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Experimental procedures for TF Perturb-Seq (UT Southwestern)

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We use this protocol and it's working

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Abstract

Optimized procedures and best practices for Perturb-Seq data production, developed at the UT Southwestern Characterization Center

Part 1: sgRNA plasmid library construction

1

1.1 Synthesize sgRNA oligo pool using **Twist Bioscience**

sgRNA sequences were derived from this study: Replogle JM, Bonnar JL, Pogson AN, Liem CR, Maier NK, Ding Y, Russell BJ, Wang X, Leng K, Guna A, Norman TM, Pak RA, Ramos DM, Ward ME, Gilbert LA, Kampmann M, Weissman JS, Jost M. Maximizing CRISPRi efficacy and accessibility with dual-sgRNA libraries and optimal effectors. Elife. 2022 Dec 28;11:e81856. doi: 10.7554/eLife.81856.

Lenti-Guide(10X) backbone:
GTGGAAAGGACGAAACACCGNNNNNNNNNNNNNNNNNNNgtttaagagctaagctggaaacagc

1.2 Resuspend sgRNA oligo pool with low EDTA TE buffer. [0.5 ng/ul. ~ 0.024uM]

1.3 Perform PCR to amplify double-stranded sgRNAs using **library-fwd** and **library-rev** primers to yield 139-bp fragments.

Primers (synthesized from IDT):
library-fwd: taacttgaaagtatttcgatttcttggctttatatatcttGTGGAAAGGACGAAACACCG
library-rev: gttgataacggactagccttatttaaacttgctatgctgtttccagcttagctcttaaac

Set up 4 tubes of 50 ul PCR reactions shown below (total 3.2 ng sgRNA oligos):
2.5 ul 10 uM **library-fwd** + **library-rev** primers
1.6 ul sgRNA oligo pool (0.5 ng/ul. ~ 0.024uM)
20.9 ul H2O
25 ul 2X KAPA HotStart Readymix (Roche, Cat. # 7958935001)

PCR program:
95C for 30s
5 cycles(98C for 20s, 59C for 15s, 72C for 10s)
5 cycles(98C for 20s, 65C for 15s, 72C for 10s)
72 for 60s
4 forever

Combine reactions and run 1.2% Agarose gel to purify 139 bp products with Qiagen MinElute Gel Extraction Kit. Purify with 2 MinElute Spin Columns and elute with 12 ul EB buffer for each column (Total 24 ul). Measure DNA concentration with Qubit kit.

1.4 Digest 15 ug LentiGuide(10X)-BFP-Puro (LW203) in 200 ul reaction volume with 15 ul NEB Esp3I for 3h and run 0.7% agarose gel to purify the vector.

Plasmid map of LentiGuide(10X)-BFP-Puro (LW203): **https://benchling.com/s/seq-cp12NBXnoIGQY6NSXY5b?m=slm-GTRPOQU0bCAenT5kHEgB**

Purify with three QIAquick Spin Columns (for DNA size between 70 bps to 10 kbs) and elute with 35 ul EB buffer for each column. Combine three elutions together(105 ul total). Measure DNA concentration with Qubit kit.

1.5 Assemble the double-stranded sgRNA library into the lentiviral backbone with the NEBuilder HiFi DNA Assembly(NEB, Cat. # E2621X).

Set up 2 tubes of 80 ul reactions shown below:

500 ng Esp3I digested LentiGuide(10X)-BFP-Puro
20 ng sgRNA PCR library
Add water to 50 ul
Add 50 ul NEB HIFI DNA assembly Master Mix

Incubate at 50 degrees for 60 min.

Purify assembled DNA with 0.8X SPRIselect beads(Beckman Coulter, Cat. # B23318). Pipette mix 160 ul (0.8X) SPRIselect beads with 200 ul PCR reactions. Incubate at room temperature for 5 min. Keep the tube on the magnet until the supernatant is clear. Wash SPRIselect beads three times with 500 ul 80% ethanol. Elute in 20 ul EB buffer. Measure DNA concentration with Qubit kit.

