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Research Techniques Made Simple: CRISPR Genetic Screens

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Abstract

CRISPR and Cas proteins, often referred to as CRISPR/Cas, are the components of a bacterial genome editing system that can be used to perturb genes in cells and tissues. A classic application is to use CRISPR/Cas to generate genetic loss-of-function. When performed at large scale and combined with deep sequencing techniques, CRISPR-based perturbations can be performed in a high throughput setting to screen many candidate genomic elements for their roles in a phenotype of interest. Here, we discuss major considerations in the design, execution, and analysis of CRISPR screens. We focus on CRISPR knockout screens but also review adaptations to the CRISPR/Cas system that highlight the versatility of the system to make other types of experimental genetic changes as well. We also discuss examples of CRISPR genetic screens in investigative dermatology and how they may be used to answer key scientific questions in the field.

INTRODUCTION

The human genome contains over 20,000 protein-coding genes, and their disruption underlies many diseases, underscoring the need to comprehensively understand their biological functions. One approach to studying gene functions and biological phenotypes is to perform a genetic screen, which aims for systematic functional interrogation of many candidate elements in a single experiment (Doench, 2018; Ford et al., 2019; Sanjana, 2017; Schuster et al., 2019). In a typical cell culture–based screen, systematic loss-of-function of a set of candidates is applied to identify elements contributing to a phenotype of interest. RNA interference (RNAi) and transposon-based technologies have been used successfully, but since their development, CRISPR/Cas-based tools have become a preferred method for genetic screens (Doench, 2018; Ford et al., 2019; Guitart et al., 2016; Schuster et al., 2019).

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

GENE KNOCKOUT USING CRISPR IN POOLED HIGH-THROUGHPUT SCREENS

CRISPR-based screens demonstrate improved versatility, efficacy, and lower off-target effects compared with approaches such as RNAi (Ford et al., 2019; Guitart et al., 2016; Schuster et al., 2019). For a comprehensive description of the fundamentals of CRISPR-mediated genome editing, we refer to a previous Research Techniques Made Simple article (Guitart et al., 2016). In brief, the bacterial Cas enzyme (usually Cas9) is guided to a genomic DNA target by a single guide RNA (sgRNA), an approximately 20-nucleotide sequence that specifies the genomic target, such as a protein-coding gene. Once present at the target, Cas9 catalyzes a double-strand DNA (dsDNA) break. Cells repair the dsDNA break, most commonly by nonhomologous end joining (NHEJ). Because NHEJ is error-prone, small insertions or deletions (indels) are introduced during repair, which leads to frameshifts and/or a premature stop codon at the target that result in loss-of-function. The classical CRISPR-based nuclease approach is therefore also referred to as CRISPR knockout (CRISPR-ko).

By using one sgRNA, CRISPR-ko of a single target can be achieved. However, by using multiple sgRNAs designed to target distinct genes, the investigator can generate a sgRNA library, which allows high-throughput genetic screens (Figure 1). Construction of a library containing sgRNAs against all protein-coding genes enables a genome-wide screen, whereas a smaller library containing sgRNAs against preselected genes enables targeted assessment of a specific gene set. During a CRISPR screen (Figure 1), the sgRNA library is introduced into a cell population in a manner such that each cell receives only one sgRNA. As a result, each cell within the bulk population undergoes a single knockout event, but the targeted elements differ between cells. After subjecting the CRISPR-ko cells to assays that enable positive or negative selection for a phenotype of interest, the effect of a gene knockout can be quantitated by assessing the relative enrichment or depletion of the causative sgRNA compared with its abundance in the starting population (Figures 1 and 2).

Here, we provide an overview of the key steps in designing a CRISPR-ko screen. Furthermore, we review adaptations to the CRISPR/Cas system that extend the genomic targets that can be studied, discuss examples of CRISPR genetic screens in investigative dermatology, and provide examples of prominent CRISPR screens from other research fields.

