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Integrated design, execution, and analysis of arrayed and pooled CRISPR genome-editing experiments

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS M.C.C., M.H., and L.P. conceived this project. M.H. and J.-P.C. created CRISPOR. L.P., M.C.C., D.E.B., and G.-C.Y. created CRISPResso. M.C.C. and D.E.B. performed the experiments.

M.C.C., D.E.B., S.H.O., N.E.S., O.S., G.-C.Y., F.Z., and L.P. analyzed the experimental data. M.C.C., M.H., and L.P. wrote the manuscript with input from all authors.

COMPETING INTERESTS

The authors declare no competing interests.

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Abstract

CRISPR (clustered regularly interspaced short palindromic repeats) genome-editing experiments offer enormous potential for the evaluation of genomic loci using arrayed single guide RNAs (sgRNAs) or pooled sgRNA libraries. Numerous computational tools are available to help design sgRNAs with optimal on-target efficiency and minimal off-target potential. In addition, computational tools have been developed to analyze deep-sequencing data resulting from genome-editing experiments. However, these tools are typically developed in isolation and oftentimes are not readily translatable into laboratory-based experiments. Here, we present a protocol that describes in detail both the computational and benchtop implementation of an arrayed and/or pooled CRISPR genome-editing experiment. This protocol provides instructions for sgRNA design with CRISPOR (computational tool for the design, evaluation, and cloning of sgRNA sequences), experimental implementation, and analysis of the resulting high-throughput sequencing data with CRISPResso (computational tool for analysis of genome-editing outcomes from deep-sequencing data). This protocol allows for design and execution of arrayed and pooled CRISPR experiments in 4–5 weeks by non-experts, as well as computational data analysis that can be performed in 1–2 d by both computational and noncomputational biologists alike using web-based and/or command-line versions.

INTRODUCTION

The CRISPR nuclease system is a facile and robust genome-editing system that was originally identified as the driver of prokaryotic adaptive immunity to allow for resistance to bacteriophages^{1–3}. This system has been subsequently repurposed for eukaryotic genome editing by heterologous expression of the CRISPR components in eukaryotic cells. Site-specific cleavage by Cas9 requires an RNA molecule to guide nucleases to specific genomic loci to initiate double-strand breaks (DSBs)^{1,2,4}. Site-specific cleavage requires Watson–Crick base pairing of the RNA molecule to a corresponding genomic sequence upstream of a protospacer-adjacent motif (PAM)^{1,2}. The required RNA molecule for genome-editing experiments consists of a synthetic fusion of the prokaryotic tracrRNA and CRISPR RNA (crRNA) to create a chimeric sgRNA⁵. In contrast to Cas9, the Cpf1 nuclease does not require a tracrRNA and engenders DSBs downstream of its PAM sequence. Cpf1 requires a crRNA to create DSBs, which results in 5' overhangs⁴.

CRISPR mutagenesis relies on engagement of endogenous DNA repair pathways after nuclease-mediated DSB induction has occurred. The principal repair pathways include nonhomologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ repair is an error-prone pathway, which results in a heterogeneous spectrum of insertions/deletions (indels) primarily in the range of 1–10 bp^{1,2,6–8}. HDR relies on the co-delivery of an

extrachromosomal template to be used as a template for DNA repair following DSB, as opposed to an endogenous template such as a sister chromatid. This allows for the insertion of customized sequence into the genome^{1,2}.

Applications of the method and development of the protocol

A variety of computational tools have been developed for the design and analysis of CRISPR-based experiments. However, these tools are typically developed in isolation, without features for facile integration with one another, and/or without sufficient consideration to facilitate implementation in a laboratory setting. Here, we offer a protocol to integrate robust, publicly available tools for the design, execution, and analysis of CRISPR genome-editing experiments. Specifically, we have adapted CRISPOR⁹ and CRISPResso¹⁰ to be integrated with one another as well as streamlined for experimental implementation (Figs. 1–3). This protocol has been used in previously published works to functionally interrogate the *BCL11A* enhancer as well as to evaluate potential functional sequences within all DNase hypersensitive sites (DHSs) in proximity to the *MYB* gene^{11,12}. CRISPR mutagenesis allows for the study of both coding and noncoding regions of the genome¹³. This involves usage of one sgRNA for arrayed experiments or multiple sgRNAs for pooled experiments. Arrayed experiments are useful when a target can be mutagenized by one sgRNA. Pooled screening allows for targeting of a handful of genes up to genome-scale gene targeting^{14–17}. It also allows for saturating mutagenesis (tiling of sgRNA) experiments to identify functional sequence within noncoding regions^{11,12,18}. This protocol can be used to design and execute arrayed or pooled genome-editing experiments. Furthermore, it can also be used for the design, implementation, and analysis of pooled screens for gene targeting, saturating mutagenesis of noncoding elements, or any other targeting strategies^{11,12,19,20}. It is important to note that computational skills are required for the command-line and Docker versions of CRISPOR and CRISPResso in this protocol. Specifically, users are required to have a basic understanding of how to execute a command in a terminal and how to navigate a Unix-based file system. However, web-based versions are also available that do not require these skills.

CRISPOR: sgRNA and PCR primer design for arrayed and pooled screen experiments

CRISPOR (<http://crispor.org>) is a computational sgRNA design tool that predicts off-target cleavage sites and offers a variety of on-target efficiency scoring systems to assist sgRNA selection for more than 120 genomes using many different CRISPR nucleases (Supplementary Table 1)⁹. CRISPOR offers a variety of on- and off-target prediction scores that can aid in optimal sgRNA selection (Box 1)⁹. Analysis of on-target sgRNA efficiency can be predicted based on available scores and/or investigated experimentally by analysis of editing frequency. In addition to the numerous sgRNA efficiency prediction scores, CRISPOR offers automated primer design to facilitate PCR amplification of regions for deep-sequencing analysis to quantitate editing frequency. This involves PCR amplification of sequences flanking the DSB site for a given sgRNA. Similarly, CRISPOR offers computational prediction of off-target sites as well as PCR primers for deep-sequencing analysis of potential mutagenesis at these predicted off-target sites.

CRISPOR can also be used for the design of gene-targeted pooled screens by including exonic regions for sgRNA design as well as saturating mutagenesis screens. Alternatively, a list of gene names can be input into CRISPOR ('CRISPOR Batch') to aid in the design of large scale gene-targeted libraries, which includes non-targeting controls. This analysis includes automated design of the required oligonucleotides for library cloning.

Saturating mutagenesis involves using all PAM-restricted sgRNAs within a given region(s) in a pooled screening format to identify functional sequences^{11,12,18}. Saturating mutagenesis can be used to analyze coding and noncoding elements in the genome or a combination of the two. Screen resolution is a function of PAM frequency and can be enhanced by PAM choice and/or a combination of nucleases with unique PAM sequences¹². CRISPOR can be used to design saturating mutagenesis libraries by simply selecting all sgRNAs within the inputted region(s). This analysis also includes automated design of the required oligonucleotides for library cloning. It is particularly important to consider off-target prediction scores offered by CRISPOR for saturating mutagenesis screens, as repetitive sequences can confound screen results¹². sgRNAs with high probability of off-target mutagenesis can be excluded at the library design stage or can be appropriately handled at the analysis stage after the experiments have been performed (Box 1).

CRISPResso: analysis of deep sequencing from arrayed or pooled sgRNA experiments

CRISPResso is a computational pipeline that enables accurate quantification and visualization of CRISPR genome-editing outcomes, as well as comprehensive evaluation of effects on coding sequences, noncoding elements, and off-target sites from individual loci, pooled amplicons, and whole-genome deep-sequencing data¹⁰. The CRISPResso suite involves multiple tools for analysis, including the CRISPResso webtool and command-line version of CRISPResso. There are also multiple additional command-line-only tools in the CRISPResso suite: CRISPRessoPooled, CRISPRessoWGS, CRISPRessoCount, CRISPRessoCompare, and CRISPRessoPooledWGSCompare. The applications and features of these tools are summarized in Table 1. CRISPResso analysis offers many unique features, such as splice-site or frameshift analysis to quantify the proportion of engendered mutations that result in a frameshift when targeting coding sequence. In addition, features for indel visualization have been added to CRISPResso since its initial publication¹⁰. Notably, CRISPResso also provides a variety of features to offer users the opportunity to optimize analysis of sequencing data (Box 2).

CRISPResso analysis of an individual locus requires PCR amplification of the sequences flanking the genomic position of the DSB for a given sgRNA. The resulting deep-sequencing FASTQ file can be analyzed by CRISPResso to quantitate the indel spectrum as well as visualize individual alleles (see Fig. 4 and ANTICIPATED RESULTS). This analysis can be performed when targeting coding (Fig. 4a–d) or noncoding sequences. When targeting coding (exonic) sequence, CRISPResso can determine the frequency of in-frame and out-of-frame (frameshift) mutations produced (Fig. 4d). This type of analysis may suggest toxicity upon gene knockout by identifying an increased frequency of in-frame mutations¹². Two separate CRISPResso analyses with the same amplicon can be directly compared using the CRISPRessoCompare tool (Table 1). CRISPRessoCompare is useful in

situations such as comparison of ‘treated’ and ‘untreated’ groups, as well as comparison of different experimental conditions. It can also be used to compare indel distributions created by two different sgRNAs within the same region/amplicon¹¹.

CRISPResso analysis can be extended from a single amplicon to multiple amplicons using the CRISPRessoPooled tool (Table 1). This is useful for individual locus experiments that require multiple amplicons for analysis of the full region or when multiple genes are targeted. The CRISPResso suite can also be used to analyze whole-genome sequencing (WGS) data through CRISPRessoWGS. This requires pre-aligned WGS data in BAM format, which can be created using publicly available aligners (e.g., Bowtie2 (ref. 21) or Burrows–Wheeler Aligner^{22,23}). Similar to CRISPRessoCompare, two analyses using either CRISPRessoPooled or CRISPRessoWGS can be directly compared using CRISPRessoPooledWGSCompare (Table 1). This can be particularly useful when using multiple-amplicon sequencing data or WGS data to evaluate off-target cleavages by two different sgRNAs to identify sgRNAs with lower off-target activity.

CRISPResso also offers the ability to analyze deep-sequencing data from pooled CRISPR screens. Sequence/indel-based analysis in a pooled screening format is confounded by non-edited reads from cells containing sgRNAs targeting other regions/loci. Therefore, pooled screens are typically analyzed by enumeration of sgRNAs by PCR-amplifying sequences containing the cloned sgRNA within the lentiviral construct that has been integrated into the cell’s genome (using PCR primers specific to the lentiviral construct). Deep-sequencing data generated using PCR primers specific to the lentiviral construct can be analyzed by CRISPRessoCount for sgRNA enumeration for the purposes of calculating enrichment and/or dropout of sgRNAs under different experimental conditions^{11,12} (Figs. 2, 4e). After sgRNA enumeration, it is important to normalize the reads by taking into account the total number of reads when comparing two different deep-sequencing samples. An example strategy for performing normalization involves normalizing all samples to 1 million reads. This can be accomplished by dividing the read count for each sgRNA by the total number of reads in that sample. Then this quotient is multiplied by 1 million, for example: (sgRNA_n read count/total read count of sample)×1,000,000. This should be repeated for all samples so that each sample has 1 million total normalized reads. These normalized reads can then be used to calculate enrichment and/or dropout (‘depletion’) ratios, for example: $\log_2(\text{normalized read count of sgRNA}_n \text{ in sample X} / \text{normalized read count of sgRNA}_n \text{ in sample Y})$.

Comparison with other methods

Numerous computational tools are freely available to aid sgRNA design for a wide spectrum of PAM sequences, as well as for on-target efficiency and off-target cleavage predictions: Broad GPP Portal²⁴, Cas-Database²⁵, Cas-OFFinder²⁶, CasOT²⁷, CCTop²⁸, COSMID²⁹, CHOPCHOP^{30,31}, CRISPRdirect³², CRISPR-DO³³, CRISPR-ERA³⁴, CRISPR-P³⁵, CROP-IT³⁶, DNA Striker¹², E-CRISP³⁷, flyCRISPR³⁸, GUIDES³⁹, GuideScan⁴⁰, GT-scan⁴¹, MIT CRISPR design tool⁸, WU-CRISPR⁴², CRISPRseek⁴³, sgRNAs9 (ref. 44), and CRISPR multiTargeter⁴⁵, as well as others offered by companies such as Deskgen⁴⁶ and Benchling⁴⁶. CRISPOR offers several unique advantages for designing sgRNAs for genome-editing

experiments. First, CRISPOR integrates multiple published on-target sgRNA efficiency scores, including those from Fusi *et al.*⁴⁷, Chari *et al.*⁴⁸, Xu *et al.*⁴⁹, Doench *et al.*^{24,50}, Wang *et al.*⁵¹, Moreno-Mateos *et al.*⁵², Housden *et al.*⁵³, Prox. GC⁵⁴, -GG⁵⁵, and Out-of-Frame⁵⁶. It also offers previously published off-target prediction (MIT specificity score)⁸. Second, CRISPOR has been optimized to facilitate experimental implementation by providing automated primer design for both on-target and off-target deep-sequencing analysis. The primers and output files are further designed to be compatible for subsequent analysis by CRISPResso after the experiments have been completed. CRISPOR also offers features to automate oligonucleotide design for cloning pooled sgRNA libraries targeting both genes and noncoding regions. Taken together, CRISPOR provides an sgRNA design platform to facilitate experimental execution and downstream data analysis.

Alternative computational tools to CRISPResso exist to evaluate genome-editing outcomes from deep-sequencing data^{57–61}; however, these tools offer limited analysis functionality for pooled amplicon sequencing or WGS data as compared with the CRISPResso suite. CrispRVariants is another tool that offers functionality to analyze deep-sequencing data by quantifying mosaicism and allele-specific gene editing, as well as multisequence alignment views⁶⁰.

CRISPR genome-editing reagents have taken many forms, including DNA, RNA, protein, and various combinations of each^{62,63}. Delivery of these reagents has also been attempted using a variety of methods, including electroporation, lipid-based transfection, and viral-mediated delivery^{62,63}. For further discussion of delivery methods for genome-editing reagents, refer to Yin *et al.*⁶⁴. Pooled screening relies on the ability to deliver individual reagents to individual cells in batches⁶⁵. Electroporation and lipid-based transfection methods offer limited ability to control the number of reagents (i.e., sgRNAs) delivered per cell; however, lentiviral transduction at low transduction rates (~30–50%) results in single lentiviral integrants per cell in the majority of cases⁶⁵. Furthermore, lentivirus offers stable integration of the CRISPR reagents into each cell's genome. These features of lentivirus allow for pooled CRISPR experiments. Therefore, this protocol describes the use of lentivirus for both arrayed and pooled CRISPR experiments (Boxes 3 and 4).

Limitations of on-target prediction, off-target prediction, and indel analysis

As described above, there are many tools available for both on-target sgRNA efficiency and off-target cleavage prediction. Progress has been made toward enhancing the predictive value of these scores; however, although these predictions are useful to focus sgRNA selection for experimental design, experimental validation provides the definitive analysis of on-target and off-target mutagenesis. Alternatively, experimental approaches have also been developed for unbiased genome-wide detection of off-target cleavages^{66–72}. Continued investigation is necessary to more completely understand the rules governing sgRNA efficiency and off-target mutagenesis (see Box 1 for further discussion).

CRISPResso indel analysis can supplement other common analyses, such as analyses of gene expression or protein-level changes. Although CRISPResso is useful for quantifying editing frequency to demonstrate that editing has successfully occurred and demonstrates the full indel spectrum (substitutions, insertions, and deletions), it is often helpful to perform

other techniques to assess for changes beyond genomic DNA (gDNA) for further evaluation (i.e., gene expression or protein-level changes).

Experimental design

Target identification and nuclease choice—CRISPR genome-editing experiments require appropriate target identification to fit experimental objectives, which can include functional analysis of gene- or noncoding sequence (e.g., enhancers, CCCTC-binding factor (CTCF), or other transcription factor binding sites). Gene knockout is usually accomplished by targeting exonic sequences, but it can also be achieved through promoter disruption. Gene knockout is often complicated by alternative splicing and/or expression of multiple isoforms for a given gene of interest. It is difficult to predict the relevant isoform without prior knowledge; however, it is often possible to target exonic sequence common to all isoforms or to design sgRNAs targeting unique isoforms to aid in the identification of a relevant/functional isoform. In the case of gene families, conserved domains can be identified for disruption³⁷. Although exon targeting toward the 5′ end of the gene has been shown to be more effective for functional disruption than targeting the 3′ end⁵⁰, it is also possible to target functional protein domains. In this case, even in-frame mutations frequently disrupt protein function⁷³. Similar considerations can be applied to CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa) approaches; however, CRISPRi/CRISPRa require sgRNA targeting in close proximity to the transcriptional start site for maximal repression/activation^{17,74–80}; CRISPRi/CRISPRa approaches are not the focus of this protocol. For further discussion and a protocol for CRISPRa approaches, refer to Joung *et al.*²⁰.

Once target(s) have been identified, it is necessary to determine whether the experiment will be arrayed or pooled. Arrayed experiments are useful when a target can be mutagenized by one sgRNA (e.g., a transcription factor binding motif and gene knockout via exon mutagenesis) (Fig. 1), whereas pooled screen experiments are useful when a target(s) requires > 1 sgRNA for mutagenesis (Fig. 2). Both arrayed and pooled experiments are described in this protocol, as arrayed experiments are typically required to validate the results of pooled experiments. As with arrayed experiments, the first step of pooled experiments is target identification. This can include targeting a single locus with multiple sgRNAs (e.g., saturating mutagenesis (so-called tiling sgRNA) experiments)^{11,12,18}, targeting multiple loci by saturating mutagenesis (e.g., saturating mutagenesis of multiple DHSs)^{11,12}, and gene-targeted pooled screens (e.g., targeting multiple genes with multiple sgRNAs per gene)^{14–17}. In addition, libraries combining gene-based targeting and saturating mutagenesis approaches can also be designed. Experimental designs and workflows in this protocol are summarized in Figure 3.

Finally, each CRISPR nuclease offers a unique PAM sequence with varying frequency of occurrence in the genome depending on the location of targeting such as exons, introns, promoters, DHSs, enhancers, or repressed regions^{12,65}. The optimal nuclease can be chosen based on the density of available PAMs (e.g., for saturating mutagenesis) or proximity of PAMs to a particular genomic position (e.g., for transcription factor binding motifs)

(Supplementary Table 1). Finally, high-fidelity nucleases (e.g., HypaCas9, SpCas9-HF1, and eSpCas9) can be used to minimize the probability of off-target mutagenesis^{81–83}.

Positive, negative, and editing controls for arrayed and pooled screen CRISPR experiments—Positive and negative controls are essential for both arrayed and pooled screen experiments. Positive controls are generally experiment-specific; however, some widely used positive controls when performing experiments to identify novel essential genes or when performing dropout (‘depletion’) screens include targeting of known essential genes (e.g., ribosomal genes and housekeeping genes) (see Box 4 for further discussion of dropout screens). When targeting noncoding sequences for the effects on gene expression, one possible positive control is targeting of exonic sequences^{11,12}.

Negative controls typically take the form of nontargeting controls, which are sgRNAs without any perfect matches in a given genome and with low potential for cleavage at genomic loci with imperfect matches (selected by minimization of sites with few mismatches, which is similar to minimizing off-target effects for targeted sgRNA). Nontargeting controls are genome build-, species-, and PAM-specific (unless designed to be compatible with multiple genomes and/or PAMs). Other options for negative controls include targeting a safe harbor locus such as *AAVS1* or a gene/region known to have no effect on cellular function or the phenotype of interest. Nontargeting sgRNA can be generated ‘by hand’ via a guess-and-check approach to ensure no genomic matches (and minimal sites with few mismatches) or can be designed using previously published tools¹². For both positive and negative controls, one sgRNA is often used for an arrayed experiment and multiple sgRNAs are often used for pooled screen experiments, oftentimes comprising 1–5% of the total number of sgRNAs in the library (see Box 4 for further discussion)^{11,12}.

