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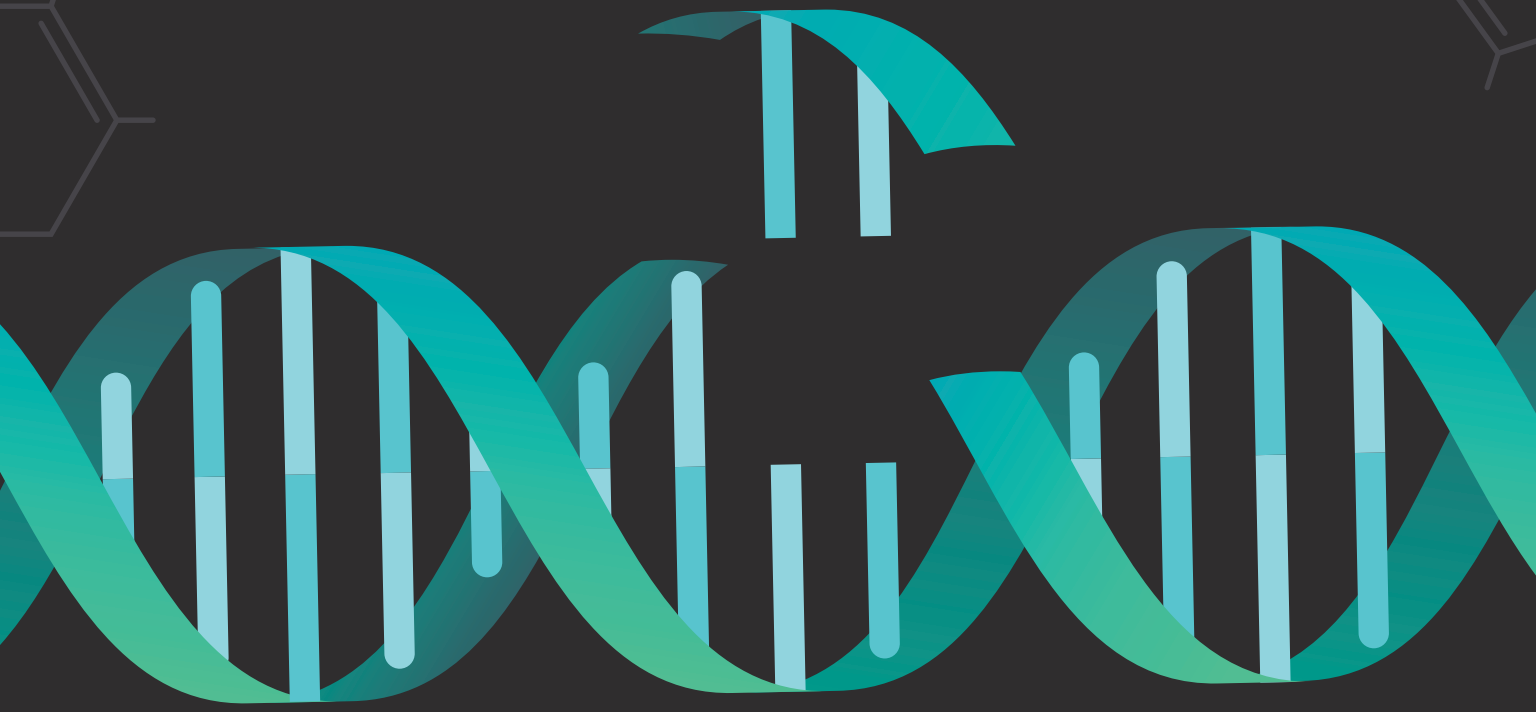

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CRISPR SCREENING 101

Genetic screens are powerful tools for understanding gene function. When performed in cell culture, each cell receives a different genetic perturbation. Researchers then identify the mutations that affected the biological process of interest by pooled screens.[1] Because they test thousands of individual perturbations in a single experiment, well-designed pooled screens can provide insights into many biological questions.

CRISPR technology allows researchers to target pre-selected genes with high specificity, and has become the preferred tool for functional geneticists to query the genome. A typical pooled CRISPR screen consists of three steps: small guide RNA (sgRNA) library development, the functional screen, and target validation.

sgRNA Library Development

When researchers embark on a screen, they first choose whether to use a readily available off-the-shelf sgRNA library or design a custom one that is tailored to their targets of interest. Here, it is important to consider scale: genome-wide screens allow unbiased identification of all genes involved in a biological process, but require the scientist to culture tens of millions of cells.[2] Depending on the scalability of the screened cell type, it may be better to design a custom sgRNA library that targets a set of genes involved in a specific pathway or biological process.

