CRISPR droplet sequencing (CROP-seq) protocol (version 2017/01/18)

Authors: Paul Datlinger (pdatlinger@cemm.oeaw.ac.at), André F Rendeiro*, Christian Schmidl*, Thomas Krausgruber, Peter Traxler, Johanna Klughammer, Linda C Schuster, Amelie Kuchler, Donat Alpar and Christoph Bock (cbock@cemm.oeaw.ac.at).

Future updates of the protocol will be shared via http://crop-seq.computational-epigenetics.org.

Part A: Pooled, amplification-free cloning of gRNA libraries

1. Validation of the CROPseq-Guide-Puro plasmid preparation

Order the CROPseq-Guide-Puro plasmid from Addgene (86708), perform plasmid DNA extraction, and validate by restriction digestion and Sanger sequencing.

Plasmid validation by restriction digestion			
Restriction enzyme	Buffer	Temperature [°C]	Expected fragments [bp]
SapI	CutSmart	37 °C	1171, 3394, 5649
SphI	NEB Buffer 2.1	65 °C	1110, 3787, 5317

Plasmid validation by Sanger sequencing		
Primer ID	Primer sequence [5' > 3']	Validates element(s)
86708_p1	GAGGGCCTATTTCCCATGATTCC	hU6, BsmBI site
86708_p2	CAAATGCATGCTCTTCAACCTC	BsmBI site, gRNA backbone, 3' LTR
86708_p3	GAGACAAATGGCAGTATTCATCCAC	EF-1a
86708_p4	TAAGTGCAGTAGTCGCCGTG	EF-1a, Puro
86708_p5	AGTCTTGTAAATGCGGGCCA	Puro
86708_p6	CACACTGAGTGGGTGGAGAC	Puro
86708_p7	AGCAACAGATGGAAGGCCTC	WPRE
86708_p8.1	TTGGGCACTGACAATTCCGT	3' LTR, filler or gRNA
86708_p10	GACGAACCACTGAATTGCCG	Psi, Gag
86708_p11	GGGAGCTAGAACGATTCGCA	RRE
86708_p12	TTCCTTGGGTTCTTGGGAGC	cPPT
86708_p13	CGAGTCAGTGAGCGAGGAAG	5' LTR

2. Preparation of CROPseq-Guide-Puro vector backbone

2.1 Plasmid digestion

Digest CROPseq-Guide-Puro plasmid and gel-purify the 8,333 bp fragment as indicated. Once prepared, the backbone fragment can be frozen at -20 °C and re-used for cloning further gRNA libraries.

Vector digestion mix							
	Per reaction		Master mix for # of digestions				
Component	Volume	Amount	4	6	8	10	12
CROPseq-Guide-Puro		5 µg					
NEB Buffer 3.1	2.5 µl	1x	10.0	15.0	20.0	25.0	30.0
BsmBI	2 µl	20 U	8.0	12.0	16.0	20.0	24.0
Nuclease-free water		to 25 µl					

Distribute 25 μl of vector digestion mix per well into a tube strip, incubate: 1 hour at 55 °C (digestion), 20 min at 80 °C (inactivation of the enzyme), hold at 10 °C.

2.2 UV-free gel extraction

<Note> Based on SNAP UV-Free Gel Purification Kit (Invitrogen cat. no. 45-0105)

Prepare required number of 0.8% agarose gels with TAE buffer containing 1.8 μg/ml Crystal Violet:
Example for one gel: 0.4 g agarose in 50 ml TAE, plus 40 μl of 2 mg/ml Crystal Violet Solution
Important: use a thin comb for more intense bands, TBE is not compatible with this kit

Load 8 μg of DNA ladder in 25 μl of water, mixed with 5 μl of 6x Crystal Violet Loading Dye

Load 25 μl of each plasmid digestion, mixed with 5 μl of 6x Crystal Violet Loading Dye

Run at 80 V and observe the separation of bands with the naked eye

Once the bands are sufficiently resolved, stop and isolate 8,333 bp fragment with gel-cutting tips

Transfer gel slice to 1.5 ml tube, weigh to estimate the agarose volume (assuming that 1 mg ~ 1 μl)

Add 2.5 volumes of Sodium Iodide Solution

Incubate at 42 °C with occasional vortexing, until the agarose is completely dissolved

Add 1.5 volumes of Binding Buffer, mix and load onto SNAP Purification Column

Centrifuge at 3,000 rcf for 30 seconds

Pour the flow through back onto the column and repeat the centrifugation, for a total of three times

After the last centrifugation step, discard the flow through

Wash two times with 400 μl of 1x Final Wash Buffer, discard flow through after second centrifugation

Centrifuge column for 1 min at > 10,000 rcf to remove residual wash buffer

Transfer the column to a fresh 1.5 ml tube and elute with 40 μl of EB buffer (10,000 rcf for 1 min)
Important: incubate for at least 1 min to allow absorption into the column

Transfer the eluate back to the column, and elute a second time as described above

Measure 1 μl of the eluate in a Qubit HS assay to determine the concentration of dsDNA

<Safe Stopping Point> The backbone fragment can be stored at -20 °C and re-used.

<Troubleshooting> If required, perform ethanol precipitation to further concentrate the DNA: pool multiple gel-purification reactions, add 1/10 volume of 3M sodium acetate, pH 5.5 and 2.5 volumes of 100% ice cold ethanol. Precipitate DNA for 30 min at -80 °C, then spin for 20 min at 20,000 rcf at 4 °C to pellet. Wash pellet two times with room temperature 70 % ethanol, leave to dry (take care not to over-dry the pellet) and elute in a smaller volume of EB buffer.

3. Assembly of gRNA-encoding ssDNA oligonucleotides into the vector backbone using Gibson Assembly

3.1 Design gRNAs

Recommended for manual design: https://benchling.com/, for automated design: https://github.com/boutroslab/cld/. Order as separate ssDNA oligos, with 18 and 35 bases of homology to the hU6 promoter and gRNA backbone for Gibson Assembly. The 5' prefix also adds a G as a constant first base for more efficient transcription from the hU6 promoter. Examples for positive controls (targeting essential genes) and negative controls (non-targeting) can be found in the original publication of CROP-seq (Supplementary Table 2).

<Example> TGGAAAGGACGAAACACCG (5' prefix, hU6 promoter and G as first base) + gRNA + GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC (3' postfix, gRNA backbone)

3.2 Gibson Assembly

Dilute gRNA-encoding ssDNA oligos to 100 μM with EB buffer. Pool all oligos contained in the library (including positive and negative controls) in equal amounts. Dilute the oligo pool 1:1000 in EB buffer, resulting in a molarity of 100 nM. Set up Gibson assembly reactions for each library and the included NEBuilder HiFi positive control, as described below.

