Oct 22, 2025

18 Loci Variant Perturb-Seq Protocol (ABE)

DOI
https://dx.doi.org/10.17504/protocols.io.3byl461mrgo5/v1

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Protocol Citation: Quang Vinh Phan, Richard Sherwood 2025. 18 Loci Variant Perturb-Seq Protocol (ABE). **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl461mrgo5/v1

Protocol status: Working
We use this protocol and it's working

Created: October 21, 2025

Last Modified: October 22, 2025

Protocol Integer ID: 230389

Keywords: using crispr base editing screening, crispr base editing screening, robust phenotypes in crispr screen, cholesterol trait, variants with strong gwas evidence, genes within these loci, identified numerous single nucleotide polymorphism, known monogenic disease gene, human genetic evidence, monogenic disease gene, crispr, numerous single nucleotide polymorphism, gene, used human genetic evidence, using cholesterol, strong gwas evidence, genome, ancestry gwa, related phenotypic assay, robust phenotype, crispr screen, phenotypic assay, variants with strong evidence, loci variant installation protocol, trait, gwa, liver eqtl, same trait, snp, various trait, human hepatocyte cell line, ldl uptake screen, impact on the transcriptome, transcriptome, loci variant perturb, wide association study, functional consequences of these variant, burden for these same trait

Funders Acknowledgements:
Pinello
Grant ID: HG012010

Abstract

Genome-wide association studies (GWAS) have identified numerous single nucleotide polymorphisms (SNPs) associated with various traits and diseases, yet understanding the functional consequences of these variants remains challenging. We have chosen a set of 18 loci associated with cholesterol traits (LDL-C and HDL-C) in a recent trans-ancestry GWAS (Graham et al 2021, Nature, GLGC consortium). Genes within these loci have coding burden for these same traits and/or are known monogenic disease genes, and importantly, targeting these genes gives robust phenotypes in CRISPR screens using cholesterol-related phenotypic assays. We have used human genetic evidence to select ~2,500 variants within these 18 loci to evaluate, including variants with strong GWAS evidence and variants with strong evidence as liver eQTLs through fine-mapping and/or linkage to sentinel variants.

This protocol describes pooled Perturb-Seq with selected variants of interest to characterize each variant on their impact on the transcriptome.

Guide library Cloning

1 **Library structure**
GGCTTTATATATCTTGTGGAAAGGACGAAACACCG(19-20-bp protospacer—remove initial G for any 20-bp protospacer with one natively)
GTTTAAGAGCTATGCTGGAAACAGCATAGCAAGTT

2 **Reconstitution of ssDNA oligo pool**

2.1 Spin down lyophilized ssDNA oligo pool

2.2 Prepare 2 ng/µL stock by resuspending in TE buffer with low EDTA (10mM Tris-Cl pH 8.0, 0.1 mM EDTA)

2.3 Store at -20 °C.

3 **Restriction digest of CRISPRv2FE-ABE8e-SpRY-BsrGI library backbone**

3.1 Cut 10 µg CRISPRv2FE-ABE8e-SpRY-BsrGI with BsmBI-V2 and BsrGI-HF

3.2 Restriction digest reaction mix overview:

	A	B
	H2O	ad 100 µl
	NEB 3.1	10 µL
	CRISPRv2FE-ABE8e-SpRY-BsrGI	10 µg
	BsmBI-v2	10 units
	BsrGI-HF	10 units

3.3 Incubate at 55 °C for 3-4 hours with BsmBI-V2, then add BsrGI-HF and incubate at 37°C for another 3-4 hours.

3.4 Run digest on 1% agarose gel with SYBR and cut out band at 13.7 kbp.

4 **Amplification of library**

Amplification of library done with the following primers:

010415_sgRNA_60bp_fw
TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG

010415_sgRNA_60bp_rv
GTTGATAACGGACTAGCCTTATTTAAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC

4.1 **Determination of optimal PCR cycles for library amplification**

Run a qPCR with 0.5 µl of 1122_18LDLlocus_ABE_gRNAlib library in 15 µl qPCR to determine optimal cycles using the primers above.

qPCR mix

	A	B
	Oligo pool	0.5 uL
	2x Q5 mix	7.5 uL
	F primer @20uM	0.375 uL
	R primer @20uM	0.375 uL
	dd H20	5.5 uL
	20x EVA Green	0.75

qPCR program

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

Repeat steps B-D for 20x

Identify the cycle number where the qPCR stops log-linear increase (this is typically 4-5 cycles after the CT), determine how much more input in PCR as in qPCR and subtract the log2 ratio (e.g. if using 0.5 uL, keep as is, if using 1 uL, subtract 1 cycle, if using 2 uL, subtract 2 cycles), to identify PCR1 cycle number.

4.2 Library amplification

Once optimal cycle number is determined, run a 100 µl PCR with the following conditions:

	A	B
	Oligo pool	0.5 uL
	2x Q5 mix	50 uL
	F primer @20uM	2.5 uL
	R primer @20uM	2.5 uL
	dd H20	44.5 uL

	A	B	C	D	E	F
	98 °C	98 °C	65 °C	72 °C	72 °C	4 °C
	30 sec	10 sec	30 sec	60 sec	5 min	remaining

Repeats steps B-D with the determined optimal cycle numbers.

Load PCR onto a 2% agarose gel with SYBR and gel purify band at 299 bp.

5 Gibson assembly

	A	B
	2xNEB HiFi assembly mix	50 ul
	Digested CRISPRv2F E-ABE8e-SpRY-BsrGI	2100 ng (0.25 pmol)

	A	B
	Purified library PCR	140 ng (0.75 pmol)
	Water	ad 100 ul

Incubate at 50°C for 1 hour.

6 **Cleanup/concentration**

6.1 Add 1 ul GlycoBlue, 2 ul 50mM NaCl, 100 ul Isopropanol to the Gibson reaction system

6.2 Vortex, incubate at room temperature for 15 min

6.3 SPIN > 15.000 g for 15 min

6.4 Carefully remove liquid without disturbing pellet

6.5 Wash with 300 ul 80% EtOH and SPIN >15.000 g for 5 min

6.6 Remove most liquid with P1000 and spin at >15,000 g for 1 min

6.7 Carefully remove all liquid with p200, making sure tube has no liquid left, and air-dry pellet 3-5 min by keeping cap open and leaving at room temp

6.8 Add 8.25 ul EB, warming at 55°C for 10min to fully resuspend

7 **Determination of Coverage and Diversity of Cloned Library**
Transformation of a small amount of the assembly mix for the determination of diversity and coverage of the cloned library.

7.1 Add 0.25 µl of the purified assembled mix into a 1.5 ml tube and gently mix with 25 µl of NEB Stable cells. Incubate for 30 minutes on ice.

7.2 Heat shock at 42°C for 30 seconds and put back on ice for 1 minute.

7.3 Add 975 µl of NEB 10-beta/Stable Outgrowth Medium and incubate at 30°C for 1 hour while shaking.

7.4 Plate 1/10, 1/100, and 1/1,000 of transformation mix onto LB agar plates (supplied with 100 µg/ml ampicillin). Incubate at 30°C overnight.

7.5 **Calculation of the Coverage**
Choose the dilution plate with colony numbers in the range between 20-200 and count the colonies. Calculate the coverage as follows:

((Counted colonies * dilution factor) / Number of library members) * 1600

A coverage of >100x is ideal.

Note: 0.25 µl of the remaining 8 uL purified assembly mix is 32x used in this test. Electroporation with Endura typically yields in a 50x higher transformation efficiency compared to NEB Stable cells, meaning that the entire remaining 8 ul of the assembly mix has 32×50=1600x the number of colonies as the test transformation with 0.25 µl.