Next, scientists choose how many sgRNAs to develop per gene: typical libraries contain three to five sgRNAs per gene, while minimal libraries have two sgRNAs per gene to screen primary cells or cell lines that cannot be cultured at the scale needed to screen genome-wide libraries. While typical and minimal libraries are usually designed for genome-wide loss-of-function screens, tiling libraries contain more than 10 sgRNAs per gene to interrogate the functional domains of a small set of targets.

Once sgRNA library design is complete, sgRNAs are batch synthesized and cloned into plasmids. Several commercial oligo-synthesis platforms exist to create custom or predesigned sgRNA libraries.[1]

Many viral and non-viral vectors exist to deliver sgRNAs into cells.[3,4] Adenoviral, retroviral or lentiviral vectors are the preferred vectors for cell-based screens, as they have a high transduction efficiency and integrate the sgRNA fragments into the target cell genome, which helps researchers bypass proliferation-mediated sgRNA loss in dividing cells.[1]

Functional Screen

Researchers choose among several Cas9 versions to knock out, knock down, overexpress, change the methylation state, or alter the sequence of their genes of interest.[5] Transfecting cells with separate lentiviruses encoding Cas9 and the sgRNA ensures that each cell obtains a single copy of both components.[2] Next, researchers can apply a variety of selection criteria including survival, reporter activity, and gene expression to isolate cells showing interesting phenotypes. Regardless of the screen type, researchers perform next generation sequencing to compare read numbers for each sgRNA between the various screening conditions and identify the most promising candidate genes for validation.[6]

Target Validation

Following candidate gene identification, researchers must confirm the gene's involvement in the studied biological process. The likelihood of a candidate being a true positive depends on the number of sgRNAs identified from the screen that target the same gene. Identifying true hits often necessitates performing a secondary screen, where researchers build a custom library that contains more sgRNAs to target their top candidate genes. Next, it is important to perform on-target validation steps to verify that a top candidate's sequence or expression level was indeed altered by the targeting sgRNA, and that it caused the observed phenotype that was screened for. These experiments can include western blotting, immunofluorescence, and target gene sequencing. Finally, restoring gene function in the mutant cell to reverse the phenotype in rescue experiments unambiguously shows the gene's involvement in the screened biological process.

CRISPR technology has enabled scientists to perform functional genetic screens more easily. The nature of the sgRNA library and the design of the functional screen affects the number and quality of candidate genes the screen identifies. Carefully considering these factors increases the confidence of identified candidates and decreases the workload in downstream validation steps.

Custom CRISPR Screens: From Design to Validation

CRISPR screens are powerful tools for functional gene annotation. A typical CRISPR screen consists of three steps: small guide RNA (sgRNA) library development, the functional screen, and target validation. Researchers must optimize every step to maintain a uniform library throughout the screening process to maximize confidence in identified candidate genes.

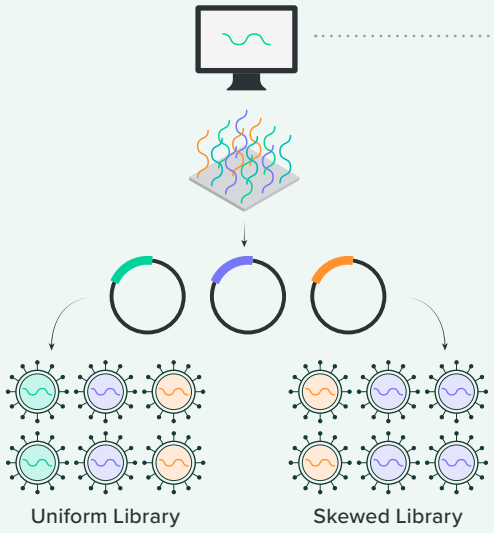
1. sgRNA Library Development

sgRNA design

High-fidelity batch synthesis of sgRNAs

Pooled cloning of plasmid library

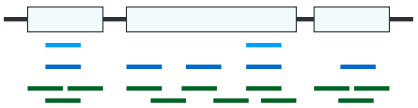
Uniform library generation of sgRNA-encoding lentiviruses



Deciding on Library Type

sgRNA library type depends on a screen's scalability and purpose. Regardless of library type, researchers can opt for a genome-wide versus custom library.