Assembly of gRNA-encoding ssDNA oligos into the vector backbone by Gibson Assembly		
		Per reaction
Component	Volume	Amount
CROPseq-Guide-Puro backbone fragment (8,333 bp)		11 fmoles
Library ssDNA oligos, pooled (100 nM)	2.0 μl	200 fmoles
NEBuilder HiFi DNA Assembly Master Mix	10 μl	1x
Nuclease-free water		to 20 μl

<Note> We use the following online tool to calculate the volume of backbone fragment required:
http://nebiocalculator.neb.com/#!/dsdnaends

Incubate at 50 °C for 1 hour, then dialyze the reaction against water:

Fill a 6 cm cell culture dish with HPLC-quality water, place membrane filter on top

Pipette 20 μl of the Gibson Assembly reaction and let float on top of the filter for 30 min

<Note> This removes salts and makes the electroporation more efficient

About 10 μl of the Gibson Assembly reaction can be recovered after the filtration

3.3 Electroporation of Lucigen Endura cells

Preparations

Prepare mixture of ice and water

Thaw one vial of Lucigen Endura electrocompetent cells on ice (takes about 20 min, sufficient for cloning one gRNA library with two electroporations)

Pre-chill two 1 mm electroporation cuvettes on ice

Place two empty 2 ml tubes on ice

Pre-warm Lucigen recovery medium at 37 °C

Pre-warm two 25 x 25 cm LB-agar plates containing 100 μg/ml carbenicillin at room temperature

Electroporation

Aliquot 25 μl of bacteria into pre-chilled 2 ml tubes, and keep on ice

Add 10 μl of de-salted Gibson Assembly to the bacteria and gently tap the tube to mix

Transfer to electroporation cuvette, tap to bring contents to the bottom, check for air bubbles

Prepare P1000 pipette filled with 1 ml of Lucigen recovery medium

Electroporate with the following settings: 25 μF, 200 Ω, 1.5 kV

Immediately after the pulse, add the prepared Lucigen recovery medium

Gently pipet up and down two times, then transfer to round-bottom tubes

Take a note of the time constant for the electroporation (should be ~4.5 msec)

Incubate for 1 hour at 37 °C for recovery

Prepare a 1:100 dilution (10 μl bacteria plus 990 μl SOC medium), and plate 100 μl on a 10 cm LB-agar dish

Plate the remaining bacteria onto the 25 x 25 cm LB-agar plate

Incubate for 20 hours at 32 °C

3.4 Plasmid preparation

The next day, count colonies on the 1:1000 dilution plate and calculate the library coverage as follows:
(number of colonies x 1000) / (number of gRNAs in library)
Retrieve bacteria from plates as follows:
Add 20 ml of pre-warmed LB medium to the plate, scrape off bacteria, and transfer to 50 ml tube
Add another 30 ml of pre-warmed LB medium, tilt plate and transfer to 50 ml tube
Spin at 4,700 rcf for 15 min to collect a bacterial pellet and discard supernatant
Proceed with plasmid extraction using the Endo-Free Plasmid Mega kit (Qiagen #12381) according to the manufacturer's instructions

4. Next-generation sequencing of gRNA sequences for library quality control or pooled screens

gRNA sequences are amplified from a CROPseq-Guide-Puro plasmid library or from the gDNA of transduced cells (pooled screen) in a single PCR reaction with specially designed primers. This step adds all sequences required for compatibility with Illumina sequencers. In addition, i7 barcodes are provided for sample multiplexing, and stagger sequences can increase library complexity for Illumina sequencing.

Primers for amplification of gRNAs for next-generation sequencing

Primer ID	Primer sequence [5' > 3']
CROPseq_libQC_i5_s1	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCTTCTTGTGGAAAGGACGAAACACCG
CROPseq_libQC_i5_s2	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCTATCTTGTGGAAAGGACGAAACACCG
CROPseq_libQC_i5_s3	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCTTGTGGAAAGGACGAAACACCG
CROPseq_libQC_i5_s4	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATCTTGTGGAAAGGACGAAACACCG
CROPseq_libQC_i5_s5	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGATCTTGTGGAAAGGACGAAACACCG
CROPseq_libQC_i5_s6	AATGATACGGCGACCACCGAGATCTACACACACTCTTTCCCTACACGACGCTCTTCCGATCTATCGATCTTGTGGAAAGGACGAAACACCG
CROPseq_libQC_i7:1	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGTGTCTCAAGATCTAGTTACGCCAAGC
CROPseq_libQC_i7:2	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGTGTCTCAAGATCTAGTTACGCCAAGC
CROPseq_libQC_i7:3	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGTGTCTCAAGATCTAGTTACGCCAAGC
CROPseq_libQC_i7:4	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGTGTCTCAAGATCTAGTTACGCCAAGC
CROPseq_libQC_i7:5	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGTGTCTCAAGATCTAGTTACGCCAAGC
CROPseq_libQC_i7:6	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGTGTCTCAAGATCTAGTTACGCCAAGC

Functional elements
5' part of i5 or i7 Illumina adapter
i7 index for sample multiplexing
3' part of i5 or i7 Illumina adapter
stagger sequence to increase complexity for Illumina sequencing
primer binding site on CROPseq-Guide-Puro

To retain a good library representation and reduce bias, multiple PCR reactions should be performed for each gRNA plasmid library or gDNA preparation. The number ultimately depends on the size of the gRNA library, but we mostly use four parallel PCR reactions. These reactions can use the same i7 barcode, so that they are demultiplexed into the same file during data processing.

qPCR amplification of gRNAs from the plasmid library or gDNA of transduced cells		
		Per reaction
Component	Volume	Amount
CROPseq-Guide-Puro plasmid library or gDNA from a pooled screen		10 ng or 100 ng
Q5 Hot Start High-Fidelity 2x Master Mix	25 µl	1x
CROPseq_libQC_i5 primer (10 µM)	2.5 µl	500 nM
CROPseq_libQC_i7 primer (10 µM)	2.5 µl	500 nM
Optional: SYBR Green, 100x	0.5 µl	1x
Nuclease-free water		to 50 µl

By adding SYBR Green, the reactions can be run in a qPCR machine and stopped just before reaching the plateau phase. This ensures that the optimal number of enrichment cycles is used. When running multiple samples, the machine can be paused during the denaturation phase. Pierce the foil to remove finished reactions, and resume the amplification of the remaining samples.