7.6 **Determination of the Diversity**
Perform colony PCR on 16 colonies from any of the dilution plates to determined the diversity of the cloned library, using the following conditions:

pX330_seqfw
GAGGGCCTATTTCCCATGAT
111219_postPT_rv
CTAGGCACCGGATCAATTGC

PCR mix for one reaction:

	A	B
	NEB OneTaq 2X Master Mix with Standard Buffer	12.5 µl
	Forward Primer @20 µM	0.25 µl

	A	B
	Reverse Primer @20 µM	0.25 µl
	Water	ad 12 µl

PCR program:

	A	B	C	D	E	F
	94 °C	94 °C	55°C	72 °C	72 °C	4 °C
	30 sec	15 sec	30 sec	60 sec	5 min	remaining

Repeat steps B-D for 35x

7.7 Load 5 µl of each PCR onto a 2% agarose gel with ethidium bromide and expect bands at 635 bp for successful cloning, while 850 bp indicates plasmid background. If >2 colonies have 850 bp, then re-clone.

7.8 Purify rest of the colony PCR via QIAquick PCR Purification Kit and send in for Sanger sequencing with primer pX330_seqfw (GAGGGCCTATTTCCCATGAT). Check the cloned sgRNAs for diversity and move on to the electroporation of the assembly mix into Endura cells.

8 Electroporation of Assembly Mix in Endura Cells

8.1 Add 2 uL of assembly mix to 25uL of Lucigen Endura electrocompetent cells, repeat 4x

8.2 Electroporate using the following parameters:
- 1mm cuvette
- 10 µF
- 600 Ohms
- 1800 Volts

8.3 Immediately add 1mL recovery media from Lucigen into the electroporation cuvette.

8.4 Gently pipet bacterial suspension into a 14 ml culture tube that already contains 1 ml of Lucigen recovery media

8.5 Incubate at 30°C for 1 hour while shaking.

8.6 In a 50 ml centrifugation tube, pool all 4 electroporation mixes together and mix well by swirling.

8.7 Take out 10 µl of the pooled electroporation mix and add to 1 ml of recovery medium. Plate out 20 and 100 µl of that dilution onto pre-warmed plates (40,000 and 8,000-fold dilution, respectively). Incubate at 30°C overnight and count colonies to determine the coverage (see step 7.5 for calculation).

8.8 Transfer the pooled electroporation mix into 400 ml LB medium supplied with 100 µg/ml ampicillin. Incubate at 30°C overnight while shaking.

8.9 Centrifuge bacterial suspension as 2×200 ml aliquotes. Maxi prep on one bacteria pellet and keep the other one as backup at -20°C.

Lentivirus Production

9 **Production and Titration of Lentivirus**

9.1 **Day -1: Plate HEK293 cells for Transfection**
Plate 4×15 cm plates with HEK293FT cells at 1.625*10^7 cells per 15-cm plate in 20 mL DMEM + 10% FBS each

9.2 **Day 0: Transfection of Lentiviral Plasmid Library**

Prepare 2 separate tubes with the following components (mix for 1×15 cm dish transfection).

Tube A

	A	B
	OptiMEM	4 ml
	pMDLg/pRRE	9.7 µg
	pRSV-Rev	6.5 µg
	pcDNA3-VSV-G	3.3 µg

	A	B
	Lentiviral Plasmid Library	13 µg

Tube B

	A	B
	OptiMEM	4 ml
	TransIT-Lenti Transfection Reagent	98 µl

Combine tube A and B and mix by gently inverting. Incubate for 15 minutes at room temperature.

9.3 Gently add transfection mix dropwise to the cells.

9.4 **Day 1: First Harvest**
Collect lentiviral supernatant and store at 4°C. Replace medium with 16 ml of DMEM+10% FBS.

9.5 **Day 2: Second Harvest**
Collect lentiviral supernatant and store at 4°C. Replace medium with 16 ml of DMEM+10% FBS.

9.6 **Day 3: Final Harvest and Lentivirus Concetration**
Collect lentiviral supernatant and pool all lentivirus harvest together.

9.7 Centrifuge pooled lentiviral supernatant at 300xg for 5 minutes. Filter supernatant through a 0.45 µm filter and add 1/3 volume of Lenti-X Concentrator. Mix gently by inversion and incubate for 30 minutes or overnight at 4°C.

9.8 Centrifuge at 1,500xg for 45 minutes at 4°C.

9.9 Gently pour out the supernatant and resuspend pellet in 2 ml of DMEM+10% FBS per 15 cm. Aliquote 500 µl per cryotube and store at -80°C.

9.10 **Titration of Lentivirus**
Titrate the lentivirus on a 24 well plate using 8*10^4/well (4*10^4/cm2). Eventual experiment will use 6.25M (6.25*10^6) cells on a 15-cm (4*10^4/cm2). Each 24-well of

titration uses ~1/75 as many cells as
the 15-cm plate will.

Below is the standard lentivirus titration dose chart, although you can alter if necessary. Volumes are calculated for 4 mL total lenti and should be adjusted as necessary to account for fraction of lenti produced:

- 1/150 = 26.66 uL
- 1/300 = 13.33 uL
- 1/600 = 6.66 uL
- 1/1200 = 3.33 uL
- 1/1800 = 2.22 uL
- 1/2400 = 1.67 uL
- 1/4800 = 0.83 uL

9.11 **Day 0: Plating and Infection of HepG2 Cells**
Plate 80,000 HepG2 cells per well in a 24-well supplied with polybrene in a final concentration of 8 µg/ml. Add lentivirus in the dilution steps as stated above. Have two extra wells seeded with no virus as selection control.

9.12 **Day 2: Start of Puromycin Selection**
Replace medium with DMEM+10% FBS supplied with 500 ng/ml puromycin

9.13 **Day 3: First Passage**
Wash cells with PBS and add 75 µl of trypsin. Incubate for 5 minutes at 37°C and add 500 µl of medium supplied with puromycin. Mix detached cells with a P1000 and transfer cell suspension to a new 24-well.

9.14 **Day 5: Second Passage**
Wash cells with PBS and add 75 µl of trypsin. Incubate for 5 minutes at 37°C and add 500 µl of medium supplied with puromycin. Mix detached cells with a P1000 and transfer cell suspension to a new 24-well.

9.15 **Day 7: Count**
By now, control well with no virus should be completely dead. Count each well and detemine the lentiviral dose with the highest survival. The lentiviral dose with 50% survival from that is then the desired lentiviral dosage for the screen. Multiply the dosage with 75 to scale the lentiviral dosage to the 15 cm dish format.

10 **Lentivirus Library Infection**

10.1 **Day 0: Infection of HepG2 Cells**
Trypsinze and count HepG2 cells. Add 6.25×10^6 cells to a 15 ml centrifugation tube, add lentivirus library and mix by gently inverting. Plate mix onto 15 cm dish with a total medium volume of 20 ml supplied with 8 µg/ml polybrene.

Seed 410.000 HepG2 cells in a 6-well as selection control.

10.2 **Day 1: VPA Treatment**
Replace media with DMEM+10% FBS and 2mM VPA

10.3 **Day 3: Start of Puromycin Selection**
Replace medium with 20 ml of DMEM+10% FBS supplied with 500 ng/ml puromycin.

10.4 **Day 4: First Passage**
Split cells 1:2 to one new 15 cm dish with 20 ml of DMEM+10% FBS supplied with 500 ng/ml puromycin (ending up with one plate per replicate).

10.5 **Day 6: Second Passage**
Split cells 1:2 to two new 15 cm dish with 20 ml of DMEM+10% FBS supplied with 500 ng/ml puromycin (ending up with two plates per replicate). By now, control 6-well with no virus should be dead.

10.6 **Day 8: Seed for LDL Uptake Screen**
Seed 31.25×10^6 cells per replicate in a 15 cm dish. For bulk, seed $12,25\times10^6$ cells in a 10 cm dish. Seed both in DMEM+10% FBS.

10.7 **Day 9: Serum Depravation**
In the late afternoon, change medium to OptiMEM to start the serum depravation.