- **Minimal library** (2 sgRNAs/gene): for cell types that are difficult to culture at scale
- **Typical libraries** (3-5 sgRNAs/gene): increases statistical power of hits
- **Tiling libraries** (>10 sgRNAs/gene): interrogates a gene's functional domains.



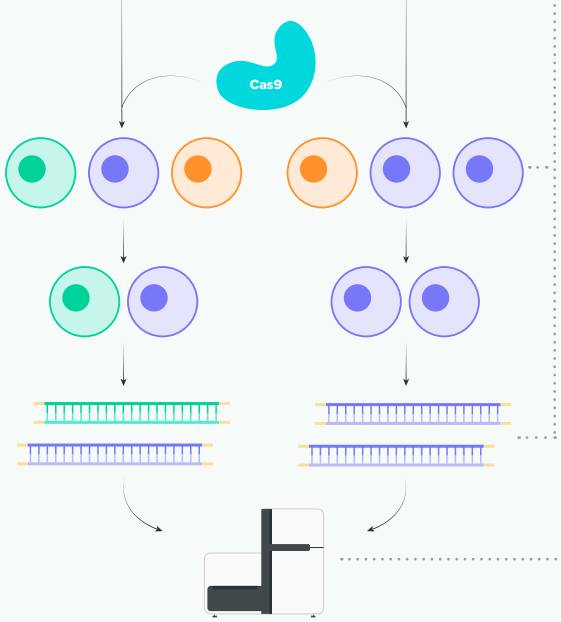
2. Functional Screen

Transduce cells with sgRNA and Cas9

Select for cells showing desired phenotype

Capture and amplify sgRNA

Sequence and identify target



Library Uniformity

Scientists must ensure that their library is fully represented at every step of the screen. Loss (green) or overrepresentation (purple) of even a small percentage of sgRNAs introduces bias through the absence of screened targets or the need to oversample to ensure all targets are represented.

Sequencing and Target Identification

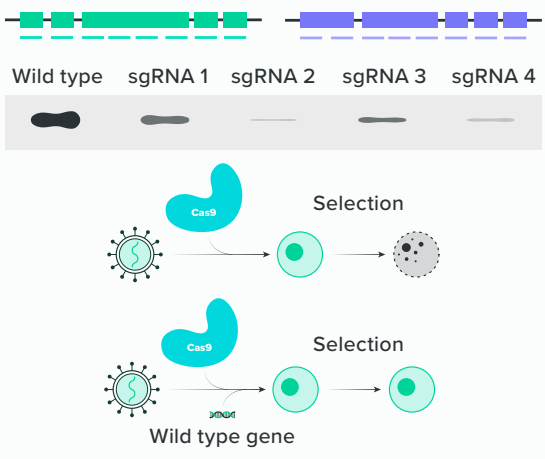
Massive parallel sequencing and bioinformatic analysis identifies enriched or depleted sgRNAs. Researchers identify top hits by mapping sgRNAs to target genes.

3. Target Validation

Secondary screen

Target analysis

Rescue experiment



Target Validation

Researchers validate top hits in three ways:

- Perform a secondary screen with a custom sgRNA library containing more sgRNAs per gene
- Confirm altered expression level of sgRNA target gene
- Perform rescue experiments with synthesized wildtype gene constructs to confirm candidate gene involvement in the screened biological process

THE GOOD, THE BAD, AND THE BIASED

The quality and uniformity of a small guide RNA (sgRNA) library will make or break a screen. Every step of library creation—sgRNA design, oligo synthesis, library cloning, and amplification—is important to ensure that the resulting library contains an equal distribution of all sgRNAs that will allow researchers to conduct an unbiased screen of selected genes. In the last decade, scientists have developed numerous resources to facilitate library creation. Platforms exist to help design and synthesize sgRNAs, minimal, typical, and tiling sgRNA libraries are available for nearly every model organism, and a flurry of viral and non-viral vectors target genes in the selected model while providing various selection and screening options.[1] While these options allow researchers to choose the reagents that best fit their screens, navigating through them can be a daunting task.

Selecting the Right sgRNA Library

The ideal sgRNA design platform should use the most recent reference genome, as gene and transcript annotations change over time.[2] In addition, it is important to sequence the screened cell line and compare it to the reference genome, as a single base pair mismatch determines sgRNA on- or off-target activity. For the same reason, selecting a high-fidelity oligo synthesis platform will ensure a uniform, high-quality library with equal sgRNA distribution.

