer 75 μ L of the supernatant (<300bp fraction) into a new tube without disturbing the pellet (store the remaining 90 μ L in a separate tube). Set aside this supernatant at room temperature for gRNA cleanup below.
4. For mRNA amplicon (pellet)
 - Add 200 μ L 80% ethanol to the pellet. Wait 30 sec.
 - Remove the ethanol and repeat the wash step one more time.
 - Centrifuge briefly and place on magnet (low). Remove trace ethanol.
 - Air dry for 2 min (do not exceed 2 min as this will decrease elution efficiency).
 - Remove from the magnet. Add 40.5 μ L Buffer EB. Pipette mix 15x.
 - Incubate 2 min at room temperature. Place the tube on magnet (high) until the solution clears.
 - Transfer 40 μ L to a fresh tube.
 - Measure concentration by Qubit dsDNA HS assay (14-81 ng/ μ L).
 - Store at 4°C for up to 72 h or at -20°C for up to 4 weeks, or proceed to PCR2.
5. For gRNA amplicon (supernatant)



- Add 25 μL of SPRI beads (1.2x) to the supernatant from step 10 and pipette mix 15x (pipette set to 80 μL).
- Incubate at room temperature for 5 min.
- Place the tube on magnet (high) until solution clears.
- Discard supernatant.
- Add 300 μL 80% ethanol to the pellet. Wait 30 sec.
- Remove the ethanol and repeat the wash step one more time.
- Centrifuge briefly and place on magnet (low). Remove trace ethanol.
- Air dry for 2 min (do not exceed 2 min as this will decrease elution efficiency).
- Remove from the magnet. Add 41 μL Buffer EB. Pipette mix 15x.
- Incubate 2 min at room temperature. Place the tube on magnet (high) until the solution clears.
- Transfer 40 μL to a fresh tube.
- Store at 4°C for up to 72 h or at -20°C for up to 4 weeks, or proceed to Part 2.4. CRISPR Screening Library Construction below.

PCR2 (mRNA)

1. Thaw inner primers, which have already been pooled.
2. Dilute all PCR1 products to 1 ng/ μL .
3. Make the mix for PCR2 in the following way (on ice):

	A	B
	mRNA PCR1 Cleanup	10 μL (10 ng)
	10 μM Partial Read 1	4 μL
	100 μM ENCODE inner primer mix	2.5 μL
	KAPA HiFi	50 μL
	Water	33.5 μL
	Total	100 μL

4. Aliquot 90 μL . Add 10 μL of respective DNA (at 10 ng).
5. Run PCR2 with the following program:

A	B	C
95°C	3 min	
98°C	20 sec	8 or 11 cycles
67°C	60 sec	
72°C	60 sec	



	A	B	C
	72°C	5 min	
	4°C	Hold	

SPRI cleanup

1. Cleanup by adding 150 μ L (1.5x) SPRI beads to the reaction and pipette mix 15x (pipette set to 200 μ L).
2. Incubate at room temperature for 5 min. Place the tube on a magnet (high) until solution clears and remove the supernatant.
3. Add 200 μ L 80% ethanol to the pellet. Wait 30 sec.
4. Remove the ethanol and repeat the wash step one more time.
5. Spin down the beads, place on magnet (low) and remove traces of ethanol.
6. Air dry for 2 min (do not exceed 2 min as this will decrease elution efficiency).
7. Remove from the magnet. Add 31 μ L Buffer EB. Pipette mix 15x.
8. Incubate at room temp for 2 min. Place the tube on magnet (low) until solution clears.
9. Transfer 30 μ L to a fresh tube.
10. Measure concentration by Qubit dsDNA HS assay (0.4-1.3 ng/ μ L).
11. Store samples at -20°C overnight or proceed with PCR3.

PCR3 (mRNA)

1. Dilute PCR2 samples to 0.5 ng/ μ L.
2. Make the PCR3 mix in the following way (on ice). Add a unique 2.5 μ L N7_XXX primer into each tube. Record primer barcodes.

A	B
mRNA PCR2 Cleanup	20 μ L (10 ng)
10 μ M P5_Truseq-R1_X	4 μ L
10 μ M N7_XXX	2.5 μ L
KAPA HiFi	50 μ L
Water	23.5 μ L
Total	100 μ L

3. Aliquot 77.5 μ L mastermix. Add 20 μ L of respective DNA (at 10 ng) and 2.5 μ L of the respective N7_XXX primer. Note down the BCs.
4. Run PCR3 with the following program:

A	B	C
95°C	3 min	



	A	B	C
	98°C	20 sec	8 cycles
	60°C	15 sec	
	72°C	45 sec	
	72°C	5 min	
	4°C	Hold	

SPRI cleanup

1. Cleanup by adding 150 µl (1.5x) SPRI beads to the reaction and pipette mix 15x (pipette set to 200 µl).
2. Incubate at room temperature for 5 min. Place the tube on a magnet (high) until solution clears and remove the supernatant.
3. Add 200 µl 80% ethanol to the pellet. Wait 30 sec.
4. Remove the ethanol and repeat the wash step one more time.
5. Spin down the beads, place on magnet (low) and remove traces of ethanol.
6. Air dry for 2 min (do not exceed 2 min as this will decrease elution efficiency).
7. Remove from the magnet. Add 30.5 µl Buffer EB. Pipette mix 15x.
8. Incubate at room temp for 2 min. Place the tube on magnet (low) until solution clears.
9. Transfer 30 µl to a fresh tube.
10. Measure concentration by Qubit dsDNA HS assay (1.6-4 ng/ul).
11. Store samples at -20°C.

Part 2.4. CRISPR Screening Library Construction

1. Follow the entire Step 4 according to the 10X protocol.
2. After making sure that the BioA traces look clean, measure concentration by Qubit dsDNA HS assay.
3. Download CSV of PN-3000483 Dual Index Plate NT Set A and ensure that the chosen wells' i7 indices are compatible with the N7_XXX barcodes of mRNA libraries.

Part 2.5. Bioanalyzer

1. Dilute gRNA samples 1:50. Dilute mRNA samples 1:4.
2. Run 1 µl on BioA. gRNA should be 306 bp.

Part 2.6. Sequencing

Read 1 (28 cycles), Read 2 (90 cycles) and Sample index i7 (8 cycles).



Protocol references

1. Schraivogel, D., Gschwind, A.R., Milbank, J.H. et al. Targeted Perturb-seq enables genome-scale genetic screens in single cells. *Nat Methods* 17, 629–635 (2020).
2. Replogle, J.M., Norman, T.M., Xu, A. et al. Combinatorial single-cell CRISPR screens by direct guide RNA capture and targeted sequencing. *Nat Biotechnol* 38, 954–961 (2020).