10.8 **Day 10: Single RNA Seq - Gel Bead-in-Emulsion Preparation**

10xGenomics Single Cell RNA Kit: GEM-X Universal 5' Gene Expression v3 4-plex (CG000772)

Cell Preparation

1. Get beads out of -80c to allow to equilibrate to room temperature (at least 30 minutes)
2. Chill 5 mL PBS in advance on ice.
3. Aspirate medium, add 10 ml of room temp PBS, wash by gently rocking the plate back and forth, and aspirate PBS completely
4. Add 4 ml of 0.25% Trypsin-EDTA, distribute the Trypsin across the entire plate by gently rocking the plate back and forth, and incubate in a humidified incubator for 5 minutes at 37°C
5. With a 10 ml serological pipet, add 8 ml of DMEM+10% FCS medium to the plate and hold the plate at a 30° angle facing towards you. Using the same 10 ml serological pipet, take up the cell suspension and discharge at the top of the plate. Repeat this rinsing motion a few times until the white viscous film is gone and cell suspension appears homogeneous. Transfer cell suspension into a 15 ml tube. Filter through 50-

mL filter-cap FACS tube(s) then transfer back to 15 mL tube and centrifuge at 1,000 rpm for 5 minutes at 4°C.

6. Aspirate supernatant and resuspend in 1 ml ice-cold PBS+0.4%BSA with a 1000 ul pipette by gently pipetting up and down 10-15x. Add another 1 ml of ice-cold PBS+0.4% BSA.

7. Among 2 blue filter-cap FACS tubes, carefully apply about 500 ul of cell suspension onto the membrane of a blue cap FACS tube each and pulse spin for 10 seconds (centrifuge will hit about 2,300 rpm at the end). Cells will form a pellet at the bottom of each FACS tube.

8. Gently resuspend the pellet in the same supernatant by pipetting up and down with a 1000 ul pipet 10x and combine both in a single FACS tube.

9. With the 1000 ul pipette, push the cell suspension through the membrane of a fresh blue filter-cap FACS tube. Repeat until the entire cell suspension went through the filter.

10. Repeat step 6 again to ensure single cell suspension for processing. Keep cells on ice.

11. Count cells and make a fresh cell suspension with the concentration of **1,500 live cells per ul (1.5M cells per ml)** in 1 ml total volume in PBS+0.4% in 5 ml tubes

Prepare Master Mix

Master Mix *Add reagents in the order listed*	PN	4X + 10% (µl)	8X + 10% (µl)
RT Reagent E	2001106	18.9	37.8
CRISPR Poly-dT Primer Mix B	2001145	2.6	5.3
Reducing Agent B	2000087	2.2	4.4
RT Enzyme E	2001146	7.9	15.8
Total	-	**31.7**	**63.4**

1. Add 7.2 ul of master mix into each tube of a 8-tube PCR strip on ice.

Assemble Chip Holder

1. Get the gasket (light blue rubber part) - **DO NOT TOUCH** the smooth side of the gasket
2. Attach the gasket by holding the tongue (curved end, to the right) and hook the gasket on the left-hand tabs of the holder. Gently pull the gasket toward the right and hook it on the two right-hand tabs.
3. Open the chip holder

4. Remove the chip from the sealed bag. Use the chip within less than 24 hours after opening.
5. In the open chip holder, align the notch of the chip (upper left corner)
6. Slide the chip to the left until the guide on the holder is inserted into the chip. Depress the right hand side of the chip until the spring-loaded chip engages (audible click sound)
7. Keep the assembled unit with the attached gasket open until ready for and while dispensing reagents/cells into wells

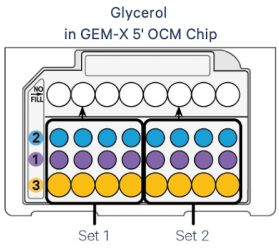
Load GEM Chip

a. If loading only one set (each set = 4 samples), add 50% glycerol solution to each unused well in row 1, 2, and 3 of the second set

If running fewer than 4 samples in a set, DO NOT load glycerol to the unused wells in that set. Refer to Sample Multiplexing Guidelines on page 43 for guidance on mock samples.

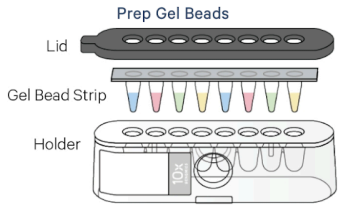
- **15 µl** in each unused well in row labeled 1
- **18 µl** in each unused well in row labeled 2
- **70 µl** in each unused well in row labeled 3

DO NOT add 50% glycerol solution to the wells in top row labeled NO FILL.
DO NOT use any substitute for 50% glycerol solution.



1. Ensure that the gel beads are equilibrated to room temperature 30 minutes before loading the chip

- Snap the tube strip holder with the Gel Bead strip into a 10x Vortex Adapter. Vortex **30 sec**. Gel Beads are color coded to indicate two sets of 4 Gel Beads each, where each color represents a unique set of barcodes.
- Centrifuge the Gel Bead strip for **~5 sec**.
- Confirm there are no bubbles at the bottom of the tubes & the liquid levels are even.
- Place the Gel Bead strip back in the holder. Blue tube should be loaded on the left. Secure the holder lid.



Colors indicate Gel beads with different barcode whitelists.

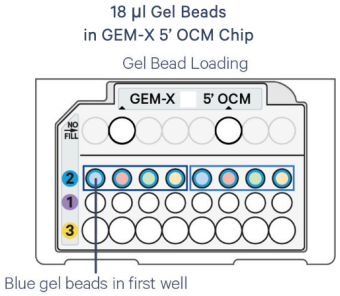
To the PCR strip holding the master mix prepared in step 1.3.1, add to each tube **4.3 ul of 1xPBS**, mix by pipetting and centrifuge briefly

1. Gently mix prepared stock cell suspensions (1,500 cells per ul) with a P1000 and **add 5.5 ul of cell suspension** into each tube of master mix and PBS (total volume 17 ul in each tube)
2. Using a multi channel (P20), gently mix the master mix and cell suspension. Avoid bubbles!

3. Using the same pipette tip, dispense **15 ul** of master mix/cell suspension into the bottom center of each well in **row labeled 1** without introducing bubbles
4. Wait 30 sec and proceed with next step immediately

Load Row Labeled 2

- Puncture the foil seal of the Gel Bead tubes. Check orientation to ensure blue is loaded in the first well of the set.
- Using a multichannel pipette (P20), slowly aspirate **18 µl** Gel Beads.
- Dispense into the bottom center of each well in **row labeled 2** without introducing bubbles. Load the gel beads such that the blue gel beads are loaded in the first well of the set.
- Wait **30 sec**.
- If a mock sample is loaded, the corresponding gel bead should still be loaded.



5. Using a multichannel pipette (P200), dispense 70 ul of Partitioning Oil B into wells in **row labeled 3.**

Run Chromium X Series

1. Close the lid of the chip holder. DO NOT touch the smooth side of the gasket. DO NOT press down on the top of the gasket. Keep horizontal to avoid spillage and wetting the gasket.
2. Press the eject button to eject the tray.
3. Place the assembled chip in the tray. Press the button again to retract the tray
4. Confirm GEM-X 5P OCM program on screen. Press the play button.
5. Run will take about 4-5 minutes. Immediately proceed with the next step.
6. Have a PCR tube strip ready on ice
7. Press the eject button and remove the chip holder
8. Discard the gasket. Open the chip holder and inspect the GEM.
9. Fold the lid of the chip holder back until it clicks and expose the wells at 45 degrees.
10. **Slowly** aspirate 100 ul GEMs from top row “No fill” **Well 2.**
11. GEMs should appear opaque and uniform in both the recovery wells. **Over the course of 20 sec**, dispense GEMs into a tube of the pre-chilled PCR strip on ice with the pipette tip against the sidewall of the tube.

Reverse Transcription

a. Incubate in a thermal cycler with the following protocol.

Lid Temperature	Reaction Volume	Run Time
48°C	125 µl	~55 min
Step	**Temperature**	**Time *hh:mm:ss***
1	48°C	00:45:00
2	85°C	00:05:00
3	4°C	Hold

b. Store at **4°C** for up to **72 h** or at **−20°C** for up to **a week**, or proceed to the next step.