An editing control can also be included to ensure proper functioning of the CRISPR reagents, particularly nuclease function (see Boxes 3 and 4 for further discussion). One possibility is to include a construct that expresses GFP together with an sgRNA targeting GFP to assess for functional Cas9 expression by flow cytometry^{12,50}. This is particularly informative when using cell lines with stable Cas9 expression and can be helpful for troubleshooting low or absent editing rates. Typically, only one reporter system/sgRNA to serve as an editing control is needed for both arrayed and pooled experiments.

Synthesis of individual or multiple pooled sgRNA libraries—After designing relevant sgRNAs, CRISPOR automates the design of full-length oligonucleotides for pooled screen applications such as saturating mutagenesis (Step 1A and 1C) and gene-targeted libraries generated from a gene list (Step 1B). Barcodes allow for oligonucleotides for multiple unique libraries to be synthesized on the same programmable microarray (Steps 16 and 17), which reduces cost by avoiding purchase of multiple microarrays for multiple libraries. Specifically, the barcodes offer the ability for individual PCR amplification of unique libraries from a single batch of oligonucleotides synthesized on the same microarray. The number of libraries that can be included on a single programmable microarray is limited by the microarray’s oligonucleotide capacity; however, there is theoretically no limit to the number of possible barcodes that can be used (and thus no limit to the number of libraries that can be generated from a single pool of oligonucleotides). Barcodes for ten libraries are

provided in Supplementary Table 2. To create additional or new barcodes, generate 10–13 bp of sequence distinct from the sequences to be amplified and the other barcodes used. It is also important to ensure that the barcode results in a primer melting temperature (T_m) compatible with the IsPCR1 reaction (Step 19; see Supplementary Table 3 for examples). Homologous sequence to the lentiGuide-Puro plasmid is required because batch sgRNA library cloning is performed using Gibson assembly (Steps 27 and 28), which relies on homologous flanking sequence for successful cloning.

Sequencing of arrayed experiments and pooled screens—Sanger sequencing is initially required to confirm successful cloning of an individual sgRNA for an arrayed experiment using a U6 sequencing primer (5'-CGTAACTTGAAAGTATTTTCGATTTCTTGGC-3' (ref. 62)). For a pooled screen experiment, deep sequencing is required to confirm successful library cloning.

Deep sequencing can be used to analyze editing outcomes for arrayed experiments. In contrast, deep sequencing is typically required to analyze pooled screen experiments. The choice for appropriate deep-sequencing platform (e.g., MiSeq or HiSeq) should include consideration of the required number of reads for the experiment as well as cost. For an arrayed experiment for indel enumeration/analysis by CRISPResso (Step 83), the number of reads needed to adequately represent a locus may vary widely based on experimental goals; however, > 1,000 reads at a given locus is reasonable. For a pooled sgRNA library experiment, 100–1,000× coverage of the number of sgRNAs in the library is reasonable. The desired read length should be chosen on the basis of the length of the amplicon generated by the primers used in Step 75. Once the deep-sequencing platform has been selected, submit barcoded samples for deep sequencing (Step 82; these represent different barcodes than those described in the 'Synthesis of individual or multiple pooled sgRNA libraries' section).

Amplicon length should be compatible with the read length as described above. 100- and 200-bp amplicons are reasonable for paired-end deep sequencing with 75- or 150-bp reads, respectively. Because sequencing quality is lower toward the end of the reads, there must be some overlap of read pairs to allow reliable merging. For example, for a 200-bp amplicon, it is suggested to use 125- to 150-bp reads in order to have sufficient overlap. Some considerations for determining amplicon length include the cost of sequencing for a given read length, the size in base pairs of the region to be sequenced, and the length of reliable sequencing (due to unreliable sequence at read ends). If an amplicon length is too short, it may result in inadequate coverage of the target region, as well as miss larger indels. By contrast, if the amplicon is longer than twice the read length, no overlap will exist between reads, making the merging steps of paired-end reads impossible.

MATERIALS

REAGENTS

Cloning/transformation

- E. cloni 10G Elite electrocompetent cells with recovery medium (Lucigen, cat. no. 60052)

- Endura electrocompetent cells with recovery medium (Lucigen, cat. no. 60242)
- Gibson assembly master mix (New England BioLabs, cat. no. E2611S)
- Gene Pulser/MicroPulser electroporation cuvettes, 0.1-cm gap (Bio-Rad, cat. no. 1652089)
- Fast Digest Esp3I (Thermo Fisher, cat. no. FD0454)
- NEB stable competent *E. coli* (high efficiency) (New England BioLabs, cat. no. C3040H)
- Lysogeny broth (LB) medium base (Thermo Fisher, cat. no. 12780052)
- Dehydrated agar (Fisher Scientific, cat. no. DF0140-01-0)
- Thermosensitive alkaline phosphatase (TSAP; Promega, cat. no. M9910)
- Nuclease-free water (Fisher Scientific, cat. no. AM9937)
- T4 polynucleotide kinase (New England BioLabs, cat. no. M0201S)
- Ampicillin sodium salt (Sigma-Aldrich, cat. no. A9518)
- SOC (Super Optimal broth with Catabolite repression) medium (Thermo Fisher, cat. no. 15544034)
- Quick Ligation Kit (New England BioLabs, cat. no. M2200S)
- Individual oligonucleotides (e.g., Integrated DNA Technologies, Bio-Rad, custom order)
- Oligonucleotide pool (e.g., CustomArray, Twist Bioscience, custom order)
- QIAquick PCR Purification Kit (Qiagen, cat. no. 28104)

Gel electrophoresis

- SYBR Safe DNA Gel Stain (Thermo Fisher, cat. no. S33102)
- Amresco Agarose I (VWR, cat. no. 97062-250)
- 1-kb Plus DNA Ladder (Thermo Fisher, cat. no. 10787018)
- 50× Tris-acetate-EDTA (TAE) buffer (Boston Bioproducts, cat. no. BM-250)

PCR

- Phusion Hot Start Flex DNA Polymerase (New England BioLabs, cat. no. M0535S)
- Q5 High-Fidelity DNA Polymerase (New England BioLabs, cat. no. M0491S)
- Herculase II Fusion DNA Polymerase (Agilent Genomics, cat. no. 600675)
- DMSO (Sigma-Aldrich, cat. no. D8418)
- QIAquick Gel Extraction Kit (Qiagen, cat. no. 28704)
- MinElute PCR Purification Kit (Qiagen, cat. no. 28004)

- Qubit dsDNA HS Assay Kit (Thermo Fisher, cat. no. Q32854)
- DNeasy Blood & Tissue Kit (Qiagen, cat. no. 69504)

Plasmids/plasmid preparation

- lentiGuide-Puro (Addgene, plasmid ID 52963; ref. 19)
- lentiCas9-Blast (Addgene, plasmid ID 52962; ref. 19)
- pXPR_011 (Addgene, plasmid ID 59702; ref. 50)
- lenti-Cas9-VQR-Blast (Addgene, plasmid ID 87155; ref. 12)
- lenti-Cas9-VQR-GFP_activity_reporter (Addgene, plasmid ID 87156; ref. 12)
- Qiagen Plasmid Maxi Kit (Qiagen, cat. no. 12163)
- AccuPrep Plasmid Mini Extraction Kit (Bioneer, cat. no. K-3030)

Lentivirus production

- pCMV-VSV-G (Addgene, plasmid ID 8454)
- psPAX2 (Addgene, plasmid ID 12260)
- Polyethylenimine (PEI), branched (Sigma-Aldrich, cat. no. 408727)
- Sucrose (Sigma-Aldrich, cat. no. S0389)
- Steriflip-HV filter units, 0.45 μ m, PVDF, radio-sterilized (Millipore, cat. no. SE1M003M00)
- Stericup-GP filter units, 0.22 μ m, polyethersulfone, 500 ml, radio-sterilized (Millipore, cat. no. SCGPU05RE)
- Phosphate-buffered saline, 1 \times , without calcium, magnesium (Lonza, cat. no. 17-516F)
- DMEM (Life Technologies, cat. no. 11995-073)
- HEK293FT cells (Thermo Fisher Scientific, cat. no. R70007)

! CAUTION Cell lines should be regularly checked to ensure that they are authentic and are not infected with mycoplasma.

- Lenti-X qRT-PCR Titration Kit (Takara Bio, cat. no. 631235)
- Lenti-X p24 Rapid Titer Kit (Takara Bio, cat. no. 632200)

EQUIPMENT

- Corning untreated 245-mm² bioassay dishes (Fisher Scientific, cat. no. 431111)
- Ultracentrifuge tube, thin-wall, polypropylene, 38.5 ml, 25 \times 89 mm (Beckman Coulter, cat. no. 326823)
- Falcon 50-ml conical centrifuge tubes (Fisher Scientific, cat. no. 14-432-22)

- Corning tissue culture (TC)-treated culture dishes, 15 cm, round (Fisher, cat. no. 08-772-24)
- Penicillin–streptomycin (Life Technologies, cat. no. 15140122)
- Solid glass beads (Fisher Scientific, cat. no. 11-312-10B)
- Electroporation system (e.g., Bio-Rad’s Gene Pulser MXcel, Gene Pulser Xcell, and MicroPulser electroporators)
- Corning Falcon bacteriological Petri dishes with lids (Fisher Scientific, cat. no. 08-757-100D)
- MiSeq or HiSeq sequencing system (Illumina) or equivalent sequencing platform
- Qubit fluorometer (Thermo Fisher Scientific)
- UV-visible spectrophotometer (NanoDrop)
- Gel visualization system (Alpha Innotech)
- Thermocycler (Bio-Rad)
- UV-light transilluminator (Fisher Scientific)
- UV-filter face mask (Fisher Scientific) ! **CAUTION** Wear gloves, lab coat, and face shield to avoid harm to eyes and skin caused by UV light.
- Microcentrifuge tubes, polypropylene (VWR, cat. no. 87003-294)
- 8-Strip PCR tubes, 0.2 ml (Fisher Scientific, cat. no. 14-222-250)
- Sterile culture tube with attached dual-position cap (VWR, cat. no. 89497-812)
- Ultracentrifuge (Beckman Coulter)
- SW32Ti rotor with compatible swinging buckets (Beckman Coulter, cat. no. 369694)
- Falcon cell scraper with 40-cm handle and 3.0-cm blade (Corning, cat. no. 353087)

COMPUTING EQUIPMENT

- Computer with at least 8 GB of RAM, 1 TB of disk space, and a recent central processing unit (CPU) with two or more cores (command-line and Docker version only). Windows (Docker only), OSX, and Linux platforms are supported.
- Anaconda Python 2.7 (<https://www.continuum.io/downloads> (CRISPResso is not compatible with Python 3))
- Docker (<https://www.docker.com/community-edition#/download>)
- Sequence analysis software, e.g., free software such as ApE (<http://biologylabs.utah.edu/jorgensen/wayned/ape/>), SerialCloner (http://serialbasics.free.fr/Serial_Cloner.html), or commercial software such as Thermo Scientific Vector NTI (<https://www.thermofisher.com/us/en/home/life-science/>)

cloning/vector-nti-software.html), Lasergene DNASTAR (<https://www.dnastar.com/t-allproducts.aspx>), CLC Main Workbench (<https://www.qiagenbioinformatics.com/products/clc-main-workbench>), or MacVector (<http://macvector.com>)

- CRISPResso command-line software (<https://github.com/pinellolab/CRISPResso>)
 - ▲ **CRITICAL** Refer to the ‘Equipment Setup’ section for detailed installation instructions and troubleshooting advice for CRISPResso.
- CRISPOR command-line software: <http://crispor.org/downloads>
 - ▲ **CRITICAL** Refer to the ‘Equipment Setup’ section for detailed installation instructions and troubleshooting advice for CRISPOR.
- FASTQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>)

REAGENT SETUP

Preparation of sgRNA cloning, sequencing, and primer oligonucleotides—

When ordering individual oligonucleotides for sgRNA cloning, add ‘CACC’ onto the sense strand of the sgRNA (5′ CACCNNNNNNNNNNNNNNNNNNNNN 3′) and ‘AAAC’ onto the antisense strand of the sgRNA (5′ AAACNNNNNNNNNNNNNNNNNNNNN 3′). The two oligonucleotides are designed as reverse complements because they will be annealed together in Step 4. The ‘CACC’ and ‘AAAC’ sequences are required for cloning of the phosphorylated/annealed oligonucleotides into the Esp3I-digested/dephosphorylated lentiGuide-Puro plasmid (Steps 3–10). The addition of a ‘G’ at the 5′ end of the sgRNA sequence can increase transcription from the U6 promoter (5′ CACCGNNNNNNNNNNNNNNNNNNNNN 3′ and 5′ AAACNNNNNNNNNNNNNNNNNNNNNC 3′); this is not required if the 5′ end of the sgRNA sequence is already a ‘G’. Using CRISPOR, the sequences for sgRNA cloning into lentiGuide-Puro can be downloaded by following the ‘Cloning/PCR primers’ link below the sgRNA sequence. The ready-to-order oligonucleotide sequences are available from the sgRNA results table by selecting the ‘lentiGuide-Puro (Zhang lab)’ plasmid under the ‘U6 expression from an Addgene plasmid’ heading. Dissolve the obtained oligonucleotides in nuclease-free water at a concentration of 100 μM. Store at 20 °C for up to 2 years.

TAE electrophoresis solution—Dilute 50× TAE buffer stock in dH₂O to obtain a 1× working solution. Store at room temperature (25 °C) for up to 6 months.

Ampicillin solution—Dissolve ampicillin in a 1:1 mix of 100% ethanol and dH₂O to a final concentration of 100 mg/ml. Store at –20 °C for up to 4–6 months. ▲ **CRITICAL** Protect from light.

HEK293FT cell culture medium—HEK293FT cell culture medium is DMEM with glucose/sodium pyruvate supplemented with 10% (vol/vol) FBS and 1% (vol/vol) penicillin–streptomycin. Sterilize with a 0.22-μm filter and store it at 4°C for up to 4 weeks. !

CAUTION The HEK293 cells used should be regularly checked to ensure that they are not infected by mycoplasma.

PEI solution—Dissolve PEI in nuclease-free water at 10 µg/µl with pH 7.4. This solution can be stored at 4 °C for years.

20% (wt/vol) sucrose solution—Dissolve sucrose in PBS to create a 20% (wt/vol) solution by mass. Sterilize with a 0.22-µm filter and store at 4 °C for up to 1 year.

EQUIPMENT SETUP

Installation of Docker—Docker is a virtualization technology that allows for packaging software with all dependencies into files called containers to be executed on Windows, Linux, or OSX. This allows for the creation and distribution of a ‘frozen’ version of the software that will always run independent of updates or changes to libraries or the required dependencies on the host machine. In this case, you only need to install Docker and do not need to install any dependencies. Download and install Docker from this link: <https://docs.docker.com/engine/installation/>.

Sharing disk volumes with Docker—By default, Docker containers cannot share data with the machine on which they run. For this reason, it is necessary to map local folders to folders inside the Docker container. Mapping allows the container to read the input data files to process/write the output files into a folder on the disk of the local machine. First, it is necessary to check whether Docker has permission to access the disk(s) on your machine where analysis data are to be stored and processed. By default, any subfolder within your home directory is automatically shared; however, this behavior may change with future versions of Docker. Check that the drive(s) you want to be available to the container is/are selected in the ‘Settings.../ Shared Drives’ panel (Supplementary Fig. 1a).

To map a local folder (i.e., a folder on the local disk) to a container folder, Docker has a special option with this syntax:

```
-v local_folder:container_folder
```

You can specify this option multiple times if it is necessary to map more than one folder. In our examples, the folders to map are all subfolders of the home folder of the user ‘user’, i.e., /home/user/. In addition, some of the Docker commands will be used with the option

```
-w /DATA
```

to specify the working directory (i.e., where the command will be executed) to allow for the use of relative paths and to shorten the commands.

Allocation of memory for Docker containers—It is necessary to allocate enough memory to the container. The default assigned by Docker may depend on the version and on

the machine on which it is run. To run the CRISPOR/CRISPResso containers, we suggest assigning Docker 6–8 GB of RAM. For analysis involving large sequences or many sites, it may be necessary to increase the amount of allocated memory under the ‘Settings.../Advanced’ panel (Supplementary Fig. 1b). The execution may halt with an error if enough memory is not allocated to the container (e.g., with ‘/bin/bash: line 1: 14 Killed/crisporWebsite/bin/Linux/bwa bwasmw’). In this instance, the memory allocated to the Docker container is not sufficient to run CRISPOR, and the process is terminated. Therefore, increase the memory as discussed above and run the same command again.

CRISPResso installation—The CRISPResso utility can be used for analysis of deep-sequencing data of a single locus/amplicon on a local machine or proprietary server, using a command-line version or a webtool freely available at <http://crispresso.rocks/>. The webtool version of CRISPResso does not require installation. For installation of the command-line version of CRISPResso or installation of CRISPResso with Docker, see the instructions below.

Installation of the command-line version of CRISPResso: To install CRISPResso on a local machine, it is necessary to install some dependencies before running the setup script. Download and install Anaconda Python 2.7, following the instructions at this link:

<http://continuum.io/downloads>.

Open a terminal and type

```
conda config --add channels r
conda config --add channels defaults
conda config --add channels conda-forge conda
config --add channels bioconda
conda install CRISPResso fastqc
```

Close the terminal and open a new one; this will set the PATH variable. Now, you are ready to use the command-line version of CRISPResso.

If you have installed an old version of CRISPResso, please remove it with the following command to avoid conflicts:

```
rm -Rf /home/user/CRISPResso_dependencies
```

Installation of CRISPResso with Docker: To install CRISPResso with Docker, it is necessary to verify that Docker is installed before running the setup script (see ‘Installation of Docker’ within the ‘Equipment Setup’ section). After verification of Docker installation, type the following command:

```
docker pull pinellolab/crispor_crispresso_nat_prot
```

Check whether the container was downloaded successfully by running the command

```
docker run pinelloolab/crispor_crispresso_nat_prot
CRISPResso --help
```

If CRISPResso was properly installed, you will see the Help for the command-line version of CRISPResso.

PROCEDURE

sgRNA design using CRISPOR ● TIMING 1–4 h

- 1| Design sgRNAs from a single genomic locus using the CRISPOR webtool by following option A. sgRNAs can be selected manually from the CRISPOR output using on- and off-target predictions (Box 1). Alternatively, follow option B for the ‘CRISPOR Batch’ gene-targeting assistant webtool for gene-targeted pooled library generation from a gene list. If multiple genomic loci are required for sgRNA design or the command-line version is preferred, use option C for the command-line version of CRISPOR.

▲ **CRITICAL STEP** CRISPOR does not offer the ability to design repair templates for HDR experiments. If the experimental design requires a repair template, other protocols can provide further instruction⁶².

(A) sgRNA design for arrayed and pooled experiments using the CRISPOR webtool ● TIMING 1–4 h

- i. Open the CRISPOR webtool (<http://crispor.tefor.net/>).
- ii. Input the target DNA sequence. The webtool requires a sequence input (< 2 kb) or genomic coordinates.

▲ **CRITICAL STEP** The input sequence must be a genomic sequence as opposed to a cDNA sequence, as the latter can include sequences that are not in the genome due to splicing. You can obtain genomic sequences using a website such as the UCSC Genome Browser (<https://genome.ucsc.edu>, click on ‘View - DNA’ after searching for a gene or genomic region) or Ensembl (<http://www.ensembl.org>, click on ‘Export data – Text’ after searching for a gene or genomic region). From the UCSC Genome Browser, the current sequence in view can be sent directly to CRISPOR via the menu entry ‘View – In external tools’.

? TROUBLESHOOTING

- iii. Select the relevant genome assembly (e.g., hg19, mm9) and PAM sequence for the nuclease (e.g., NGG for *Streptococcus pyogenes* Cas9).

▲ **CRITICAL STEP** It is important to pick the appropriate assembly to be consistent with the genomic coordinates of the DNA sequence provided in Step 1A(ii) and for accurate prediction of off-target sites.