Overview of the methodology

The design of a pooled genomic CRISPR-ko screen (Figure 1) is characterized by four key steps (Ford et al., 2019), each with specific considerations (Table 1) influencing practical execution and screen results.

Gene set to study and sgRNA library design

A first step in designing a CRISPR screen is to define the set of genes to study. The number of elements included in the screen determine the size, complexity, and cost of the experiment. A genome-wide CRISPR screen has the advantage of being comprehensive and

avoids pretest selection bias. In addition, several validated genome-wide CRISPR sgRNA libraries are publicly available and can save the investigator from the task of designing and building their own library. Addgene is a nonprofit repository that distributes predesigned CRISPR libraries (www.addgene.org/crispr/libraries/).

Two prominent examples of genome-wide CRISPR-ko libraries are the Genome-scale CRISPR knockout (GeCKO) (Sanjana et al., 2014; Shalem et al., 2014) and Brunello (Doench et al., 2016) libraries, which both target all protein-coding genes in the human genome. These libraries can be ordered as pooled plasmids or directly as ready-to-use lentiviral particles. Within investigative dermatology, a genome-wide CRISPR-ko screen using the GeCKO library was used to identify genes whose loss is involved in resistance to the therapeutic cancer drug vemurafenib (Shalem et al., 2014).

A genome-wide screen can be both labor- and resource-intensive, and a more focused screen may be appropriate for scientific objectives where reasonable filters can be applied to narrow down the screening candidate list. For instance, RNA sequencing data can be used to identify only the set of genes that are expressed in the condition or cell type of interest. Another approach is to focus on a certain class of elements, such as transcription factors, kinases, or RNA binding proteins (Doench, 2018). Each of these approaches results in a more directed and manageable screening strategy but may require a custom-designed CRISPR library. For example, a targeted CRISPR library was used to screen for kinases that have a role in IL-17–mediated inflammatory signaling in primary keratinocytes (Slivka et al., 2019).

The overall sgRNA library size is principally determined by the number of candidate genes and the number of sgRNAs per target. Each sgRNA varies in its knockout effectiveness and target specificity. If multiple different sgRNAs targeting the same gene lead to consistent outcomes, the confidence of the finding increases. Therefore, including multiple sgRNAs per target improves the sensitivity and specificity of a CRISPR-ko screen (Doench et al., 2016). Predesigned and validated genome-wide human and mouse CRISPR libraries typically include >3–4 independent sgRNAs per gene (Doench, 2018). Online tools can assist in the design of effective sgRNAs (Table 1 in Doench, 2018), or the investigator can select specific sgRNAs from previously designed libraries. Additionally, the sgRNA library should contain negative and positive controls. Negative controls are typically nontargeting sgRNAs whose sequences do not match any sites in the genome. These nontargeting sgRNAs can be used to assess neutral variations in sgRNA abundance in the screen (Figure 2). Positive controls are sgRNAs that target essential (housekeeping) genes such as ribosomal or proteasomal subunits. These positive controls should be depleted in CRISPR-ko screens and serve as benchmarks to judge the confidence of the screen. The library can be synthesized by commercial vendors as an oligonucleotide pool and cloned into target vectors (e.g., lentiviral plasmids).

Cells of interest and CRISPR library delivery

CRISPR screens can be performed in primary cells, such as keratinocytes, melanocytes, and fibroblasts (Fenini et al., 2018; Slivka et al., 2019; Sun et al., 2015). However, primary cells can be difficult to transfect, generate lower gene-editing efficiency, or may have cell

limitations that are incompatible with long-term library screens (Ford et al., 2019). For these reasons, use of transformed or immortalized cell lines, such as 293T or HeLa cells, are sometimes favored for their technical tractability.

CRISPR-based genome editing requires two components, the Cas9 protein and the sgRNA, which contains both a scaffold and a target-specific spacer sequence. There are several options to deliver these components into cells, each with their specific advantages and disadvantages (Table 2 in Ford et al., 2019). Some delivery methods can give rise to undesired effects, such as cytotoxicity or innate immunity responses (Kim et al., 2018). Therefore, choice and optimization of the preferred delivery method should be evaluated for the cell type of choice.