Off-the-shelf libraries are convenient since researchers can order or request them from various sources, but there are a few factors to consider when selecting this library type. Like sgRNA design platforms, a premade library depreciates over time due to the dynamic nature of the genome. To facilitate downstream analyses, it is a good idea to re-analyze the target sequences of an existing library's sgRNAs before starting a screen.[3] In addition, scientists should verify the compatibility of the library and the screened cell line to make sure that sgRNA target sites do not contain any polymorphisms in their cell model.

Off-the-shelf libraries may be distributed in small aliquots that scientists need to amplify prior to a screen's start. Researchers should verify that the obtained aliquot is a complete representation of the original library. A recent comparison of two off-the-shelf library aliquots showed a loss of approximately 1% of sgRNAs compared to the original library.[4] Such loss of representation introduces bias and compromises a screen from the start.

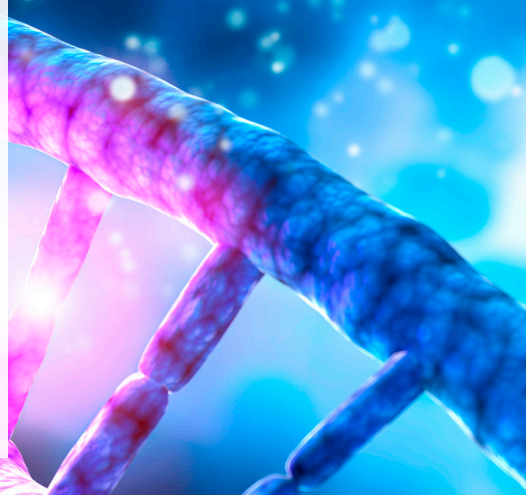
In contrast to genome-wide libraries that generally target a single transcript per gene, custom libraries can be optimized for the screened cell type. Sequencing the cell model's transcriptome can identify relevant isoforms and exclude non-expressed genes.[3] This way, researchers can increase their screen's efficiency and feasibility, which helps offset the increased cost of a custom library.

Maintaining an Unbiased Library

Designing or selecting a sgRNA library that is best suited for the cellular model and the screen's biological question is key to ensure the screen will yield promising candidates. The perfect sgRNA library is highly uniform, meaning it contains an equal representation of each sgRNA regardless of the library's size. Maintaining a well-balanced library is key to minimize bias-inducing factors and maintain library uniformity at all steps of a pooled screen. Ideally, scientists should avoid amplifying a library, as this complex process often decreases sgRNA distribution.[4] If a library needs amplification, researchers should limit the number of PCR cycles.[5] They should also scale each step of the cloning process to the library's size, obtaining 100 to 1000 bacterial colonies per library.[6] Next, scientists should culture bacteria so that there is no inter-colony competition to avoid amplification bias. Finally, they should deep sequence the plasmid DNA to confirm the amplified library's sgRNA distribution.

A poorly constructed or amplified library can cause an increase in empty vectors or a loss of uniformity, meaning that a subset of the library's sgRNAs becomes under- or overrepresented. This increases the cost and decreases the feasibility of a screen because it requires researchers to screen more cells to obtain full coverage of their library while it simultaneously increases downstream validation efforts. Scientists can increase the number of sgRNAs per gene to offset a biased library, but this will decrease screen feasibility because it significantly increases the number of cells that need to be cultured, screened, and sequenced. Incorporating steps to maintain high-quality libraries will ensure that the amplified library is an accurate reproduction of the starting library and will prevent library-induced bias from affecting the candidate genes identified by a screen.

THE RESULTS ARE IN: APPLICATIONS OF CUSTOM CRISPR SCREENS



CRISPR-based technology has revolutionized genetics research, and scientists are continuously developing new strategies and technologies to further increase the feasibility and applicability of CRISPR screens. Focused custom libraries permit researchers to screen more relevant cell types that are typically more difficult to culture at scale and allow them to tailor a library to answer specific gene functionality questions.

For example, scientists conduct CRISPR-based screens to identify a cancer cell's genetic vulnerability for drug discovery purposes. Konstantinos Tzelepis and colleagues used a CRISPR dropout screen to identify genes that are essential for acute myeloid leukemia (AML) cell survival.[1] To ensure specificity to AML, the team compared sgRNA-mediated lethality of five independent AML cell lines to two non-AML cell lines. Tzelepis identified 492 AML-specific essential genes and validated a selected subset with genetic and pharmacological inhibition. Independent teams have since confirmed that the screen's top hit, KAT2A, is required for AML stem cell maintenance.[2] During their screen, Tzelepis discovered that a subset of lentiviral-transfected cells acquired Cas9 mutations and failed to express the protein. Hence, researchers should sub-clone Cas9 expressing cells and verify both protein expression and function with a positive control before they start a functional screen.