- iv. Click on the ‘Submit’ button to run the CRISPOR analysis. Select the ‘Cloning/PCR primers’ link underneath each sgRNA sequence for automated primer design for future indel analysis by CRISPResso at on- and off-target loci. If you are conducting a saturating mutagenesis screen, the list of the oligonucleotide pool sequences for Gibson assembly, sequencing primers for validation, sequencing amplicons for CRISPResso, and the full list of sgRNA sequences can be downloaded by following the link ‘Saturating Mutagenesis Assistant’ at the top of the CRISPOR output page. The four files provided by the Saturating Mutagenesis Assistant can be downloaded by selecting from the drop-down menu adjacent to ‘output file’. The outputs are described in Table 2. Alternatively, the full list of sgRNA sequences for saturating mutagenesis can also be found by following the ‘Cloning/PCR primers’ link under each sgRNA sequence and then following the link provided under the ‘Saturating mutagenesis using all guides’ heading.

▲ **CRITICAL STEP** CRISPOR allows for PCR amplicon lengths of 50–600 bp. The melting temperature (T_m) is calculated for each primer pair.

(B) Gene-targeted library design generated from a gene list using the CRISPOR Batch gene-targeting assistant webtool ● TIMING 1–4 h

- i. To generate a gene-targeted library using the CRISPOR Batch gene-targeting assistant, go to <http://crispor.tefor.net/> and click on the ‘CRISPOR Batch’ link.
- ii. Select the source genome-wide library for the extraction of sgRNA sequences using the drop-down menu adjacent to ‘Lentiviral screen library’. References are provided for each library to assist with appropriate source library selection.
- iii. Select the desired ‘number of guides per gene’ (maximum of six sgRNAs/gene) using the drop-down menu. Note that some libraries have fewer than six sgRNAs per gene. If more sgRNAs are requested than are available for a given gene with the library, the maximum number of sgRNA sequences available within the library will be output instead.
- iv. Enter the ‘number of non-targeting control guides’ to include in the library (maximum = 1,000). See the ‘Experimental design’ section for further discussion of controls.
- v. Select a barcode for library preparation as described in Step 16 (see ‘Synthesis of individual or multiple pooled sgRNA libraries’ within the ‘Experimental design’ section for further discussion of barcodes).
- vi. Copy and paste the gene list into the entry field. Genes should be entered as official gene symbols, with only one entry per line. Refer to the HUGO Gene Nomenclature Committee (HGNC) (<https://www.genenames.org/>) and Mouse Genome Informatics (MGI) (<http://www.informatics.jax.org/>) for official gene symbols for human and mouse, respectively.

▲ **CRITICAL STEP** This entry is case-insensitive because the libraries are already species-specific. If an entry is not found in the database of genome-wide

sgRNAs, sgRNAs for the entered gene will display a warning and will not be included in the output.

? TROUBLESHOOTING

- vii. Click on ‘Submit’ for gene-targeted library generation and download the output library.

(C) sgRNA design using command-line CRISPOR ● TIMING 1–4 h

- i. Type the following command into a terminal to download the latest version of the Docker container for this protocol:

```
docker pull pinellolab/crispor_crispresso_nat_prot
```

- ii. Verify that the container was downloaded successfully by running the following command:

```
docker run pinellolab/crispor_crispresso_nat_prot crispor.py
```

- iii. Obtain the CRISPOR assembly identifier of the genome (e.g., hg19 or mm9). If you are unsure, the full list of assemblies is available at <http://crispor.tefor.net/genomes/genomeInfo.all.tab>. This protocol uses ‘hg19’ in its examples; however, this should be switched to the relevant identifier for the assembly of interest as appropriate.

- iv. Execute the following commands to download the relevant pre-indexed genome in the folder created in the previous step. Here, we are assuming that the user will store the pre-indexed genomes in the folder /home/user/crispor_genomes.

```
mkdir -p /home/user/crispor_genomes
docker run -v /home/user/crispor_genomes:/crisporWebsite/genomes \
pinellolab/crispor_crispresso_nat_prot downloadGenome hg19 \
/crisporWebsite/genomes
```

- v. Prepare the input genomic DNA (gDNA) sequences in FASTA format (see a description of FASTA format at <https://www.ncbi.nlm.nih.gov/blast/fasta.shtml>).

▲ **CRITICAL STEP** It is important to pick the appropriate genome assembly to be consistent with the genomic coordinates of the input DNA sequences. In particular, it is essential for accurate prediction of off-target sites.

- vi. (Optional) Generate a FASTA file with the exonic sequences. If you have a list of genes and you want to target their exons, go to the UCSC Genome Browser page <http://genome.ucsc.edu/cgi-bin/hgTables>.
- vii. Set the following options (refer to the description of each item in the ‘Table Browser’ to assist with setting each parameter). Of note, the ‘Table’ parameter

will offer different options depending on the chosen ‘Track’. Step 1C(viii–xii) is based on the example entry from Step 1C(vii):

UCSC Genome Browser table option	Example entry (adjust as appropriate)
Clade	Mammal
Genome	Human, assembly: hg19
Group	Genes and gene predictions
Track	Gencode V19
Table	Basic (wgEncodeGencodeBasicV19)
Output format	Sequence
Output file	selected_genes_exons.fasta
File type returned	‘plain text’

- viii. Press the button ‘paste list’ and enter the list of transcript identifiers for each gene.
- ix. Press the button ‘get output’. A new page will open.
 - ▲ **CRITICAL STEP** The ‘Send output to’ Galaxy, GREAT, or GenomeSpace checkboxes are off by default; however, they will remain checked if you have ever checked any of them in the past. Make sure that none of the checkboxes are selected.
- x. Select the option ‘genomic’ and press ‘submit’. A new page will open.
- xi. Select ‘CDS: Exons’ and ‘One FASTA record per region (exon, intron, etc.)’.
- xii. Press the button: ‘get sequence’ and save the file as selected_genes_exons.fasta.
- xiii. Run CRISPOR over your single- or multi-fasta input file by entering the following commands. Here, we assume that this file is stored in /home/user/crispor_data/crispor_input.fasta and contains a sequence with id > REGION_1.

```
docker run \
-v /home/user/crispor_data:/DATA \
-v /home/user/crispor_genomes:/crisporWebsite/genomes \
-w /DATA \pinellolab/crispor_crispresso_nat_prot \
crispor.py hg19 crispor_input.fasta crispor_output.tsv --
satMutDir=.
```

The sgRNA sequences will be written to the file crispor_output.tsv and four files for each sequence ID in the FASTA file will be created as in the web version in the folder /home/user/crispor_data/ (see Table 2 for output files).

- xiv. To select a subset of sgRNAs from the file REGION_1_satMutOligos.tsv as generated in Step 1A(iv) or 1C(xiii) (Table 2), select the lines corresponding to

sgRNAs with the desired scores/attributes (Box 1) and save them to a new file called REGION_1_satMutOligos_filtered.tsv. Filter the sgRNAs by running the following commands:

```
docker run -v /home/user/crispor_data:/DATA -w /DATA pinello/
crispor_crispresso_nat_prot bash -c "join -t '$\t' -1 1 -2 1
REGION_1_satMutOligos_filtered.tsv REGION_1_ontargetAmplicons.tsv -
o
2.1,2.2,2.3 > CRISPRessoPooled_amplicons.tsv"
docker run -v /home/user/crispor_data:/DATA -w /DATA pinello/
crispor_crispresso_nat_prot bash -c "join -t '$\t' -1 1 -2 1
REGION_1_satMutOligos_filtered.tsv REGION_1_ontargetAmplicons.tsv -
o 2.3
| sed 1d > CRISPRessoCounts_sgRNA.tsv"
docker run -v /home/user/crispor_data:/DATA -w /DATA pinello/
crispor_crispresso_nat_prot bash -c "join -t '$\t' -1 1 -2 1
REGION_1_satMutOligos_filtered.tsv REGION_1_ontargetPrimers.tsv -o
2.1,2.2,2.3,2.4,2.5,2.6,2.7 >
REGION_1_ontargetPrimers_filtered.tsv"
```

This will create a filtered version of the files described in Table 2 to use for designing amplicons and for performing CRISPResso analysis.

▲ **CRITICAL STEP** If the input file to CRISPOR was a multi-fasta file (Step 1C(xiii)), repeat this step for the other regions as appropriate.

Synthesis and cloning of individual sgRNA into a lentiviral vector ● TIMING 3 d to obtain sequence-confirmed, cloned sgRNA lentiviral plasmid

- 2| Individually resuspend lyophilized primer oligonucleotides at 100 μ M in nuclease-free water.
 - **PAUSE POINT** Resuspended oligonucleotides can be stored at -20 °C for years.
- 3| Set up the phosphorylation reaction:

Components	Amount (μ l)	Final concentration
Oligonucleotide 1 (at 100 μ M)	1	–
Oligonucleotide 2 (at 100 μ M)	1	
10 \times Ligation buffer	1	1 \times
T4 polynucleotide kinase (10 units/ μ l)	1	10 U
Nuclease-free water	To 10	

- 4] Perform phosphorylation and annealing of the oligonucleotides in a thermocycler:

Step	Temperature (°C)	Time	Comment
1	37	30 min	
2	95	5 min	
3	95–25	30 s	Ramp down 5 °C/min

■ **PAUSE POINT** Resuspended phosphorylated/annealed oligonucleotides can be stored at – 20 °C for years.

- 5] Prepare the digestion reaction by combining the following components in a 0.2-ml or 1.5-ml tube.

Components	Amount	Final concentration
lentiGuide-Puro plasmid	1 µg	–
10× FastDigest buffer	2 µl	1×
DTT (20 mM)	1 µl	1 mM
Esp31 restriction enzyme	1 µl	
Nuclease-free water	To 20 µl	

- 6] Digest the sgRNA plasmid (lentiGuide-Puro) with Esp31 (an isoschizomer of BsmBI) restriction enzyme for 15 min at 37 °C.
- 7] Add 1 µl of TSAP to dephosphorylate the reaction for 15 min at 37 °C, followed by heat inactivation for 15 min at 74 °C.
- 8] Perform gel purification of the Esp31-digested/dephosphorylated lentiGuide-Puro plasmid. See MATERIALS for gel purification kit and follow the manufacturer's instructions. For further information on gel purification, refer to this previously published protocol⁸⁴.
- 9] Determine the concentration of the Esp31-digested/dephosphorylated lentiGuide-Puro plasmid after gel purification using a UV-visible spectrophotometer (e.g., NanoDrop) or other equivalent method, following the manufacturer's instructions.
- 10] Perform a ligation reaction for 5–15 min at room temperature using 30–70 ng of Esp31-digested/dephosphorylated lentiGuide-Puro plasmid (generated in Steps 5–9) with the phosphorylated–annealed oligonucleotides produced in Steps 2–4 diluted 1:500 with nuclease-free water.

Components	Amount	Final concentration
2× Quick ligase reaction buffer	5 µl	1×
Diluted oligonucleotides (from Step 4)	1 µl	
LentiGuide-Puro plasmid (from Steps 5 to 9)	30–70 ng	1×
Quick ligase	1 µl	10 U
Nuclease-free water	To 10 µl	

- 11] Heat-shock-transform the ligation reaction into stable competent *E. coli* by incubating samples at 42 °C for 45 s, followed by a 10-min incubation on ice. Add 250 µl of SOC medium and incubate at 37 °C with 250-r.p.m. shaking for 1 h. Use of recombination-deficient *E. coli* is important to minimize recombination events of repetitive elements in lentiviral plasmid (e.g., long terminal repeats). For more information about performing heat-shock transformation of competent *E. coli*, refer to previously published protocols^{85,86}.
- 12] Plate the transformation product from Step 11 on a 10-cm ampicillin-resistant (0.1 mg/ml ampicillin) agar plate. Incubate at 32 °C for 14–16 h. It is recommended that transformation products be incubated at 32 °C to reduce recombination between lentiviral long terminal repeats, but incubation at 37 °C can also be performed.
- 13] Picking three to five colonies is reasonable for individual incubation of each colony in 2 ml of ampicillin-resistant (0.1 mg/ml ampicillin) LB medium at 32 °C for 14–16 h with shaking at 250 r.p.m. It is recommended that transformation products be incubated at 32 °C to reduce recombination between lentiviral long terminal repeats, but incubation at 37 °C can also be performed.

? TROUBLESHOOTING

- 14] Perform mini-scale plasmid preparation of each 2-ml culture from Step 13 (see MATERIALS for a mini-scale plasmid preparation kit; follow the manufacturer's instructions).
 - **PAUSE POINT** Successfully cloned sgRNA plasmids can be stored long term (years) at – 20 °C before creating lentivirus (Steps 49–71).
- 15] Sanger-sequence each colony-derived plasmid with the U6 sequencing primer to identify correctly cloned plasmids (see 'Experimental design' section for U6 primer sequence)⁶². Subsequent midi- or maxi-scale plasmid preparation may be required for lentivirus production (Steps 49–71).

Synthesis and cloning of pooled sgRNA libraries into a lentiviral vector ● TIMING 2–4 d to obtain cloned sgRNA lentiviral plasmid library

- 16] Use the sgRNAs from Step 1 to design full-length oligonucleotides (96–99 bp) flanked by barcodes and homologous sequence to the lentiGuide-Puro plasmid (Supplementary Table 2).

- 17| Order the designed DNA oligonucleotides on a programmable microarray (MATERIALS).
- 18| Obtain the resuspended oligonucleotides for use as the template for the PCR reaction in Step 19. The concentration will be determined by the manufacturer, so refer to the manufacturer for identifying the concentration.

■ **PAUSE POINT** Oligonucleotide pools can be stored at $-20\text{ }^{\circ}\text{C}$ for short-term storage (months to years) and at $-80\text{ }^{\circ}\text{C}$ for long-term storage (years).

- 19| Set up the barcode-specific PCR to amplify using the barcode-specific primers (Supplementary Table 3). This PCR will herein be referred to as ‘Library Synthesis PCR1’ (lsPCR1).

▲ **CRITICAL STEP** It is important to use a proofreading DNA polymerase to minimize introduction of PCR errors.

Components	Amount (μl)	Final concentration
Synthesized oligonucleotide template from Step 18	1	–
5 \times Phusion HF buffer or 5 \times Q5 reaction buffer	10	1 \times
Deoxynucleoside triphosphates (dNTPs; at 10 mM)	1	200 μM
Barcode-specific forward primer (at 10 μM)	2.5	0.5 μM
Barcode-specific reverse primer (at 10 μM)	2.5	0.5 μM
Phusion Hot Start Flex DNA Polymerase or Q5 High-Fidelity DNA Polymerase	0.5	1.0 U/50- μl PCR
Nuclease-free water	To 50	

? TROUBLESHOOTING

- 20| Perform multiple cycles (e.g., 10, 15, and 20 cycles) of lsPCR1 in a thermocycler as follows:

Cycle number	Denature	Anneal	Extend
1	98 $^{\circ}\text{C}$, 30 s		
2- x (e.g., 10, 15, 20)	98 $^{\circ}\text{C}$, 10 s	63 $^{\circ}\text{C}$, 30 s	72 $^{\circ}\text{C}$, 30 s
$x + 1$			72 $^{\circ}\text{C}$, 5 min

▲ **CRITICAL STEP** The number of PCR cycles should be limited to reduce PCR bias. It is difficult to predict the required number of cycles for a given library. Therefore, it is important to perform multiple PCRs with different numbers of cycles to empirically determine the optimal number of cycles.

■ **PAUSE POINT** The PCR product can be stored for multiple days at 4 $^{\circ}\text{C}$ in the thermocycler at the end of the PCR program. Long-term storage (months to years) should be at $-20\text{ }^{\circ}\text{C}$.

- 21| Run a fraction (1–5 μ l of the 50- μ l PCR reaction from Step 20) of each IsPCR1 product with a different number of PCR cycles on a 2% (wt/vol) agarose gel. Analyze the gel to determine if the products occur at the expected size of ~100 bp (Supplementary Fig. 2a). If the bands run at the expected size, determine the minimal number of PCR cycles required to produce a visible band (Supplementary Fig. 2a). For example, 15 cycles would be used based on the gel presented in Supplementary Figure 2a; however, it may be possible to reduce the cycle number even further (i.e., another experiment with 5 and 10 PCR cycles). Use the remaining reaction volume for the cycle number chosen for Step 22 based on the gel from this step.
- 22| Using nuclease-free water, dilute the IsPCR1 reaction chosen in Step 21 1:10.
- 23| Set up ‘Library Synthesis PCR2’ (herein referred to as IsPCR2) with the universal IsPCR2 primers listed in Supplementary Table 4. IsPCR2 amplification removes the library-specific barcodes and adds a sequence homologous to the lentiGuide-Puro plasmid for Gibson assembly-based cloning of the library.

▲ **CRITICAL STEP** It is important to use a proofreading DNA polymerase to minimize introduction of PCR errors.

Components	Amount (μ l)	Final concentration
Diluted IsPCR1 product from Step 22	1	–
5 \times Phusion HF buffer or 5 \times Q5 reaction buffer	10	1 \times
dNTPs (at 10 mM)	1	200 μ M
IsPCR2_forward primer (at 10 μ M)	2.5	0.5 μ M
IsPCR2_reverse primer (at 10 μ M)	2.5	0.5 μ M
Phusion Hot Start Flex DNA Polymerase or Q5 High-Fidelity DNA Polymerase	0.5	1.0 U/50- μ l PCR
Nuclease-free water	To 50	

- 24| Perform IsPCR2 using the same cycling conditions and multiple cycle numbers as Step 20.

■ **PAUSE POINT** The PCR product can be stored for multiple days at 4 $^{\circ}$ C in the thermocycler at the end of the PCR program. Long-term storage (months to years) should be at – 20 $^{\circ}$ C.

- 25| Run IsPCR2 products on a 2% (wt/vol) agarose gel to determine whether the products occur at the expected size of ~140 bp (Supplementary Fig. 2b). Choose the sample with the minimal number of PCR cycles that produces a visible band in order to minimize PCR bias (Supplementary Fig. 2b). For example, 15 cycles would be used based on the gel presented in Supplementary Figure 2b; however, it may be possible to reduce cycle number even further (i.e., another experiment with 5 and 10 PCR cycles).

- 26| Gel-purify the ~140-bp band. See MATERIALS for gel purification kit and follow the manufacturer's instructions. For further information on gel purification, refer to this previously published protocol⁸⁴.
- ▲ **CRITICAL STEP** If synthesizing multiple libraries simultaneously, leave empty lanes between different samples during gel electrophoresis to minimize the risk of sample contamination during gel purification.
- 27| Set up a Gibson assembly reaction using gel-purified Esp31-digested/dephosphorylated lentiGuide-Puro plasmid from Steps 5 to 9:

Components	Amount	Final concentration
Gel-purified IsPCR2 from Step 26	10 ng	
Esp31-digested/dephosphorylated lentiGuide-Puro plasmid from Steps 6 to 9	25 ng	
2x Gibson assembly master mix	10 μ l	1x
Nuclease-free water	To 20 μ l	

- 28| Incubate the Gibson assembly reaction mix in a thermocycler at 50 °C for 60 min.
- 29| Thaw one 25- μ l aliquot of electrocompetent bacterial cells on ice until they are completely thawed (~10–20 min).
- 30| Mix thawed electrocompetent bacterial cells by gently tapping on the side of the tube.
- 31| Add 25 μ l of thawed electrocompetent bacterial cells to a prechilled 1.5-ml microcentrifuge tube on ice.
- 32| Add 1 μ l of the Gibson assembly reaction mixture from Step 28 to 25 μ l of thawed electrocompetent bacterial cells (from Step 31).

▲ **CRITICAL STEP** It is reasonable to aim for > 50x representation of the sgRNA library. Depending on the achieved transformation efficiency as calculated in Step 41 and the number of sgRNAs in the library, it may be necessary to set up more than 1 transformation reaction from Step 32. It is important to set up multiple reactions using 1 μ l of Gibson assembly reaction mixture and 25 μ l of thawed electrocompetent bacterial cells. Increasing the amount of Gibson assembly reaction mixture beyond 1 μ l for a given electroporation can alter the chemistry of the electroporation and reduce transformation efficiency.