For many cell types, the preferred delivery is by utilizing lentivirus, which can stably integrate into the genome of the host and express Cas9 and RNA components. One important technical consideration is to determine how Cas9 is introduced into cells. Simultaneous delivery of both Cas9 and sgRNAs is a simple, one-step approach but can create variability in Cas9 protein expression among cells. Variability of Cas9 protein levels affects CRISPR-ko efficiency. An alternative approach is to establish or purchase cell lines stably expressing Cas9, such as from ATCC (www.atcc.org).

Most library vectors include antibiotic resistance and/or fluorescence markers, allowing for selection of successfully infected cells. When using lentivirus to deliver the sgRNA library, viral titers and infection efficiencies should be determined. CRISPR libraries should be infected at low infection efficiencies to maximize the number of cells receiving a single sgRNA. This results in a single perturbation event per cell, an assumption that underlies accurate screening analysis and identification of candidates (Figure 1). In general, infection efficiencies of 20–60% are recommended (Doench, 2018; Ford et al., 2019).

It is essential to determine the total number of cells needed to perform the CRISPR screen. A 1× representation indicates that the number of cells infected matches the number of sgRNAs. For a reliable CRISPR screen, sgRNA representation of 300–1000× assures that all screening sgRNAs are present in the cell population (Doench, 2018; Ford et al., 2019; Schuster et al., 2019; Yau and Rana, 2018). A CRISPR screen with 50,000 sgRNAs and an infection efficiency of 40% requires a starting number of 125,000 cells to achieve approximately 1× representation after selection (125,000 × 0.40 = 50,000) and 125 million cells for 1,000× representation. To maintain library representation, >50 million cells need to be propagated throughout the screen. Factoring in biological and technical replicates, it is easy to envision how a screen can become an intensive effort. These prescreen planning steps should be performed to accurately estimate the resources (lentivirus, cells, culture space, plasticware, culture media, etc.) that will be needed.

Choosing the phenotype assay

The next step in a CRISPR screen is to choose or design an assay that provides a basis for positive or negative selection of cells in the screened population. As every sgRNA inflicts a genetic perturbation, the response of each genetic perturbation occurs within the bulk

population of cells, and the effects are ultimately identified by changes in sgRNA abundance (Figure 1).

Most CRISPR screens are combined with assays that exert a selective stress on cell fitness. Cells with lower fitness decrease in abundance. Relative differences in cell fitness can be accelerated by applying the desired selective pressure, such as a drug or UVR. Although common, CRISPR screens are not just limited to cell fitness assays. Other groups have conducted CRISPR screens on cells engineered with a fluorescence reporter that activates upon triggering a desired phenotype (e.g., expression of cytokines) and combined their screen with FACS. Selection for a desired phenotype might require developing a novel assay. For instance, to select for cells with an active *Hedgehog* signaling pathway, researchers created an assay in which active Hedgehog signaling confers resistance to the antibiotic blasticidin, which allowed for their selection (Figure 2 in [Breslow et al., 2018]). Such a FACS-based phenotypic assay has also been applied in a CRISPR-ko screen in primary keratinocytes to screen for kinases affecting the expression of the cytokine IL-8 (Slivka et al., 2019).

Measuring and quantifying screen output

In the case of lentiviral-based screening, the sgRNA sequence delivered to a cell is integrated into the genome and serves as a unique identifier for that cell. sgRNAs that target genes involved in the phenotype of interest will be either enriched or depleted after the screen (Figures 1 and 2). To measure changes in sgRNA abundance, sgRNA sequences are amplified from genomic DNA isolated before and after the screen. Using primers flanking sgRNA sequences in bulk genomic DNA, deep sequencing is performed to assess sgRNA abundances (Yau and Rana, 2018).