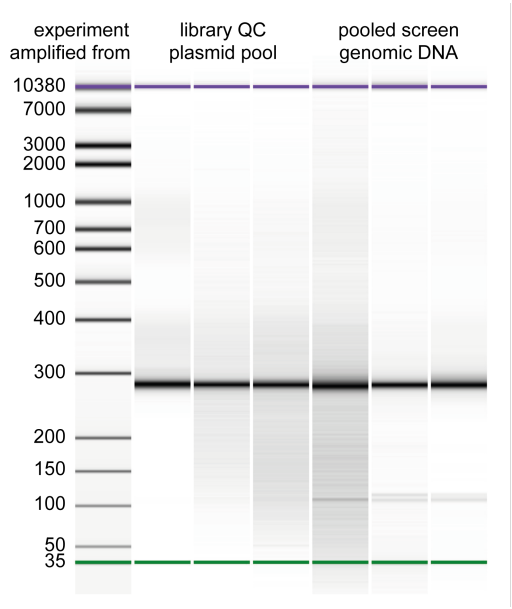
Incubate as follows:

98 °C for 30 sec (initial denaturation)
40 cycles { 98 °C for 10 sec
72 °C for 45 sec }
stop qPCR reactions just before they reach the plateau phase

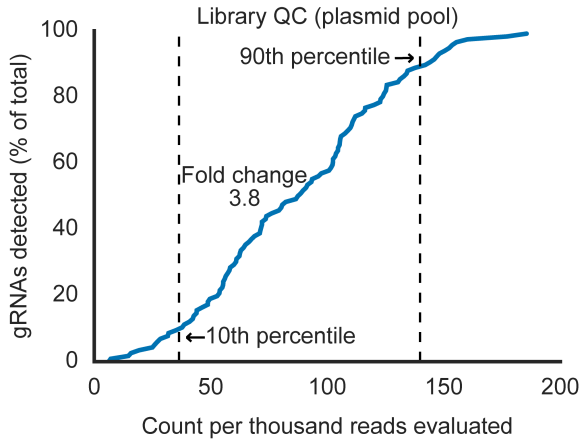
Clean PCR products with a 2.0x AMPure XP bead clean-up
Quality control on an Agilent Bioanalyzer High Sensitivity DNA chip should show a single band at 282 bp (an example is shown on the following page, in the *Expected results* section)
Measure the dsDNA concentration in a Qubit HS assay
Dilute library to 4 nM with EB buffer + 0.1% Tween based on size and concentration measurements
Sequence on an Illumina MiSeq machine, using a 150-cycle v3 flowcell with a read configuration of 167 bases (read 1) and 8 bases (i7 index). While this is the cheapest option, NextSeq and HiSeq instruments can be used instead

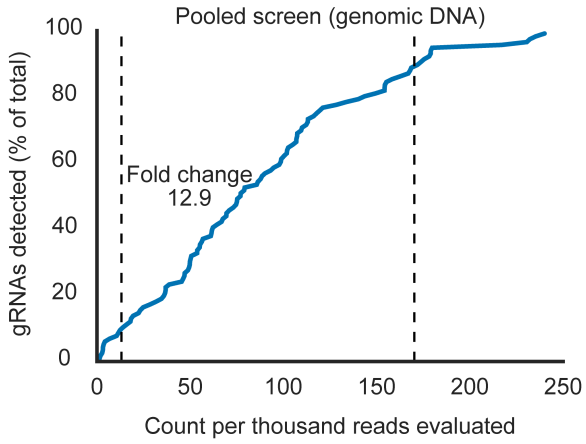
Part A: Expected results for the pooled, amplification-free cloning of gRNA libraries

gRNAs amplified from the plasmid pool during library quality control, or from genomic DNA in a classical pooled screen. The 282 bp PCR product is compatible with Illumina sequencing without further processing.



Typical representation of the gRNA library in the plasmid pool (left) and when amplified from genomic DNA in a pooled screen (right, day 10 post transduction).





Part B: Lentivirus production and cell culture for CROP-seq screens

5. Production of lentiviral particles

Prerequisites:

Plasmid pool of gRNA library cloned as described in part A, and validated by NGS

Plasmid preparations of the 3rd generation lentiviral packaging plasmids pMDLg/pRRE (Addgene 12251), pRSV-Rev (Addgene 12253), and pMD2.G (Addgene 12259)

Cell line(s) stably expressing Cas9 after transduction with lentiCas9-Blast (Addgene 52962). The lentivirus production and transduction protocols below can be used to produce this cell line

<Note> The required scale of the lentivirus production depends on the size of the gRNA library and the planned experiments. For most applications 10 cm dishes, or even 6-well plates should suffice, due to the relatively high titers achieved with the CROPseq-Guide-Puro construct. Numbers for both formats are provided below (color-coded).

<Important> Dispose of lentivirus-containing materials according to your institute's biosafety guidelines. Persons performing experiments with lentivirus must be properly trained.

Prepare lentivirus packaging medium: Opti-MEM I, 5% FCS, 200 μM sodium pyruvate, no antibiotics

Seed HEK293T cells at 7 million cells per 10 cm dish in 12 ml of lentivirus packaging medium, or at 1.2 million cells per well on a 6-well plate in 2 ml of lentivirus packaging medium

The next morning proceed with the transfection

Prepare transfection mixtures A and B as indicated below

Transfection of HEK293T cells for lentivirus production					
		10 cm dish		6-well plate	
Transfection mix	Component	Volume	Amount	Volume	Amount
A	CROPseq-Guide-Puro gRNA library, plasmid pool		10.2 μg		1.7 μg
	pMDLg/pRRE		5.4 μg		0.9 μg
	pRSV-Rev		5.4 μg		0.9 μg
	pMD2.G		5.4 μg		0.9 μg
	Opti-MEM I	1.5 ml		250 μl	
	P3000 enhancer reagent	36 μl		6 μl	
B	Opti-MEM I	1.5 ml		250 μl	
	Lipofectamine 3000 reagent	42 μl		7 μl	

Prepare lipid-DNA complexes by combining transfection mixes A and B, and incubating for 20 min at room temperature

In the meantime, remove 6 ml, or 1 ml of medium from the HEK293T cells

Add 3 ml or 500 μl of lipid-DNA complexes to the HEK293T cells, and gently agitate

After 6 h of incubation at 37 °C and 5% CO_2 exchange medium for 12 ml or 2 ml of fresh lentivirus packaging medium

Collect lentivirus-containing medium after 24 h, store at 4 °C, add 12 ml or 2 ml of fresh lentivirus packaging medium

Collect lentivirus-containing medium after 48 h, pool with supernatant obtained the previous day and filter through a 0.45 μm filter to remove cells

Aliquot lentivirus preparation into cryotubes and freeze at -80 °C
Proceed with titration of the lentivirus preparation and pooled screening

6. Optional - Titration of lentivirus preparations

<Note> To save time, transductions for pooled screens can be performed with variable volumes of the virus preparation. All transduction conditions are then selected for 2 days, and the condition with the desired MOI can be chosen for the screen.