1.6 Electroporation with Lucigen Endura Duo competent cells.

Electroporate 25 ul E.coli + 2 ul ligation DNA (>50 ng) with 0.1 cm cuvette with BioRAD MicroPulser Electroporator. Add 2 ml SOC recovery media, transfer the E.coli to a 14 ml tube. Electroporate another 25 ul E.coli + 2 ul ligation DNA (>50 ng) with 0.1 cm cuvette with BioRAD MicroPulser Electroporator. Add 2 ml SOC recovery media, transfer the E.coli to a 14 ml tube.

Culture at 37 degree shaker for 1 h.

Spread 0.05, 0.01, 0.0005, 0.0005 ul Ecoli containing media together with 50 ul LB to Agarose plates and culture at 37 degree overnight. The next day, count the plated E.coli colonies to ensure that there are >1000 clones per sgRNA in 2 ml of culture.

Transfer 4 ml of E.coli culture (stored at 4 degree O/N) to a flask with 300 ml fresh LB + 75 ug/ml ampicillin and culture at 37 degree shaker for 16 hours. Pellet the E.coli. Extract plasmids with Zymo Midiprep kit. Elute plasmids with 250 ul + 200 ul elution buffer. Measure DNA concentration.

Part 2: Bulk sequencing of sgRNAs

2 **Goal**: This section describes procedures to construct next-generation sequencing libraries of sgRNAs, either from the plasmid library described in Part 1 or from genomic DNA where sgRNAs have been integrated into cells. Typically, we sequence sgRNAs from 3 samples:

a. the plasmid library from Part 1;
b. genomic DNA from iPSCs/hESCs before differentiation;
c. genomic DNA from differentiating cells, ~5 days before harvest

2.1 For genomic DNA samples, prepare genomic DNA from >2M cells (or 1000X sgRNA number) using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Cat. # 69504).

2.2 Prepare PCR amplicon for NGS bulk sequencing (v2 with 10X TT index):
Primers needed:
Fwd-v2: ACACTCTTTCCCTACACGACGCTCTTCCGATCTgaaagtatttcgatttcttggct
Rev : GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTaagttgataacggactagcc
10X TT set A index or IDT-synthesized TT set A index (A1 ~ A10)

Map of sgRNA PCR amplicon v2 Benchling file: **https://benchling.com/s/seq-ev18tEY07pX59CNZ075A?m=slm-2MGRsOieiH7dX2Ms3Ecn**

Prepare 1st round of PCR
Prepare master mix of 8 ~ 12 tubes of PCR reactions(100 ul per reaction) (calculate PCR reaction volume according to the size of sgRNA library. 6pg genomic DNA per human cell.)

5 ul 10 uM Fwd-v2 primer
5 ul 10 uM Rev primer
0.125 ug sgRNA plasmid library (or 0. 5 ug genomic DNA),add water to 40 ul.
50 ul 2X KAPA HIFI HS RM (Roche, Cat No.: 7958935001)

PCR protocol:
95ºC for 2 min,
21 cycles for genomic DNA or 8 cycles for plasmids (27 cycles for genomic DNA or 15 cycles for plasmids for direct Nanopore sequencing): 98ºC for 20 s, 55ºC for 20s ,72ºC for 20s
72ºC for 1 min
4ºC forever

Mix all 100 ul PCR reactions together. Take 100 ul and add 60 ul (0.6X) SPRIselect to remove plasmids. After 5 minute incubation, keep the tube on the magnet until the supernatant is clear. Transfer 150 ul supernatant to a new tube and add 60 ul SPRIselect. Incubate at room

temperature for 5 min. Keep the tube on the magnet until the supernatant is clear. Wash three times with 300 ul 80% ethanol. Elute PCR product in 40 ul EB buffer.

Prepare 2nd round PCR

Set up 2nd round master mix of 1 PCR reaction.
20 ul 10X plate TT set A primer (or **5u**l 10 uM IDT-synthesized TT set A 1 ~ 10 primers **+ 15 ul** H2O)
30 ul PCR1 elution
50 ul 2X KAPA HotStart ReadyMix

PCR protocol:
98ºC for 45s
8 cycles: 98ºC for 20s, 62ºC for 10s, 72ºC for 10s
72ºC 1 min
4ºC forever

Post PCR SPRIselect purification
Mix 70 ul(0.7X) SPRIselect with 100 PCR products to remove large fragments.
Transfer 150 ul supernatant to 30 ul(final 1.0X) SPRIselect tube to purify 275bps PCR products.
Elute with 35 ul EB. Spec DNA using a Qubit fluorometer and an Agilent TapeStation.