? TROUBLESHOOTING

- 33| Carefully pipette 25 μ l of the electrocompetent bacterial cell/Gibson assembly reaction mixture from Step 32 into a chilled 0.1-cm-gap electroporation cuvette without introducing bubbles. Quickly flick the cuvette downward to deposit the cells across the bottom of the cuvette's well.

▲ **CRITICAL STEP** Minimize introduction of air bubbles, as they can interfere with plasmid electroporation efficiency.

- 34| Using an electroporator, electroporate the sample at 25 μ F, 200 Ω , and 1,500 V. The expected time constant during electroporation is \sim 4.7 (typical range of 4.2–4.8).

? **TROUBLESHOOTING**

- 35| Add 975 μ l of recovery or SOC medium to the cuvette as soon as possible after electroporation (to a final volume of 1 ml in the cuvette). Pipette up and down enough times to resuspend the cells within the cuvette (probably a single pipetting up and down will be sufficient). Recovery medium is preferred here; however, SOC medium can be used in its place.

▲ **CRITICAL STEP** Electroporation is toxic to bacterial cells. Addition of recovery or SOC medium as quickly as possible enhances bacterial cell survival after electroporation.

- 36| Transfer the cell mixture from Step 35 (\sim 1 ml) to a culture tube already containing 1 ml of SOC medium.
- 37| Shake each culture tube (containing a total of 2 ml of recovery/SOC medium) at 250 r.p.m. for 1 h at 37 $^{\circ}$ C.

▲ **CRITICAL STEP** If multiple transformations were set up in Step 32 using the same Gibson assembly reaction mixture, the 2-ml culture mixes generated in Step 37 can be pooled/mixed together so that one set of dilution plates is sufficient for calculation of transformation efficiency (Step 38).

- 38| Remove 5 μ l from the 2-ml mixture (or 5 μ l from a larger volume, if samples were pooled/mixed as described in Step 37) from Step 37 and add it to 1 ml of SOC medium. Mix well and plate 20 μ l of the mixture (20,000 \times dilution) onto a prewarmed 10-cm ampicillin-resistant (0.1 mg/ml ampicillin) agar plate and then plate the remaining 200 μ l (2,000 \times dilution) onto a separate 10-cm ampicillin-resistant (0.1 mg/ml ampicillin) agar plate. These two dilution plates can be used to estimate transformation efficiency, which will help ensure full library representation of the sgRNA plasmid library. It is reasonable to aim for $>$ 50 \times representation of the sgRNA library.

▲ **CRITICAL STEP** Adjust the dilutions as necessary to obtain plates with a colony density that allows for accurate counting of the number of colonies on the 10-cm plates.

- 39| Plate the remaining 2-ml transformation mixture (or 2 ml from a larger volume, if samples were pooled/mixed) from Step 37 onto a prewarmed square 24.5-cm ampicillin-resistant (0.1 mg/ml ampicillin) agar plate using beads to ensure even spreading. Spread the liquid culture until it is largely absorbed into the agar and will not drip when inverted.

▲ **CRITICAL STEP** If multiple samples were mixed/pooled together in Step 37, it is still important to only plate 2 ml onto each square 24.5-cm ampicillin-resistant agar plate, as volumes > 2 ml may not be fully absorbed into the agar.

- 40| Invert all three (or more) plates (2,000× dilution plate, 20,000× dilution plate, and square 24.5-cm nondilution plate(s)) from Steps 38 and 39, and grow for 14–16 h at 32 °C. It is recommended to incubate transformations at 32 °C to reduce recombination between lentiviral long terminal repeats, but incubation at 37 °C can also be performed.
- 41| Count the number of colonies on the two dilution plates from Step 38. Multiply this number of colonies by the dilution factor (2,000× or 20,000×) and by the increased area of the square 24.5-cm plate (~7.6-fold increase in area) for estimation of the total number of colonies on the square 24.5-cm plate. It is reasonable to aim for > 50× representation of the sgRNA library.

? TROUBLESHOOTING

- 42| Select ~10–20 colonies from the dilution plates (from Step 41) for screening by mini-scale plasmid preparation and subsequent Sanger sequencing (as in Steps 14 and 15) to determine whether the sgRNA library has been PCR-amplified. Given that the oligonucleotides for multiple libraries can be synthesized on the same programmable microarray (Steps 16 and 17), it is important to confirm that the correct library intended for amplification has been obtained. This is also useful for preliminary evaluation of library representation. The expectation is to identify unique sgRNA sequences among all sequenced mini-scale plasmid preparations, given that the probability of obtaining the same sgRNA from a full library of sgRNAs should be low.

▲ **CRITICAL STEP** This step is intended to offer qualitative re-assurance that library synthesis is proceeding in the expected manner by determining that the correct library has been PCR-amplified and offering an initial assessment of library representation. Ultimately, the deep-sequencing step (Step 48) provides a more comprehensive assessment of library composition.

? TROUBLESHOOTING

- 43| Pipette 10 ml of LB medium onto each square 24.5-cm plate from Steps 39 and 40 and scrape the colonies off with a cell scraper. The LB medium aids in removal of the colonies from the agar.
- 44| Pipette the LB medium/scraped bacterial colony mixture into a preweighed 50-ml tube and repeat the procedure a second time on the same plate with an additional 5–10 ml of LB to maximize removal of bacterial colonies.
- 45| Centrifuge the LB medium/scraped bacterial colony mixture at 400g for 5 min at room temperature to pellet the bacteria and then discard the supernatant.
- 46| Weigh the bacterial pellet in the tube (and subtract the preweighed tube to determine the weight of the bacterial pellet) to determine the proper number of

columns for maxi-scale plasmid preparation of the library. Each column can support ~0.45 g of bacterial pellet.

? TROUBLESHOOTING

- 47| Perform a sufficient number of maxi preps, following the manufacturer's instructions and combine the eluted library plasmid DNA in a 1.5-ml tube.
- 48| (Optional) To confirm successful library representation, it is recommended to deep-sequence the batch-cloned plasmid library produced in Steps 16–47 by following the deep-sequencing procedure found in Steps 73–82 and using library plasmid DNA as the template DNA for laPCR1 in Step 75.

Lentivirus production from individual sgRNA plasmid or pooled sgRNA plasmid library ●

TIMING 4 d

! CAUTION Take all necessary precautions for the handling of lentivirus and ensure proper disposal of lentiviral waste. Lentivirus is capable of integrating into the genome of human cells. This caution should be exercised during Steps 49–71.

- 49| Passage and maintain HEK293 cells in a 15-cm round plate with 16 ml of HEK293 medium as previously described⁶².
- 50| Perform transfection when HEK293 cells reach ~80% confluency in a 15-cm round plate using polyethylenimine (PEI) as a transfection reagent. The plasmid/PEI ratio should be 1 µg of the total transfected plasmid to 3 µg of PEI. Total plasmid consists of the sum of micrograms of VSV-G + micrograms of psPAX2 + library plasmid.
- 51| Mix PEI, VSV-G, psPAX2, and library plasmid in 1 ml of filtered DMEM without supplements in a sterile microcentrifuge tube:

Reagent	Amount (µg)
VSV-G plasmid	8.75
psPAX2 plasmid	16.25
Individual sgRNA plasmid or sgRNA library plasmid from Step 15 or 47	25
Branched PEI (at 10 µg/µl)	150

? TROUBLESHOOTING

- 52| Invert the microcentrifuge tube several times to mix. Allow the tube to incubate at room temperature for 20–30 min.

▲ CRITICAL STEP The DMEM/plasmid DNA/PEI mixture should change from translucent to opaque during this incubation period. If the color change does not occur, it is likely that one (or more) of the components is missing, and Steps 51 and 52 should be repeated.

- 53| Add the full volume (~1 ml) of DMEM/plasmid DNA/PEI mixture dropwise to the ~80% confluent HEK293 cells from Steps 49 and 50 and incubate the mixture at 37 °C for 16–24 h.
- 54| Replace the medium with 16 ml of fresh HEK293 medium 16–24 h after transfection in Step 53.
- 55| *Lentiviral supernatant harvest no. 1.* 24 h after replacing the medium in Step 54, collect the 16 ml of medium that now contains lentiviral particles (herein referred to as ‘viral supernatant’) in a 50-ml tube. Replace the medium with 16 ml of fresh HEK293 medium. Store the viral supernatant at 4 °C until Step 56 is performed. The viral supernatant can be stored at 4 °C for up to 7 d before ultracentrifugation; however, storage at 4 °C may result in reduction of viral titer.
- 56| *Lentiviral supernatant harvest no. 2.* 24 h after lentiviral supernatant harvest no. 1, collect the 16 ml of viral supernatant and put it into the same 50-ml tube from Step 55 (should now contain ~32 ml of viral supernatant). Appropriately discard the plate of HEK293 cells.
- 57| Centrifuge the collected viral supernatant from Step 56 at 500–700g for 5 min at 4 °C to pellet the HEK293 cells and other debris.
- 58| Filter the viral supernatant through a 0.45- μ m 50-ml filter.

? TROUBLESHOOTING

- 59| Physical or functional titering can be performed. Physical titering involves detection of viral DNA (e.g., qPCR-based amplification of the viral genome, see MATERIALS) or viral proteins (e.g., ELISA for p24, see MATERIALS). Functional titering involves determination of the transduction rate using cells. Either physical or functional titering can be performed based on experimental needs. Refer to Kutner *et al.* for various titering protocols⁸⁷.
- 60| If sufficient viral titer is obtained with the viral supernatant from Step 59, the filtered viral supernatant can be stored at –80 °C. It is recommended to freeze aliquots with volumes appropriate for future experimental needs to minimize freeze–thaw cycles, as they result in a reduction of viral titer. Aliquots also help to ensure consistent titer for each use of the same batch of virus. If insufficient viral titer is achieved based on the desired multiplicity of infection, it can be improved via ultracentrifugation (Steps 61–71).

■ **PAUSE POINT** The filtered viral supernatant can be stored at 4 °C for up to 7 d before ultracentrifugation; however, storage at 4 °C may result in reduction of viral titer. If ultracentrifugation is required (Steps 61–71), it is recommended to perform ultracentrifugation as soon as possible (minimize storage time at 4 °C). Lentiviruses can be stored at –80 °C for months. Longer storage will result in a decrease of viral titer.

(Optional) Ultracentrifugation of viral supernatant ● TIMING 2.5 h

- 61| Transfer the filtered viral supernatant to ultracentrifugation tubes.
- 62| Add 4–6 ml of 20% (wt/vol) sucrose solution to the bottom of the ultracentrifugation tube to create a sucrose layer (also known as a ‘sucrose cushion’) below the viral supernatant. This sucrose layer helps to remove any remaining debris from the viral supernatant, as only the high-density viral particles can pass through the sucrose layer to pellet, whereas the low-density debris remains in the supernatant.

? TROUBLESHOOTING

- 63| Add sterile PBS to the top of the ultracentrifuge tube until the final liquid volume within the ultracentrifuge tube is ~2–3 mm from the top.

▲ **CRITICAL STEP** The volume must be within 2–3 mm of the top of the tube to prevent the tube from collapsing because of the force of ultracentrifugation.

- 64| Place ultracentrifuge tubes into ultracentrifuge buckets.
- 65| Weigh all ultracentrifuge buckets containing ultracentrifuge tubes before ultracentrifugation to ensure appropriate weight-based balancing. Use sterile PBS to correct weight discrepancies.
- 66| Centrifuge at 100,000*g* for 2 h at 4 °C in an ultracentrifuge.
- 67| Remove ultracentrifuge tubes from ultracentrifuge buckets. The sucrose gradient should still be intact.

? TROUBLESHOOTING

- 68| Discard the supernatant by inverting the ultracentrifugation tube. Allow the inverted ultracentrifuge tube to stand on sterile paper towels to dry for 1–3 min.

? TROUBLESHOOTING

- 69| Revert the ultracentrifugation tube to an upright orientation and add 100 µl of sterile DMEM supplemented with 1% (vol/vol) FBS.
- 70| Incubate for 3–4 h (or overnight) on a rotator/rocker at ~10–15 rotations per minute at 4 °C to resuspend the lentiviral pellet.

■ **PAUSE POINT** Resuspension at 4 °C can be left overnight on a rotator/rocker.

- 71| Store concentrated lentivirus in a microcentrifuge tube at –80 °C. Owing to loss of lentiviral titer with repeated freeze–thaw cycles, consider aliquoting at this point with volumes appropriate for experimental needs (i.e., aliquot size with enough lentivirus for one experiment). Aliquots frozen at the same time can be assumed to have the same titer, which can be advantageous for multiple experiments using the same lentivirus.

■ **PAUSE POINT** Lentiviruses can be stored at -80°C for months. Longer storage will result in a decrease of viral titer.

Execution of arrayed or pooled screen experiments ● TIMING 1–2 weeks

- 72| Perform arrayed or pooled screen experiments. Experiments/screens using lentivirus should be performed as appropriate for the experimental design/objectives and the cells being used for study. Refer to Boxes 3 and 4 for discussions about and considerations for performing an arrayed or pooled screen experiment.

■ **PAUSE POINT** After experiments/screens have been completed, cell pellets can be stored at -20 or -80°C for weeks to months before processing for deep-sequencing analysis of the samples (Steps 73–82).

Deep sequencing of arrayed or pooled screen experiments ● TIMING 1 d

- 73| Extract gDNA from fresh cell pellets after completion of genome-editing screens/experiments or from frozen pellets from previous experiments. See MATERIALS for genomic DNA extraction kit and follow the manufacturer's instructions.

▲ **CRITICAL STEP** For arrayed experiments intended for indel analysis by CRISPResso (Step 83), it is recommended to include a non-edited sample from the same cell type. This sample will be a useful negative control when performing CRISPResso indel analysis/enumeration. Specifically, a non-edited sample is useful in regard to optimization of the CRISPResso analysis parameters summarized in Box 2. Furthermore, a non-edited sample can be useful in identifying single-nucleotide polymorphisms (SNPs) or other variants present in the cells under study that can confound CRISPResso analysis. If a variant/indel is identified in the non-edited sample, the CRISPResso settings can be altered to account for the variant/indel during CRISPResso indel enumeration (Box 2).

- 74| Determine the concentration of each gDNA sample using a UV-visible spectrophotometer (e.g., NanoDrop) or other equivalent method using the manufacturer's instructions.

▲ **CRITICAL STEP** The amount of gDNA can vary based on experimental needs for an arrayed experiment and based on the number of sgRNAs in the library for a pooled experiment. On average, a genome from a single cell is $\sim 6.6\text{ pg}^{88}$. Use adequate gDNA to represent the desired number of cells. For an arrayed experiment for indel analysis of a single locus using CRISPResso, $> 1,000$ cells at a given locus is a reasonable number. For a pooled sgRNA library experiment, $100\text{--}1,000\times$ coverage of the sgRNAs in the library is reasonable. For example, $6.6\text{ }\mu\text{g}$ of gDNA is estimated to represent 1 million cells. For a library with $1,000$ sgRNAs, $6.6\text{ }\mu\text{g}$ of gDNA would provide $1,000\times$ coverage.

- 75|** Set up the ‘Library Analysis PCR1’ reaction (herein referred to as laPCR1). Use lentiGuide-Puro-specific primers (Supplementary Table 5) to enumerate the sgRNAs present for enrichment/dropout analysis or locus-specific primers to quantitate indels (Supplementary Table 6). lentiGuide-Puro-specific primers are suggested in Supplementary Table 5; however, other primer pairs to PCR-amplify from the lentiGuide-Puro construct are possible.

▲ CRITICAL STEP It is important to use a proofreading DNA polymerase to minimize introduction of PCR errors.

▲ CRITICAL STEP Optimization of DMSO concentration may be required (typically 1–10% (vol/vol)). 8% (vol/vol) is used in the reaction below.

▲ CRITICAL STEP It is important to sequence the cloned plasmid library as generated in Steps 16–47. Sequencing of the plasmid library allows for confirmation of successful cloning/representation of the library and can be used as an initial time point for a dropout (‘depletion’) screen (see Box 4 for details of this type of screen). Given that plasmids contain many fewer base pairs than a full genome, ≥ 50 pg of plasmid DNA will be sufficient to represent a pooled plasmid library.

? TROUBLESHOOTING

Components	Amount (μ l)	Final concentration
gDNA or plasmid DNA	<i>X</i>	–
5 \times Herculase II reaction buffer	10	1 \times
dNTPs (at 100 mM)	1	2 mM
lentiGuide_forward or Locus_forward primer (at 10 μ M)	2.5	0.5 μ M
lentiGuide_reverse or Locus_reverse primer (at 10 μ M)	2.5	0.5 μ M
DMSO	4	8%
Herculase II Fusion DNA Polymerase	0.5	
Nuclease-free water	To 50	

- 76|** Perform multiple cycles (e.g., 10, 15, and 20 cycles) of laPCR1 in a thermocycler as follows. Gradient PCR may be required to determine the optimal annealing temperature (Supplementary Fig. 2c).

Cycle number	Denature	Anneal	Extend
1	95 $^{\circ}$ C, 30 s		
2- <i>x</i> (e.g., 10, 15, and 20)	95 $^{\circ}$ C, 10 s	60 $^{\circ}$ C, 30 s	72 $^{\circ}$ C, 30 s
<i>x</i> + 1			72 $^{\circ}$ C, 5 min

? TROUBLESHOOTING

■ **PAUSE POINT** The PCR product can be stored at 4 °C in a thermocycler at the end of the PCR program for multiple days. Longer-term storage (months to years) should be at – 20 °C.

- 77| Run a fraction (1–5 µl of the 50-µl PCR reaction from Steps 75 and 76) of the laPCR1 product with a different number of PCR cycles on a 2% (wt/vol) agarose gel.

▲ **CRITICAL STEP** The number of PCR cycles should be limited to reduce PCR bias. It is difficult to predict the required number of cycles for a given library. Therefore, it is important to perform multiple PCRs with different cycle numbers to empirically determine the optimal number of cycles.

? TROUBLESHOOTING

- 78| Set up ‘Library Analysis PCR2’ (herein referred to as laPCR2) using the barcode-specific primers (Supplementary Tables 7 and 8). Each sample will need a unique Illumina Nextera index to allow demultiplexing of multiple samples sequenced together. Set up two PCRs for each sample.

▲ **CRITICAL STEP** Two separate 10-µl reactions are performed, as opposed to a single 20-µl reaction, to minimize PCR bias.

Components	Amount (µl)	Final concentration
laPCR1 product (diluted 1:5 in nuclease-free water) from Steps 75–77	1	-
5× Herculase II reaction buffer	2	1×
dNTPs (at 100 mM)	0.1	1 mM
Forward primer (at 2 µM)	1	0.2 µM
Reverse primer (at 2 µM)	1	0.2 µM
Herculase II Fusion DNA Polymerase	0.1	
Nuclease-free water	To 10	

- 79| Perform laPCR2 using the same cycling conditions and multiple cycle numbers as in Step 76.

▲ **CRITICAL STEP** The number of PCR cycles should be limited to reduce PCR bias. It is difficult to predict the required number of cycles for a given library. Therefore, it is important to perform multiple PCRs with different cycle numbers to empirically determine the optimal number of cycles.

■ **PAUSE POINT** The PCR product can be stored at 4 °C in a thermocycler at the end of the PCR program for multiple days. Longer-term storage (months to years) should be at – 20 °C.

- 80| Combine both 10-µl reactions from the same sample for a total volume of 20 µl. Run laPCR2 products on a 2% (wt/vol) agarose gel to determine whether the products occur at the expected size (expected size varies based on primers used in laPCR1). Choose the sample with the minimal number of PCR cycles that

produces a visible band in order to minimize PCR bias (Supplementary Fig. 2d). For example, 20 cycles would be used based on the gel presented in Supplementary Fig. 2d; however, it may be possible to reduce the cycle number even further (i.e., another experiment with 5, 10, and 15 cycles). Then gel-purify the relevant band of the expected size.

▲ **CRITICAL STEP** Leave empty lanes between different samples to minimize the risk of sample contamination during gel purification.

