



Dissecting Molecular Phenotypes Through FACS-Based Pooled CRISPR Screens

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Abstract

Pooled CRISPR screens are emerging as a powerful tool to dissect regulatory networks, by assessing how a protein responds to genetic perturbations in a highly multiplexed manner. A large number of genes are perturbed in a cell population through genomic integration of one single-guide RNA (sgRNA) per cell. A subset of cells with the phenotype of interest can then be enriched through fluorescence-activated cell sorting (FACS). SgRNAs with altered abundance after phenotypic enrichment allow identification of genes that either promote or attenuate the investigated phenotype. Here we provide detailed guidelines on how to design and execute a pooled CRISPR screen to investigate molecular phenotypes. We describe how to generate a custom sgRNA library and how to perform a FACS-based screen using readouts such as intracellular antibody staining or Flow-FISH to assess phosphorylation levels or RNA abundance. Through the variety of available perturbation systems and readout options many different molecular and cellular phenotypes can now be tackled with pooled CRISPR screens.

Key words CRISPR screen, Embryonic stem cell, Signaling, MAPK pathway, Intracellular antibody staining, Flow-FISH, RNA, sgRNA library

1 Introduction

Pooled CRISPR screens are a powerful tool for the unbiased identification of genes mediating a certain phenotype. Prior to the advent of the CRISPR/Cas9 technology, short-hairpin RNA pools were used for mRNA downregulation in pooled RNAi screens, but high variability in knock-down efficiency and off-target activity restricted their use [1, 2]. More recently, the combination of the Cas9 endonuclease together with pooled sgRNA libraries led to a broader adoption of pooled screens as a discovery tool in forward genetics [3, 4]. The number of available perturbation systems and phenotypes that can be investigated has rapidly expanded since then. Gene overexpression (CRISPR activation) and downregulation (CRISPR inhibition) through coupling catalytically inactive Cas9 (dCas9) to transcriptional effector domains was successfully used in CRISPR screens [5, 6]. Moreover,

in addition to the investigation of viability phenotypes, a variety of molecular phenotypes can now be studied through fluorescence-activated cell sorting (FACS) [7].

CRISPR screens have been used to identify protein-coding genes, noncoding RNAs, and more recently also cis-regulatory elements that control a specific phenotype or gene of interest [3, 7–11]. In addition, also single-cell RNA sequencing (scRNA-seq) has been used as readout in CRISPR screens, which allows omission of the phenotypic enrichment step [12–14]. However, since the technical considerations for scRNA-seq screens are rather different and they, so far, cannot be applied on a large scale and require extensive computational efforts to limit background noise and reduce the number of false positives, they are not covered here. In this protocol we will provide detailed guidelines for screen design and execution of enrichment-based pooled CRISPR screens, which can be applied to numerous systems, cell types, and experimental questions. Moreover, general considerations are additionally given at various steps that allow the adaptation of the protocol to different experimental requirements.

In a pooled CRISPR screen, a population of cells is transduced with one sgRNA per cell to induce perturbation of a single gene. After enrichment of cells with the phenotype of interest the relative abundance of each sgRNA is quantified by deep sequencing. Importantly, the sgRNA library is usually delivered by lentiviral transduction, which allows a precise control of how many sgRNAs are expressed in each cell (usually only one) and enables quantification of the relative number of cells carrying each sgRNA in a population, through sequencing of the genomically integrated sgRNA vector.

In pooled CRISPR screens a sufficiently strong perturbation must be induced with a single sgRNA per cell. In knock-out screens this can be achieved with high efficiency by inducing frameshift mutations. However, due to the fact that one third of repair events will generate in-frame mutations, this strategy generates a mixture of wildtype, homozygous, and heterozygous mutant cells, thus potentially reducing screen sensitivity. Cas9 nevertheless remains the most potent loss-of-function perturbation system to date, but is usually limited to protein-coding genes. An alternative strategy for loss-of-function that also allows interrogation of noncoding genes, such as long noncoding RNAs (lncRNAs), is CRISPR interference (CRISPRi), where dCas9 is fused with a repressor domain, such as KRAB or the more recently developed KRAB-MeCP2 fusion protein [5, 15]. To interrogate the function of RNAs also RNA-targeting CRISPR/Cas systems, such as Cas13/CasRx, are being established, and might be promising screening tools in the future [16, 17].

Through the development of CRISPR activation (CRISPRa) systems, also gain-of-function screens have become possible. However, typically, overexpression with a single sgRNA recruiting a

simple dCas9 effector fusion protein is not sufficiently strong. Therefore CRISPRa screens have made use of systems that recruit multiple effector domains with one sgRNA, such as the SunTag or SAM systems [5, 6]. Also other potent CRISPRa systems, such as VPR, might potentially be used in a screen setting [18]. A general limitation of CRISPRa and also CRISPRi screens is that perturbation strength varies significantly across sgRNAs and genes [19].

Pooled CRISPR screens rely on the ability to physically separate a subset of cells with the phenotype of interest. For phenotypes that affect proliferation or viability, cells can be enriched or depleted through prolonged culture (for example [20, 21]). This approach can be easily scaled up and is therefore used in most genome-wide screens. Here we focus on screens that investigate molecular phenotypes and thus use FACS-sorting for phenotypic enrichment. To investigate the regulation of a specific protein, it can be tagged with a fluorescent protein. If protein tagging is not possible, the endogenous protein can be detected through antibody-mediated fluorescence staining [22, 23]. If a suitable antibody exists, this approach also allows investigation of specific post-translational modifications. A second tagging-free approach which additionally allows investigation of noncoding RNAs is based on RNA detection through Flow-FISH and can in principle be applied to any sufficiently abundant RNA [24–26].

To ensure high screen sensitivity, sufficient library representation, also called coverage, must be maintained at all steps of a screen. Coverage is defined as the average number of cells or molecules per sgRNA present in a sample. Maintenance of sufficient coverage is important to ensure that all sgRNAs are detected in all samples, which is a prerequisite for the subsequent statistical analysis. Similar coverage should be maintained throughout the different steps of the screen and is defined as follows:

1. Library cloning: Number of independent ligation and transformation events per sgRNA.
2. Lentiviral transduction: Number of cells transduced with each sgRNA.
3. Cell splitting: Number of cells plated per sgRNA.
4. Phenotypic enrichment: Number of cells per sgRNA in the selected/sorted population.
5. Sequencing library preparation: Number of genomically integrated sgRNA cassettes (= number of cells) that are amplified in the PCR.
6. Sequencing: Number of reads per sgRNA.