Conceptually, a positive screen hit for a gene will result in multiple independent sgRNA abundances changing concordantly (Figures 1 and 2). A detailed review of CRISPR screen analysis is beyond the scope of this review, but many web-based and command-line analysis tools are available (Box 3 in [Schuster et al., 2019]).

LIMITATIONS, APPLICATIONS, AND FUTURE DIRECTIONS

CRISPR-ko screens can identify novel roles for genes contributing to a phenotype. However, even after a well-designed and executed screen, it is important to validate screen hits using an alternative knockdown method, such as RNAi, and/or by using complementary functional experiments. Ongoing innovations are expanding the application of CRISPR/Cas genetic screens to primary cells, tissue, and even in vivo models (Chow and Chen, 2018).

Although this review focuses on CRISPR-ko screens that classically target protein-coding genes, it is worthwhile to note that other genome elements can be studied as well. CRISPR screens have been used to study enhancers (Korkmaz et al., 2016) and microRNAs (Kurata and Lin, 2018). Additionally, the versatility of CRISPR/Cas has increased dramatically by re-engineering Cas proteins, such as the catalytically dead Cas9 protein (dCas9). By fusing dCas9 to different effector domains (Table 2), the variety of genomic elements that can be interrogated (especially those not reliably perturbed by small indels) can be expanded. As

a result, CRISPR has been applied to study the role of long noncoding RNAs (Liu et al., 2017). Recently, we performed a CRISPR screen using dCas9 to identify long noncoding RNAs contributing to epidermis formation (Cai et al., 2020).

In the future, CRISPR screens could be applied to address other questions in investigative dermatology. Can we identify novel therapeutic targets in keratinocyte cancers, melanomas, and other genetic skin diseases? What are the noncoding genomic regions that contribute to skin disease? For questions like these, CRISPR screens offer a powerful alternative way for new discoveries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CRISPR-ko	CRISPR knockout
dCas9	dead Cas9
dsDNA	double-strand DNA
GeCKO	Genome-scale CRISPR knockout
indel	insertion or deletion
NHEJ	nonhomologous end joining
RNAi	RNA interference
sgRNA	single guide RNA

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SUMMARY POINTS

Advantages

- The versatility and programmability of CRISPR/Cas genome editing enables high throughput genetic screens.
- CRISPR genetic screens enable a systematic evaluation of many genetic elements in a single experiment.
- The availability of predesigned CRISPR libraries provides opportunities to quick-start a CRISPR screen using prevalidated single guide RNAs.
- The wide variety of CRISPR toolsets enables the study of many classes of genetic element (proteins, microRNAs, noncoding genes, and enhancers).

Limitations

- CRISPR screens can be labor- and resource-intensive.
- Screen readouts might require the development of an assay that allows selection for a phenotype of interest.
- CRISPR screen hits need to be validated by complementary, independent functional techniques.

MULTIPLE CHOICE QUESTIONS

1. What is the main reason to perform a high throughput genetic screen, such as with CRISPR?

- **A.** It allows you to measure the effect of a gene in many different conditions.
- **B.** It provides a systematic assessment of genotype-phenotype relations in a systematic manner.
- **C.** It can be executed in every cell line because of the endogenous expression of CRISPR/Cas9 in mammalian cells.

2. What is the main outcome parameter of a CRISPR-mediated genetic screen?

- **A.** Changes in gene knockout frequencies.
- **B.** Changes in lentiviral multiplicity of infection.
- **C.** Changes in single guide RNA (sgRNA) abundance.

3. Which of the following parameters most likely ensures only one genetic perturbation per cell?

- **A.** An infection efficiency of 100%.
- **B.** An infection efficiency of 50%.
- **C.** An infection efficiency of 0%.

4. What directly contributes most to a higher statistical certainty of a CRISPR screen output?

- **A.** Increasing the number of independent sgRNAs per target.
- **B.** Amplifying the sgRNA library to high titer.
- **C.** Decreasing the number of cells in the screen.