Seed HEK293T cells onto 24-well plates at 50,000 cells per well in 500 µl of complete culture medium (DMEM, 10% FCS, penicillin-streptomycin)
Grow overnight at 37 °C and 5% CO_2 so that cells reach 30% to 50% confluence
Exchange culture medium for 450 µl complete culture medium, supplemented with 8 µg/ml polybrene
Thaw one vial of the lentivirus preparation from storage at -80 °C
In a 96-well plate, prepare a 1:5 dilution series of the lentivirus preparation in complete culture medium with polybrene, ranging over 10 wells (dilution factors from 1:5 to 1:9,765,625)
Test the original stock, and each dilution in duplicate, by adding 50 µl per well to the 24-well plate with HEK293T cells (this requires 2 x (1 + 10) = 22 wells).
The remaining two wells serve as untransduced controls. Add 50 µl complete culture medium containing polybrene to test for toxicity of polybrene to cells (possible for some cell types).
24 h post transduction exchange the medium for 500 µl complete culture medium without polybrene
48 h post transduction start selection with puromycin and renew selective medium every 2-3 days. The table below provides common antibiotic concentrations we use for puromycin selection, but ultimately the concentration will depend on the cell line used.

Examples of selection conditions used in our lab Cell line	c(Puromycin) [µg/ml] to select for gRNA	c(Blasticidin) [µg/ml] to select for Cas9
K562	2	30
Jurkat	2	25
MV4-11	1	20
KBM7	0.5	20
HEK293T	2.25	22.5

As soon as cells in the untransduced controls are dead, remove medium and wash once with 1x PBS
Stain colonies for 15 min in a solution of 1% (w/v) crystal violet in 10% ethanol
Wash several more times with 1x PBS, count colonies for two dilution factors, and two replicates each

Calculate the virus titer (transducing units/ml) as:
(average number of resistant colonies) x (dilution factor of well) x (1,000 µl/50 µl)

Example for a typical gRNA library:
56 and 66 colonies were counted for the well with dilution factor 15,625
((56 + 66) / 2) x (15,625) x (1,000 µl/50 µl) = 1.9 x 10^7 TU/ml

7. Transduction of suspension cells for pooled CROP-seq screens

Seed suspension cells in 6-well plates at 5 million cells per well in 2 ml of complete culture medium (depends on cell line), supplemented with 8 μg/ml polybrene and blasticidin (we recommend to keep up selection for lentiCas9-Blast throughout the screen, as it is easily silenced; please refer to the table above for suggestions on blasticidin concentrations for various cell lines).

Option 1 (virus titer was measured): Add calculated volume of lentivirus preparation for the desired MOI to one well, use a second well as untransduced control.

Option 2 (virus titer unknown): Transduce five wells with different volumes of the lentivirus preparation (e.g. 250 μl or 50 μl of the original stock, or 50 μl of 1:3, 1:6 and 1:12 dilutions). The sixth well is left untransduced.

Immediately after addition of lentiviral particles, centrifuge the plate for 45 min at 1,200 rcf and 37 °C (spinfection), followed by over night incubation at 37 °C and 5% CO_2.

24 h post transduction, pellet cells in 15 ml tubes, remove supernatant. Transfer cells to T75 cell culture flasks in 30 ml of fresh complete culture medium supplemented with blasticidin (still only selecting for lentiCas9-Blast). 48 h post transduction, start selection for the gRNA library by adding puromycin directly to the flask. Renew selective medium containing blasticidin and puromycin every 2-3 days for a total of 7-10 days to allow for efficient genome editing. If option 2 was chosen, select the flask with about 30% surviving cells after 2 days of puromycin selection.

8. Transduction of adherent cells for pooled CROP-seq screens

Seed adherent cells in 15 cm dishes at 5 million cells per plate in 20 ml of complete culture medium (depends on cell line) supplemented with 8 μg/ml polybrene and blasticidin (we recommend to keep up selection for lentiCas9-Blast throughout the screen, as it is easily silenced; please refer to the table above for suggestions on blasticidin concentrations for various cell lines).

Option 1 (virus titer was measured): Add calculated volume of lentivirus preparation for the desired MOI to one plate, use a second plate as untransduced control.

Option 2 (virus titer unknown): Transduce five plates with different volumes of the lentivirus preparation (e.g. 250 μl or 50 μl of the original stock, or 50 μl of 1:3, 1:6 and 1:12 dilutions). A sixth plate is used as the untransduced control.

After addition of lentiviral particles, swirl the plate gently to mix and incubate over night at 37 °C and 5% CO_2. 24 hours post transduction, exchange medium for fresh complete culture medium supplemented with blasticidin (selecting for lentiCas9-Blast). At 48 h post transduction, start selection for the gRNA library by adding puromycin directly to the plate. Renew selective medium every 2-3 days for a total duration of 7-10 days to allow for efficient editing. If option 2 was chosen, select the plate with about 30% surviving cells after 2 days of puromycin selection.

Part C: Single-cell RNA-seq based on Drop-seq

Modified from: Macosko et al. 2015 and additional protocols (http://mccarrolllab.com/dropseq/)

9. Droplet microfluidics

9.1 Prepare cells

Collect cells by centrifugation at 300 rcf for 5 min

<Note> Centrifugation speed and time might need to be adjusted for other cell types

Wash once with 1 ml of PBS-0.01% BSA (make fresh on the day of the run)

Remove supernatant and resuspend in 1 ml of PBS-0.01% BSA

Filter through a 40 μm cell strainer to obtain a single-cell suspension

Measure cell concentration twice using a cell counter

<Note> We use a CASY device, but any alternative method can be used

Dilute to 220 cells per μl using PBS-0.01% BSA in a tube that allows easy uptake by the syringes

Check concentration of diluted cells twice

9.2 Prepare beads

Mix 1.6 ml of Drop-seq lysis buffer (stored at 4 °C) with 80 μl of DTT (stored at room temperature)

Dilute Drop-seq beads as follows

Resuspend Drop-seq beads (stored at 4 °C) by very gentle shaking

Immediately transfer the equivalent of 225,000 beads to a 1.5 ml tube

Centrifuge for 1 min at 1,000 rcf

Remove supernatant and take up beads in 1.5 ml of lysis buffer with DTT (150 beads per μl)

Transfer to a tube that allows easy uptake by the syringes

9.3 Drop-seq

<General comment> To load syringes, cut a 200 μl pipet tip without filter on both ends (to reduce shear force and to facilitate attachment to the syringe). After loading, invert the syringe, pull up whatever is left in the tip, discard tip, tap or push to remove air bubbles and attach a safe-lock needle and tubing using tweezers (be careful not to puncture your fingers!). Press syringe until tubing is free of air bubbles.

Load oil into a 10 ml syringe

Load cells into a 3 ml syringe

Place Drop-seq microfluidic device on microscope stage

Attach tubing for oil and cells and connect the outlet to a 50 ml tube (labelled “waste”)

Prepare a second 50 ml tube for collecting droplets once the run is stable

Load beads

<Critical step> Loading the beads is a critical step, as they can easily settle or clump. This can clog the needle or lead to an uneven bead concentration throughout the run.