Typically, a coverage between 100 and 1000 is used [3, 5] and simulations have confirmed that this range supports high performance [27]. The required coverage strongly depends on the width

of the distribution of the cloned sgRNA library [28]. The distribution width can be quantified as the fold change between the 90th and 10th percentile. Simulations have shown that a 200× coverage is sufficient for a distribution width of 2.5, while 400× is required for a tenfold distribution width [28].

Here we provide detailed instructions on how to perform a CRISPR-KO screen, using intracellular antibody staining of phospho-Mek for phenotypic readout. We have used this assay to identify genes that modulate the differentiation-promoting MAPK signaling pathway in murine embryonic stem cells [23]. We also provide instructions on how to perform phenotypic enrichment based on RNA levels quantified by Flow-FISH, which we have recently used to investigate the regulation of the Xist lncRNA [25].

2 Materials

2.1 Cloning and PCR

1. Synthesized oligo pool (e.g., Genscript or Twist Bioscience).
2. lentiGuide-Puro sgRNA expression vector (Addgene, plasmid ID 52963).
3. BsmBI restriction enzyme (alternatively Esp3I, New England BioLabs).
4. Phusion Hot Start Flex DNA Polymerase (New England BioLabs).
5. KAPA HiFi HS Ready Mix (Roche).
6. dNTP Mix: 10 mM each.
7. PCR purification kit (e.g., Qiagen QIAquick PCR Purification Kit).
8. Gel extraction kit (e.g., QIAquick Gel extraction kit).
9. LE Agarose.
10. 10× TBE Buffer (Thermo Fisher Scientific).
11. DNA Ladder 100 bp (New England Biolabs).
12. SYBR Safe DNA Gel Stain-400 (Thermo Fisher Scientific).
13. Gel Loading Dye, Purple (6×, New England BioLabs).
14. Gibson Assembly Master Mix (New England Biolabs).
15. Pellet Paint Co-precipitant (Merck KGaA).
16. NaOAc: 3 M solution in H₂O (Invitrogen).
17. Ethanol 100%.
18. NaCl.
19. MegaX DH10B T1R Electrocomp. Cells (Thermo Fisher Scientific).
20. LB medium: 10 g NaCl, 10 g Tryptone, 5 g Yeast Extract, 1 ml 1 M NaOH dissolved in 1 l ddH₂O.

21. Ampicillin.
22. SOC medium (Thermo Fisher Scientific).
23. Plasmid Maxi prep (e.g., Nucleobond Xtra MidiPlus, Macherey-Nagel).
24. Agar Agar.
25. Agar plates (1 l LB medium +15 g Agar Agar).
26. Primer Sequences (*see* Table 1).

2.2 Cell Culture

1. HEK293FT (Thermo Fisher).
2. ViraPower™ Lentiviral Packaging Mix: pLP1, pLP2, VSVG (Thermo Fisher Scientific).
3. HEK293T culture medium: DMEM, 10% FBS.
4. Opti-MEM I Reduced Serum (Thermo Fisher Scientific).
5. Lipofectamine 2000 (Thermo Fisher Scientific).
6. Lenti-X-Concentrator (Takara).
7. Cell line stably expressing Cas9 (e.g., 1.8 ESCs, transduced with lentiCas9-Blast, Addgene #52962).
8. Puromycin (Sigma-Aldrich).
9. Polybrene Infection/Transfection Reagent (Merck KGaA).
10. 10× PBS (Sigma-Aldrich).
11. Trypsin (Thermo Fisher Scientific).
12. ESC Culture medium: DMEM, 15% ES-grade FBS, 0.1 mM β -mercaptoethanol, 1000 U/ml LIF.

2.3 Antibody Staining and Flow-FISH

1. Staining media: PBS, 1% BSA.
2. Sorting buffer: PBS, 1% FBS, 1 mM EDTA.
3. Paraformaldehyde: 3% solution in H₂O.
4. Methanol: 100%.
5. Bovine Serum Albumin (BSA, Sigma-Aldrich).
6. Secondary fluorescently labeled antibody (e.g., AlexaFluor-647 Goat anti-Rabbit, Thermo Fisher Scientific).
7. PrimeFlow RNA assay (ThermoFisher) and gene-specific target probes.
8. Conical bottom 96-well plate.

2.4 Genomic DNA Extraction

1. UltraPure SDS Solution (Thermo Fisher Scientific).
2. Tris-EDTA (TE) buffer solution (Sigma-Aldrich).
3. Lysis Buffer: 1% SDS, 0.2 M NaCl, 5 mM DTT in TE Buffer.
4. RNase A: 10 mg/ml (Thermo Fisher Scientific).
5. Proteinase K: 20 mg/ml solution in H₂O (Sigma-Aldrich).

Table 1
Primer sequences. Illumina barcodes are represented in bold

Name	Sequence
OG113	TAAC TTGAAAGTATTT CGATTTCTTGGCTTTATATATCTTGTGGAAAGGAC GAAACACCG
OG114	ACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAG CTCTAAAAC
OG115	AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTT CG
OG116	CTTTAGTTTGTATGTCTGTTGCTATTATGTCTACTATTCTTTCC
OG125	AATGATACGGCGACCACCGAGATCTACACTCTTTC CC TACACGACGCTCT TCCGATCTTCTTGTGGAAAGGACGAAACACCG
OG126_bc01	CAAGCAGAAGACGGCATA CAGATATC ACGGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc02	CAAGCAGAAGACGGCATA CAGATCG ATGTGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc03	CAAGCAGAAGACGGCATA CAGATTTAG GCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc04	CAAGCAGAAGACGGCATA CAGATTG ACCAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc05	CAAGCAGAAGACGGCATA CAGATAC AGTGGTGACTGGAGTTCAGACGTG GCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc06	CAAGCAGAAGACGGCATA CAGATGCC AATGTGACTGGAGTTCAGACGTG GCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc07	CAAGCAGAAGACGGCATA CAGATC AGTCGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc08	CAAGCAGAAGACGGCATA CAGATACT TGAGTGACTGGAGTTCAGACGTG TGCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc09	CAAGCAGAAGACGGCATA CAGATG ATCAGGTGACTGGAGTTCAGACGTG GCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
OG126_bc10	CAAGCAGAAGACGGCATA CAGATTAG CCTTGTGACTGGAGTTCAGACGTG GCTCTTCCGATCTTCTACTATTCTTTCC CC TGCACTGT
LR256	CTGTGAATAAGATGTCCATT
LR257	ACACTCTCGTTATTTGTCAT
LR21	ACTATCATATGCTTACCGTAA C