81| Quantitate DNA concentration by Qubit or another equivalent method, following the manufacturer's instructions. Refer to the manufacturer's instructions for the sequencing platform to be used (Step 82) for the minimum amount of DNA required. If an inadequate amount of DNA is obtained, repeat Steps 75–81.

82| Sequence the libraries on a MiSeq, HiSeq, or equivalent sequencing platform (see 'Experimental design' for details).

▲ **CRITICAL STEP** It can be useful to discuss experimental goals with the sequencing facility or individual performing the sequencing prior to sequencing samples to help ensure that the desired number of reads are obtained to adequately represent the sequenced locus/loci or sgRNA library.

CRISPResso analysis of deep-sequencing data ● TIMING 10–120 min

83| The CRISPResso utility can be used for analysis of deep-sequencing data of a single locus/amplicon on a local machine or proprietary server, using a command-line version or a webtool freely available at <http://crispresso.rocks/>. Installation instructions are provided in the 'Equipment Setup' section for command-line and Docker versions. No installation is required for the webtool. The following step will present detailed instructions for workflows using the webtool (option A), command-line (option B), or Docker (option C) versions:

(A) Analysis of deep-sequencing data using the CRISPR esso webtool ● TIMING 10–120 min

▲ **CRITICAL** An illustrative video of the entire process is provided here: <http://www.youtube.com/embed/dXblIiAe00?autoplay=1>.

- i. Open a web browser and go to the CRISPResso page <http://crispresso.rocks/>.
- ii. Select the option for single-end (one FASTQ file) or paired-end reads (two FASTQ files) based on the type of deep sequencing performed. Analysis of paired-end reads requires overlapping sequence, as the paired reads will be merged. Upload the relevant FASTQ file(s) (as .fastq or .fastq.gz) for analysis. An example data set to test the tool with previously generated FASTQ files is provided at <http://crispresso.rocks/help> (see the 'Try it now!' section).

? TROUBLESHOOTING

- iii. Input the amplicon sequence (required), sgRNA sequence (optional), and coding sequence(s) (optional input for when targeting exonic sequence). The reads

uploaded in the previous step will be aligned to the provided amplicon sequence. If an sgRNA sequence is provided, its position will be indicated in all output analyses. If a coding sequence is provided, it will allow CRISPResso to perform frameshift analysis. The provided exonic sequence must be a subsequence of the amplicon sequence and not the sequence of the entire exon.

? TROUBLESHOOTING

- iv. Adjust the parameters for window size (bp around each side of cleavage site) to quantify NHEJ edits (if sgRNA sequence is provided), minimum average read quality (Phred33 scale), minimum single-bp quality (Phred33 scale), exclude bp from the left side of the amplicon sequence for the quantification of the mutations, exclude bp from the right side of the amplicon sequence for the quantification of the mutations, and trimming adapter (see Box 2 for a discussion of these parameters).
- v. Submit samples for analysis and download analysis reports.

(B) Analysis of deep-sequencing data using command-line CRISPR esso ● TIMING 10–120 min

- i. Gather the required information and files (see Step 1A(iv) or 1C(xiii); the CRISPOR utility will output all the required information to run CRISPResso).

Input	Example entry (adjust as appropriate) or description
FASTQ files for analysis (.fastq or .fastq.gz)	reads1.fastq.gz reads2.fastq.gz
Reference amplicon sequence	AATGTCCCCCAATGGGAAGTTCATCTGGCACTGCCACAGGTGA GGAGGTCATGATCCCCTTCTGGAGCTCCCAACGGGCCGTGGTCT GGTTCATCATCTGTAAGAATGGCTTCAAGAGGCTCGGCTGTGGTT
sgRNA(s) used	TGAACCAGACCACGGCCCGT
Expected repaired amplicon sequence	Only provide this if it is necessary to quantify HDR efficiency
Desired single-base-pair or average read quality in Phred33 score	We suggest using 20 for single-base-pair quality and 30 for the average read quality
Exonic sequence (for frameshift analysis)	Only provide this if the reference amplicon sequence contains exonic sequence
Experiment name	BCL11A_exon2

- ii. Open a terminal and run the CRISPResso utility by running the following command. Here, we are assuming that the FASTQ files reads1.fastq.gz and reads2.fastq.gz are stored in the folder /home/user/amplicons_data/.

```
CRISPResso \
-r1 /home/user/amplicons_data/reads1.fastq.gz \
-r2 /home/user/amplicons_data/reads2.fastq.gz \
-a AATGTCCCCCAATGGGAAGTTCATCTGGCACTGCCACAGGTGAGG\
```

```

AGGTCATGATCCCCTTCTGGAGCTCCCAACGGGCCGTGGTCTGGTTCAT\
CATCTGTAAGAATGGCTTCAAGAGGCTCGGCTGTGGTT \
-g TGAACCAGACCACGGCCCGT \
-s 20 \
-q 30 \
-n BCL11A_exon2

```

▲ **CRITICAL STEP** Be sure to check whether or not the sequencing files were trimmed for adapters (refer to Box 2 for further discussion of the trimming process and options).

▲ **CRITICAL STEP** The command is based on the example data from Step 83B(i) and must be amended before use. After the execution of the command, a new folder with all the results will be created; in this example, the folder will be in `/home/user/amplicons_data/CRISPResso_on_BCL11A_exon2`. A summary of the different events discovered in the sequencing data is presented in the `Alleles_frequency_table.txt` file, and in several illustrative plots (.pdf files). Refer to the online documentation for the full description of the output: <https://github.com/pinellolab/CRISPResso>.

? TROUBLESHOOTING

(C) Analysis of deep-sequencing data using CRISPR esso with Docker ● TIMING 10–120 min

- i. Gather the required information and files as in Step 83B(i).
- ii. Assuming that the FASTQ files `reads1.fastq.gz` and `reads2.fastq.gz` are stored in the folder `/home/user/amplicons_data/`, run the command

```

docker run \
-v /home/user/amplicons_data:/DATA \
-w /DATA pinellolab/crispor_crispresso_nat_prot \
CRISPResso \
-r1 reads1.fastq.gz \
-r2 reads2.fastq.gz \
-a AATGTCCCCCAATGGGAAGTTCATCTGGCACTGCCACAGGTGAGG\
AGGTCATGATCCCCTTCTGGAGCTCCCAACGGGCCGTGGTCTGGTTCAT\
CATCTGTAAGAATGGCTTCAAGAGGCTCGGCTGTGGTT \
-g TGAACCAGACCACGGCCCGT \
-s 20 \
-q 30 \
-n BCL11A_exon2

```

After the execution of the command, a new folder with all the results will be created; in this example, the folder will be in `/home/user/amplicons_data/CRISPResso_on_BCL11A_exon2`. A summary of the different events discovered

in the sequencing data is presented in the Alleles_frequency_table.txt file, and in several illustrative plots (.pdf files). Refer to the online documentation for the full description of the output: <https://github.com/pinello/CRISPResso>.

? TROUBLESHOOTING

Analysis of a pooled sgRNA experiment using CRISPRessoCount ● TIMING 20 min–4 h

▲ **CRITICAL** CRISPRessoCount is a utility for the enumeration of sgRNA (Fig. 2; Box 4). It is necessary to use the command-line or Docker version of CRISPResso to run CRISPRessoCount. CRISPRessoCount can enumerate sgRNAs from a user-generated list or can empirically identify all sgRNAs present in the FASTQ file.

- 84| Gather the required data and information (adjust the entries as appropriate for the experiment):

Input	Example entry (adjust as appropriate) or description
FASTQ files for sgRNA enumeration analysis (.fastq or .fastq.gz)	sample_screen.fastq.gz
File containing only the sgRNA sequences to enumerate, one per line. This is an optional input. If not provided, all unique sgRNA sequences will be enumerated	library_NGG.txt (or the file created by CRISPOR, see Step 1A(iv) or 1C(xiii))
The sgRNA scaffold sequence immediately downstream of the cloned sgRNA sequence	GTTTTAGAGCTAGAAATAGC
sgRNA length	20
Desired single-base-pair or average read quality in Phred33 score	We suggest 20 for single-base-pair quality and 30 for the average read quality
Experiment name	SAMPLE_SCREEN

- 85| Run the CRISPRessoCount analysis. The following step will present detailed instructions for running CRISPRessoCount from the command line (option A) or Docker (option B). Here, we are assuming that the FASTQ file and the optional sgRNA file are stored in the folder home/user/sgRNA_data/.

(A) Using the command-line version for CRISPR essoCount analysis ● TIMING 10–120 min

- i. Run the CRISPRessoCount analysis with the following commands:

```
CRISPRessoCount -r /home/user/sgRNA_data/sample_screen.fastq.gz \
-s 20 -q 30 -f library_NGG.txt \
-t GTTTTAGAGCTAGAAATAGC -l 20 --name SAMPLE_SCREEN
```

(B) Using the Docker container for CRISPR essoCount analysis ● TIMING 10–120 min

- i. Run the CRISPRessoCount analysis with the following commands:

```
docker run \
```

```

-v /home/user/sgRNA_data:/DATA \
-w /DATA \
pinellolab/crispor_crispresso_nat_prot \
CRISPRessoCount -r sample_screen.fastq.gz \
-s 20 -q 30 -f library_NGG.txt \
-t GTTTTAGAGCTAGAAATAGC -l 20 --name SAMPLE_SCREEN

```

After the execution of the command, a new folder with all the results will be created; in this example, the folder will be in /home/user/amplicons_data/CRISPRessoCount_on_SAMPLE_SCREEN. This folder contains two files, the execution log (CRISPRessoCount_RUNNING_LOG.txt) and a file (CRISPRessoCount_only_ref_guides_on_sample_screen.fastq.gz.txt) containing a table with the raw and normalized counts for each sgRNA in the input FASTQ file:

Guide sequence	Read counts	Read (%)	Reads per million (RP M)
CTCTGCCCTTCTGACATTGT	2,592	0.312315496488	3123.15496488
ATGTGAGCATATGTATTCAT	2,553	0.30761630499	3076.1630499
CCTGCTATGTGTTCTCTGTTT	2,455	0.2958080802	2958.080802
TTCTCGTGCCTCAGCCTCCT	2,430	0.292795777957	2927.95777957
ACCCTGTGTATTTACACAT	2,349	0.283035918692	2830.35918692
TTCCATTAATACACAATGT	2,235	0.269299820467	2692.99820467

- 86|** Calculate the enrichment and/or dropout ('depletion') between two conditions, and calculate the \log_2 ratio using the normalized sgRNA counts (this takes into account the total number of reads within each sequenced sample) using the 'Reads_Per_Millions_(RPM)' column for the two conditions. Refer to the 'CRISPResso: Analysis of deep sequencing from arrayed or pooled sgRNA experiments' section for further discussion of read normalization.

▲ **CRITICAL STEP** The \log_2 transformation is not necessary, but is often used when displaying the data, as it will center the data at ~ 0 for an equal ratio; positive values will represent enrichment and negative values will represent depletion/dropout.

? TROUBLESHOOTING

CRISPR esso analysis using CRISPRessoPooled ● TIMING 20 min–4 h

▲ **CRITICAL** CRISPRessoPooled is a utility for analyzing and quantifying targeted sequencing CRISPR experiments involving sequencing with pooled amplicons. To use CRISPRessoPooled, it is necessary to use the command-line version of CRISPResso (see Step 83B). Although CRISPRessoPooled can be run in different modes (amplicon only, genome only, and mixed), in this protocol, we use the most reliable mode for quantification, called 'mixed mode'. In this mode, the amplicon reads are aligned to both reference amplicons and the genome to discard ambiguous or spurious reads. For more details

regarding the different running modes, consult the online help (help is located below the list of files at the following link): <https://github.com/pinellolab/CRISPResso>.

- 87|** Gather the required data and information, including FASTQ files that contain reads for a pooled experiment with multiple sgRNAs, for example:

Input	Example entry (adjust as appropriate) or description
FASTQ files for analysis (.fastq or .fastq.gz)	Reads_pooled_1.fastq.gz Reads_pooled_2.fastq.gz
Reference amplicon sequences used in the pooled experiment	
sgRNA sequences used, one for each amplicon	
Expected repaired amplicon sequence	Only provide this if it is necessary to quantify HDR efficiency
Desired single-base-pair or average read quality in Phred33 score	We suggest 20 for single-base-pair quality and 30 for the average read quality
Exonic sequence (for frameshift analysis)	Only provide this if the reference amplicon sequence contains exonic sequence
Amplicon names	All the names should be unique
A global name for the pooled analysis	Pooled_amplicons

▲ CRITICAL STEP Be sure to check whether or not the sequencing files were trimmed for adapters (refer to Box 2 for further discussion of the trimming options).

- 88|** Create a folder for the reference genome and genomic annotations (this step is necessary only one time):

```
mkdir -p /home/user/crispresso_genomes/hg19
```

- 89|** Download the desired genome sequence data and precomputed index from https://support.illumina.com/sequencing/sequencing_software/jgenome.html. In this example (Steps 90 and 91), we download the hg19 assembly of the human genome.

- 90|** Move to the folder containing the genome:

```
cd /home/user/crispresso_genomes/hg19
wget ftp://igenome:G3nom3s4u@ussd-ftp.illumina.com\
/Homo_sapiens/UCSC/hg1938/Homo_sapiens_UCSC_hg19.tar.gz
```

- 91|** Extract the data with the following command:

```
tar -xvzf Homo_sapiens_UCSC_hg19.tar.gz \
```



```
Homo_sapiens/UCSC/hg19/Sequence/Bowtie2Index \
--strip-components 5
```

- 92] Create an amplicon description file or use the amplicon file created by CRISPOR in Step 1A(iv) or 1C(xiii). This file is a tab-delimited text file with up to five columns (the first two columns are required) (Supplementary Fig. 3a):

Column no.	Description
1	An identifier for the amplicon (must be unique)
2	Amplicon sequence used in the design of the experiment
3 (optional)	sgRNA sequence used for this amplicon, without the PAM sequence. If not available, enter 'NA'
4 (optional)	Expected amplicon sequence in the case of HDR. If more than one, separate by commas and not spaces. If not available, enter 'NA'
5 (optional)	Subsequence(s) of the amplicon corresponding to coding sequences. If more than one, separate by commas and not spaces. If not available, enter 'NA'

Here, we are assuming that the file is saved in the folder `/home/user/pooled_amplicons_data/` and is called `AMPLICONS_FILE.txt`

- 93] Open a terminal and run the CRISPRessoPooled utility from the command line (option A) or Docker (option B). Here, we are assuming that the FASTQ files `Reads_pooled_1.fastq.gz` and `Reads_pooled_2.fastq.gz` are stored in the folder `/home/user/pooled_amplicons_data/`

(A) Using the command-line version for CRISPR essoPooled analysis ● TIMING 10–120 min

- i. Run CRISPRessoPooled by executing the following commands:

```
CRISPRessoPooled \
-r1 /home/user/pooled_amplicons_data/Reads_pooled_1.fastq.gz \
-r2 /home/user/pooled_amplicons_data/Reads_pooled_2.fastq.gz \
-f /home/user/amplicons_data/AMPLICONS_FILE.txt \
-x /home/user/crispresso_genomes/hg19/genome \
-s 20 -q 30 \
--name Pooled_amplicons
```

? TROUBLESHOOTING

(B) Using the Docker container for CRISPR essoPooled analysis ● TIMING 10–120 min

- i. Run CRISPRessoPooled by executing the following commands:

```
docker run \
-v /home/user/amplicons_data:/DATA -w /DATA \
```

```
-v /home/user/crispresso_genomes/:/GENOMES \
pinellolab/crispor_crispresso_nat_prot \
CRISPRessoPooled \
-r1 Reads_pooled_1.fastq.gz \
-r2 Reads_pooled_2.fastq.gz \
-f AMPLICONS_FILE.txt \
-x /GENOMES/hg19/genome \
-s 20 -q 30 \
--name Pooled_amplicons
```

After the execution of the command in the previous step, a new folder with all the results will be created. In this example, the folder will be in /home/user/amplicons_data/CRISPRessoPooled_on_Pooled_amplicons. In this folder, the user can find the following files:

Output	Description
REPORT_READS_ALIGNED_TO_GENOME_AND_AMPLICONS.txt	This file contains the same information provided in the input description file, plus some additional columns: <ul style="list-style-type: none"> • Amplicon_Specific_fastq.gz_filename: name of the file containing the raw reads recovered for the amplicon • n_reads: number of reads recovered for the amplicon • chr_id: chromosome of the amplicon in the reference genome • bpstart: start coordinate of the amplicon in the reference genome • bpend: end coordinate of the amplicon in the reference genome • Reference_Sequence: sequence in the reference genome for the region mapped for the amplicon
MAPPED_REGIONS (folder)	This folder contains all the .fastq.gz files for the discovered regions
Set of folders with CRISPResso reports	CRISPResso analysis for the recovered amplicons with enough reads
SAMPLES_QUANTIFICATION_SUMMARY.txt	This file contains a summary of the quantification and the alignment statistics for each region analyzed (read counts and percentages for the various classes: unmodified, NHEJ, and HDR)
CRISPRessoPooled_RUNNING_LOG.txt	Execution log and messages for the external utilities called

? TROUBLESHOOTING

CRISPResso analysis using CRISPRessoWGS ● TIMING 20 min–4 h

94| Gather the required data and information:

Input	Example entry (adjust as appropriate) or description
Genome-aligned WGS data in BAM format	sample_WGS.bam
The sequence of the reference genome used for the alignment, in FASTA format (Steps 88–91)	Human hg19 genome assembly in FASTA format
(Optional) gene annotation file (Step 1C(vi–xii))	gencode_v19.gz
Coordinates of the regions to analyze	REGIONS_FILE.txt
Expected repaired amplicon sequence	Only provide this if it is necessary to quantify HDR efficiency
Desired single-base-pair or average read quality in Phred33 score	We suggest 20 for single-base-pair quality and 30 for the average read quality
Exonic sequences (for frameshift analysis)	Only provide this if the reference amplicon sequence contains exonic sequence
Region names	All the names should be unique
A global name for the WGS analysis	WGS_regions

- 95] Create a regions description file. This file is a tab-delimited text file with up to seven columns (four are required) and contains the coordinates of the regions to analyze and some additional information (Supplementary Fig. 3b):

Column no.	Description
1	Chromosome of the region in the reference genome
2	Start coordinate of the region in the reference genome
3	End coordinate of the region in the reference genome
4	An identifier for the region (must be unique)
5 (optional)	sgRNA sequence used for this genomic segment without the PAM sequence. If not available, enter 'NA'
6 (optional)	Expected genomic segment sequence in case of HDR. If more than one, separate by commas and not spaces. If not available, enter 'NA'
7 (optional)	Subsequence(s) of the genomic segment corresponding to coding sequences. If more than one, separate by commas and not spaces. If not available, enter 'NA'

Here, we are assuming that the file is saved in the folder /home/user/wgs_data/ and it is called REGIONS_FILE.txt.

- 96] Open a terminal and run the CRISPRessoWGS utility. The following step will present detailed instructions for the command-line version (option A) and Docker (option B). Here, we are assuming that the BAM file is stored in the folder /home/user/wgs_data/ and was aligned using the human hg19 reference genome.