- Expected Qubit fluorometer concentration: 1 to 30 ng/ul.
- Expected the library size on a TapeStation: 275 bps

2.3 **Submit the libraries for NGS illumina sequencing or do Nanopore sequencing shown below**
Nanopore sequencing according to the modified nanopore protocol (Ligation sequencing amplicons - Native Barcoding Kit 24 V14 (SQK-NBD114.24)). Briefly, repair and prepare the input amplicon DNA, ligate native barcodes and sequencing adaptors, load the libraries into either MinION or PromethION flowcell. The two modifications are changing “0.4X AMPure XP beads (AXP)” to “**1.0X** AMPure XP beads(AXP)” in Native barcode ligation part 11 and Changing “Add 20 ul of resuspended AMPure XP beads (AXP)” to “Add **50** ul of resuspended AMPure XP beads (AXP)” in Adapter ligation and clean-up part 9.

Part 3: Transfection of Adherent Cells for Virus Packaging

3 Goal: Package sgRNA lentivirus.

3.1 The day before transfection, seed 293T (WT) cells at 5.5X106 cells per 10cm dish.

3.2 Check cell health. Do not proceed if cell density or health is not correct for transfection.

3.3 Mix the DNA in a sterile tube. Scale according to the number of plates to be transfected.

	A	B
	DNA	Amount for 10-cm dish
	transgene	8 ug
	psPAX2	6ug
	pMD2G	2ug

Transfection

3.4 Add 1ml Opti-MEM per plate, mix/vortex.

3.5 Add 64ul Transporter 5 transfection reagent per plate, vortex 5 seconds, gently spin down.

3.6 Incubate at room temperature for 20 minutes.

3.7 Gently pipette up and down 3 times to mix, then gently add 1ml per plate to cells, spreading around the plate.

3.8 Gently mix the dish.

3.9 Change the media the next day.

Part 4: Virus collection and concentration

4 **Goal:** Generate, harvest, and concentrate sgRNA lentivirus.

4.1 **Collect virus 48 hours after the media change:** For ultracentrifugation (UC), pre-chill Beckman Optima XL-100K centrifuge to 4C.

4.2 Draw media with virus up into 10 ml or larger syringe.

4.3 Attach a 0.45ul filter, filter the virus into sterile tube(s) of appropriate size and type.

4.4 If concentrating:
1. Filter in ultracentrifuge tubes at 25-30ml per tube. Supplement with DMEM if needed.
2. Balance the ultracentrifuge buckets and contents.
3. Concentrate virus with the SW28 Rotor at 25000 rpm @ 4C for 90 minutes.
4. Pour out supernatant
5. Resuspend in appropriate volume of complete mTeSR+CloneR2.

4.5 Aliquot virus if needed.

4.6 Bleach everything that has contacted virus using 50% bleach.

4.7 If titering, use the virus fresh for titer infection (Part 6).

4.8 Store remaining virus at 4C for large-scale infection (Part 7).

Part 5: Single-cell suspension of ESC/iPSCs

5 **Goal**: Detach cultured ESCs and iPSCs into a single-cell suspension for lentiviral infection.

5.1 Remove media from cells, wash with 2ml PBS, remove PBS.

5.2 Add 1ml TrypLE Select and incubate at 37C for 15min.

5.3 Pipette the cells to dissociate them, then add 4ml PBS to neutralize.

5.4 Spin at 300 g for 5min. Carefully remove supernatant.

5.5 Resuspend pellet in media+Rock Inhibitor. Pipette thoroughly to break up remaining cell clusters and to fully separate cells from Matrigel.

5.6 Spin at 300 g for 5min. Carefully remove supernatant. When aspirating, watch for the “pop” from the top of the pellet that indicates the Matrigel layer of the pellet being removed.

5.7 Resuspend pellet in an appropriate volume of media+Rock Inhibitor.

Part 6: Measure lentiviral infection in hESCs/iPSCs

6 **Goal**: Measure the titer of a lentivirus infected into H9/WTC11 cells.

6.1 Culture target cells to 80% confluency (~2M cells per well). Check cell health and density. Do not proceed if cells look abnormal or overgrown.