6. Phenol/chloroform/Isoamyl alcohol mixture 125:24:1 (Roth).

7. Qubit dsDNA HS Assay Kit-100 (Thermo Fisher Scientific).

2.5 Equipment

1. ECM 399 electroporator (BTX).
2. Spectralphotometer NanoDrop™ 2000 (Peqlab).
3. Safe Imager™ 2.0 Blue-Light Transilluminator (Thermo Fisher Scientific).
4. Hybridization oven capable of maintaining temperature at 40 °C (for Flow-FISH).
5. EVE™ Automated Cell Counter, NanoEnTek (VWR).
6. Qubit Fluorometer (Thermo Fisher Scientific).
7. Illumina sequencer.

3 Methods

In this section we describe every step of a FACS-based pooled lentiviral CRISPR screen, where molecular phenotypes are detected by intracellular staining of proteins or RNA, using the lentiGuide-Puro sgRNA expression vector (Fig. 1). Subheadings 3.1 and 3.2 provide detailed instructions for design and cloning of the sgRNA library, respectively. Subheading 3.3 covers packaging of the sgRNA library in lentiviral particles and transduction with the library. Phenotypic enrichment using antibody staining or RNA detection by Flow-FISH is described in Subheading 3.4. Subheadings 3.5 and 3.6 explain the preparation of sequencing libraries and statistical data analysis, respectively.

3.1 SgRNA Library Design

1. Download a genome-wide CRISPR knock-out library from the Addgene website (<https://www.addgene.org/crispr/libraries/>) (*see Note 1*).
2. Decide which genes should be targeted by the library and how many sgRNAs should be included for each gene (*see Note 2*). Extract the sequences from the genome-wide library accordingly. In addition, add at least 100–1000 non-targeting or safe-targeting control sgRNAs (*see Note 3*). Make sure to also include positive control genes in the library, which are known to affect your phenotype of interest.
3. Add homology arms for library cloning by extending the sgRNA sequences as follows:
TGGAAAGGACGAAACACCG[sgRNA]GTTTTAGAGC
TAGAAATAGCAAGTTAAAATAAGGC
4. Synthesize the sequences as a custom oligo pool. Several sgRNA libraries can be synthesized in the same pool (*see Note 4*).