11 Post GEM-RT Cleanup

1. Add 125 ul Recovery Agent to each sample at room temperature. **DO NOT pipette mix or vortex the biphasic mixture!** Gently invert the tubes and Incubate for 2 minutes.
2. The resulting biphasic mixture contains Recovery Agent/Partitioning Oil (pink) and **aqueous phase (clear - this is your sample)**, with no persisting emulsion (opaque).
3. Note: If the biphasic separation is incomplete:
4. Firmly secure cap on the tube and mix by inverting tubes 5x, centrifuge briefly, and proceed with next step
5. Slowly remove and discard **125 ul** of recovery agent/partitioning oil (pink) from the bottom of the tube. **DO NOT aspirate any aqueous sample.**
6. Prepare Dynabeads Cleanup mix
7. Vortex Dynabeads for about 30 sec **immediately** before adding to the mix
8. Pipette full liquid volume in the Dynabead tube with a pipette tip to verify that beads have not settled. If clumps are present, pipette mix to resuspend completely. DO NOT centrifuge!
9. Prepare master mix according to 1x shown below:

Dynabeads Cleanup Mix *Add reagents in the order listed*	PN	1X (µl)	2X + 10% (µl)
Cleanup Buffer	2000088	182	400.5
Dynabeads MyOne SILANE	2000048	8	17.5
Reducing Agent B	2000087	5	11
Nuclease-free Water	—	5	11
Total		**200**	**440**

Vortex cleanup mix and add **200 ul** of mix to each sample tube. Pipette mix 10x.

1. Incubate for 10 min at room temperature (keep caps open). Pipette mix again 5 minutes after start of incubation.
2. In the meantime, prepare elution solution 1. See mix below:

Elution Solution I *Add reagents in the order listed*	PN	1X (µl)	10X (µl)
Buffer EB	—	98	980
10% Tween 20	—	1	10
◯ **Reducing Agent B**	2000087	1	10
Total		**100**	**1000**

After the 10 minutes incubation, place tubes into a 10x magnetic separator (high position) until solution clears (or about 5 minutes)

1. Remove supernatant
2. Add 300 ul of 80% Ethanol to the bead pellet while on the magnet. Incubate for 30 sec
3. Remove ethanol
4. Add 200 ul of 80% Ethanol to the bead pellet while on the magnet. Incubate for 30 sec
5. Remove ethanol
6. Centrifuge briefly. Place on the 10x magnetic separator (low position)
7. Carefully remove remaining ethanol with a P10 or P20. Air dry for 1 min.
8. Remove tube from the magnet and add 35.5 ul Elution Solution 1.
9. Gently pipette mix for 15x. There should be no clumps left - if so, keep pipetting until fully resuspended.
10. Incubate for 1 minute at room temperature
11. Place tube on the magnet (low position) until the solution clears (or 5 minutes)
12. Transfer 35 ul sample to a new PCR tube. Proceed with cDNA amplification.

cDNA Amplification

cDNA Amplification Reaction Mix *Add reagents in the order listed*		PN	1X (µl)	2X + 10% (µl)
○	**Amp Mix** DO NOT use the Library Amp Mix PN-2000531 (if provided)	2000047/ 2000103	50	110
●	**cDNA Primers**	2000089	15	33
	Total		**65**	**143**

1. Add 65 ul cDNA Amplification Reaction mix to the 35 ul sample (cleaned up earlier) and pipette mix 15x. If necessary, centrifuge briefly.
2. Run samples in a thermocycler with the following program

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25-50 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	63°C	00:00:30
4	72°C	00:01:00
5	Go to Step 2, see table below for total # of cycles	
6	72°C	00:01:00
7	4°C	Hold

cDNA Cleanup - SPRIselect

1. Vortex and resuspend the SPRI beads. Add **60 ul** of SPRI beads (0.6x) to each sample and mix 15x by pipetting.
2. Incubate for 5 minutes
3. Place tube on magnet
4. Transfer 75 ul of supernatant to a new tube without disturbing the pellet. Keep supernatant on ice. **Save the other 85 ul as back up.** Keep tube with the beads on the magnet. **DO NOT discard the pellet!** Immediately proceed to the pellet cleanup.

Pellet Clean Up - Gene Expression

1. Add 200 ul of 80% Ethanol to the pellet. Incubate for 30 seconds.

2. Remove ethanol carefully
3. Add 200 ul of 80% Ethanol to the pellet. Incubate for 30 seconds.
4. Remove ethanol carefully (total of 2 washes)
5. Centrifuge briefly and place tube back into magnet
6. Carefully remove remaining ethanol with a P10 or P20. Air dry for 2 minutes. Do not exceed 2 minutes!
7. Remove tube from magnet and resuspend in 40.5 ul EB buffer. Mix by pipetting 15x (pipet set to 35 ul)
8. Incubate for 2 minutes
9. Place tube back into magnet and incubate for 5 minutes
10. Transfer 40 ul of supernatant to a new tube. Keep sample on ice.

Transfer Supernatant Clean Up - CRISPR

1. Vortex and resuspend the SPRI beads. Add **30 ul** of SPRI beads to each of the 75 ul transferred supernatants (1.2x) and mix 15x by pipetting.
2. Incubate for 5 minutes
3. Place tube on magnet
4. Remove supernatant
5. Add 200 ul of 80% Ethanol to the pellet. Incubate for 30 seconds.
6. Remove ethanol carefully
7. Add 200 ul of 80% Ethanol to the pellet. Incubate for 30 seconds.
8. Remove ethanol carefully (total of 2 washes)
9. Centrifuge briefly and place tube back into magnet
10. Carefully remove remaining ethanol with a P10 or P20. Air dry for 2 minutes. Do not exceed 2 minutes!
11. Remove from magnet and resuspend pellet in 50.5 ul EB buffer by pipetting 15x (pipet set to 35 ul)
12. Incubate for 2 minutes at room temperature
13. Place tube back into the magnet and incubate for 5 minutes
14. Transfer 50 ul supernatant of sample to a new tube.

Gene Expression Library Construction

12 Prepare a thermal cycler with the following incubation protocol:

Lid Temperature	Reaction Volume	Run Time
65°C	50 µl	~35 min
Step	**Temperature**	**Time** *hh:mm:ss*
Pre-cool block *Pre-cool block prior to preparing the Fragmentation Mix*	4°C	Hold
Fragmentation	32°C	00:05:00
End Repair & A-Tailing	65°C	00:30:00
Hold	4°C	Hold

1. Vortex Fragmentation buffer. Verify there is no precipitate.
2. Prepare Fragmentation mix on ice. Add reagents in the order listed. Pipette mix and centrifuge briefly.
3. Transfer only 10 ul of the cDNA sample of the **Gene Expression clean up** (pellet cleanup, from **step 2.4.1** earlier) to a new PCR strip. Store the remaining cDNA sample at -20c.
4. Add 40 ul of the Fragmentation mix to each of 10 ul samples.
5. Pipette mix 15x on ice.
6. Transfer into pre-cooled thermocycler (at 4c) and press resume to start reaction.

GEX Post Fragmentation, End Repair & A-tailing Double Sided - SPRI select

1. Vortex to resuspend SPRI beads. Add 30 ul of breads (0.6x) to each sample. Mix by pipetting 15x
2. Incubate for 5 minutes at room temperature
3. Place on magnet and incubate for 5 minutes
4. Remove 80 ul supernatant. DO NOT discard any beads!
5. With the tube still on the magnet, add 125 ul 80% Ethanol and incubate for 30 seconds
6. Remove ethanol
7. With the tube still on the magnet, add 125 ul 80% Ethanol and incubate for 30 seconds
8. Remove ethanol (total 2 wash steps)
9. Centrifuge briefly and place tubes back on the magnet
10. Carefully remove remaining ethanol and air dry for 1 minute
11. Remove tube from magnet and resuspend in 50.5 ul EB Buffer by pipetting 15x
12. Incubate for 2 minutes
13. Place tube back on the magnet and incubate for 5 minutes
14. Transfer 50 ul of supernatant to a new PCR tube

GEX Adaptor Ligation

Prepare Adaptor ligation mix.