Remove plunger from 3 ml syringe, then insert magnetic mixing disc and push plunger back

Set plunger of the empty syringe to 1.5 ml, attach to the pump

Set up the magnet to 1 jump per second and keep switched on

Detach empty syringe from pump

Use a P1000 to gently mix the bead solution (careful not to form air bubbles)

Load the syringe while gently agitating the bead solution; immediately invert the syringe to avoid clogging the needle (hold syringe with needle facing upwards)

Gently push the bead solution through the tubing, then connect tubing for bead solution to the

microfluidic device

Finally, attach the syringe to the pump with the needle facing downwards (the magnet is still running and starts mixing immediately)

Set flow rate to 1.6 ml/h for cells and beads, 8 ml/h for the oil, and make sure the pump is set to the specific syringe type

Start the pumps in the order cells > beads > oil

<Note> Cells are started first to avoid a backflow of lysis buffer contained in the bead solution

<Troubleshooting> If you want to stop the run for whatever reason, the order is beads > cells > oil

Check droplets from time to time under the microscope by adding a few drops from the outlet tubing onto a glass slide. *<Note> About 5% of droplets should contain a bead. Cells are lysed within seconds and are therefore not visible.*

Start collecting droplets in a 50 ml tube if the droplet size is homogenous

As the run progresses, the mixing disk will stop jumping because it gets blocked by the plunger (approximately when the plunger reaches the 0.4 ml marking). A typical run lasts for 35-40 min, filling the 50 ml tube to 7.5 – 10 ml. Make sure to retrieve the mixing disc from the bead syringe at the end.

Proceed with droplet breakage on the same day. When performing multiple runs per day, 50 ml tubes containing droplets can be stored at 4 °C and further processed together.

9.4 Droplet breakage

Remove as much oil below the droplet layer as possible

Add 30 ml of 6x SSC buffer

Add 1 ml of perfluoroctanol

Shake forcefully by hand 6 times to break the droplets

Bead capture

Label the top side of a 0.22 μm filter unit, remove plunger from a 20 ml syringe, attach syringe to 0.22 μm filter unit, place on a fresh 50 ml tube and pour the bead-containing sample into the syringe

Then place plunger back and filter as usual

Discard 50 ml tube with flowthrough and the syringe, but keep the filter containing the beads

<Note> The beads are retained in the 0.22 μm filter unit, due to their relatively large diameter

Washing beads

Place filter unit (top side up) on a fresh 50 ml tube, pour 6x SSC buffer into a reservoir, take up 20 ml from the reservoir with a fresh syringe, attach to filter unit on 50 ml tube and wash the beads

Repeat once for a total of two washes

Keep the wash fraction and filter, and discard the syringe

<Note> Beads are still retained in the filter unit, and only washed in the process

Eluting beads

Invert the filter unit and place it on a fresh 50 ml tube

Cut a 200 μl tip without filter on both ends, it will serve as an adapter to connect the bottom side of the 0.22 μm filter to a 10 ml syringe

Then elute beads three times with 10 ml of 6x SSC buffer

<Note> Beads have now been collected in the 50 ml tube in a total volume of 30 ml

Concentrating beads

Spin 50 ml tubes for wash and elution fractions for 2 min at 1,250 rcf with brake speed set to 50% (or use 'soft' setting if available)

<Note> Reducing the brake speed is critical, to prevent kicking beads back into solution

Compare pellets between wash and elution fraction, discard wash fraction unless there is a pronounced pellet as well

Remove supernatant from the elution fraction until only about 1 ml remains in the tube
Wash bead pellet once with 10 ml 6x SSC buffer, centrifuging with the above settings
Remove the supernatant carefully with a P1000 pipette
Take up the pellet in 200 µl of 5x reverse transcription (RT) buffer and transfer to a 1.5 ml tube
Spin 1.5 ml tube at 1,250 for 2 min with brake speed set to 50% (or 'soft' setting)
Remove as much of the RT buffer as possible without disturbing the beads
Immediately proceed with the reverse transcription

10. Preparation of cDNA library

10.1 Reverse transcription

Prepare reverse transcription master mix as indicated in the table below. The volumes provided already compensate for 10% dead volume.

Reverse transcription master mix									
		Per reaction		Master mix for # of Drop-seq runs					
Component	Stored at	Volume	Amount	1	2	3	4	5	6
Nuclease-free water	RT	75 µl	to 200 µl	82.5	165	247.5	330	412.5	495
Maxima 5x RT buffer	-20 °C	40 µl	1x	44	88	132	176	220	264
20% Ficoll PM-400	4 °C	40 µl	4%	44	88	132	176	220	264
10 mM dNTPs	-20 °C	20 µl	1 mM	22	44	66	88	110	132
Recombinant RNase inhibitor	-20 °C	5 µl	200 U	5.5	11	16.5	22	27.5	33
50 µM template switching oligonucleotide	-80 °C	10 µl	2.5 µM	11	22	33	44	55	66
Maxima H-RTase	-20 °C	10 µl	2000 U	11	22	33	44	55	66

Resuspend bead pellet (one per run) from step **9.4** in 200 µl of reverse transcription master mix
Transfer to a 96-well plate and incubate in a thermocycler with heated lid
30 min at 25 °C
90 min at 42 °C
Storage at 4 °C
<Safe stopping point> The finished reverse transcription reaction can be stored at 4 °C over night or left at 4 °C in the thermocycler.

10.2 Exonuclease I treatment

Wash 1 (centrifuge 96-well plate for 2 min at 1,250 rcf with brake speed set to 50% throughout)
Wash beads once with 200 µl of TE-SDS
Wash beads twice with 200 µl of TE-TW
Wash beads once with 200 µl of 10 mM Tris, pH 8.0
Prepare exonuclease I master mix as indicated in the table below. The indicated volumes already account for 10% dead volume

Exonuclease I master mix		/reaction		Master mix for # of Drop-seq runs					
Component	Stored at	Volume	Amount	1	2	3	4	5	6
Nuclease-free water	RT	170 µl	to 200 µl	187	374	561	748	935	1122
10x Exonuclease I buffer	-20 °C	20 µl	1x	22	44	66	88	110	132
Exonuclease I	-20 °C	10 µl	200 U	11	22	33	44	55	66

Remove supernatant carefully, and resuspend the bead pellet in 200 µl of exonuclease I master mix
Incubate for 45 min at 37 °C
Wash 2 (centrifuge 96-well plate for 2 min at 1,250 rcf with brake speed set to 50% throughout)
Wash beads once with 200 µl of TE-SDS
Wash beads twice with 200 µl of TE-TW
<Safe stopping point> Sample can be stored in TE-WE at 4 °C