(A) Using the command-line version for CRISPR essoWGS analysis ● TIMING 10–120 min

- i. Run CRISPRessoWGS by executing the following commands:

```
CRISPRessoWGS\
```

```
-b /home/user/wgs_data/sample_WGS.bam \
-f /home/user/wgs_data/REGIONS_FILE.txt \
-r /home/user / crispresso_genomes/hg19/hg19.fa \
-s 20 -q 30 \
--name WGS_regions
```

? TROUBLESHOOTING

(B) Using the Docker container version for CRISPR essoWGS analysis ● TIMING 10–120 min

- i. Run CRISPRessoWGS by executing the following commands:

```
docker run \
-v /home/user / wgs_data:/DATA -w /DATA \
-v /home/user / crispresso_genomes:/GENOMES \
pinellolab/crispor_crispresso_nat_prot \
CRISPRessoWGS \
-b sample_WGS.bam \
-f REGIONS_FILE.txt \
-r /GENOMES/hg19.fa \
-s 20 -q 30 \
--name WGS_regions
```

After the execution of the command, a new folder with all the results will be created; in this example, the folder will be in `/home/user/wgs_data/CRISPRessoWGS_on_WGS_regions`. In this folder, the user can find the following files:

Output filename	Description
REPORT_READS_ALIGNED_TO_SELECTED_REGIONS_WGS.txt	<p>This file contains the same information provided in the input description file, plus some additional columns:</p> <ul style="list-style-type: none"> • <code>sequence</code>: sequence in the reference genome for the region specified • <code>n_reads</code>: number of reads recovered for the region • <code>bam_file_with_reads_in_region</code>: file containing only the subset of the reads that overlap, also partially, with the region. This file is indexed and can be easily loaded, for example, onto the Integrative Genomics Viewer (IGV; http://software.broadinstitute.org/software/igv/) for visualization of single reads or for the comparison of two conditions • <code>fastq.gz_file_trimmed_reads_in_region</code>: file containing only the subset of reads fully covering the specified regions and

Output filename	Description
	trimmed to match the sequence in that region. These reads are used for the subsequent analysis with CRISPResso
ANALYZED_REGIONS (folder)	This folder contains all the BAM and FASTQ files, one for each region analyzed
A set of folders with the CRISPResso report on the regions provided in the input	Refer to description of the output of CRISPResso (http://software.broadinstitute.org/software/igv/)
CRISPRessoWGS_RUNNING_LOG.txt	Execution log and messages for the external utilities called

? TROUBLESHOOTING

Direct comparison of CRISPResso analyses using CRISPRessoCompare and CRISPRessoPooledWGSCSCompare ● TIMING 20 min–4 h

- 97| Gather the required data and information: two completed CRISPResso, CRISPRessoPooled or CRISPRessoWGS analyses:

Input	Example entry (adjust as appropriate) or description
Path to two completed CRISPResso, CRISPRessoPooled, or CRISPRessoWGS analyses	/home/user/results/CRISPResso_on_sample1
	/home/user/results/CRISPResso_on_sample2
	or
	home/user/results/CRISPRessoPooled_on_sample1
	home/user/results/CRISPRessoPooled_on_sample2
	or
A name to use for the report	/home/user/results/CRISPRessoWGS_on_sample1
	/home/user/results/CRISPRessoWGS_on_sample2
	comparison_sample1_sample2

- 98| Open a terminal and run the CRISPRessoCompare or CRISPRessoPooledWGSCSCompare utility from the command line (option A) or Docker (option B). Here, we are assuming that output folders from those utilities were saved in /home/user/results/.

(A) Using the command-line version for CRISPRessoCompare or CRISPRessoPooledWGSCSCompare analysis ● TIMING 10–120 min

- i. Execute the following command for CRISPRessoCompare:

```
CRISPRessoCompare \
/home/user/results/CRISPResso_on_sample1 \
/home/user/results/CRISPResso_on_sample2 \
-n comparison_sample1_sample2
```

Or execute the following command for CRISPRessoPooledWGSCSCompare:

```
CRISPRessoPooledWGSCompare \
/home/user/results/CRISPRessoPooled_on_sample1 \
/home/user/results/CRISPRessoPooled_on_sample2 \
-n comparison_sample1_sample2
```

(B) Using the Docker container version for CRISPRessoCompare or CRISPRessoPooledWGSCCompare analysis ● TIMING 10–120 min

- i. Execute the following command for CRISPRessoCompare:

```
docker run \
-v /home/user/results:/DATA -w /DATA \
pinelloolab/crispor_crispresso_nat_prot \
CRISPRessoCompare \
CRISPResso_on_sample1 \
CRISPResso_on_sample2 \
-n comparison_sample1_sample2
```

Or execute the following command for CRISPRessoPooledWGSCCompare:

```
docker run \
-v /home/user/results:/DATA -w /DATA \
pinelloolab/crispor_crispresso_nat_prot \
CRISPRessoPooledWGSCompare \
CRISPRessoPooled_on_sample1 \
CRISPRessoPooled_on_sample2 \
-n comparison_sample1_sample2
```

The syntax for the utility CRISPRessoPooledWGSCCompare is exactly the same for results obtained with CRISPRessoWGS.

After the execution of the command CRISPRessoPooledWGSCCompare, a new folder with subfolders (one for each region) will be created. In this example, the folder will be in /home/user/results/CRISPRessoPooledWGSCCompare_on_comparison_sample1_sample2.

In the folder created by CRISPRessoPooledWGSCCompare, the user will see the following files:

Output filename	Description
COMPARISON_SAMPLES_QUANTIFICATION_SUMMARIES.txt	This file contains a summary of the quantification for each of the two conditions for each region and their differences (read counts and percentages for the various classes:

Output filename	Description
	unmodified, NHEJ, MIXED NHEJ–HDR and HDR)
A set of folders with CRISPRessoCompare reports on the common regions with enough reads in both conditions	Please refer to the table below for the description of the files contained in this folder
CRISPRessoPooledWGSCompare_RUNNING_LOG.txt	Detailed execution log

A single folder will be created if CRISPRessoCompare was used. In the folder created by CRISPRessoCompare, the user will see the following files:

Output filename	Description
Comparison_Efficiency.pdf	A figure containing a comparison of the editing frequencies for each category (NHEJ, MIXED NHEJ–HDR and HDR), as well as the net effect of subtracting the second sample (second folder in the command line) provided in the analysis from the first sample (first folder in the command line)
Comparison_Combined_Insertion_Deletion_Substitution_Locations.pdf	A figure showing the average profile for the mutations for the two samples in the same scale and their differences with the same convention used in the previous figure (first sample–second sample)
CRISPRessoCompare_RUNNING_LOG.txt	Detailed execution log

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

● TIMING

Step 1, sgRNA design using CRISPOR: 1–4 h

Steps 2–15, synthesis and cloning of individual sgRNA into a lentiviral vector: 3 d to obtain sequence-confirmed, cloned sgRNA lentiviral plasmid

Steps 16–48, synthesis and cloning of pooled sgRNA libraries into a lentiviral vector: 2–4 d to obtain cloned sgRNA lentiviral plasmid library

Steps 49–60, lentivirus production from individual sgRNA plasmid or pooled sgRNA plasmid library: 4 d

Steps 61–71, (optional) ultracentrifugation of viral supernatant: 2.5 h

Step 72, execution of arrayed or pooled screen experiments: 1–2 weeks

Steps 73–82, deep sequencing of arrayed or pooled screen experiments: 1 d

Step 83, CRISPResso analysis of deep-sequencing data: 10–120 min

Steps 84–86, analysis of a pooled sgRNA experiment using CRISPRessoCount: 20 min–4 h

Steps 87–93, CRISPResso analysis using CRISPRessoPooled: 20 min–4 h

Steps 94–96, CRISPResso analysis using CRISPRessoWGS: 20 min–4 h

Steps 97 and 98, direct comparison of CRISPResso analyses using CRISPRessoCompare and CRISPRessoPooledWGSCompare: 20 min–4 h

ANTICIPATED RESULTS

Indel enumeration/analysis of a deep-sequencing amplicon generated by locus-specific PCR primers can be performed by CRISPResso for an arrayed experiment (Fig. 4a–d). As a reference for assessment of locus-specific edits, we provide data demonstrating the quantification of editing (Fig. 4a) and distribution of combined insertions/deletions/substitutions, to show successful editing of *BCL11A* exon 2 with indels flanking the DSB site (Fig. 4b–c). In this example, 36.1% of reads had a mutation present, whereas 63.9% of reads were unmodified (Fig. 4a). Mutation position analysis demonstrated that the majority of indels clustered at the predicted sgRNA DSB site (Fig. 4b), and indel size analysis demonstrated that most of the identified indels were small (< 10 bp) (Fig. 4c). Frameshift analysis was performed because coding sequence was targeted, which revealed a predominance of frameshift mutations (79.3% of reads) as compared with in-frame mutations (20.7% of reads) (Fig. 4d). The distribution of all identified alleles can be directly visualized as well (Supplementary Fig. 4). Using CRISPRessoCompare for direct comparison of the sample treated with CRISPR/Cas9 with a non-edited control can offer reassurance that the identified mutations are likely to have resulted from CRISPR-mediated mutagenesis. Specifically, this analysis was performed by sequencing the mutagenized sample and a wild-type control, followed by analysis via CRISPRessoCompare (Supplementary Fig. 5).

As a reference for assessment of a pooled CRISPR screen experiment, data are provided from two separate saturating mutagenesis experiments for identification of functional sequence within the *BCL11A* enhancer^{11,12}. All sgRNAs were designed within the core of the *BCL11A* enhancer. All sgRNAs were designed within *BCL11A* exon 2 to serve as positive controls and nontargeting controls were included as negative controls. sgRNAs were batch-cloned into pLentiGuide-puro, and lentivirus was produced. HUDEP-2 erythroid cells with stable Cas9 expression were transduced at low multiplicity. After puromycin selection and allowing 1–2 weeks for editing to occur, phenotypic selection occurred by FACS. Specifically, cells with high and low fetal hemoglobin (HbF) were sorted by FACS. gDNA was extracted from the high- and low-HbF sorted cell pellets. PCR was performed with primers specific to the pLentiGuide-Puro construct. After deep sequencing, FASTQ files were analyzed by CRISPRessoCount to determine read counts for each sgRNA in the library. Read counts were subsequently normalized for each sample. Normalized read counts from the high-HbF sample were divided by the normalized read counts from the low-HbF sample for each sgRNA, followed by a log₂ transformation to provide an enrichment score. The enrichment score for each sgRNA was plotted at the genomic position for the predicted

DSB (Fig. 4e). Notably, this analysis shows pooled experiments using NGG- and NGA-restricted sgRNA (Supplementary Table 1), and demonstrates reproducibility of the experimental results using this procedure across multiple studies. On the basis of the analysis presented in Figure 4e, enrichment of sgRNA based on HbF levels can be observed for sgRNAs targeting *BCL11A* coding sequence as compared with the nontargeting controls. Notably, the nontargeting sgRNAs cluster around an enrichment score of zero, suggesting no effect on HbF. The sgRNAs targeting the *BCL11A* enhancer demonstrate comparable enrichment to targeting *BCL11A* coding sequence and demonstrate substantial enrichment as compared with the nontargeting controls. Taken together, these saturating mutagenesis pooled screen experiments suggest that functional sequence exists within the *BCL11A* enhancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1 | Selection of sgRNA based on on-target and off-target prediction scores

CRISPOR provides two separate types of predictions: on-target and off-target scores. Off-target scores try to estimate the off-target cleavage potential, which depends on the sequence similarity between the on-target sequence and predicted off-target sequences. Predictions are first calculated on the level of individual putative off-target sites using the cutting frequency determination (CFD) off-target score²⁴. An off-target site with a high off-target score is more likely to be cleaved than one with a low score. The scores of all off-target sites of a guide are then summarized into the 'guide specificity score'. It ranges from 0 to 100; a guide with a high specificity score is expected to have fewer off-target sites and/or off-target sites with lower cleavage frequencies than one with a lower score. It was previously shown that guides with specificity scores > 50 never lead to more than around a dozen off-targets and none had a total off-target cleavage > 10% in whole-genome assays⁹. These guides are shown in green by CRISPOR.

On the level of the individual site, off-targets with a CFD score < 0.02 are very unlikely to be cleaved based on previous analysis that demonstrated only a few true off-target events with such a score (Haeussler *et al.*⁹). For off-target sites that are cleaved, the CFD score is well-correlated with editing frequency, but predicted off-target sites are known to be primarily false positives. As such, for most guides, more than 95% of predicted off-target sites will not show any cleavage detectable with the sequencing depths in use at present. In addition, as summarized in Haeussler *et al.*⁹, a few guides with low specificity scores (in the range of 10–30) lead to fewer than three to five detected off-targets, so low specificity scores do not always translate to strong off-target effects.

The other type of prediction is on-target scoring, which tries to estimate on-target cleavage efficiency and depends only on the target sequence. CRISPOR offers eight algorithms in total, with two scores identified as most predictive when compared on independent data sets (refer to Haeussler *et al.* for further details)⁹. The algorithm by Doench *et al.*²⁴ (herein referred to as the 'Doench 2016 score') is optimal for cell-culture-based assays in which the guide is expressed from a U6 promoter as described in this protocol. The score by Moreno-Mateos *et al.*⁵² (CRISPRscan) is best for guides expressed *in vitro* with a T7 promoter (e.g., for mouse/rat/zebrafish oocyte injections, not discussed further here). On-target scoring is not very reliable, as the Pearson correlation between cleavage and the Doench 2016 score is only ~0.4. Despite this, there is an enrichment for top-scoring guides. For example, for guides in the top 20% of Doench 2016 scores, 40% of them are in the top quartile for efficiency, which is a 1.5-fold enrichment of top quartile guides as compared with random guide selection.

By default, guides in CRISPOR are sorted by guide specificity score and off-targets are sorted by CFD score. The relative importance of off-target specificity versus on-target efficiency depends on the experiment. In some cases, such as when targeting a narrow region, one has limited options, and a guide with low specificity (i.e., many/high-probability off-targets) is the only one available. In this case, more time and effort may be needed to screen for off-target effects. To provide an estimate of the number of off-target sites to test, previous work showed that most off-target cleavages for guides with a

specificity of > 50 are detected by screening the 96 predicted off-target sites with the highest CFD scores⁹.

Both off- and on-target scores have been precalculated for coding sequences (and ± 200 -bp flanking coding sequences) of most common model organisms and are shown when hovering with the computer mouse over a guide in the UCSC Genome Browser track 'CRISPR' in the group 'Genes and Gene Predictions' at <http://www.genome.ucsc.edu/cgi-bin/hgTracks?db=hg19>. The sequence shown in the UCSC Genome Browser can be sent directly to CRISPOR by selecting 'View – In external tools' in the Genome Browser menu. In the UCSC track, guides are colored by predicted on-target efficiency, whereas they are colored by off-target specificity on crispor.org.

It can be particularly important to use off-target scoring prediction to aid in saturating mutagenesis experiments for sgRNA depletion or dropout¹². sgRNAs with highly probable off-target sites may deplete/drop out from screens due to cellular toxicity resulting from multiple cleavages, as opposed to a biological effect from mutagenesis of the on-target site.

Box 2 | Optimization of parameters for CRISPResso analysis

CRISPResso allows many parameters to be set/adjusted based on the desired analysis to be performed. Details on all the parameters are available in the online help section (<http://crispresso.rocks/help>). Here, we discuss the key parameters that can significantly affect the quantification.

Amplicon sequence (-a) (required; Step 83)

This is the sequence expected to be observed without any edits. The sequence must be reported without adapters and barcodes.

sgRNA sequence (-g) (optional; Step 83)

This is the sequence of the sgRNA used, and it should be a subsequence of the amplicon sequence (or its reverse complement). Although this parameter is optional, it is required to specify it to enable the window mode (see the parameter `--window_size` in order to reduce false positives in the quantification). It is important to remember that the sgRNA must be input as the sgRNA sequence (usually 20 nt) immediately upstream of the PAM sequence for Cas9 species (Supplementary Table 1). For other nucleases, such as Cpf1, enter the sequence (usually 20 nt) immediately downstream of the PAM sequence and explicitly set the cleavage offset (see the parameter: `--cleavage_offset`).

Coding sequence (-c) (optional; Step 83)

The subsequence of the amplicon sequence covering one or more coding sequences. Without this sequence, frameshift analysis cannot be performed (Step 83).

Window size (-w) (optional; Step 83)

This parameter allows for the specification of a window(s) in bp around each sgRNA to quantify indels. This can help limit sequencing/amplification errors and/or non-editing polymorphisms (e.g., SNPs) from being inappropriately quantified by CRISPResso's analysis. The window is centered on the predicted cleavage site specified by each sgRNA. Any indels that do not overlap and/or substitutions that are not adjacent to the window are excluded from analysis. A value of 0 will disable this filter (default: 1).

Cleavage offset (--cleavage_offset) (optional; Step 83)

This parameter allows for the specification of the cleavage offset to use with respect to the provided sgRNA sequence. The default is -3 and is suitable for *S. pyogenes* Cas9. For alternative nucleases (Supplementary Table 1), other cleavage offsets may be appropriate. For example, set this parameter to 1 if using Cpf1.

Average read and single-bp quality (--min_average_read_quality and --min_single_bp_quality) (optional; Step 83)

These parameters allow for the specification of the minimum average quality score or the minimum single-bp score for inclusion of a read in subsequent analysis. The scale used is Phred33 (default: 0, minimum: 0, and maximum: 40)⁸⁹. The PHRED score represents the confidence in the assignment of a particular nucleotide in a read. The maximum score of 40 corresponds to an error rate of 0.01%. This average quality of a read is useful to filter

out low-quality reads. More-stringent filtering can be performed by using the single-bp quality; any read with a single-bp quality below the threshold will be discarded. A reasonable value for this parameter is > 20 .

Identity score (`--min_identity_score`) (optional; Step 83)

This parameter allows for the specification of the minimum identity score for the alignment (default: 60.0). For a read to be considered properly aligned, it should pass this threshold. We suggest lowering this threshold to < 50.0 only if large insertions or deletions are expected in the experiment ($> 40\%$ of the amplicon length) (Step 83).

Exclude ends of the read (`--exclude_bp_from_left` and `--exclude_bp_from_right`) (optional; Step 83)

Artifacts are sometimes present at the ends of the reads due to imperfect adapter trimming or a drop in quality scores. For example, if the ends of reads appear to show indels or appreciable editing frequencies, this may be due to sequencing artifacts, as opposed to true indels from genome editing. Therefore, to exclude these regions at the ends of reads, these parameters allow for the exclusion of a few bp from the left and/or right of the amplicon sequence during indel quantification (default: 15) (Step 83).

Trimming of adapters (`--trim_sequences`) (optional; Step 83)

This parameter enables the trimming of Illumina adapters with Trimmomatic (default: False). For custom adapters, the user can customize the Trimmomatic execution using the parameter `--trimmomatic_options_string` (check the Trimmomatic manual for more information on the different flags and options: http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic/TrimmomaticManual_V0.32.pdf)⁹⁰. It is important to check with your sequencing facility to determine whether the reads were trimmed for adapters or not. If this is not possible, we suggest using the software FASTQC to determine whether reads were already trimmed (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). To perform this analysis, open a terminal and type `fastqc`. This command will open the main software window. After opening the FASTQ file(s) to analyze with FASTQC, a graphical report will be automatically generated. In the graphical report, find the section 'Adapter content'. If the reads are not properly trimmed, the adapter used will be reported. If a custom adapter was used, find the section 'Overrepresented sequences' instead, where all the sequences that are present in more than 0.1% reads are reported. A subsequence present in more than 10% of the reads is usually an indication that the reads were not properly trimmed. An example of FASTQC report is available here: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/RNA-Seq_fastqc.html#M9.

Box 3 | Technical and experimental considerations for performing an arrayed CRISPR genome-editing experiment

Here are some practical guidelines for executing an arrayed CRISPR experiment. An experimental schematic can be found in Figure 1 and an example workflow in Figure 3:

Generation of cells with stable CRISPR nuclease expression

For cell lines, it is convenient to generate lines with stable CRISPR nuclease expression for arrayed experiments. This can be accomplished by transducing cells with a lentiviral CRISPR nuclease with subsequent selection for transduced cells, such as the usage of lentiviral Cas9 with blasticidin resistance (the focus of the remainder of the discussion here will be on the usage of *S. pyogenes* Cas9; however, the same principles apply to other CRISPR nucleases, including Cpf1). It is recommended that a ‘kill curve’ be created to determine the optimal concentration of blasticidin for the cells used before beginning the experiment. It is also important to determine the duration of selection required to complete cell death by blasticidin, as assessed by cell viability over time with blasticidin selection. Stable expression of Cas9 can be confirmed via western blot. Alternatively, stable expression can be confirmed by assessing Cas9 function using a reporter system, such as the previously described constructs that provide GFP and an sgRNA targeting GFP to assess for functional Cas9 through flow cytometry (MATERIALS)^{12,50}. It is possible to screen for clones with high Cas9 expression and/or high Cas9 activity, as assessed by a reporter; however, it is not required. If stable Cas9 expression cannot be generated, such as with usage of primary cells with limited culture duration, cotransduction of Cas9 and sgRNAs can be performed with double selection (blasticidin for Cas9 and puromycin for sgRNA). Cotransduction can occur simultaneously or can occur on back-to-back days. Selection by blasticidin and/or puromycin is typically performed 24–48 h after transduction.