Adaptor Ligation Mix *Add reagents in the order listed*	PN	1X (µl)	2X + 10% (µl)
Ligation Mix	2001109	40	88
DNA Ligase	220110/220131	10	22
Total		**50**	**110**

Add 50 ul of Adapter ligation mix to 50 ul of sample. Mix by pipetting 15x

1. Incubate samples in a thermocycler with the following settings

Lid Temperature	Reaction Volume	Run Time
30°C (lid may be turned off if the instrument does not enable 30°C)	100 µl	15 min

Step	Temperature	Time *hh:mm:ss*
1	20°C	00:15:00
2	4°C	Hold

GEX Post Ligation Cleanup - SPRIselect

1. Vortex SPRI beads and add 80 ul of beads (0.8x) to each Gene expression sample (post ligation) and mix via pipetting for 15x
2. Incubate for 5 minutes at room temperature
3. Place tube in magnet and incubate for 5 minutes
4. Carefully remove supernatant
5. Add 200 ul 80% Ethanol and incubate for 30 seconds
6. Remove ethanol.
7. Add 200 ul 80% Ethanol and incubate for 30 seconds
8. Remove ethanol (total wash steps of 2)
9. Centrifuge briefly and place tubes back on the magnet
10. Carefully remove any remaining ethanol with either at P10 or P20. Air dry for 2 minutes.
11. Remove tube from magnet and resuspend in 30.5 ul EB buffer and mix via pipetting 15x
12. Incubate for 2 minutes
13. Place tube back into magnet and incubate for 5 minutes
14. Carefully transfer 30 ul supernatant to a new tube.

Perform the following qPCR:

· First, mix 0.3 uL of eluted library with 1.2 uL of Elution Solution (or dH2O). This will give a 5x dilution of sample and allow for repeat qPCRs if necessary without depleting more precious sample in case something goes wrong. Then, prepare the following qPCR:

10 uL 2X Q5 mastermix
1 uL 20X EvaGreen
0.5 uL 20 uM 092922_P5_anchor AATGATACGGCGACCACCGAGA
0.5 uL 20 uM NEBNext i7 index primer (note that any i7 primer is OK)
0.5 uL 5x-diluted scRNA-seq-seq library
7.5 uL dH20

Perform the following qPCR protocol
98 deg for 45s
25 cycles of:
98 deg for 20s
54 deg for 30s
72 deg for 20s
Then:
72 deg for 60s
4 deg forever
Run the qPCR product on a 2% EtBr gel after it plateaus.

In this qPCR, we use 0.5 uL of a 5x diluted, so equivalent of 0.1 uL of template. For the actual PCR you will use 30 uL. This means we are using 300x less template than we will in the actual PCR. 300=2^~8, so your qPCR will come up 8 cycles later than you would expect your PCR with 30 uL input to come up. Usually, you would run a PCR to the middle to latter half of the exponential phase, so in this case, find the middle to latter half of the exponential phase and subtract 8 cycles to determine PCR cycle count. Hopefully it comes to a similar cycle count to what is recommended in the 10x protocol.

GEX Sample Index PCR

Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000431 Dual Index Plate TT Set A well ID) used. See list below:

	index_name	index(i7)	index2_workflow_a(i5)	index2_workflow_b(i5)
	SI-TT-A1	GTAACATGCG	AGTGTTACCT	AGGTAACACT
	SI-TT-A2	GTGGATCAAA	GCCAACCCTG	CAGGGTTGGC
	SI-TT-A3	CACTACGAAA	TTAGACTGAT	ATCAGTCTAA

	SI-TT-A4	CTCTAGCGAG	TATCTTCATC	GATGAAGATA
	SI-TT-A5	GTAGCCCTGT	GAGCATCTAT	ATAGATGCTC
	SI-TT-A6	TAACGCGTGA	CCCTAACTTC	GAAGTTAGGG
	SI-TT-A7	TCCCAAGGGT	TACTACCTTT	AAAGGTAGTA
	SI-TT-A8	CGAAGTATAC	GAACTTGGAG	CTCCAAGTTC
	SI-TT-A9	AAGTGGAGAG	TTCCTGTTAC	GTAACAGGAA
	SI-TT-A10	CGTGACATGC	ATGGTCTAAA	TTTAGACCAT
	SI-TT-A11	CGGAACCCAA	GATTCGAGGA	TCCTCGAATC
	SI-TT-A12	CACCGCACCA	GACTGTCAAT	ATTGACAGTC
	SI-TT-B1	ACAGTAACTA	ACAGTTCGTT	AACGAACTGT
	SI-TT-B2	TCTACCATTT	CGGGAGAGTC	GACTCTCCCG
	SI-TT-B3	CACGGTGAAT	GTTCGTCACA	TGTGACGAAC
	SI-TT-B4	GTAGACGAAA	CTAGTGTGGT	ACCACACTAG
	SI-TT-B5	TCGGCTCTAC	CCGATGGTCT	AGACCATCGG
	SI-TT-B6	AATGCCATGA	TACGTAATGC	GCATTACGTA
	SI-TT-B7	GCCTTCGGTA	CCAACGATTT	AAATCGTTGG
	SI-TT-B8	GCACTGAGAA	TATGCGTGAA	TTCACGCATA
	SI-TT-B9	TATTGAGGCA	CAGGTAAGTG	CACTTACCTG
	SI-TT-B10	GCCCGATGGA	AATCGTCTAG	CTAGACGATT
	SI-TT-B11	TCTTACTTGC	TGACCTCTAG	CTAGAGGTCA
	SI-TT-B12	CGTCAAGGGC	TAGGTCACTC	GAGTGACCTA
	SI-TT-C1	TGCGCGGTTT	CAAGGATAAA	TTTATCCTTG
	SI-TT-C2	CAATCCCGAC	CCGAGTAGTA	TACTACTCGG
	SI-TT-C3	ATGGCTTGTG	GAATGTTGTG	CACAACATTC
	SI-TT-C4	TTCTCGATGA	TGTCGGGCAC	GTGCCCGACA
	SI-TT-C5	TCCGTTGGAT	ACGTTCTCGC	GCGAGAACGT
	SI-TT-C6	ACGACTACCA	ACGACCCTAA	TTAGGGTCGT
	SI-TT-C7	CGCGCACTTA	CCTGTATTCT	AGAATACAGG

	SI-TT-C8	GCTACAAAGC	CACGTGCCCT	AGGGCACGTG
	SI-TT-C9	TATCAGCCTA	GTTTCGTCCT	AGGACGAAAC
	SI-TT-C10	AGAATGGTTT	GAGGGTGGGA	TCCCACCCTC
	SI-TT-C11	ATGGGTGAAA	CTTGGGAATT	AATTCCCAAG
	SI-TT-C12	TCGTCAAGAT	GCAACTCAGG	CCTGAGTTGC
	SI-TT-D1	TGCAATGTTC	GCTTGTCGAA	TTCGACAAGC
	SI-TT-D2	TTAATACGCG	CACCTCGGGT	ACCCGAGGTG
	SI-TT-D3	CCTTCTAGAG	AATACAACGA	TCGTTGTATT
	SI-TT-D4	GCAGTATAGG	TTCCGTGCAC	GTGCACGGAA
	SI-TT-D5	TGGTTCGGGT	GTGGCAGGAG	CTCCTGCCAC
	SI-TT-D6	CCCAGCTTCT	GACACCAAAC	GTTTGGTGTC
	SI-TT-D7	CCTGTCAGGG	AGCCCGTAAC	GTTACGGGCT
	SI-TT-D8	CGCTGAAATC	AGGTGTCTGC	GCAGACACCT
	SI-TT-D9	TGGTCCCAAG	CCTCTGGCGT	ACGCCAGAGG
	SI-TT-D10	ATGCGAATGG	ACAAGTGTCG	CGACACTTGT
	SI-TT-D11	CGAATATTCG	CTGGAAGCAA	TTGCTTCCAG
	SI-TT-D12	GAATTGGTTA	ACTCTAGTAG	CTACTAGAGT
	SI-TT-E1	TTATTCGAGG	CTGTCCTGCT	AGCAGGACAG
	SI-TT-E2	ATGGAGGGAG	ATAACCCATT	AATGGGTTAT
	SI-TT-E3	ACCAGACAAC	AGGAACTAGG	CCTAGTTCCT
	SI-TT-E4	AACCACGCAT	ATTCAGGTTA	TAACCTGAAT
	SI-TT-E5	CGCGGTAGGT	CAGGATGTTG	CAACATCCTG
	SI-TT-E6	TTGAGAGTCA	AACCTGGTAG	CTACCAGGTT
	SI-TT-E7	GTCCTTCGGC	TCATGCACAG	CTGTGCATGA
	SI-TT-E8	GAGCAAGGGC	ATTGACTTGG	CCAAGTCAAT
	SI-TT-E9	TGTCCCAACG	TCGATGTCCA	TGGACATCGA
	SI-TT-E10	CACAATCCCA	ATATCCACAA	TTGTGGATAT
	SI-TT-E11	TCCGGGACAA	GTGAATGCCA	TGGCATTCAC