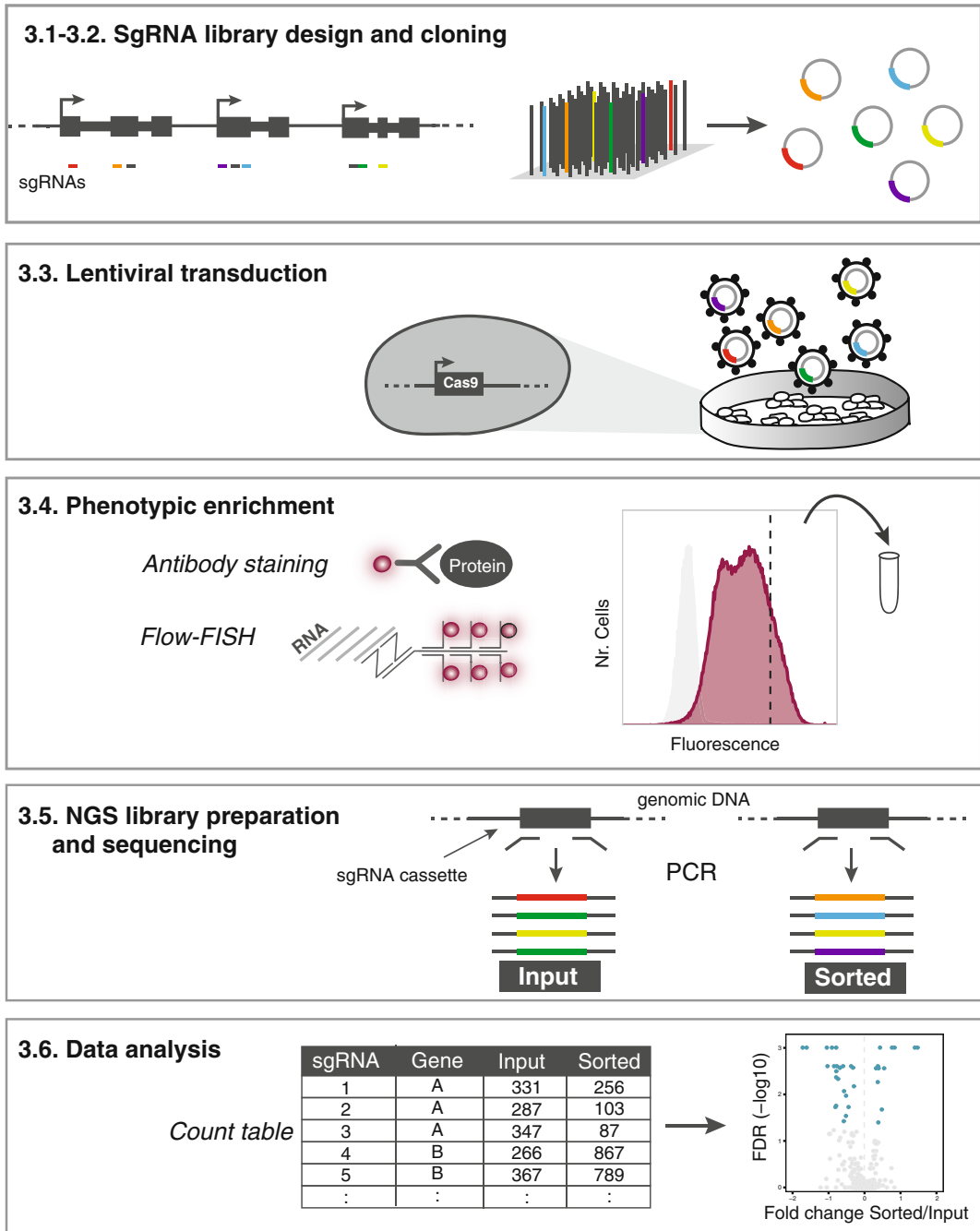


Fig. 1 Protocol overview. The sgRNA library is designed, synthesized in a pooled fashion on a microarray, and cloned into the lentiviral sgRNA expression vector lentiGuide-Puro (Subheadings 3.1 and 3.2). The cloned sgRNA library is packaged in lentiviral particles and used to transduce cells expressing the perturbation system (Subheading 3.3). Cells with the phenotype of interest are enriched by FACS using intracellular antibody staining or RNA detection by Flow-FISH (Subheading 3.4). Genomic DNA is extracted from the sorted and input samples. The genomically integrated sgRNA cassette is amplified by PCR, tagged with sample barcodes and sequencing adaptors, and subjected to NGS (Subheading 3.5). Reads are mapped to the sgRNA library to produce a count table and screen hits are identified by statistical analysis (Subheading 3.6)

Components	Amount	Final concentration
5× Phusion HF Buffer	10 µl	1×
10 mM dNTPs	1 µl	200 µM
DMSO	1.5 µl	3%
10 µM Forward Primer (OG113)	2.5 µl	0.5 µM
10 µM Reverse Primer (OG114)	2.5 µl	0.5 µM
Oligo Pool	1–5 ng	
Phusion Hot Start Flex DNA Polymerase	1 µl	2 units
Nuclease-free water	Add to 50 µl	

- Run the PCR with the following cycle parameters.

Cycle	Denature	Anneal	Extend
0	98 °C 30 s	–	–
1–14	98 °C 15 s	63 °C 15 s	72 °C 15 s
15	–	–	72 °C 5 min

- Pool PCR reactions and purify with a PCR Purification Kit to concentrate the PCR reactions. Elute in 30 µl Nuclease-free water.
- Gel-purify the pooled PCR product (140 bp) from a 2% agarose gel (LE) to eliminate primer dimers (approx. 120 bp).
- Digest 2.5 µg of lentiGuide-Puro plasmid (*see Note 5*) with 2.5 µl of BsmBI for 1 h at 55 °C or overnight at 37 °C in a 50 µl reaction.
- Gel-purify the digested plasmid (approx. 8.3 kb, filler sequence is approx. 1.9 kb).
- Determine the concentration of the digested plasmid and the PCR product via Nanodrop.
- Set up a Gibson assembly reaction as follows using the gel-purified lentiGuide-Puro plasmid from **step 6** and the purified PCR amplicon from **step 4**, and one negative control reaction without insert. Perform 1–2 Gibson reactions per 10,000 sgRNAs.

Component	Amount
BsmBI digested and gel-purified lentiGuide-Puro	100 ng
PCR-amplified oligo pool	7 ng
2× Gibson assembly master mix	10 μl
Nuclease-free water	Add to 20 μl

9. Incubate in a thermocycler at 50 °C for 1 h and quench on ice.
10. Pool Gibson reactions and bring to 200 μl with nuclease-free water.
11. Add 1.5 μl of pellet paint, 20 μl 3 M NaOAc, and 600 μl 100% EtOH.
12. Incubate samples at −80 °C for 30 min (or overnight at −20 °C).
13. Centrifuge for 30 min at 20,000 × *g* and 4 °C to pellet DNA, followed by two washes with ice-cold 80% EtOH.
14. Air-dry pellet and resuspend in 5 μl nuclease-free water per Gibson reaction.
15. Freeze the eluted DNA for 3 h at −80 °C (this step might increase transformation efficiency).
16. Add maximally 9 μl of the Gibson assembly reaction to 20 μl of thawed electrocompetent MegaX DH10B T1R *E.coli* and incubate 30 min on ice. Perform enough electroporation reactions to transform the entire Gibson assembly reaction.
17. Pipette the bacterial cells with the Gibson reaction into a pre-chilled electroporation cuvette and tap gently on a surface to remove bubbles. Place the cuvette in an electroporator and electroporate the sample at 2.0 kV, 200 Ω, and 25 μF.
18. Add 1 ml of prewarmed recovery or SOC medium to the cuvette quickly after electroporation (make sure to pipette up and down to resuspend all bacterial cells from the bottom of the cuvette).
19. Transfer bacterial cells to a culture tube and shake at 250 rpm for 1 h at 37 °C. Pool electroporation reactions at this step if multiple reactions were performed.
20. Bring sample to a volume of 10 ml with LB medium supplemented with ampicillin (0.1 mg/ml) and mix well.
21. To estimate library coverage plate 10 μl (1:1000 dilution) and 100 μl (1:100 dilution) of the mixture on pre-warmed Ampicillin-supplemented (0.1 mg/ml) agar plates. Additionally, make a 1:100 dilution of the mixture (10 μl in 990 μl LB medium) and plate 1 μl (1:1,000,000 dilution), 10 μl (1:100,000 dilution), and 100 μl (1:10,000 dilution) on three

- additional ampicillin agar plates and incubate overnight at 37 °C.
22. Inoculate 500 ml of LB medium plus ampicillin (0.1 mg/ml) with the remaining bacteria and incubate overnight on a shaker at 37 °C 250 rpm.
 23. The next day count the colonies in the agar plates and multiply by the dilution factor. The average number of colonies divided by the library size yields the coverage of the cloned sgRNA library. The coverage of library cloning should be 100–1000× (see **Note 6** and example in Table 2).
 24. Pick 10 colonies, carry out mini-scale plasmid preparations and sequence the insert through Sanger sequencing with primer LR21. At least eight out of the ten colonies should carry an identifiable sgRNA sequence from the cloned library.
 25. Pellet bacterial cells from **step 22** by centrifugation for 20 min at $20,000 \times g$ at 4 °C and carry out a plasmid Maxiprep.