10.3 SMART PCR to enrich cDNA library
Pellet beads by centrifuging for 2 min at 1,250 rcf with the brake speed set to 50%
Remove supernatant and resuspend bead pellet in 400 µl of nuclease-free water
Estimate bead concentration
<Note> Drop-seq beads have a very high sedimentation rate, make sure to mix thoroughly immediately before pipetting bead-containing solution.
Prepare PCR tube strip, containing 7.5 µl of 6x gel loading dye per well (one well per bead sample)
<Note> The gel loading dye helps to evenly distribute beads in the counting chamber later on
Mix bead solution by pipetting up and down with a P200
Quickly remove 37.5 µl of bead suspension, mix with the 6x gel loading dye in the tube strip and load 20 µl into each of the two chambers of a disposable Incyto C-Chip with Fuchs-Rosenthal grid
Count >4 squares for both chambers and calculate the average number of beads per square
Calculate total amount of beads per tube as
[(Average number of beads per square) x 5,000 x 1.2] * 0.3625 = x beads in original tube
where 5,000 is the chamber volume factor, 1.2 the dilution factor (gel loading dye) and multiplication by 0.3625 converts to the total amount of beads left in the original tube (now containing 362.5 µl)
Dilute bead solution to 220 beads per µl as follows
Pellet beads by centrifuging for 2 min at 1,250 rcf with the brake speed set to 50%
Remove supernatant and resuspend in (x / 220) = y µl of nuclease-free water, where x is the total number of beads in the pellet, as calculated above
Calculate number of PCR reactions as
y / (20+1) = n PCR reactions
where y is the volume used for diluting beads, 20 is the volume for 4,400 beads used in a single PCR reaction, and +1 accounts for 5% dead volume during pipetting
Prepare SMART PCR master mix as indicated in the table below

SMART PCR master mix		/reaction	
Component	Stored at	Volume	Amount
Nuclease-free water	RT	4.6 µl	to 50 µl
100 µM SMART PCR primer	-20 °C	0.4 µl	0.8 µM
2x Kapa HiFi HotStart ReadyMix	-20 °C	25 µl	1x

Aliquot 20 μl of beads into 96-well plates

<Note> This corresponds to 4,400 beads or approximately 220 cells per PCR

Add 30 μl of SMART PCR master mix per well

Incubate PCR as follows

95 °C for 3 min

4x { 98 °C for 20 sec,
65 °C for 45 sec,
72 °C for 3 min }

10x { 98 °C for 20 sec,
67 °C for 20 sec,
72 °C for 3 min }

72 °C for 5 min

Storage at 4 °C

<Note> Depending on the cell type, the number of PCR cycles might need optimization. These cycle numbers have worked well on cell lines.

10.4 Purification, quality control and pooling of SMART-PCR enriched cDNA

Purification of SMART PCR products

<Note> Perform with multichannel pipettes in 96-well format

Add 30 μl of room temperature AMPure XP beads to each PCR reaction (0.6x bead to sample ratio)

Incubate for 10 min at room temperature

Place on magnet for 5 min

Wash twice with 80% ethanol (5 sec per wash)

Leave to dry for 5 min

Elute in 10 μl nuclease-free water

Run 1 μl of randomly selected wells on a Bioanalyzer High Sensitivity Chip according to the manufacturer's instructions. This should confirm high quality of the cDNA (peak maximum >1000 bp without bands that would indicate degradation), and low or undetectable amounts of primer dimer formation (peak at 150 bp).

<Troubleshooting> If a pronounced primer dimer peak is present at 150 bp, repeat the 0.6x AMPure XP bead clean-up one more time. Usually, this removes the peak entirely.

Measure the dsDNA concentration for all wells using a Qubit HS DNA assay

Based on these measurements, pool all SMART PCRs for a particular Drop-seq run in equal amounts

Measure the dsDNA concentration of each pool using a Qubit HS DNA assay, this concentration is needed for the Nextera XT library preparation.

<Safe stopping point> Enriched cDNA pools can be kept at 4 °C, or stored long-term at -20 °C

11. Nextera XT library preparation

11.1 Tagmentation

In a PCR tube strip, prepare 1 ng of pooled cDNA per Drop-seq run in a total volume of 5 μl of nuclease-free water

Preheat a thermocycler to 55 °C

Transfer Nextera XT Neutralization Buffer from storage at 4 °C to room temperature

Prepare Nextera XT tagmentation master mix as indicated in the table, the volumes are already accounting for 10% dead volume

Nextera XT tagmentation master mix								
			Master mix for # of Drop-seq runs					
Component	Stored at	Volume	1	2	3	4	5	6
Nextera TD buffer	-20 °C	10 µl	11	22	33	44	55	55
Amplicon tagment enzyme	-20 °C	5 µl	5.5	11	16.5	22	27.5	27.5

Add 15 µl of tagmentation master mix per well, and pipette up and down to mix
Incubate at 55 °C for 5 min
Immediately add 5 µl of Neutralization Buffer to stop the reaction
Incubate at room temperature for 5 min

11.2 Nextera XT PCR enrichment

Prepare Nextera XT PCR master mix as indicated in the table below. The volumes provided already account for 10% dead volume lost during pipetting.

Nextera XT PCR master mix								
		/ reaction	Master mix for # of Drop-seq runs					
Component	Stored at	Volume	1	2	3	4	5	6
Nuclease-free water	RT	8 µl	8.8	17.6	26.4	35.2	44	44
Nextera PCR mix	-20 °C	15 µl	16.5	33	49.5	66	82.5	82.5
10 µM New-P5-SMART PCR hybrid oligo	-20 °C	1 µl	1.1	2.2	3.3	4.4	5.5	5.5
10 µM Nextera N70X oligo	-20 °C	1 µl	1.1	2.2	3.3	4.4	5.5	5.5

Add 25 µl of Nextera XT PCR master mix to the tagmentation reaction, bringing the volume to 50 µl
Incubate PCR

95 °C for 30 sec
10x { 95 °C for 10 sec,
55 °C for 30 sec,
72 °C for 30 sec }
72 °C for 5 min
Storage at 4 °C

11.3 Purification of the final library

Clean-up 1 (perform with multichannel pipettes in the PCR tube strip)

Add 30 µl of room temperature AMPure XP beads to each PCR reaction (0.6x bead to sample ratio)
Incubate for 10 min at room temperature
Place on magnet for 5 min
Wash twice with 80% ethanol (5 sec per wash)
Leave to dry for 5 min
Elute in 32 µl of nuclease-free water, then transfer 30 µl to a fresh PCR tube strip

Clean-up 2

<Note> Both clean-up steps are required to remove PCR primers that might interfere with sequencing
Add 30 µl of room temperature AMPure XP beads to each PCR reaction (1.0x bead to sample ratio)
Incubate for 10 min at room temperature
Place on magnet for 5 min

Wash twice with 80% ethanol (5 sec per wash)
Leave to dry for 5 min
Elute in 10 μl of nuclease-free water, then transfer to 1.5 ml tubes for long-term storage at -20 °C

12. Library quality control and next-generation sequencing

12.1 Quality control and dilution of the CROP-seq library

Run 1 μl of the final library on a Bioanalyzer High Sensitivity DNA chip according to the manufacturer's instructions.