Focus on the Immune System

CRISPR-based screens have also identified regulators of immune function. Eric Shifrut and his team screened primary human T cells for genes that regulate proliferation upon T cell activation.[3] To avoid establishing stable cell lines that express Cas9, the scientists electroporated Cas9 protein directly into primary cells. They also performed single-cell RNAseq of the screened T cells to better understand the CRISPR-induced consequences during T cell activation. This data revealed four promising targets for immunomodulatory cancer treatments. The team's follow-up experiments showed that loss of SOCS1, TCEB2, RASA2, or CBLB enhances T cell proliferation and cancer cell targeting efficiency in vitro.

Zeyu Chen and colleagues recently adapted CRISPR screens to study immune function in vivo.[4] The team isolated Cas9-expressing cells from mice and transfected them with sgRNA-containing retroviruses to target transcription factors. They next transferred the manipulated Tcells into virally-infected mice to identify sgRNAs that strengthened the mouse's immune response. In further studies, they showed that FLI1, the screen's top hit, is a key regulator of the effector T cell response. In sum, both screens identified promising therapeutic candidates to improve cancer immunotherapies.

The future of CRISPR screens

Most cell-based screens perturb a single gene and analyze its effect on survival or proliferation rate, but scientists are optimizing technologies to screen a wider range of phenotypes. For example, Valentina Diehl and colleagues developed covalently-closed circular-synthesized (3Cs) multiplexing, a method to generate a uniform sgRNA library that targets two genes in the same cell.[5] In a fluorescence-based screen to assess autophagy activation and flux, Diehl performed a pair-wise dropout screen with a 3Cs library to identify genetic interactions between human autophagy genes. The team identified *PI3KR4*, *ATG5*, and *ATG7* as autophagy hub genes, and discovered an important buffering function for *AMBRA1* in Torin1-induced autophagy. Because *AMBRA1* was never identified from monogenic genetic autophagy screens, this finding highlights the strength of combinatorial screens to identify genes with unknown roles in well-studied pathways.

To further enhance CRISPR screen versatility, Joseph Replogle and his team developed a barcoding technology to simultaneously capture mRNA and sgRNA from the same cell and sequence them on a single platform.[6] Researchers can apply this single-cell CRISPR screening method, which incorporates a capture sequence into the sgRNA sequence, to both single and combinatorial genetic perturbations, increasing the amount of information that scientists can obtain from a single experiment.

The convenience and speed with which researchers can conduct CRISPR-based screens to query the entire genome has convinced scientists in most disciplines of its benefits. While most CRISPR screens target a single gene in cell culture, scientists are developing novel methods to increase the feasibility and flexibility of CRISPR-screens, both in vivo and in vitro. Such advances, combined with the flexibility to create high-quality custom libraries that fit a research question, will enable scientists to detect subtle phenotypes and will increase the chance of finding truly novel hits due to the screen's custom nature. Together, this will allow researchers to accelerate gene function annotation in their favorite model organism across disciplines.

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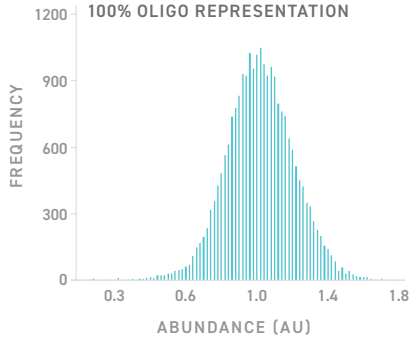

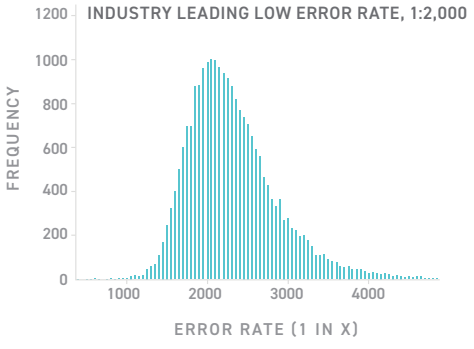

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