6.2 4-5 hours before infection, replace culture media with media containing CloneR2.

6.3 Prepare the cells per the Single Cell Suspension protocol (Part 5). Resuspend in ~1ml per 2 wells collected.

6.4 Count and aliquot appropriate number of cells - 1M cells per condition. Spin at 300*g for 5 minutes at room temperature. Remove supernatant completely.
Check cell viability. Repeat wash if viability is <80%. Do not proceed if still <80% live.

6.5 Resuspend each 1M cell pellet in 1ml media+CloneR2. Transfer to a clean well of a 6well Ultra-Low Attachment Tissue Culture plate.

6.6 Add virus - 10ul, 50ul, 200ul is usually a good starting point. Gently tap/shake the plate to mix. If using concentrated virus, spike in 10% virus with a different fluorophore to help estimate MOI.

6.7 Incubate cells in humidified 37C/5% CO2 for 3 hours, either on a plate shaker or tapping/shaking the plate every ~30 minutes to keep the cells in suspension.

6.8 After 3 hours, add 3ml media+CloneR2 to each well to get 4ml total.

6.9 Plate half of the cells per well to new 6well Matrigel coated plates. Incubate the cells in a humidified 37C/5% CO2 incubator.

6.10 Change the media the next day.

6.11 Measure titer 2 days later:
a. Collect cells per the Single Cell Suspension protocol. Resuspend cell pellet at ~10M cells per ml (~100-200ul per well).
b. Measure BFP+ cells using a flow cytometer.
c. Calculate the appropriate amount of virus for full-scale infection, taking into account a ~10% loss in infectivity due to storing the virus in 4C.

Part 7: Large-scale lentiviral infection of hESCs/iPSCs

7 **Goal**: Infect a sgRNA lentivirus into H9/WTC11 cells.

Notes: The below protocol is essentially the same as the titer infection, but with more cells and a single virus quantity. This infection is 2 days after the titer infection, using the same batch of virus.

7.1 Culture target cells to 80% confluency (~2M cells per well).
Check cell health and density. Do not proceed if cells look abnormal or overgrown.

7.2 4-5 hours before infection, replace culture media with media containing CloneR2.

7.3 Prepare the cells per the Single Cell Suspension protocol (Part 5). Resuspend in ~1ml per 2 wells collected.

7.4 Count and aliquot appropriate number of cells. Spin at 300*g for 5 minutes at room temperature. Remove supernatant completely.
Check cell viability. Repeat wash if viability is <80%. Do not proceed if still <80% live.

7.5 Resuspend cell pellet in 1ml media+CloneR2 per 1 M cells.

7.6 Add the appropriate amount of virus. Invert the tube several times to mix.

7.7 Transfer to a clean 6well Ultra-Low Attachment Tissue Culture plate at 1M cells per well.

7.8 Incubate cells in humidified 37C/5% CO2 for 3 hours, either on a plate shaker or tapping/shaking the plate every ~30 minutes to keep the cells in suspension.

7.9 After 3 hours, combine all cells and add media+CloneR2 to 250k cells per ml.

7.10 Plate 2ml (500k cells) per well to new 6-well Matrigel coated plates. Incubate the cells in a humidified 37C/5% CO2 incubator.

7.11 Change the media the next day.

7.12 Incubate the cells in a humidified 37C/5% CO2 incubator until sorted. Media change every 1-2 days. Split as needed, maintaining complexity of the cell library.

a. Terminate experiment if:

i. Viability of cells is <50% or is too low to maintain library complexity

ii. Cells appear unhealthy

iii. Expression of marker fluorophore is much lower than expected

b. Take a representative picture before the first split to estimate integration efficiency

Part 8: Sort for BFP+ hESCs/iPSCs

8 **Goal**: Enrich for lentivirally infected cells by sorting for BFP+ cells

8.1 **Cell Sorting:** Prepare the cells per the Single Cell Suspension protocol (Part 5). Resuspend cells in media+Rock Inhibitor to ~10M cells/ml.

8.2 Meanwhile, prepare 15ml conical tubes to sort into by adding 2ml media+Rock Inhibitor and thoroughly coating the inside of the tube.