<Note> To increase the likelihood that fragments contain the gRNA sequence, CROP-seq uses a slightly larger library fragment size. This is achieved by adding more cDNA as input for the Nextera XT tagmentation reaction. Sometimes, the final library might appear a bit skewed towards larger fragments, which does not have a negative impact on the sequencing results.

Estimate the average size of the library from the Bioanalyzer profile.

<Note> For symmetrical libraries the peak maximum can be used; for skewed libraries the average fragment size is estimated based on the fragment distribution. We usually use 575 bp.

Measure the concentration of dsDNA in a Qubit DNA HS assay, according to the standard protocol
Calculate the molarity of your library as

c(dsDNA)[ng/ul] / (average size [bp] x 660 g / mol) x 1,000,000 = molarity of library [nM]

where 660 g / mol is the molecular weight of a DNA basepair and 1,000,000 is a conversion factor
Dilute your library with EB-0.01% Tween to reach a molarity of 4 nM

12.2 Next-generation sequencing on Illumina HiSeq 3000/4000 instruments

Drop-seq Custom Read1 Primer is spiked into the HP10 primer solution, located in column 11 of the cBot Reagent Plate at 1 μM final concentration. High sequence complexity needed for optimal base calling performance is achieved by adding 20-30% PhiX as spike-in. Cluster generation and Read 1 primer hybridization should be completed using the Illumina cBot protocol 'HiSeq_3000_4000_HD_Exclusion_Amp_v1.0'.

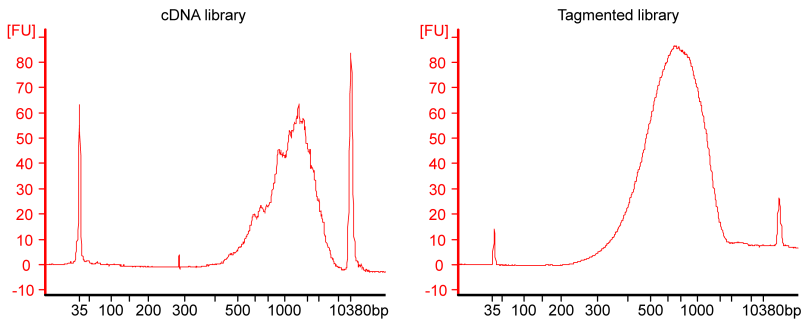
12.3 Next-generation sequencing on Illumina NextSeq 500 instruments

Libraries are sequenced on an Illumina NextSeq 500 instrument using the 75 cycle High Output v2 kit. Load 1.8 pM library without PhiX spike-in and provide Drop-seq Custom Read1 Primer at 0.3 μM in position 7 of the reagent cartridge. Set the read configuration to 20 bases (Custom Read1), 8 bases (Index1) and 64 bases (Read2).

<Note> When setting up the run in the Illumina Basespace, the read length of only 20 bases for Custom Read1 results in a warning. We ignore this warning and proceed with the run, because any additional bases would just read from the A-tail, thus reducing sequencing quality. However, when sequencing with this setup, the data has to be demultiplexed manually.

Part C: Expected results for single-cell RNA-seq by Drop-seq

Typical Bioanalyzer profiles for SMART-PCR enriched cDNA and final Nextera XT libraries



Measured cell doublet rate for the described Drop-seq setup, measured in a species mixing experiment on HEK293T (human) and 3T3 (mouse) cell lines. The mixing ratio was 1:1.

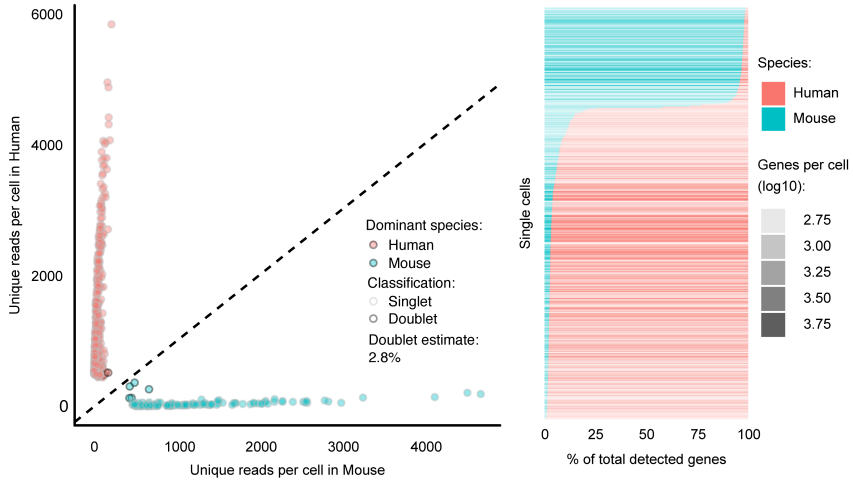


Table summarizing sequencing results for a typical NextSeq 500 run

LANE	READ	TILES	DENSITY (K / MM^2)	CLUSTER PF (%)	PHAS/PREPHAS (%)	READS	READS PF	%≥Q30
1	1	216	213 ±12	88.77 ±0.71	0.049 / 0.075	138,319,728	122,812,304	94.70
	2 (I)	216	213 ±12	88.77 ±0.71	0.000 / 0.000	138,319,728	122,812,304	81.42
	3	216	213 ±12	88.77 ±0.71	0.321 / 0.312	138,319,728	122,812,304	58.91
2	1	216	213 ±5	89.03 ±1.68	0.044 / 0.076	137,876,464	122,789,656	94.86
	2 (I)	216	213 ±5	89.03 ±1.68	0.000 / 0.000	137,876,464	122,789,656	81.99
	3	216	213 ±5	89.03 ±1.68	0.317 / 0.323	137,876,464	122,789,656	59.42
3	1	216	221 ±6	89.46 ±0.96	0.063 / 0.076	143,051,776	127,979,232	94.89
	2 (I)	216	221 ±6	89.46 ±0.96	0.000 / 0.000	143,051,776	127,979,232	83.66
	3	216	221 ±6	89.46 ±0.96	0.272 / 0.249	143,051,776	127,979,232	64.95
4	1	216	215 ±5	88.86 ±1.49	0.045 / 0.082	139,777,456	124,227,784	94.77
	2 (I)	216	215 ±5	88.86 ±1.49	0.000 / 0.000	139,777,456	124,227,784	81.89
	3	216	215 ±5	88.86 ±1.49	0.302 / 0.319	139,777,456	124,227,784	59.87

Appendix: Materials

Reagents required for Part A (Pooled, amplification-free cloning of gRNA libraries)