Table 2

Example calculation for coverage. Here we give an example how sufficient coverage can be ensured at all steps of the screen. The calculations below are for an sgRNA library of 1000 sgRNAs (library size), where 15% of cells were sorted after antibody staining and a 300× coverage is maintained

Step	Material required
PCR to amplify oligo pool (Subheading 3.2, step 1)	Amplify 1–5 ng ($\sim 6\text{--}30 \times 10^9$ molecules)
Gibson assembly and transformation (Subheading 3.2, step 21)	$\geq 3 \times 10^5$ transformants
Lentiviral transduction (Subheading 3.3, step 13)	Treat $\geq 9 \times 10^5$ cells with viral supernatant (MOI = 0.3) → $\geq 3 \times 10^5$ cells transduced with one sgRNA
Selection and expansion (Subheading 3.3, step 15)	Always plate $\geq 3 \times 10^5$ cells
Cell harvesting (Subheading 3.4, step 1)	Use $\geq 1.2 \times 10^7$ cells for staining
Antibody staining (Subheading 3.4, steps 6–11)	$\sim 30\text{--}50\%$ loss ($\geq 6 \times 10^6$ cells left) take 6×10^5 cells as input control
Single-cell gating (Subheading 3.4, step 12)	$\sim 30\%$ loss ($\geq 4 \times 10^6$ cells left)
Cell sorting (15%) (Subheading 3.4, step 12)	$\sim 85\%$ loss ($\geq 6 \times 10^5$ cells left)
Genomic DNA extraction (Subheading 3.5, steps 5–9)	$\sim 20\text{--}50\%$ loss (DNA from $\geq 3 \times 10^5$ cells left)
NGS library prep (Subheading 3.5, steps 12–17)	Amplify DNA from $\geq 3 \times 10^5$ cells $\approx 1.8 \mu\text{g}$ [a mouse cell contains 6 pg DNA, a human cell 7 pg DNA]
Sequencing (Subheading 3.5, step 18)	Sequence $\geq 4.3 \times 10^5$ reads [assuming 70% alignment rate]

26. In order to confirm good library representation by NGS, add sequencing adaptors and samples barcodes by PCR as follows.

Components	Amount	Final concentration
2× Ready Mix Kapa	25 µl	1×
10 µM Forward Primer (OG125)	1.5 µl	0.3 µM
10 µM Reverse Primer (OG126)	1.5 µl	0.3 µM
Plasmid library	100 ng	
Nuclease-free water	Add to 50 µl	

27. Run the PCR with the following cycle parameters.

Cycle	Denature	Anneal	Extend
0	95 °C 3 min	–	–
1–12	98 °C 20 s	55 °C 15 s	72 °C 15 s
13	–	–	72 °C 1 min

28. Gel-purify the PCR amplicon of 351 bp.
29. Subject the purified PCR amplicon to Illumina sequencing as outlined in Subheading 3.5, step 18.
30. For analysis follow **steps 1–8** in Subheading 3.6. Calculate the distribution width given as the fold change between the 90th and the 10th percentiles (*see* Fig. 3 for an example). The distribution width should ideally be <4, but at least <8.

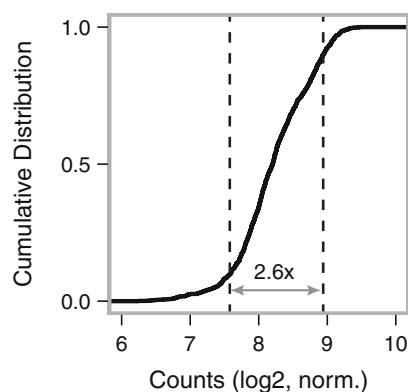


Fig. 3 Cumulative distribution of a cloned sgRNA library. Vertical dashed lines indicate the tenth and 90th percentiles. The distribution width, given by the fold change between the 90th and 10th percentiles, is indicated

3.3 Lentiviral Transduction of the sgRNA Library

Lentiviral transduction must be performed in a laboratory with the appropriate safety level. Wear appropriate protective gear.

1. Calculate the amount of virus that you have to produce to ensure sufficient coverage of the sgRNA library (*see* example calculation in Table 2).
2. Thaw low-passage Hek293T cells and passage at least twice before seeding for transfection the following day. Transfect Hek293T plates when cells are 90% confluent. To determine the number of plates that must be transfected, perform a test transduction with the sgRNA library beforehand.
3. For lipofection of a 10 cm dish, prepare Mix A and Mix B as follows.

<i>Mix A</i>	
Plasmid pLP1 (Midi prep)	6.3 µg
Plasmid pLP2 (Midi prep)	3.1 µg
Plasmid VSVG (Midi prep)	2.1 µg
Plasmid library	10.5 µg
OptiMEM	1 ml
<i>Mix B</i>	
Lipofectamine 2000	60 µl
OptiMEM	1 ml

4. Incubate both mixtures for 5 min at room temperature and combine them, followed by another 20 min of incubation. Add the mixture drop-wise to the cells.
5. Change medium 6 h after transfection.
6. Harvest Hek293T supernatant 48 h after transfection, which now contains the lentiviral particles, centrifuge at $1800 \times g$ for 15 min at 4 °C, and store at -80 °C. Aliquot as required since longer storage and repeated freeze-thaw cycles will decrease the viral titer.
7. Optional: For cells that are difficult to transduce, viral supernatant can be concentrated by using the lenti-X Concentrator.
8. To measure the viral titer, seed the cell type that will be used in the screen in two 6-well plates.
9. The next day, add lentiviral supernatant at different concentrations to the cells together with 8 ng/µl of polybrene. Add tenfold dilutions (10^{-2} to 10^{-6}) of viral supernatant to 5 wells of each plate and regular growth medium to the remaining well (non-transduced control).
10. Two days after transduction, start selection with puromycin (*see* Note 7).

11. Change medium regularly and count colonies approx. 8 days after transduction, when all cells in the non-transduced control have died. To calculate the number of transducing units (TU) per ml, average the number of colonies for each dilution in the two plates and multiply by the respective dilution factor.
12. Thaw the cell line chosen for the screening assay, expressing the perturbation system of choice (*see Note 8*), and passage twice.
13. Transduce cells with the sgRNA library with a multiplicity of infection (MOI) of 0.3. To this end, calculate the amount of viral supernatant to be added per flask (in ml) as follows:

$$[\text{Number of cells per flask}] \times 0.3 / \text{Viral titer (TU/ml)}$$

Calculate the total number of cells that must be treated with the viral supernatant to maintain sufficient coverage as $3 \times [\text{desired coverage}] \times [\text{library size}]$ (*see Table 2* for example calculation).

