Protocol for 5' Direct Capture Perturb-seq of Unmodified sgRNAs.

5' direct capture perturb-seq platform is suitable for use with standard sgRNA sequences (no engineered capture sequence). Although oJR160 should anneal to most spCas9 perturbation systems, a wide variety of sgRNA variants are currently in use, so it is necessary to check the compatibility of oJR160 obefore proceeding. Additionally, if running multiple lanes on the 10x Genomics Chromium Controller, oJR165 variants with unique i7 indices will be necessary to demultiplex libraries during sequencing.

This protocol follows the Chromium Single Cell V(D)J Reagent Kits User Guide (10x Genomics, CG000086). The Chromium Single Cell 3' Reagent Kits v3 User Guide with Feature Barcoding technology for CRISPR screening (10x Genomics, CG000184) serves as a reference for the cDNA SPRI cleanup.

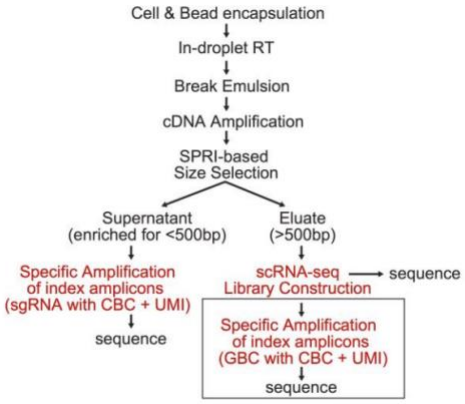
Primer designs for 5' direct capture Perturb-seq:

Primer ID	Primer sequence (5' to 3')
oJR160 (RT primer)	Design: 10X sequence–guide annealing AAGCAGTGGTATCAACGCAGAGTACCAAGTTGATAACGGACTAGCC
oJR163 (Amplification primer)	Design: P5–TruSeq Read 1 / Index 2 (on bead sequence) AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
oJR165 (Amplification primer)	Design: P7–**i7 index**–Nextera Read 2/Index 1–Shared Nextera Read 2/Annealing–annealing region CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGTACCAAGTTGATAACGGACTAGCC

1) **Perform droplet capture with the 10x Genomics Chromium Single Cell V(D)J Reagent Kits.** Prior to droplet formation, we add oJR160 (guide-specific RT primer) to the RT Master Mix**.** We add 5 pmols oJR160 (0.5 uL of 10 uM) directly to the 68.3 uL of RT Master Mix used for each 10x lane.

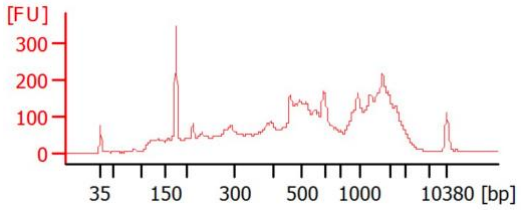
2) **cDNA amplification**. oJR160 has an adapter identical to the adapter sequence on the Poly-dT RT Primer (10x Genomics, PN-2000007). This adapter servers as a primer binding site for the Non-Poly(dT) primer (10x Genomics, PN-220106) during cDNA amplification, and thus allows amplification of reverse transcribed guides to occurs concurrently with standard cDNA amplification.

3) **cDNA cleanup and separation of cDNA libraries fractions.** For reference, see the schematic overview below and step 2.3 of our 3' direct capture Perturb-seq protocol (10x Genomics, CG000184) which generally mirrors the 5' direct capture workflow. Briefly, guide-containing cDNA amplicons (168 bp) can be enriched by size selection. To do this, we perform a 0.6X left-sided SPRI cleanup reaction and collect both the beads (which carry material for preparing the gene expression library below in step 7) and the supernatant (which is enriched for guide-derived cDNA amplicons). Use the supernatant, we complete a 0.6X-1.2X double-sided SPRI selection in order to collect the guide-containing fraction and proceed to step 4.



4) **Quantify the guide-enriched cDNA fraction.** The expected size of the guide-containing cDNA amplicons (with adapters, CBC, UMI) is 168 bp. In K562 cells, a peak is apparent in traces produced by a Bioanalyzer High Sensitivity DNA Analysis (Agilent) (representative plot below). To determine how much template to add to the specific amplification PCR in the next step, we quantify the mass of 145 bp to 1000 bp products in the sample.

   Sample Bioanalyzer trace, with peak at 168 bp:



5) **Specific amplification of guide-containing cDNA amplicons.** Using 5 ng of the guide-enriched cDNA fraction per reaction, we perform 4 PCRs with KAPA HiFi HotStart ReadyMix and 0.6 uM of primers **oJR163** and **oJR165**, as below. We purify the resulting guide (index) sequencing libraries with a 0.8X SPRI cleanup reaction to remove primers and elute in 40 uL.

Components	Volume for 1 reaction (uL)	Volume for 4.25 reactions (uL)
10 uM P5-primer	3	12.75
10 uM P7-primer	3	12.75
Template DNA (5 ng)	depends on template concentration	depends on template concentration
2X Kapa HiFi Master Mix	25	106.25
Water	depends on template concentration	depends on template concentration
Total	50	212.5

Step	Time	Temperature	Cycles
Initial Denaturation	3 minutes	95°C	1
Denature	15 seconds	98°C	12X
Anneal/Elongate	10 seconds	70°C	
Final Elongation	1 minutes	72°C	1
Hold	hold	4°C	1

6) **Quantification of the final guide (index) sequencing libraries.** We measure the concentration of the final guide sequencing libraries using a Bioanalyzer High Sensitivity DNA Analysis (Agilent) at a 1:10 dilution. The expected size of the peak is 250 bp.

7) **Prepare the corresponding gene expression library.** Gene expression libraries are prepared from material collected in step 3. For this, refer to the Chromium Single Cell V(D)J Reagent Kits User Guide (10x Genomics, CG000086).

8) **Sequencing.** We sequence the guide (index) sequencing libraries as ~10% spike-ins added to gene expression libraries. The Illumina Read 2 will sequence the sgRNA constant region before sequencing the sgRNA targeting region. Therefore, it is always necessary to sequence the sgRNA library alongside a higher diversity library (such as the gene expression library) or with PhiX. We recommend using a Read 2 of 98 bp to ensure that the read sequences through the sgRNA targeting region.