	SI-TT-E12	CGTCCACCTG	CATTCATGAC	GTCATGAATG
	SI-TT-F1	AAGATTGGAT	AGCGGGATTT	AAATCCCGCT
	SI-TT-F2	AAGGGCCGCA	CTGATTCCTC	GAGGAATCAG
	SI-TT-F3	GAGAGGATAT	TTGAAATGGG	CCCATTTCAA
	SI-TT-F4	CCCACCACAA	ACCTCCGCTT	AAGCGGAGGT
	SI-TT-F5	CGGCTGGATG	TGATAAGCAC	GTGCTTATCA
	SI-TT-F6	TTGCCCGTGC	GCGTGAGATT	AATCTCACGC
	SI-TT-F7	AATGTATCCA	AATGAGCTTA	TAAGCTCATT
	SI-TT-F8	CTCCTTTAGA	GACATAGCTC	GAGCTATGTC
	SI-TT-F9	GTCCCATCAA	CGAACGTGAC	GTCACGTTCG
	SI-TT-F10	CCGGCAACTG	CGGTTTAACA	TGTTAAACCG
	SI-TT-F11	TTCACACCTT	TAGTGTACAC	GTGTACACTA
	SI-TT-F12	GAGACGCACG	CTATGAACAT	ATGTTCATAG
	SI-TT-G1	TGTAGTCATT	CTTGATCGTA	TACGATCAAG
	SI-TT-G2	CATGTGGGTT	GATTCCTTTA	TAAAGGAATC
	SI-TT-G3	ATGACGTCGC	AGGTCAGGAT	ATCCTGACCT
	SI-TT-G4	GCGCTTATGG	GCCTGGCTAG	CTAGCCAGGC
	SI-TT-G5	ATAGGGCGAG	TGCATCGAGT	ACTCGATGCA
	SI-TT-G6	GCGGGTAAGT	TAGCACTAAG	CTTAGTGCTA
	SI-TT-G7	GTTTCACGAT	TTCGGCCAAA	TTTGGCCGAA
	SI-TT-G8	TAAGCAACTG	CTATACTCAA	TTGAGTATAG
	SI-TT-G9	CCGGAGGAAG	TGCGGATGTT	AACATCCGCA
	SI-TT-G10	ACTTTACGTG	TGAACGCCCT	AGGGCGTTCA
	SI-TT-G11	GATAACCTGC	CATTAGAAAC	GTTTCTAATG
	SI-TT-G12	CTTGCATAAA	ATCAGGGCTT	AAGCCCTGAT
	SI-TT-H1	ACAATGTGAA	CGTACCGTTA	TAACGGTACG
	SI-TT-H2	TAGCATAGTG	CGGCTCTGTC	GACAGAGCCG
	SI-TT-H3	CCCGTTCTCG	GACGGATTGG	CCAATCCGTC

	SI-TT-H4	AGTTTCCTGG	TGCCACACAG	CTGTGTGGCA
	SI-TT-H5	AGCAAGAAGC	TTGTGTTTCT	AGAAACACAA
	SI-TT-H6	CCTATCCTCG	GAATACTAAC	GTTAGTATTC
	SI-TT-H7	ACCTCGAGCT	TGTGTTCGAT	ATCGAACACA
	SI-TT-H8	ATAAGGATAC	ATAGATAGGG	CCCTATCTAT
	SI-TT-H9	AGAACTTAGA	CGAGTCCTTT	AAAGGACTCG
	SI-TT-H10	TTATCTAGGG	AAAGGCTCTA	TAGAGCCTTT
	SI-TT-H11	ACAATCGATC	TGACGGAATG	CATTCCGTCA
	SI-TT-H12	TGATGATTCA	GTAGGAGTCG	CGACTCCTAC

1. Add 50 ul Amp Mix (PN-2000047/2000103) or Library Amp Mix (PN-2000531) (either of them will work for gene expression library construction) to 30 ul of sample.
2. Add 20 ul of an individual Dual Index TT Set A to each sample and note the well ID used. Pipette mix 5x

Run the following program, note that the cycle number has to be adjusted to the cDNA input (see section **2.5 Post cDNA Amplification QC and Quantification - Gene Expression Part Only -** in short: 25% of the total cDNA yield is the input cDNA)

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~30 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	54°C	00:00:30
4	72°C	00:00:20
5	Go to step 2, see below for # of cycles	
6	72°C	00:01:00
7	4°C	Hold

The table recommends a starting point for optimization. The total cycles should be optimized based on 25% carry forward cDNA yield/input calculated during cDNA QC & Quantification (step 2.4).

Recommended Cycle Numbers

cDNA Input	Total Cycles
0.25-50 ng	14-16
50-250 ng	12-14
250-600 ng	10-12
600-1,100 ng	8-10
1,100-1,500 ng	6-8
>1,500 ng	5

Post Sample Index PCR Double Sided Size Selection - SPRIselect

1. Vortex to resuspend the SPRI beads and add 60 ul SPRI beads (0.6x) to each sample and pipet mix for 15x
2. Incubate for 5 minutes
3. Place tube in magnet and incubate for 5 minutes
4. Transfer 150 ul of supernatant to a new tube (Keep the supernatant!)
5. Vortex to resuspend the SPRI beads and add 20 ul SPRI beads (0.8x) to the transferred supernatant and pipet mix for 15x
6. Incubate for 5 minutes
7. Place tube into magnet and incubate for 5 minutes
8. Remove 165 ul supernatant. DO NOT discard any beads!
9. With the tube still in the magnet, add 200 ul of 80% Ethanol to the pellet and incubate for 30 seconds.
10. Remove the ethanol

11. With the tube still in the magnet, add 200 ul of 80% Ethanol to the pellet and incubate for 30 seconds.
12. Remove the ethanol (total of 2 wash steps)
13. Centrifuge briefly. Place tube back into the magnet.
14. Carefully remove residual ethanol with a P10 or P20. Air dry for 1 minute
15. Remove tube from magnet. Resuspend beads in 35.5 ul EB Buffer by pipetting 15x
16. Incubate for 2 minutes
17. Place tube on the magnet and incubate for 5 minutes
18. Transfer 35 ul of supernatant to a new tube.
19. Run sample on D1000 Tapestation and verify clean peak at around 400 bp

CRISPR Screening Library Construction

13 **Guide RNA cDNA Cleanup - SPRIselect**

1. Vortex SPRI beads and add 50 ul of beads (1x) to 50 ul transferred supernatant cleanup from step (xxX) and mix via pipetting 15x.
2. Incubate for 5 minutes
3. Place on magnet and incubate for 5 minutes
4. Remove supernatant
5. Add 200 ul of 80% Ethanol to the pellet and incubate for 30 seconds
6. Remove Ethanol.
7. Add 200 ul of 80% Ethanol to the pellet and incubate for 30 seconds
8. Remove Ethanol (total of 2 wash steps)
9. Carefully remove any remaining ethanol. Air dry for 2 minutes
10. Remove tube from magnet and resuspend pellet in 40.5 ul EB Buffer.
11. Incubate for 2 minutes
12. Place tube on magnet and incubate for 5 minutes
13. Transfer 40 ul of supernatant to new tube.