14. Start puromycin selection 2 days after transduction and include a non-transduced control.
15. Culture cells until selection is completed (all cells in the non-transduced control have died) and expand until you reach the cell number required for phenotypic enrichment (usually 1–2 weeks after transduction). During splitting, always maintain the coverage by plating a sufficient number of cells, given by $[\text{desired coverage}] \times [\text{library size}]$ (*see Table 2* for example calculation).

3.4 Phenotypic Enrichment

Here we describe the protocol used for intracellular staining of phosphorylated Mek, using paraformaldehyde and methanol for cell fixation and permeabilization. Other staining protocols that are compatible with the subsequent DNA extraction steps, such as RNA Flow-FISH, can also be used (*see Note 9*).

1. Harvest a sufficient number of cells and prepare a single-cell suspension with trypsin (*see Note 10*).
2. Dilute the trypsinized cells 1:1 in ice-cold PBS. Dilute again 1:1 in ice-cold 3% Paraformaldehyde in PBS (*see Note 11*) and incubate for 10 min on ice. Resuspension of cells during trypsinization and fixation should be done gently enough as not to lyse the cells, but effectively enough as to avoid cell clumping. This step should be optimized for each particular cell type.
3. Aliquot a fraction of the cells for the fixed input control and treat in parallel to the samples used for staining until **step 7**, then store at $-80\text{ }^{\circ}\text{C}$. Determine the number of cells for the input control as at least.

$$2 \times [\text{Desired coverage}] \times [\text{library size}]$$

4. Aliquot a fraction of the cells to stain with the secondary antibody only, skipping **steps 8** and **9**. For this, 10^6 cells should suffice. This will serve as background control for the staining.
5. Centrifuge for 5 min at $500 \times g$ and at 4°C .
6. Resuspend cells in ice-cold Methanol, incubate for 10 min on ice ($0.5\text{ ml per }10^6\text{ cells}$), and centrifuge for 5 min at $500 \times g$.
7. Wash cells twice with staining media ($2\text{ ml per }10^6\text{ cells}$) and block for 10 min at room temperature in the second wash before centrifugation.
8. Dilute primary antibody in staining media and incubate cell suspension for 30 min at room temperature ($100\ \mu\text{l per }10^6\text{ cells}$). Antibody dilution should be optimized beforehand.
9. Dilute cells in staining media ($2\text{ ml per }10^6\text{ cells}$) and centrifuge for 5 min at $500 \times g$. Wash once more with staining media.
10. Dilute the fluorophore-labeled secondary antibody in staining media and incubate for 15 min at room temperature ($100\ \mu\text{l per }10^6\text{ cells}$). This step can be skipped if working with a fluorescently labeled primary antibody. From this point onwards, store sample in the dark or wrap tube with aluminum foil.
11. Dilute cells in staining media ($2\text{ ml per }10^6\text{ cells}$) and centrifuge for 5 min at $500 \times g$. Wash cells twice with staining media before flow cytometry.
12. Sort the desired high and/or low fraction. As a guideline sort 15–25% of cells with high and/or low signal (*see Note 12*).
13. Freeze cell pellets at -80°C in aliquots of 1–5 Mio cells. The number of cells to be collected should be at least

$$2 \times [\text{Desired coverage}] \times [\text{library size}]$$

(*see Table 2* for example calculation).

3.5 NGS Library Preparation and Deep Sequencing

1. Thaw cell pellets and resuspend each aliquot in $250\ \mu\text{l}$ of Lysis Buffer (*see Note 13*). This protocol is optimized for samples with $1\text{--}5 \times 10^6$ cells. If working with larger cell numbers, scale the reagents accordingly.
2. Incubate samples overnight at 65°C .
3. The next morning, add $20\ \mu\text{l}$ of RNAse A (10 mg/ml) and incubate for 1 h at 37°C .
4. Add $5\ \mu\text{l}$ of Proteinase K (20 mg/ml) and incubate for 1 h at 50°C .
5. Add $275\ \mu\text{l}$ (1 vol) Phenol/Chloroform/Isoamyl alcohol, vortex for 1 min, and centrifuge the sample at $16,000 \times g$ for 10 min at room temperature.
6. Transfer the aqueous phase to a new tube and add 1 ml of 100% EtOH, $90\ \mu\text{l}$ of 5 M NaCl, and $1\ \mu\text{l}$ of Pellet paint. Mix sample thoroughly and incubate at -80°C for 1 h.

7. Pellet DNA by centrifuging at $16,000 \times g$ for 15 min at 4°C .
8. Wash pellets twice with 70% EtOH.
9. Air-dry pellet and resuspend in 50–100 μl of H_2O .
10. Quantify samples using Qubit, following manufacturer's instructions.
11. Run 1 μg of isolated DNA on a 0.8% agarose gel to assess DNA quality. The DNA should appear as a high molecular weight band.
12. Set up the first PCR (PCR1, *see* Fig. 4), which amplifies the genomically integrated sgRNA cassette. Calculate the total amount of genomic DNA that has to be amplified in order to obtain proper library coverage as outlined in **Note 14**. Set up multiple PCR reactions if required.