Arrayed sgRNA experiment execution

Unlike a pooled screen experiment as described in Box 4, copy number (i.e., number of viral integrants per cell) may not be an important consideration for many experiments, as only one sgRNA is being used. Therefore, it may be important only for certain applications for cells to have one copy of the sgRNA (i.e., comparing sgRNA efficiencies) or have a mixture of ≥ 1 copy (e.g., experiments aiming to simply have maximal editing frequency). Therefore, lentiviral transduction of Cas9-expressing cells can occur at low multiplicity (goal transduction rate of 30–50%) to ensure single integrants (i.e., one sgRNA per cell)^{91–93}. Alternatively, lentiviral transduction of Cas9-expressing cells can occur at high multiplicity (transduction rate > 50%) to obtain a higher rate of transduced cells with a resulting heterogeneous mix of sgRNA copy number. For an arrayed experiment with one sgRNA, multiple integrants per cell do not result in ‘passenger’ sgRNAs and associated false positives. Lentiviral transduction efficiency is affected by cell density, volume, incubation time, and multiplicity of infection (MOI). Therefore, it is important to keep all of these factors constant to ensure consistent lentiviral transduction rates. Notably, this underscores the importance of aliquoting lentivirus to ensure consistent lentiviral titer, as titer is decreased by freeze–

thaw cycles (see Steps 60 and 71). A decrease in cell density, a decrease in media volume, and an increase in MOI will all increase transduction rates. Similar to blasticidin, it is recommended to create a kill curve and determine the duration of selection. Longer exposure to the CRISPR/Cas9 reagents results in increased editing rates¹⁴. A reasonable experiment duration is 1–2 weeks¹⁴; however, this may vary based on the experimental system used. It is possible to use a Cas9 nuclease activity reporter as described above to more accurately determine when editing has plateaued in the experimental system. At the end of the experiment, cell pellets can be made to proceed with deep sequencing (Steps 73–82).

Enhancement of lentiviral transduction

Multiple methods exist to enhance lentiviral transduction. Reagents such as polybrene, rapamycin, protamine sulfate, and prostaglandin E2 have been shown to enhance lentiviral transduction rates⁹⁴. In addition, lentiviral spin-infection (centrifugation during transduction) can enhance transduction rates⁹⁵. These types of methodologies to enhance lentiviral transduction can be useful to reduce the amount of lentivirus required for experiments, can help achieve the desired transduction rates in the setting of low lentiviral titer, and can increase efficiency for difficult-to-transduce cells. It is important to determine whether any of these reagents/methods lead to cellular toxicity.

Box 4 | Technical and experimental considerations for performing a pooled CRISPR genome-editing experiment

Here are some practical guidelines for executing a pooled screen using an sgRNA library with 1,000 sgRNAs with a goal of 1,000× representation of the sgRNA library. An experimental schematic can be found in Figure 2 and an example workflow in Figure 3:

Generation of cells with stable CRISPR nuclease expression

For cell lines, it is convenient to generate lines with stable CRISPR nuclease expression for pooled experiments. This can be accomplished by transducing cells with a lentiviral CRISPR nuclease, with subsequent selection for transduced cells, such as the usage of lentiviral Cas9 with blasticidin resistance (the focus of the remainder of the discussion here will be on the usage of *S. pyogenes* Cas9; however, the same principles apply to other CRISPR nucleases, including Cpf1). It is recommended that a kill curve be created to determine the optimal concentration of blasticidin for the cells used before beginning the experiment. It is also important to determine the duration of selection required to complete cell death by blasticidin. Stable expression of Cas9 can be confirmed via western blot. Alternatively, stable expression can be confirmed by assessing Cas9 function using a reporter system, such as the previously described constructs that provide GFP and an sgRNA targeting GFP to assess for functional Cas9 through flow cytometry (MATERIALS)^{12,50}. It is possible to screen for clones with high Cas9 expression and/or high Cas9 activity, as assessed by a reporter; however, it is not required. If stable Cas9 expression cannot be generated, such as with usage of primary cells with limited culture duration, cotransduction of Cas9 and sgRNAs can be performed with double selection (blasticidin for Cas9 and puromycin for sgRNA). Cotransduction can occur simultaneously or can occur on back-to-back days. Selection by blasticidin and/or puromycin is typically performed 24–48 h after transduction.

sgRNA library representation

Library representation refers to estimating how frequently each sgRNA in the library is included in the experiment. 1,000× representation of a library suggests that 1,000 cells were transduced by the median sgRNA in the library at the beginning of the experiment. Suboptimal representation may lead to the absence of certain sgRNAs from the screen experiment and thus can cause sgRNAs to appear falsely depleted from the experiment. The desired level of representation may reflect the expected uniformity of genome-editing outcome and biologic phenotype, as well as the distribution of sgRNA abundance within the library. For example, true-positive sgRNAs expected to have uniform genetic and biological effect and narrowly distributed abundance may require lower representation to be accurately identified as hits. To represent a 1,000 sgRNA library at 1,000×, 1 million cells must be transduced (1,000 sgRNAs × 1,000 cells each = 1,000,000 cells).

Transduction at low multiplicity

Lentiviral transduction of Cas9-expressing cells at low multiplicity ensures single integrants (i.e., one sgRNA per cell)^{91–93}. As such, the goal transduction rate is 30–50%.

Transduction rates > 50% increase the risk of multiple integrants per cell, which can result in ‘passenger’ sgRNAs and associated false positives. Lentiviral transduction efficiency is affected by cell density, volume, incubation time, and MOI. Therefore, it is important to keep all of these factors constant to ensure consistent lentiviral transduction rates. Notably, this underscores the importance of aliquoting lentivirus to ensure consistent lentiviral titer, as titer is decreased by freeze–thaw cycles (Steps 60 and 71). The example screen requires transduction of 1,000,000 cells to represent the sgRNA library at 1,000×; however, this refers to the number of transduced cells. As the transduction rate must be between 30 and 50%, this requires using 2,000,000–3,333,333 cells at the beginning of the experiment, which will be reduced to 1,000,000 cells upon selection by puromycin for successful transductants.

Determination of screen conditions

For this example, the goal will be a 40% transduction rate. Therefore, the experiment will require 2,500,000 cells ($2,500,000 \times 0.4 = 1,000,000$). As previously described, lentiviral transduction efficiency is affected by cell density, volume, incubation time, lentiviral titer, and MOI. Constant titer will be assumed due to appropriate lentivirus aliquoting (Steps 60 and 71). Increasing media volume results in lower transduction. Therefore, it is useful to limit the amount of media. In addition, it can be simpler to perform the transduction in ‘parts’ (e.g., ten separate transductions of 250,000 cells). A reasonable approach would include transducing ten wells in a 24-well plate of 250,000 cells in 500 μ l of medium (cell density of 500,000 cells/ml). The use of a 24-well plate and the cell density of 500,000 cells/ml are reasonable approaches; however, these may need to be optimized for the cells used for study and/or experimental goals. Transductions should be performed at different MOIs to determine the MOI needed to achieve the goal transduction rate of 40%.

Enhancement of lentiviral transduction

Multiple methods exist to enhance lentiviral transduction. Reagents such as polybrene, rapamycin, protamine sulfate, and prostaglandin E2 have been shown to enhance transduction rates⁹⁴. In addition, lentiviral spin-infection (centrifugation during transduction) can enhance transduction rates. These types of methodologies to enhance lentiviral transduction can be useful to reduce the amount of lentivirus required for experiments, can help achieve the desired transduction rates in the setting of low lentiviral titer, and can increase efficiency for difficult-to-transduce cells. It is important to determine whether any of these reagents/methods lead to cellular toxicity. If a reagent/method is used, it should be used for all transduction samples. In addition, the reagent/method should be used when determining the MOI to achieve the goal transduction rate (40% in this case).

Pooled sgRNA screen experiment execution

For this example, it will be assumed that an MOI of 1 results in a 40% transduction rate. To perform the screen, ten wells in a 24-well plate with 250,000 cells in 500 μ l of medium (cell density of 500,000 cells/ml) are transduced at an MOI of 1. 24 h after transduction, the ten wells can be pooled into one larger well or flask. Before selection

for successful transduction, it is useful to remove a subset of cells from the pooled transductions to determine transduction efficiency to ensure that ~40% transduction was empirically achieved. A reasonable approach would include pooling all ten wells together for a total of 2,500,000 cells in 5 ml of medium 24 h after transduction. ~25,000–50,000 cells can be removed from this 5-ml cell mixture to be used to determine transduction rate by splitting the cells into with and without puromycin conditions to confirm the transduction rate by cell counts post selection. After removal of ~25,000–50,000 cells, puromycin selection should be initiated on all the cells in the well/flask. It is recommended that a kill curve be created to determine the optimal concentration of puromycin for the cells used before beginning the screen experiment. It is also important to determine the duration of selection required to complete cell death by puromycin. Longer exposure to the CRISPR/Cas9 reagents results in increased editing rates¹⁴. A reasonable screen duration is 1–2 weeks¹⁴; however, this may vary based on the experimental system used. It is possible to use a Cas9 nuclease activity reporter, as described above, to more accurately determine when editing has plateaued. At the end of the experiment, cell pellets can be made in order to proceed with deep sequencing (Steps 73–82).

Types of screens

Screens typically rely on either positive or negative selection. Common screen strategies involve determination of enrichment or dropout (‘depletion’) of sgRNAs. This can be achieved by deep sequencing either the plasmid pool or cells at an early time point in the experiment to serve as the initial time point for comparison. It has been previously shown that there is no difference between using the plasmid pool versus cells from an early time point in the experiment for this purpose^{14,96}. Deep sequencing of samples at the end of the experiment can then be compared with that at this initial time point. The sgRNA presence can be determined by enumerating sgRNAs using CRISPRessoCount (Figs. 2 and 3, Steps 84–86). Using enrichment/dropout strategies, screens can be performed for applications such as drug/toxin resistance/susceptibility or FACS-based selection (using antibody or cell reporter). A summary of representative published screens is provided in Joung *et al.*²⁰.

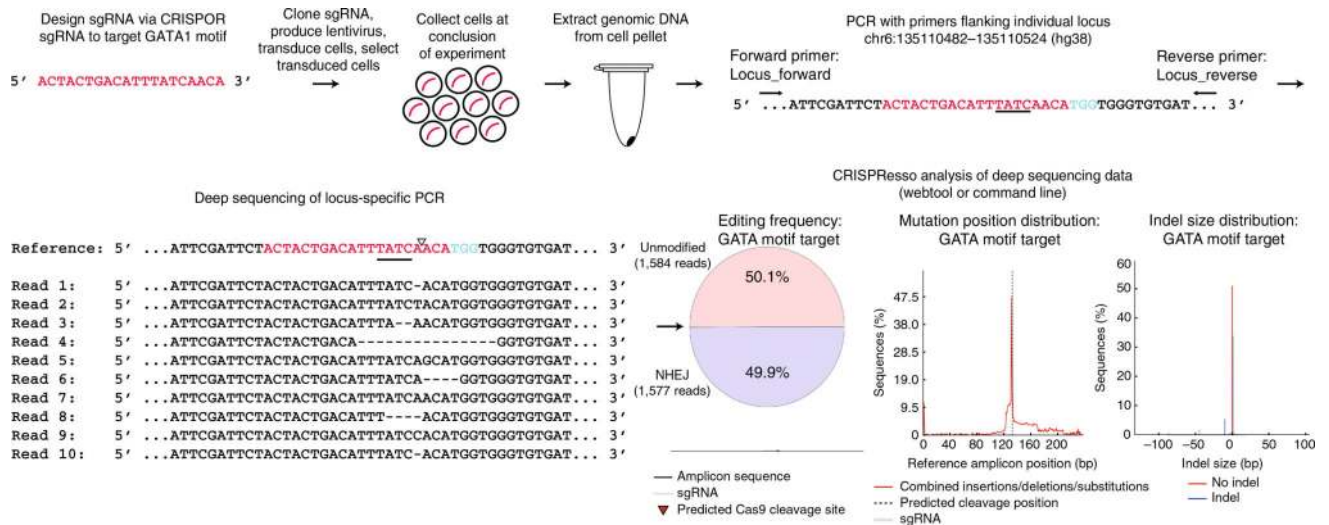


Figure 1.

Schematic of an arrayed genome-editing experiment. Arrayed genome-editing experiments are performed by designing one sgRNA using CRISPOR. This schematic demonstrates the design of an sgRNA to mutagenize a GATA motif. After designing the optimal sgRNA, it is cloned into pLentiGuide-puro, lentivirus is produced, cells are transduced, and successful transductants are selected (successful transduction is indicated by red curved lines). After conclusion of the experiment, cells are pelleted, and genomic DNA is extracted. Locus-specific PCR primers are used to amplify regions flanking the double-strand break site. Deep sequencing of the amplicon generated by locus-specific PCR is subsequently performed. Quantification of editing frequency and indel distribution is determined by CRISPResso. The GATA motif is underlined, the triangle indicates the double-strand break position, and the PAM sequence is shown in blue.

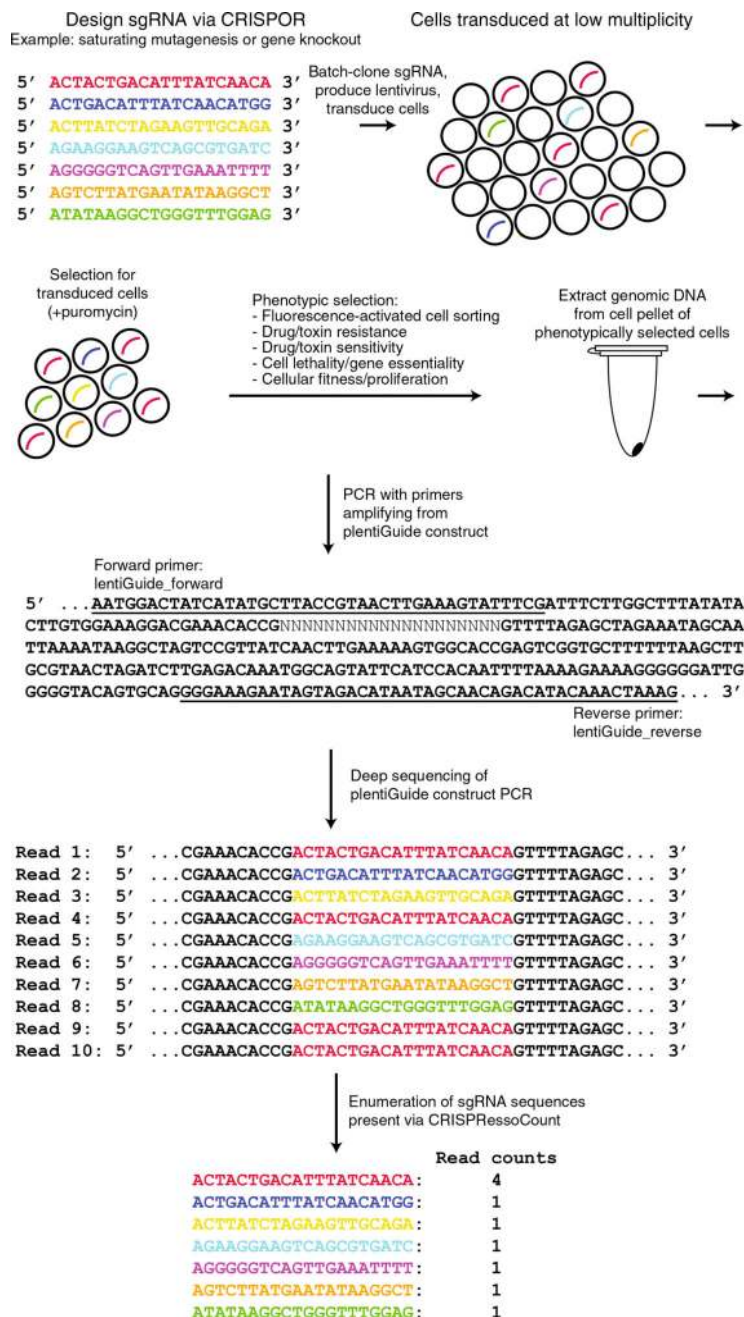


Figure 2. Schematic of a pooled genome-editing experiment. Pooled genome-editing experiments are performed by designing multiple sgRNA using CRISPOR. After designing the sgRNAs, they are batch-cloned into pLentiGuide-puro, lentivirus is produced, cells are transduced at low multiplicity, and successful transductants are selected (successful transduction is indicated by curved lines). Phenotypic selection is performed (e.g., FACS, drug/toxin resistance, drug/toxin sensitivity, cell lethality/gene essentiality, cellular fitness/proliferation). After conclusion of the experiment, cells are pelleted, and genomic DNA is extracted. PCR primers specific (primer sequence is underlined) to the pLentiGuide-Puro

construct are used to amplify regions flanking the cloned sgRNA sequence. Deep sequencing of the amplicon generated by construct-specific PCR is subsequently performed. sgRNAs present within the sample are enumerated by CRISPRessoCount.

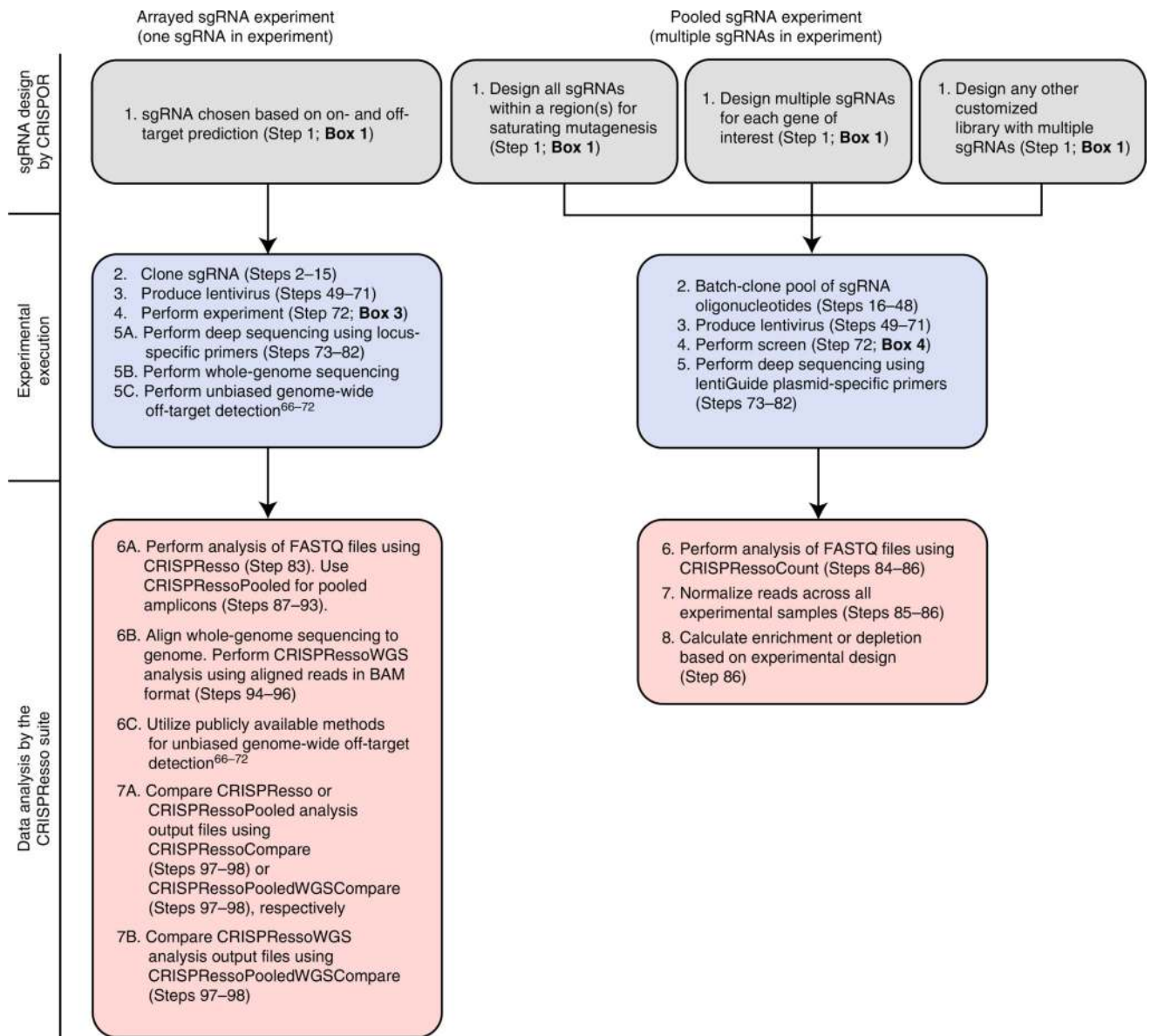


Figure 3. Design, experimental execution, and data analysis workflows for arrayed and pooled genome-editing experiments. sgRNA design steps by CRISPOR are shown in gray, experimental execution steps are shown in blue, and data analysis steps by CRISPResso are shown in red/pink.