Feature PCR

1. Prepare the following mix on ice.

Feature PCR Mix *Add reagents in the order listed*	PN	1X (µl)	2X + 10% (µl)
○ **Amp Mix**	2000047	50	110
● **Feature SI Primers 4**	2000592	45	99
Total		**95**	**209**

1. Transfer only 5 ul from the Guide RNA cDNA cleanup to a PCR tube and add 95 ul of Feature PCR mix. Gently mix via pipetting.
2. Run samples with the following PCR program:

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~20 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:10
4	72°C	00:00:10
5	Go to step 2, repeat 9X for a total 10 cycles	
6	72°C	00:01:00
7	4°C	Hold

Post Feature PCR Cleanup - SPRIselect

1. Vortex SPRI beads and add 100 ul of beads to sample. Mix via pipetting 15x
2. Incubate for 5 minutes at room temperature
3. Place tube in magnet and incubate for 5 minutes
4. Remove supernatant
5. Add 300 ul of 80% Ethanol and incubate for 30 seconds
6. Remove ethanol.
7. Add 300 ul of 80% Ethanol and incubate for 30 seconds
8. Remove ethanol (total of 2 wash steps)
9. Carefully remove any remaining ethanol. Air dry for 2 minutes
10. Remove tube from magnet and resuspend in 30.5 ul EB buffer by pipetting 15x
11. Incubate for 2 minutes
12. Place tube in magnet and incubate for 5 minutes
13. Transfer 30 ul supernatant to a fresh tube.

Sample Index PCR

Choose the appropriate sample index sets to ensure that no sample indices overlap in a multiplexed sequencing run. Record the 10x sample index name (PN-3000431 Dual Index Plate TT Set A well ID) used. See list below:

	index_name	index(i7)	index2_workflow_a(i5)	index2_workflow_b(i5)

	SI-TT-A1	GTAACATGCG	AGTGTTACCT	AGGTAACACT
	SI-TT-A2	GTGGATCAAA	GCCAACCCTG	CAGGGTTGGC
	SI-TT-A3	CACTACGAAA	TTAGACTGAT	ATCAGTCTAA
	SI-TT-A4	CTCTAGCGAG	TATCTTCATC	GATGAAGATA
	SI-TT-A5	GTAGCCCTGT	GAGCATCTAT	ATAGATGCTC
	SI-TT-A6	TAACGCGTGA	CCCTAACTTC	GAAGTTAGGG
	SI-TT-A7	TCCCAAGGGT	TACTACCTTT	AAAGGTAGTA
	SI-TT-A8	CGAAGTATAC	GAACTTGGAG	CTCCAAGTTC
	SI-TT-A9	AAGTGGAGAG	TTCCTGTTAC	GTAACAGGAA
	SI-TT-A10	CGTGACATGC	ATGGTCTAAA	TTTAGACCAT
	SI-TT-A11	CGGAACCCAA	GATTCGAGGA	TCCTCGAATC
	SI-TT-A12	CACCGCACCA	GACTGTCAAT	ATTGACAGTC
	SI-TT-B1	ACAGTAACTA	ACAGTTCGTT	AACGAACTGT
	SI-TT-B2	TCTACCATTT	CGGGAGAGTC	GACTCTCCCG
	SI-TT-B3	CACGGTGAAT	GTTCGTCACA	TGTGACGAAC
	SI-TT-B4	GTAGACGAAA	CTAGTGTGGT	ACCACACTAG
	SI-TT-B5	TCGGCTCTAC	CCGATGGTCT	AGACCATCGG
	SI-TT-B6	AATGCCATGA	TACGTAATGC	GCATTACGTA
	SI-TT-B7	GCCTTCGGTA	CCAACGATTT	AAATCGTTGG
	SI-TT-B8	GCACTGAGAA	TATGCGTGAA	TTCACGCATA
	SI-TT-B9	TATTGAGGCA	CAGGTAAGTG	CACTTACCTG
	SI-TT-B10	GCCCGATGGA	AATCGTCTAG	CTAGACGATT
	SI-TT-B11	TCTTACTTGC	TGACCTCTAG	CTAGAGGTCA
	SI-TT-B12	CGTCAAGGGC	TAGGTCACTC	GAGTGACCTA
	SI-TT-C1	TGCGCGGTTT	CAAGGATAAA	TTTATCCTTG
	SI-TT-C2	CAATCCCGAC	CCGAGTAGTA	TACTACTCGG
	SI-TT-C3	ATGGCTTGTG	GAATGTTGTG	CACAACATTC
	SI-TT-C4	TTCTCGATGA	TGTCGGGCAC	GTGCCCGACA

	SI-TT-C5	TCCGTTGGAT	ACGTTCTCGC	GCGAGAACGT
	SI-TT-C6	ACGACTACCA	ACGACCCTAA	TTAGGGTCGT
	SI-TT-C7	CGCGCACTTA	CCTGTATTCT	AGAATACAGG
	SI-TT-C8	GCTACAAAGC	CACGTGCCCT	AGGGCACGTG
	SI-TT-C9	TATCAGCCTA	GTTTCGTCCT	AGGACGAAAC
	SI-TT-C10	AGAATGGTTT	GAGGGTGGGA	TCCCACCCTC
	SI-TT-C11	ATGGGTGAAA	CTTGGGAATT	AATTCCCAAG
	SI-TT-C12	TCGTCAAGAT	GCAACTCAGG	CCTGAGTTGC
	SI-TT-D1	TGCAATGTTC	GCTTGTCGAA	TTCGACAAGC
	SI-TT-D2	TTAATACGCG	CACCTCGGGT	ACCCGAGGTG
	SI-TT-D3	CCTTCTAGAG	AATACAACGA	TCGTTGTATT
	SI-TT-D4	GCAGTATAGG	TTCCGTGCAC	GTGCACGGAA
	SI-TT-D5	TGGTTCGGGT	GTGGCAGGAG	CTCCTGCCAC
	SI-TT-D6	CCCAGCTTCT	GACACCAAAC	GTTTGGTGTC
	SI-TT-D7	CCTGTCAGGG	AGCCCGTAAC	GTTACGGGCT
	SI-TT-D8	CGCTGAAATC	AGGTGTCTGC	GCAGACACCT
	SI-TT-D9	TGGTCCCAAG	CCTCTGGCGT	ACGCCAGAGG
	SI-TT-D10	ATGCGAATGG	ACAAGTGTCG	CGACACTTGT
	SI-TT-D11	CGAATATTCG	CTGGAAGCAA	TTGCTTCCAG
	SI-TT-D12	GAATTGGTTA	ACTCTAGTAG	CTACTAGAGT
	SI-TT-E1	TTATTCGAGG	CTGTCCTGCT	AGCAGGACAG
	SI-TT-E2	ATGGAGGGAG	ATAACCCATT	AATGGGTTAT
	SI-TT-E3	ACCAGACAAC	AGGAACTAGG	CCTAGTTCCT
	SI-TT-E4	AACCACGCAT	ATTCAGGTTA	TAACCTGAAT
	SI-TT-E5	CGCGGTAGGT	CAGGATGTTG	CAACATCCTG
	SI-TT-E6	TTGAGAGTCA	AACCTGGTAG	CTACCAGGTT
	SI-TT-E7	GTCCTTCGGC	TCATGCACAG	CTGTGCATGA
	SI-TT-E8	GAGCAAGGGC	ATTGACTTGG	CCAAGTCAAT