Components	Amount	Final concentration
2 \times Ready Mix Kapa	25 μl	1 \times
10 μM Forward Primer (OG115)	1.5 μl	0.3 μM
10 μM Reverse Primer (OG116)	1.5 μl	0.3 μM
Genomic DNA	2 μg	
Nuclease-free water	Add to 50 μl	

13. Run the PCR with the following cycle parameters.

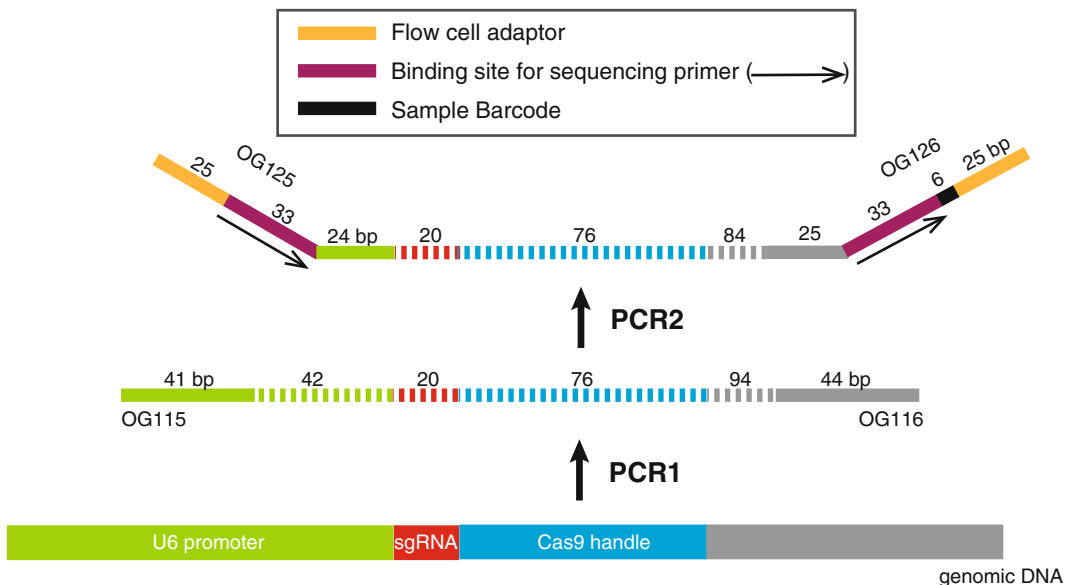


Fig. 4 Preparation of NGS library. The genomically integrated sgRNA cassette is amplified by primers OG115/116 (PCR1) and sequencing adaptors and barcodes are added in a second PCR with primers OG125/126

Cycle	Denature	Anneal	Extend
0	95 °C 3 min	–	–
1–20	98 °C 20 s	55 °C 15 s	72 °C 30 s
21	–	–	72 °C 1 min

14. Pool all PCR reactions for each sample and load 20 µl on a 1% agarose gel to verify efficient amplification. You should observe a band of 317 bp that is clearly visible, but might be faint in intensity.
15. Set up the second PCR (PCR2, *see* Fig. 4) reaction to add barcodes and adaptors for Illumina sequencing. To allow sample multiplexing on the sequencer, each sample must be amplified with a different OG126 primer, which contains the sample barcode.

Components	Amount	Final concentration
2× Ready Mix Kapa	25 µl	1×
10 µM Forward Primer (OG125)	1.5 µl	0.3 µM
10 µM Reverse Primer (OG126)	1.5 µl	0.3 µM
PCR product from step 12	2.5 µl	
Nuclease-free water	Add to 50 µl	

16. Run the PCR with the following cycle parameters.

Cycle	Denature	Anneal	Extend
0	95 °C 3 min	–	–
1–11	98 °C 20 s	55 °C 15 s	72 °C 15 s
12	–	–	72 °C 1 min

17. Gel-purify the PCR amplicon of 351 bp.
18. Subject the purified PCR amplicon to Illumina sequencing. Read at least 44 bp with a single-end sequencing run, aiming for a minimal read number per sample of $2 \times [\text{desired coverage}] \times [\text{library size}]$ (*see* **Note 15**).

3.6 Data Analysis

1. Go to <https://usegalaxy.eu/> and make an account.
2. Upload the demultiplexed sequencing files (fastq) for Read 1 as a collection as type “fastqsanger.gz”.
3. Perform “FastQC Read Quality reports” on your data.
4. Inspect the output “Webpage Results”. On the page “Per Base Sequence Content” you should see mixed sequences from position 25 onwards (compare Fig. 4).

5. Prepare a tab-delimited text file with the sgRNA library, consisting of three columns with the sgRNA name, sgRNA sequence, and name of targeted gene. The first line should contain the column names “sgRNA ID”, “Sequence”, and “Gene”. Upload the file as type “tabular”.
6. Use “MAGeCK count” to align the fastq files to the sgRNA library. In the output option set “Output count summary file” and “Output normalized counts file” to “yes”. Define short sample labels under “Specify sample labels” because these will be used in the design matrix to define the input and selected populations in **step 10**.
7. The output table named “sgRNA count summary” contains alignment rates and information on sgRNA dropout (column “Zerocounts”) for all samples. The alignment rate should be at least 70% and zerocounts should be <0.5% (*see Note 16*).
8. Download the normalized counts and plot the cumulative distribution for each sample, e.g., in R Studio, and analyze whether all sgRNAs are detected across samples (*see Note 17*).
9. Compare the cumulative distribution of NTC counts. Calculate the distribution width given as the fold change between the 90th and the 10th percentiles. If the distribution width for NTCs stays fairly constant throughout the screen, sufficient coverage was maintained at all steps.
10. For statistical analysis, prepare a tab-delimited text file with the design matrix of your screen. The matrix contains one row per sample. The matrix contains a column (“samples”) with the sample names, which were defined in the alignment step, a second column (“baseline”) with a “1” in every row, and an additional column for each fraction that was sorted with “0” or “1”. *See Table 3* for an example.
11. Upload the design matrix to Galaxy as type “tabular”.

Table 3
The design matrix for a screen with two replicates, where both low and high fractions are sorted

Samples	Baseline	High	Low
input_R1	1	0	0
high_R1	1	1	0
low_R1	1	0	1
input_R2	1	0	0
high_R2	1	1	0
low_R2	1	0	1

12. Perform statistical analysis with “MAGeCK mle”, using the design matrix and the MAGeCK count output “sgRNA counts” as input.
13. The output “Gene Summary” contains beta values (effect size) and false discovery rates (FDR) for all genes in the library. Significance is often defined as FDR lower than 0.05–0.1.