8.3 Strain the cells through a 40um strainer into the tubes to sort from.

8.4 Using a FACS instrument, sort for BFP+ cells. Collect into the prepared 15ml tubes.

8.5 Spin at 300 g for 5min. Carefully remove supernatant.

8.6 Resuspend pellets in the appropriate amount of media+CloneR2.

8.7 Plate cells in 6-well plate - 250k to 1M cells per well

8.8 Incubate the cells in a humidified 37C/5% CO2 incubator until differentiation. Media change every 1-2 days. Split as needed, maintaining complexity of cell library.

8.9 Two weeks after infection, differentiate the cells to cardiomyocytes using the appropriate protocol.

Part 9: Protocol for cell hashing followed by 10X single-cell RNA-Seq

9 **Goal**: Harvest cells and perform cell hashing for superloading of 10X RNA-Seq libraries

9.1 Harvest differentiated cardiomyocytes into single-cell suspension.

9.2 Resuspend 10M - 13M harvested cells in 1ml staining buffer (1 % BSA in 50% PBS / 50% culture media) in 1.5 ml Eppendorf tube

9.3 Dilute 1ul (0.5 ug) TotalSeq™-C anti-human Hashtag Antibody (BioLegend) to 5 ul staining buffer in 0.2 ml PCR strip on ice. Repeat for a total of 12 unique Hashtag antibodies.

9.4 Aliquot 50 ul of cell suspension to each Hashtag-Ab PCR tube and incubate at 4°C with rotation for 20 min.

9.5 Add 150 ul staining buffer to each tube and centrifuge at 275 x g for 5 min.

9.6 Remove supernatant and add 200 ul staining buffer to each tube. Mix and transfer cell suspensions to a 15 ml tube with 5 ml staining buffer on ice. Centrifuge the 15 ml tube at 275 g for 5 min.

9.7 Wash the cells in the 15 ml tube with a 7 ml ice-cold staining buffer once.

9.8 Wash the cells in the 15 ml tube with a 7 ml ice-cold 1% BSA in PBS once. Count cell concentration to determine how much volume should be added in the next step.

9.9 Resuspend cells in ice-cold 1% BSA in PBS at a concentration of 5 ~ 6 M cells/ml.

9.10 Filter the cell suspension with 30 um Pre-Separation filters (Miltenyi Biotec).

9.11 Count the cells. Ensure cell viability is >70% and cells are in a single-cell suspension.

9.12 There are no super-loading 10X 5' HT kits for CRISPR Screening & Cell Multiplexing available in the market. 5' HT libraries were prepared with 10X 5' HT reagent kits with Feature Barcode technology for CRISPR Screening & Cell Surface Protein. But super-loading the cells in protocol CG000513 part **1.2 Load Chromium Next GEM Chip N (**shown below):

1. For cardiomyocytes, load 173k cells to target 60k recovered cells
2. For other stem-cell derived cells, load 115k cells to target 60k recovered cells

Part 10: Construction of scRNA-Seq libraries

10 **Goal**: Construct sgRNA and HTO libraries during scRNA-Seq.

10.1 Prepare transcriptome, sgRNA, and HTO libraries according to 10X CG000513 protocol with some modifications

10.2 For transcription libraries follow the protocol CG000513 **Step 5: 5' Gene Expression Library Construction.**

10.3 For sgRNA libraries fellow the protocol CG000513 **Step 6: CRISPR Screening Library Construction**, except 4 times of the input sgRNA cDNA recommended were used:

1. CG000513 step 6.1.j ~ 6.1.m: Elute with 20.5 ul instead of 40.5 ul.
2. CG000513 part 6.2.b: Add 10 ul (instead 5 ul) Guide RNA cDNA Cleanup for each PCR reaction. Add 40 ul (instead 45 ul) Feature S1 primers 4 for each PCR reaction.
3. CG000513 part 6.2.e and 6.4.d Increase the PCR cycles from 10 to 11.

10.4 For HTO libraries follow the protocol CG000513 **Step 7: Cell Surface Protein Library Construction** without modification. TotalSeq™ anti-human Hashtag reagents (BioLegend) are designed to label most human cells, using a combination of two clones that recognize cell surface protein CD298 and β2 microglobulin, respectively.

10.5 Measure the library concentration with Qubit reagents(Thermo Fisher Scientific) and analyze the peak size of the libraries with TapeStation System(Agilent Technologies).