5. Which of the following most accurately indicates a CRISPR screen hit?

- **A.** Decreased abundance of multiple sgRNAs targeting the same genomic element.
- **B.** Decreased mRNA expression of a candidate gene at the end of the screen.
- **C.** Markedly increased gain of abundance of a single sgRNA targeting a candidate.

See online version of this article for a detailed explanation of correct answers.

DETAILED ANSWERS

1. What is the main reason to perform a high throughput genetic screen, such as with CRISPR?

Answer: B. By using libraries of different sgRNAs, CRISPR screens allow direct perturbations of a large number of genes, which are tested in a phenotypic assay.

2. What is the main outcome parameter of a CRISPR-mediated genetic screen?

Answer: C. The sgRNA sequence delivered to a cell serves as a unique identifier for that cell and the gene that is targeted.

3. Which of the following parameters most likely ensures only one genetic perturbation per cell?

Answer: B. Infection efficiencies of 20–60% are recommended, which maximizes the number of cells receiving a single sgRNA.

4. What directly contributes most to a higher statistical certainty of a CRISPR screen output?

Answer: A. True positive screen hits should have multiple, independent sgRNAs targeting the same gene change consistently, which improves screen sensitivity and specificity.

5. Which of the following most accurately indicates a CRISPR screen hit?

Answer: A. CRISPR screens are assessed by sgRNA abundances and independent sgRNAs targeting the same gene that change consistently are true screen hits.

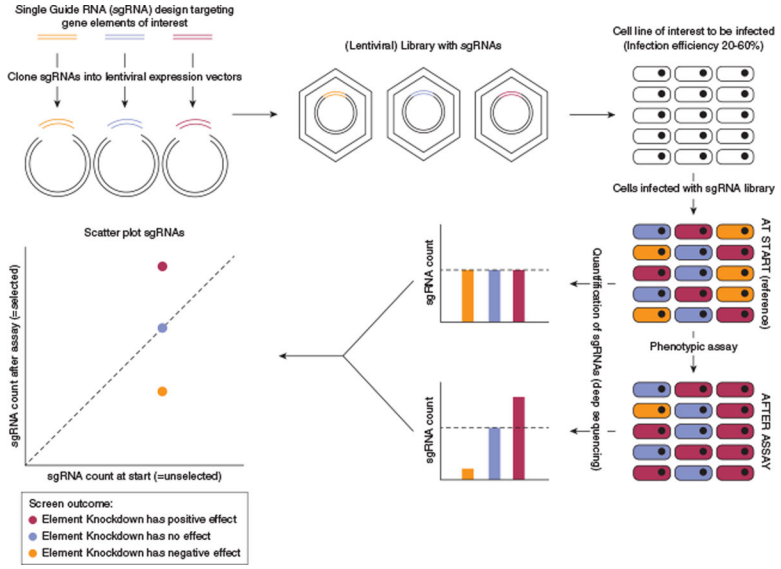

Figure 1. Overview of the key steps during a typical CRISPR-ko screen.

CRISPR sgRNA libraries can be ordered commercially, obtained through public repositories (e.g., Addgene), or custom-designed. For custom CRISPR libraries, pooled sgRNAs can be ordered as a DNA oligonucleotide pool. After PCR amplification, the library is cloned into a delivery vector (e.g., a lentiviral vector). Following packaging into lentiviral particles, the CRISPR library is infected into target cells at an infection efficiency of 20–60% to maximize the percentage of cells transduced with a single sgRNA. After infection, cells are selected with an antibiotic to deplete noninfected cells. A subset of cells is then collected as reference (at start, reference and/or unselected). An assay is then applied to select for cells displaying a desired phenotype, and cells are harvested at the endpoint and optionally at intermediate timepoints. Genomic DNA of both reference and endpoint cells is isolated and primers flanking the sgRNAs are deep sequenced from bulk DNA to measure the abundance of each sgRNA (sgRNA count) at each timepoint. sgRNA abundances can be visualized by plotting the sgRNA counts pre- and post-screen. Negative control sgRNAs (without biological targets) should appear around the dotted line, representing no change. CRISPR-ko, CRISPR knockout; sgRNA, single guide RNA.