4 Notes

1. Several genome-wide sgRNA libraries for mouse and humans have been designed for knock-out, CRISPRa and CRISPRi screens [29–32]. Libraries can be ordered as a whole from Addgene or can be used as a basis to design a custom library by extracting selected sgRNA sequences. To design additional sgRNAs to target genes not represented in these genome-wide libraries a large number of different computational tools are available, for example <http://guides.sanjanalab.org/#/> for KO screens and <http://guidescan.com/>, which can be used to target promoters for CRISPRa/i screens (–400 bp to –50 bp around TSS for CRISPRa or –50 bp to +300 bp for CRISPRi).
2. Typically, each gene is targeted by 3–10 sgRNAs. A higher number of sgRNAs per gene makes a screen more robust to nonfunctional guides and can be used for statistical analysis similar to replicate experiments. CRISPRa and CRISPRi screens generally require larger sgRNA numbers since here guide activity is less predictable. Additionally, since CRISPRa and CRISPRi target the TSS and not the coding sequence, often more sgRNAs are required because many mammalian genes have several TSSs.
3. In knock-out screens, cells transduced with NTCs are often found to have a growth advantage, probably due the absence of Cas9-induced DNA damage. So-called safe-targeting guides have thus been suggested as a better alternative for NTCs [30]. For focused screens, where only a small number of genes are targeted that are known to be associated with the phenotype of interest, it is important to include enough non- or safe-targeting controls that can be used for normalization.
4. To synthesize two different sgRNA libraries on the same microarray, add a sublibrary-specific sequence (ATGGACATCTTATT CACAG for library 1 and TGACAAATAACGAGAGTGT for library 2) to the 3'-end of the synthesized oligos during library design. Perform **steps 1–4** in Subheading 3.2 twice to first amplify each sublibrary with primers OG113/LR256 (library 1) and OG113/LR257 (library 2) for 12 cycles, and then use

100 ng to add homology overhangs with primers OG113/OG114 for 6 cycles.

5. The sgRNA expression vector lentiGuide-Puro (Addgene #52963) was specifically designed to yield high viral titers, but can also be substituted with lentiCRISPRv2 (Addgene #52961), which co-expresses Cas9, but has a significantly lower titer [33]. CRISPRi and CRISPRa screen sometimes use vectors with an optimized version of the sgRNA scaffold (e.g., Addgene #60955) [5, 34].
6. If coverage during library cloning is insufficient, sgRNAs are missing from the cloned library, or sgRNA representation is uneven, the conditions during cloning were probably not optimal or it was performed at a too small scale. To improve efficiency you can increase the number of Gibson reactions per library and pool them before EtOH clean-up, increase the amount of Gibson reaction added to the electrocompetent cells after EtOH clean-up, or increase the number of electroporation reactions.
7. Determine the optimal puromycin concentration for the cell type used beforehand by measuring a kill curve.
8. Cas9 is typically delivered via lentiviral transduction (e.g., lentiCas9-Blast, Addgene #52962) shortly before transduction with the library to minimize the negative effects due to generation of double-strand breaks. However, the use of clonal Cas9-expressing cell lines might increase screen sensitivity [35] and the negative effects associated with constitutive Cas9 expression can be mitigated by implementing drug-inducible CRISPR/Cas systems [36]. CRISPRa/i screens are in general performed in clonal cell lines, which express the perturbation system of choice and exhibit high perturbation strength.
9. To use an RNA expression phenotype as screen readout, stain the RNA of interest using Flow-FISH with PrimeFlow RNA assay according to the 96-well-plate protocol provided by the manufacturer. It is advisable to choose Alexa 647 (Type 1 Primeflow probes) as fluorophore since it provides the best signal-to-noise ratio. Leave out the target probe from one well to serve as background fluorescence control. Additionally, it is recommended to carry out a positive control with a β -Tubulin target probe, to make sure all the steps of the protocol are executed successfully. Before target probe hybridization, pellet and freeze fixed input control cells at -80°C . We often observe that subsequent PCR reactions are inhibited in cells that we stained by Flow-FISH. In this case reduce the amount of DNA per PCR reaction.

10. It is recommended to determine the required cell number beforehand by measuring the loss of material during all subsequent steps (staining, live cell gating, sorting, DNA extraction). As a guideline start with $10 \times [\text{Desired coverage}] \times [\text{library size}] / [\text{sorted fraction}]$.
11. For the detection of very dynamic antigens such as phosphoproteins, keep the time between the removal of the culture medium and fixation as short as possible and perform all steps on ice with ice-cold reagents. Trypsinization can be carried out on ice by increasing the incubation time; this step should be optimized beforehand depending on the cell type. Verify under a microscope that no cell clumps remain.
12. Sorting stringency is an important parameter of the phenotypic enrichment step, which should be rather permissive to maintain full library representation and sufficient coverage. Too stringent sorting of the target cell population will lead to higher enrichment values and detection of more true positive hits, but will also increase the number of false positives. With less stringent sorting by contrast, the number of false positives will decrease, but also screen sensitivity will be reduced. Simulations have shown that an optimal balance between these effects is achieved with a sorting stringency of approx. 25% [27]. We have successfully used sorting stringencies between 15 and 25% in FACS-based screens. We generally sort both cells with the high and low signal, allowing analysis of both positive and negative selection, but sorting cells with high signal is often more powerful.
13. Overnight incubation at 65 °C is necessary for DNA de-crosslinking. We have found that addition of DTT to the lysis buffer increases DNA yields of fixed samples.
14. A diploid mouse cell contains approx. 6 pg of DNA (7 pg for human cells). The amount of DNA to be amplified is thus given by $6 \text{ pg} \times [\text{coverage}] \times [\text{library size}]$. Try to amplify as much DNA per reaction as possible without inhibiting the PCR reaction. This amount might vary between samples and conditions. Perform a series of test PCRs (try 0.2–2 µg DNA) to optimize conditions before amplifying large amounts of DNA. If $< 2 \text{ µg}$ DNA is amplified per reaction, the amount of PCR product to be added to PCR 2 can be increased accordingly and the PCR product can be concentrated by PCR purification if necessary.
15. The first 24 bp of each read will be identical (*see* Fig. 4) resulting in low sample complexity. To ensure sufficient sequencing quality the samples should be multiplexed with a different sample type, or 20–30% of PhiX DNA should be added during

sequencing. An alternative approach to increase library complexity is to use a pool of staggered forward primers, as described for example in [12].

16. A low alignment rate might be observed, if the sequencing quality is low. Try increasing the amount of PhiX DNA to increase sample complexity or multiplex with different library type.
17. The distribution might broaden compared to the plasmid library and some guides might get lost, since sgRNAs that affect cell viability or proliferation will be counterselected during extended culture.

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