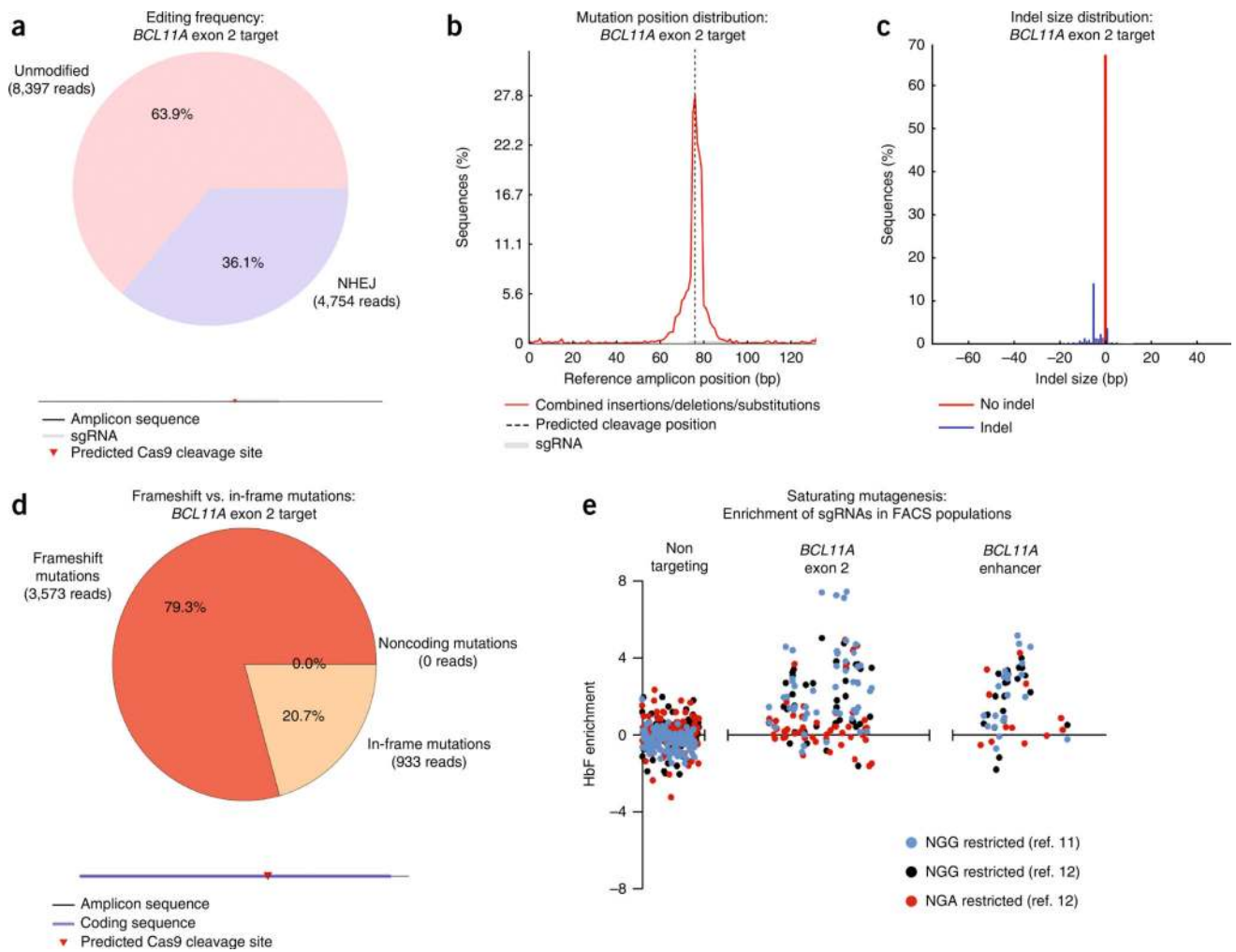


Figure 4. Locus-specific deep-sequencing analysis of coding and noncoding targeting by CRISPResso. **(a)** Frequency distribution of alleles with indels (shown in blue) and without indels (shown in pink) for an sgRNA targeting *BCL11A* exon 2. **(b)** All reads with sequence modifications (insertions, deletions, and substitutions) are mapped to a position within the *BCL11A* exon 2 reference amplicon. The vertical dashed line indicates the position of predicted Cas9 cleavage. The position of the sgRNA is shown in gray. **(c)** Distribution of indel sizes when targeting *BCL11A* exon 2. Percentage of unmodified sequences is shown in red and percentages of modified sequences are shown in blue. **(d)** Frameshift analysis of *BCL11A* exon 2 coding sequence targeted reads. Frameshift mutations are shown in red and in-frame mutations are shown in tan. **(e)** sgRNA enrichment based on analysis of fetal hemoglobin (HbF) levels when performing saturating mutagenesis of *BCL11A* exon 2 and analysis of the functional core of the *BCL11A* enhancer using NGG- and NGA-restricted sgRNAs from two previously published studies 11,12. Nontargeting sgRNAs are pseudomapped with 5-bp spacing.

Table 1

CRISPResso analysis suite.

Name	Format	Purpose	Input file formats	Comments
CRISPResso	Webtool	Analysis of single amplicon/locus deep sequencing	.fastq or .fastq.gz	http://www.crispresso.rocks
CRISPResso	Command-line version	Analysis of single amplicon/locus deep sequencing	.fastq or .fastq.gz	Large file support; batch mode capability
CRISPRessoPooled	Command-line version	Analysis of pooled amplicon experiments	fastq or .fastq.gz	—
CRISPRessoWGS	Command-line version	Analysis of WGS data or pre-aligned reads	.bam	Useful to interrogate any region of the genome for off-target effects
CRISPRessoCompare	Command-line version	Comparison of two CRISPResso analyses	Output for CRISPResso analysis on two different samples	Useful to compare treated and untreated samples or to compare different experimental conditions
CRISPRessoPooledWGSCompare	Command-line version	Compare experiments involving several regions analyzed by either CRISPRessoPooled or CRISPRessoWGS	Output from CRISPRessoPooled or CRISPRessoWGS analysis on two different samples	—
CRISPRessoCount	Command-line version	Enumerate sgRNAs present within a given sample	.fastq or .fastq.gz	Useful to perform enrichment or dropout ('depletion') analysis for a pooled screen

Table 2

Four output files from CRISPOR sgRNA design analysis.

Output number	Filename	Description	File columns
1	REGION_1_sat MutOligos.tsv	This file contains the sequences to order from a custom oligonucleotide pool supplier (MATERIALS)	<ul style="list-style-type: none"> • GuideId: the identifier of the sgRNA sequence in the input sequence. It consists of the position of the PAM and the strand, e.g., '4rev' • targetSeq: the sgRNA sequence, including the PAM • mitSpecScore: the MIT Guide Specificity score (0–100, higher score = lower off-target potential) • off-target count: number of predicted off-target sites (by default at four mismatches) • targetGenomeGeneLocus: gene symbol and sequence location name, e.g., 'exon:PITX2' • Doench'16EffScore: the Doench 2016 guide efficiency score²⁴ • Moreno-MateosEffScore: the Moreno-Mateos 2015 (CRISPRscan) guide efficiency score⁵² • OligoNucleotideAdapterHandle + PrimerFw: the forward oligonucleotide to order from a supplier • OligoNucleotideAdapterHandle + PrimerRev: the reverse oligonucleotide to order from a supplier
2	REGION_1_ontarget Primers.tsv	This file contains two primers for each sgRNA that can be ordered from an oligonucleotide supplier. The primers can be used to amplify the DNA fragment around each sgRNA	<ul style="list-style-type: none"> • guideId: sgRNA identifier, see above • forwPrimer: forward primer sequence • forwPrimerTm: forward primer Tm • revPrimer: reverse primer sequence • revPrimerTm: reverse primer Tm • ampliconSequence: the genomic sequence between forward and reverse primer
3	REGION_1_ontarget Amplicons.tsv	After sequencing using the ontargetPrimers file, this file can be used as input For CRISPRessoPooled to determine the cleavage frequency of each sgRNA	<ul style="list-style-type: none"> • guideId: sgRNA identifier, see above • ampliconSequence: the genomic sequence between forward and reverse primers, see above • guideSequence: the sgRNA sequence located within the amplicon
4	REGION_1_tar getSeqs.tsv	This file contains a list of all sgRNA sequences (one per line). It can be used to quantify the relative abundance of these sgRNA in a sample of sequenced cells and is one of the input files for CRISPRessoCount	List of all sgRNA sequences (one per line)

Table 3

Troubleshooting table.

step	problem	possible reason	solution
1A(ii)	Regions >2 kb or multiple regions	Large region or multilocus design	For batch mode or genomic regions >2 kb, the command-line version can be used (Step 1C)
1A(ii)	Genome of interest not available	–	If the genome of interest is not available at crispor.org , crispor@tefor.net . It is recommended to provide a link to download the relevant genome and the gene transcript models or the National Center for Biotechnology Information (NCBI) Assembly accession
1B(vi)	One or several genes from the gene list are not included in the gene-targeted library output from the CRISPOR Batch gene-targeting assistant	This probably results from incorrect entry of the gene symbol, Entrez Gene ID, or Refseq ID	Many genes have multiple names. Be sure to use the official gene symbol. This entry is case-insensitive
13	Low cloning efficiency	Inadequate lentiGuide-Puro plasmid dephosphorylation and/or Esp31 restriction enzyme digestion	Repeat lentiGuide-Puro plasmid dephosphorylation and/or Esp31 restriction enzyme digestion (Steps 5–9). Alternatively, increase the duration of dephosphorylation and/or restriction enzyme digestion
19	PCR failure	Too much oligonucleotide template	Dilute a subset of the oligonucleotide pool from Steps 17 and 18 based on the manufacturer's recommendations or try different dilutions (e.g., 1:10 dilution with nuclease-free water)
19	PCR bias	Suboptimal DNA polymerase	Q5 High-Fidelity DNA Polymerase may result in less PCR bias as compared with Phusion Hot Start Flex DNA Polymerase, so this reagent can be considered as an alternative
32, 34, 41	Low transformation efficiency/abnormal electroporation time constant	Abnormal reaction chemistry from use of too much Gibson assembly reaction mixture	Minimize Gibson assembly reaction mixture volume added (< 2 μ l, but 0.5–1 μ l is recommended)
41	Low cloning/transformation efficiency	Recombination of lentiviral long terminal repeats	Incubation at 32 °C instead of 37 °C can minimize recombination events and result in increased transformation efficiency
		Low efficiency of Gibson assembly reaction or no identifiable cause	Perform multiple Gibson assembly reactions. Combine Gibson assembly reaction mixtures and concentrate using minimum elution kit to elute in 10 μ l of nuclease-free water (MATERIALS)
		Suboptimal electrocompetent cells	Endura electrocompetent cells have demonstrated a higher efficiency; however, consider performing direct head-to-head comparison experiment with E. Cloni electrocompetent cells
42	Overrepresentation of single or a few sgRNA sequences	This probably results from PCR biases	Repeat library synthesis, beginning with lsPCR1 (Step 19), and ensure usage of the correct primers for the lsPCR1 and lsPCR2 reactions. It is possible that the full-length oligonucleotides have been incorrectly synthesized; however, this possibility is a less likely alternative
	sgRNA from different libraries identified	This probably results from using the incorrect barcode-specific primers for the lsPCR1 reaction (Step 19) or contamination from other libraries during gel purification (Step 25 and 26)	Repeat the lsPCR1 reaction with the correct barcode-specific primers. Separate lsPCR2 samples with multiple empty lanes and use separate gel purification supplies for each sample

step	problem	possible reason	solution
	Identified sgRNA sequence(s) not present in the library being synthesized or any other barcoded libraries	This probably represents error in the oligonucleotide synthesis pipeline	Proceed with the protocol, as long as the majority (50–75%) of sgRNAs identified match sequences within the library. Contact the oligonucleotide manufacturer to inquire about its synthesis error rate
46	Low yield from maxi-scale plasmid preparation	This probably results from overloading the maxi-scale plasmid preparation column	Ensure that < 0.45 g of bacterial pellet is loaded onto each column
51	Low lentiviral titer	Mixing of plasmid DNA and PEI in DMEM with supplements	Charged proteins (e.g., from FBS) can interfere with the charge-based complexation of PEI with plasmid DNA. Repeat with filtered (0.22- μ m filter) DMEM without supplements
58	Viral supernatant becomes stuck in the filter	This is probably due to a large amount of HEK293 cells and other debris in the viral supernatant clogging the filters	After Step 57, transfer the viral supernatant to a fresh 50-ml tube and repeat centrifugation from Step 57 to further remove HEK293 cells and debris before proceeding to the filtration step
62	Disrupted or mixed sucrose/viral supernatant layers in the ultracentrifugation tube	This is probably due to the release of air bubbles while creating the layers. Alternatively, the layers are unlikely to form correctly if the pipette tip is not at the bottom of the tube while the sucrose is being dispensed	Minimize the release of air bubbles by pipetting carefully while layering sucrose at the bottom of the ultracentrifugation tube. Ensure that the pipette tip is at the bottom of the ultracentrifugation tube when layering sucrose
67	Debris at the bottom of lentivirus-containing ultracentrifuge tubes after ultracentrifugation	Absent, disrupted, or inappropriate sucrose gradient	See troubleshooting advice for Step 62. Handle lentivirus-containing ultracentrifuge tubes and buckets carefully to avoid disrupting the layers. Ensure that the sucrose solution is 20% (wt/vol) by mass
68	Low lentiviral titer	Loss of lentiviral pellet	Invert the tube as quickly as possible in a single motion/movement. Once the tube is inverted, do not revert to the upright orientation to avoid resuspension of viral pellet and subsequent loss of resuspended viral particles
75–77	PCR failure	Suboptimal DMSO concentration and/or annealing temperature	Separately perform DMSO concentration gradient and annealing temperature gradient for each primer set used (supplementary Fig. 2c)
83	Multiple sequencing files	Examination of multiple unique amplicons	Use command-line CRISPResso, as opposed to the webtool, for batch mode to expedite the analysis of all files
83	Low number of reads analyzed by CRISPResso despite high number of reads in the input file	If using paired-end reads, verify that the reads have sufficient overlapping sequence. In addition, verify that the correct reference amplicon has been provided	See considerations for amplicon design presented in the ‘Sequencing of arrayed experiments and pooled screens’ section
83	Low number of reads analyzed by CRISPResso despite high number of reads in the input file	This probably results from low-sequence-quality reads (low Phred33 scores) being removed from the analysis	Phred33-based read filtering is too stringent based on sequencing data quality. Adjust the following two parameters: ‘Minimum average read quality (phred33 scale)’ and ‘Minimum single-bp quality (phred33 scale)’. Refer to box 2 for further discussion of these parameters
83	Many indels created far from the predicted DSB position	This probably results from low sequencing quality, whereby sequencing errors are interpreted as indels. If a particular indel(s) is consistently present, it may represent a variant/mutation in the cells	Use the ‘Window size (bp around each side of cleavage site) to quantify NHEJ edits (if sgRNA sequence provided)’ feature to restrict the analysis of indels to a set interval centered around the predicted DSB position. Refer to box 2 for further discussion of this parameter

step	problem	possible reason	solution
83	CRISPResso cannot align any reads to the reference amplicon	The amplicon sequence provided is not correct, the wrong FASTQ file was uploaded, or the sample was inappropriately demultiplexed (if demultiplexing was performed)	Inspect the first few lines of your FASTQ file. The start of the reference amplicon sequence should match the start of each read (or the reverse complement of the read)
83	CRISPResso reports spurious indels localized at the end(s) of the amplicon	The reads may not be trimmed for adapters/barcodes	If reads are not already trimmed, select the adapters used for trimming under the 'Trimming Adapter' heading under the 'Optional Parameters'. Failure to trim adapters may result in false positives. For the command line version, it is necessary to add the option '--trim sequences'. Refer to box 2 for further discussion of adapter trimming
83	CRISPResso cannot align any sequence to the reference amplicon for paired-end reads if provided as a single FASTQ file	The paired-end reads are provided in a single file instead of the two that are required (a format usually referred to as interleaved)	In this case, it is necessary to use the command-line version of CRISPResso and add the option '--split_paired_end'
83	CRISPResso is not performing frameshift analysis	The subsequence of the amplicon corresponding to the exon(s) is not provided	It is required to add the option '-e' to the command line to enable frameshift analysis. In addition, the sequence of the amplicon corresponding to the exon must be a subsequence of the reference amplicon. Refer to box 2 for further discussion of this parameter
83A	Web-version analysis is very slow	It is likely that the web version is experiencing high traffic, which is causing the prolonged analysis times	The analysis speed of CRISPResso is dependent on the number of users running the analysis at the same time on the site. If the webpage is very unresponsive, we suggest using the command-line version, attempting the analysis at a later time, or providing an email address when submitting samples for analysis so that CRISPResso can email you when your analysis is complete
83A	Web version fails to complete the analysis because of the size of the uploaded FASTQ file(s)	The combined maximum size for the FASTQ file(s) must be <100 MB for the web version of CRISPResso	To analyze large file(s) (>100 MB), it is necessary to use the command-line version of CRISPResso (skip to Step 83B)
83B	The command-line version of CRISPResso is not running correctly	Be sure to install Anaconda Python 2.7, not Python 3.x, as CRISPResso is not compatible with Python 3	Install Anaconda Python 2.7, and then run the setup again
83B,C	CRISPResso analysis is taking too much time	The high number of reads analyzed is slowing down the computation	Consider speeding up the computation by adding the option '-p <number_or_processes >' to enable the parallel option feature. For example, in a machine with four cores, you can add the following flag to the command line to use all four cores (default is to use one core): '-p 4'
86	The enrichment score is not defined (contains not a number (NAN) or infinitive (INF))	If one of the two conditions has a value of 0, the ratio may be not defined	Add a pseudocount to both conditions before taking the ratio. For example, you can add 0.1 to all the scores. This can be accomplished with Excel or similar software
93	Some regions are not analyzed and reported using CRISPRessoPooled	By default, a region is analyzed and reported if it has at least 1,000 reads	If an amplicon(s) is not reported in the analysis, check whether the sequence used in the amplicon description file is correct. In addition, check the '--min_reads_to_use_region' parameter. This parameter allows for control over which amplicons have sufficient reads to be analyzed (default = 1,000).
96	Some regions are not analyzed and reported using CRISPRessoWGS	By default, a region is analyzed and reported if it has at least 10 reads	Adjust the '--min_reads_to_use_region' parameter. This parameter allows for

step	problem	possible reason	solution
			control over which regions have sufficient reads to be analyzed (default 10)

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