	SI-TT-E9	TGTCCCAACG	TCGATGTCCA	TGGACATCGA
	SI-TT-E10	CACAATCCCA	ATATCCACAA	TTGTGGATAT
	SI-TT-E11	TCCGGGACAA	GTGAATGCCA	TGGCATTCAC
	SI-TT-E12	CGTCCACCTG	CATTCATGAC	GTCATGAATG
	SI-TT-F1	AAGATTGGAT	AGCGGGATTT	AAATCCCGCT
	SI-TT-F2	AAGGGCCGCA	CTGATTCCTC	GAGGAATCAG
	SI-TT-F3	GAGAGGATAT	TTGAAATGGG	CCCATTTCAA
	SI-TT-F4	CCCACCACAA	ACCTCCGCTT	AAGCGGAGGT
	SI-TT-F5	CGGCTGGATG	TGATAAGCAC	GTGCTTATCA
	SI-TT-F6	TTGCCCGTGC	GCGTGAGATT	AATCTCACGC
	SI-TT-F7	AATGTATCCA	AATGAGCTTA	TAAGCTCATT
	SI-TT-F8	CTCCTTTAGA	GACATAGCTC	GAGCTATGTC
	SI-TT-F9	GTCCCATCAA	CGAACGTGAC	GTCACGTTCG
	SI-TT-F10	CCGGCAACTG	CGGTTTAACA	TGTTAAACCG
	SI-TT-F11	TTCACACCTT	TAGTGTACAC	GTGTACACTA
	SI-TT-F12	GAGACGCACG	CTATGAACAT	ATGTTCATAG
	SI-TT-G1	TGTAGTCATT	CTTGATCGTA	TACGATCAAG
	SI-TT-G2	CATGTGGGTT	GATTCCTTTA	TAAAGGAATC
	SI-TT-G3	ATGACGTCGC	AGGTCAGGAT	ATCCTGACCT
	SI-TT-G4	GCGCTTATGG	GCCTGGCTAG	CTAGCCAGGC
	SI-TT-G5	ATAGGGCGAG	TGCATCGAGT	ACTCGATGCA
	SI-TT-G6	GCGGGTAAGT	TAGCACTAAG	CTTAGTGCTA
	SI-TT-G7	GTTTCACGAT	TTCGGCCAAA	TTTGGCCGAA
	SI-TT-G8	TAAGCAACTG	CTATACTCAA	TTGAGTATAG
	SI-TT-G9	CCGGAGGAAG	TGCGGATGTT	AACATCCGCA
	SI-TT-G10	ACTTTACGTG	TGAACGCCCT	AGGGCGTTCA
	SI-TT-G11	GATAACCTGC	CATTAGAAAC	GTTTCTAATG
	SI-TT-G12	CTTGCATAAA	ATCAGGGCTT	AAGCCCTGAT

	SI-TT-H1	ACAATGTGAA	CGTACCGTTA	TAACGGTACG
	SI-TT-H2	TAGCATAGTG	CGGCTCTGTC	GACAGAGCCG
	SI-TT-H3	CCCGTTCTCG	GACGGATTGG	CCAATCCGTC
	SI-TT-H4	AGTTTCCTGG	TGCCACACAG	CTGTGTGGCA
	SI-TT-H5	AGCAAGAAGC	TTGTGTTTCT	AGAAACACAA
	SI-TT-H6	CCTATCCTCG	GAATACTAAC	GTTAGTATTC
	SI-TT-H7	ACCTCGAGCT	TGTGTTCGAT	ATCGAACACA
	SI-TT-H8	ATAAGGATAC	ATAGATAGGG	CCCTATCTAT
	SI-TT-H9	AGAACTTAGA	CGAGTCCTTT	AAAGGACTCG
	SI-TT-H10	TTATCTAGGG	AAAGGCTCTA	TAGAGCCTTT
	SI-TT-H11	ACAATCGATC	TGACGGAATG	CATTCCGTCA
	SI-TT-H12	TGATGATTCA	GTAGGAGTCG	CGACTCCTAC

1. Add 50 µl Amp Mix (PN-200047) to 30 µl sample (Post Feature PCR Cleanup).
2. Add 20 ul of an individual Dual Index TT Set A to each sample and note the well ID used. Pipette mix 5x

Lid Temperature	Reaction Volume	Run Time
105°C	100 µl	~25 min
Step	**Temperature**	**Time** *hh:mm:ss*
1	98°C	00:00:45
2	98°C	00:00:20
3	62°C	00:00:10
4	72°C	00:00:10
5	Go to step 2, repeat 9X for a total of 10 cycles	
6	72°C	00:01:00
7	4°C	Hold

Re-Amplify CRISPR Library

Read1 primer

10xGen_FeatureSI_Read1 GATCTACACTCTTTCC CTACACGACGC
Tm= 49c
Read2 primers
062425_10xgRNAhp_r2seqB (O3869) GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CAAGTTGATAACGGACTAGCCTTATT
Tm= 64c

1. Prepare the following qPCR

10 uL	2X Q5 mastermix
1 uL	20X EvaGreen
0.5 uL	20 uM 062425_10xgRNAhp_r2seqB (O3869)
0.5 ul	20 uM 10xGen_FeatureSI_Read1
0.5 uL	10x gRNA library
7.5 uL	dH20

Perform the following qPCR protocol
98 deg for 30s
35 cycles of:
98 deg for 10s
62 deg for 30s
72 deg for 30s
Then:
72 deg for 60s
4 deg forever

Optional
1. PCR purify
2. Run 1 uL of each reaction on D1000 Tapestation. We are looking for a spike at ~200-250 nt (note that this is shorter than the full product because you have only added partial adapters
3. The goal is to identify the gRNA primer that is most efficient (low Ct) and specific (highest ratio of product in the correctly sized spike)
4. Cycle number for PCR1 is dependent on qPCR - ideally, choose cycle number 2-3 cycles before the plateau phase

Perform PCR1

1. Prepare the following PCR reaction:

50 uL	2X Q5 mastermix
2.5 uL	20 uM 10xGen_FeatureSI_Read1
2.5 uL	20 uM 062425_10xgRNAhp_r2seqB (O3869)
30 uL	10x gRNA library
15 uL	dH20

1. Perform the following PCR protocol
98 deg for 30s
Number of cycles optimized in qPCR (2-3 cycles before plateau)
98 deg for 10s
62 deg for 30s
72 deg for 30s
Then:
72 deg for 60s
4 deg forever

Load PCR1 onto a 2%gel. Because of the 100 ul volume, carefully distribute the PCR sample along 2 wells of a 12 well comb. Carefully cut around the 200 bp band and gel purify.

Perform qPCR2 to determine PCR2 cycle number

1. Prepare the following qPCR reaction:

10 uL	2X Q5 mastermix
1 uL	20X EvaGreen
0.5 uL	20 uM NEBNext i5 index primer (note that any i5 primer is OK)
0.5 uL	20 uM NEBNext i7 index primer (note that any i7 primer is OK)
0.5 uL	10x gRNA library PCR1 (from step 4b 4)
7.5 uL	dH20

Perform the following qPCR protocol
98 deg for 30s
35 cycles of:
98 deg for 10s
72 deg for 30s
Then:
72 deg for 60s
4 deg forever

You can stop the qPCR once reaction plateaus.

- Determine the plateau phase and subtract 2-3 cycles to find the optimal cycle number for PCR2

Perform PCR2

1. Prepare the following PCR reaction:

25 uL	2X Q5 mastermix

1.25 uL	20 uM NEBNext i5 index primer (note which i5 is used for NGS sheet)
1.25 uL	20 uM NEBNext i7 index primer (note which i7 is used for NGS sheet)
2 uL	10x gRNA library
20.5 ul	Water

1. Perform the following PCR protocol
98 deg for 30s
XX cycles of:
98 deg for 10s
72 deg for 30s
Then:
72 deg for 60s
4 deg forever

1. Perform SPRI clean up using either 1-sided or 2-sided procedure pending qPCR Tapestation result. Protocol below will have to be updated.
§ To the PCR reaction, add XX ul of mixed, ROOM TEMP, SPRI beads to each sample (this is a XX SPRI).
§ Vortex briefly and incubate for 5min at room temp
§ Apply magnet to collect beads
§ Once solution is clear, use pipette to remove supernatant
§ While still on magnet, add 180ul of 80% ETOH to each sample without mixing
§ Incubate for 30 sec at room temperature
§ Remove supernatant with pipette
§ Repeat steps EtOH wash steps for a second ethanol wash
§ Allow tubes to sit at room temp so the residual ethanol can evaporate, beads will turn from shiny to matte when dry (2-5 min), then proceed
§ With tubes off the magnet, add 20ul DNA Purification Elution Buffer
§ Cap tubes and mix by vortexing
§ Incubate samples for 5 min at room temp
§ Apply magnet to samples
§ Transfer 20 uL supernatant to a fresh labeled tube.

Perform Tapestation D1000 to quantify product. You may have to perform further bead purification to ensure clean product peak.