Reagent (alphabetical order)	*Supplier*	*Cat. No.*	*Comment on use*
Absolute ethanol	Merck	64-17-5	
Agar, powder	Sigma	A5054-250G	
Agarose	Sigma	A9539-100G	
Agilent Bioanalyzer High Sensitivity DNA chip	Agilent	5067-4626	
AMPure XP beads	Beckman Coulter	A63880	
Bioassay dish, 245 x 245 x 25 mm	Sigma	D4803-1CS	Used for growing bacteria electroporated with gRNA libraries
BsmBI restriction enzyme	NEB	R0580S	
Carbenicillin, 5g	Roth	6344.2	
CROPseq-Guide-Puro	Addgene	86708	
EB buffer	Qiagen	1014609	
Electroporation cuvettes, 1 mm	BioRad	1652089	
Endo-Free Plasmid Mega kit	Qiagen	12381	For preparing plasmid containing entire gRNA libraries
Endura electrocompetent cells	Lucigen	60242-2	
Gel-cutting tips	Biostep	99-90-306	
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific	SM0311	
LB (Miller), powder	Sigma	L3522-1KG	
Membrane filter	Merck	VMWP04700	For desalting Gibson assembly reactions
NEBuilder HiFi DNA assembly master mix	NEB	E2621S	
Plasmid Plus Midi kit, 100 preparations	Qiagen	12945	For CROPseq-Guide-Puro and lentivirus packaging plasmids
Q5 Hot Start High-Fidelity 2x Master Mix	NEB	M0494L	
Qubit dsDNA HS Assay kit	Thermo Fisher Scientific	Q32854	
Round-bottom tubes	Falcon	352051	
SapI restriction enzyme	NEB	R0569S	
SNAP UV-Free Gel Purification kit	Invitrogen	45-0105	
SOC medium	NEB	B9020S	
Sodium acetate, 3M, pH 5.5	Ambion	AM9740	
SphI restriction enzyme	NEB	R0182S	
SYBR Green; 10,000x concentrate in DMSO	Thermo Fisher Scientific	S7563	
TAE buffer, 50x	Invitrogen	24710-030	
Tween	Sigma	P7949-500ML	

Reagents required for Part B (Lentivirus production and cell culture for CROP-seq screens)

Reagent (alphabetical order)	*Supplier*	*Cat. No.*	*Comment on use*
Blasticidin	Invivogen	ant-bl-5	
Crystal violet	Sigma	61135-25G	To stain resistant colonies for titer estimation
DMEM	Gibco	10569010	
DPBS, 1x	Gibco	14190-094	
FCS	Sigma	N/A	
HEK293T cell line			
Lipofectamine 3000 transfection reagents	Invitrogen	L3000015	
Opti-MEM I	Gibco	51985-034	
Penicillin-streptomycin; 5,000 U/ml	Thermo Fisher Scientific	15070063	
pMD2.G	Addgene	12259	3rd generation lentiviral packaging system
pMDLg/pRRE	Addgene	12251	3rd generation lentiviral packaging system
Polybrene	Sigma	H9268-5G	To enhance contact between virus and cells
pRSV-Rev	Addgene	12253	3rd generation lentiviral packaging system
Puromycin	Thermo Fisher Scientific	A1113803	
Sodium pyruvate	Gibco	11360-070	
Syringe filter, 0.45 µm	VWR	514-8021	For removing cells from lentivirus preparations

Reagents required for Part C (Single-cell RNA-seq based on Drop-seq)

Modified from: Macosko et al. 2015 and additional protocols (http://mccarrolllab.com/dropseq/)

Reagent (alphabetical order)	*Supplier*	*Cat. No.*	*Comment on use*
Barcoded Bead SeqB	ChemGenes	MACOSKO-2011-10	Drop-seq beads
Bovine serum albumin (BSA)	Sigma	A7030-50G	
C-Chip Disposable Hemacytometers, Incyto, Pkg. of 50	Fisher Scientific	22-600-102	For estimating bead number
Cell strainer, 100 µm (for beads)	VWR	21008-950	
Cell strainer, 40 µm (for cells)	VWR	21008-949	
dNTP solution, 10 mM	ThermoFisher Scientific	R0192	
Droplet Generation Oil for EvaGreen, QX200	BioRad	1864006	Drop-seq oil
DTT	Sigma	646563-10x.5ML	Add on day of run
EDTA, 0.5 M, pH 8.0	ThermoFisher Scientific	AM92606	
Exonuclease I	NEB	M0293L	
Ficoll PM-400	Sigma	F5415-50ML	
KAPA HiFi HotStart ReadyMix	KAPA	KK2602	
Maxima H Minus Reverse Transcriptase, 4x 50 ul	ThermoFisher Scientific	EP0753	
Microfluidic devices	FlowJem	see order sheet for details	
N-Lauroylsarcosine sodium salt solution, 20%	Sigma	L7414	
Needle BD Microlance 3 (brown)	BD	300300	
Needle Neoject 25G 0.5 x 16 mm	Hartenstein	EU23	
Nextera XT kit for 24 samples	Illumina	FC-131-1024	
Perfluoroctanol (1H,1H,2H,2H-Perfluoro-1-octanol)	Sigma	370533-25G	For droplet breakage
Polyethylene tubing, ID 0.38 mm	Scicominc	BB31695-PE/2	
Recombinant Rnase inhibitor	Takara	2313A	
SSC buffer, 6x	Promega	V4261	For droplet breakage
Syringe BD Discardit II 10 ml	BD	309110	
Syringe BD Discardit II 20 ml	BD	300296	
Syringe BD Luer-Lok 1 ml	BD	309628	
Syringe BD Luer-Lok 3 ml	BD	309657	
Syringe filter unit, Millex-GV, 0.22 µm, PVDF	Merck Millipore	SLGV033RS	
Tris, 2M, pH 7.5	Sigma	T2944-100ML	
Tween-20	Sigma	P7949-500ML	

Oligonucleotide (order of use)	*Supplier*	*Sequence*	*Comment*
Template switching oligonucleotide (TSO)	Exiqon	AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG	r … indicates RNA bases, store at -80 °C after resuspending
SMART PCR primer	Sigma	AAGCAGTGGTATCAACGCAGAGT	
New-P5-SMART PCR hybrid oligo	Sigma	AATGATACGGCGACCACCGAGATCTACACGCCTGTCC GCGGAAGCAGTGGTATCAACGCAGAGT*A*C	
Custom Read1 primer	Sigma	GCCTGTCCGCGGAAGCAGTGGTATCAACGCAGAGTAC	Custom sequencing primer for Read1 on Illumina machines

Solution (order of use)	*Recipe*
Drop-seq lysis buffer	For 40 ml (it's possible to store large stocks without DTT at 4 °C): 20 ml Nuclease-free water 12 ml 20% Ficoll PM-400 400 ul 20% N-Lauroylsarcosine sodium salt solution (Sarcosyl), *<Caution> toxic* 1.6 ml 0.5 M EDTA 4 ml 2 M Tris pH 7.5 50 ul per ml Lysis buffer 1 M DTT → add this on the day of the experiment
TE-SDS	10 mM Tris, pH 8.0 1 mM EDTA 0.5% SDS
TE-TW	10 mM Tris, pH 8.0 1mM EDTA 0.01% Tween