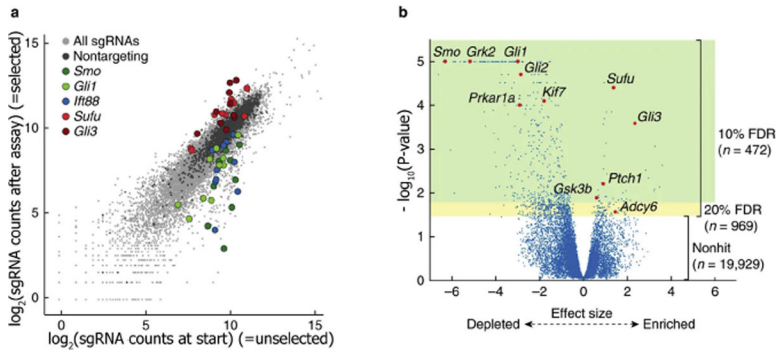

Figure 2. Example of output of a published CRISPR-ko screen.

(**a**) Scatter plot of a CRISPR screen aiming to systematically identify genes essential for Hedgehog signaling (Breslow et al., 2018). A transcriptional reporter assay allowed selection of cells in which the Hedgehog signaling pathway is active. The plot shows the abundance of sgRNAs (10 per target) at the start of the screen (x-axis; reference population) and after selection for cells with an active Hedgehog signaling pathway (y-axis). sgRNAs targeting selected genes are highlighted, similar colors indicate different sgRNAs targeting the same gene. (**b**) Volcano plot showing the effect size (x-axis) and P-values (y-axis) as calculated by the Cas9 high throughput likelihood estimator (casTLE) algorithm for this screen (Breslow et al., 2018). Select Hedgehog signaling pathway components are highlighted. Genes with P-value cut-offs corresponding to 10% FDR are highlighted in green, and those corresponding to a 20% FDR are in yellow. FDR, false discovery rate; sgRNA, single guide RNA. Reprinted with permission from Springer-Nature.

Table 1.

Key Considerations When Performing a CRISPR-ko Screen

Screen Phase	Considerations	Advantages/Disadvantages
I. Targets What genes will be studied?	Choice of library	• Genome-scale libraries have the benefit of being comprehensive but can be resource-intensive. Predesigned genome-wide CRISPR libraries are available • Targeted libraries focus on class of elements (kinases, transcription factors, etc.) or can be custom-designed and generated by the investigator
II. Model What cells should be used?	Cas9 expression	• Primary cells require delivery of both Cas9 and sgRNA, which may be technically challenging • Stably expressing Cas9 cell lines provide uniform high expression of Cas9
III. Assay How are cells screened?	Phenotype	• A classical screen results in positive or negative selection from a selective pressure (e.g., exposure to a drug) • Combining CRISPR screens with other genetic tools such as reporter cell lines can facilitate screening for diverse phenotypes
IV. Analysis How are screen results evaluated?	Measuring screen outputs	• Changes in sgRNA abundance, measured by next-generation sequencing, are a classical output of CRISPR-ko screens • A variety of validated CRISPR screen analysis pipelines are publicly available

Abbreviations: CRISPR-ko, CRISPR knockout; sgRNA, single guide RNA.

Table 2.

CRISPR Toolset for Genetic Screens

DNA Binding Protein	DNA Cleavage?	Effector	Mechanistic Result	Assess the Role of
Cas9	Yes	None	Loss-of-function (knockout)	Protein-coding genes, miRNAs, enhancers, …
dCas9	No	None	Transcriptional repression	All genes
		KRAB (CRISPR interference)	Transcriptional repression	All genes
		VP16, VP64 (CRISPR activation)	Transcriptional activation	All genes

Abbreviation: dCas9, dead Cas9.