Improved CRISPR Knockout Efficiency Using An Automated Optimization Workflow

Application Note

SYNTHEGO

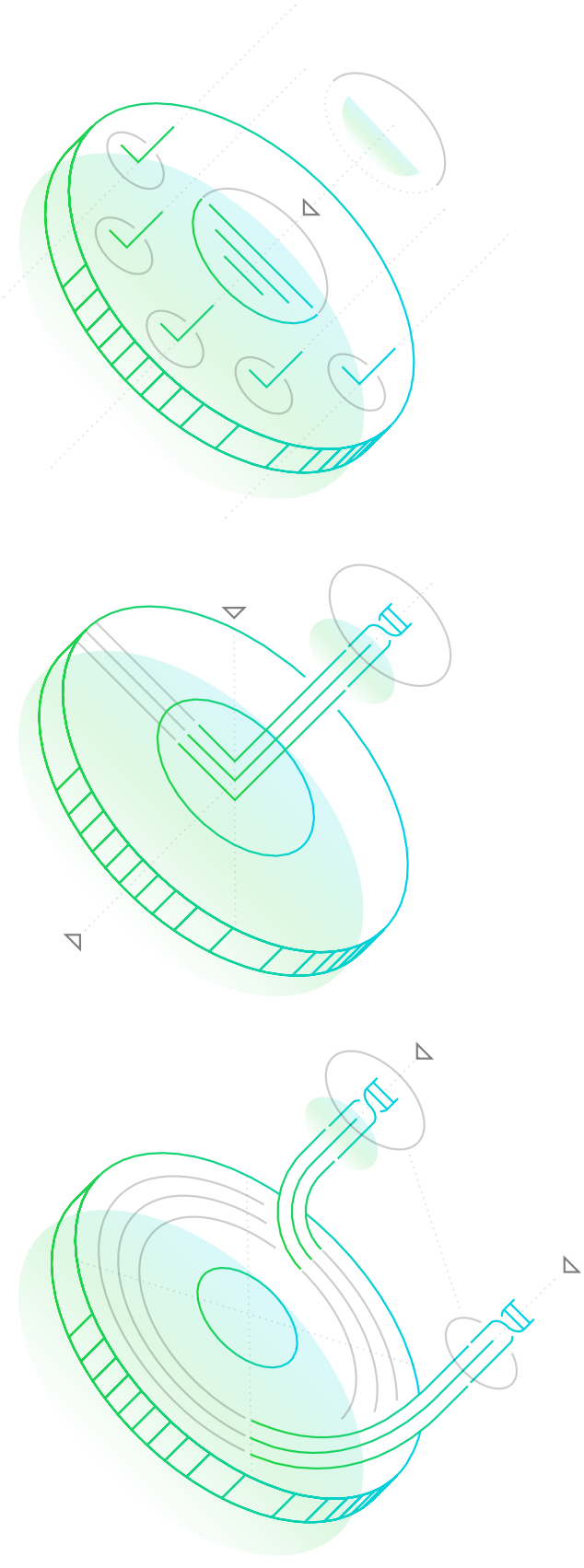
Introduction

CRISPR is an immensely powerful research tool that has revolutionized the way scientists manipulate genomes[1]. In particular, CRISPR-mediated gene knockouts play key roles in, drug discovery, understanding gene function, and other uses that improve the world around us[2] .

Generating knockouts using CRISPR-Cas9 utilize the ability of a guide RNA to deliver a Cas9 nuclease to a specific region of the genome. Upon binding to the desired region, a double-stranded break is created by Cas9. This break forces the cell to undergo non-homologous end joining to repair the double-stranded break, and in doing so, introduces insertions and/or deletions (indels) at the cut site. In many cases the insertion or deletions shifts the reading frame, leading to either a premature stop codon or a protein sequence that is non-functional and degraded by nonsense-mediated decay. This often causes a complete knockout of protein expression and/or function.

Although CRISPR-based genome engineering is easier than previous gene editing methods, the guide design, editing optimization, and analysis of a CRISPR-mediated knockout still require a significant amount of work. For instance, multiple guide RNAs generally need to be tested and several transfection conditions need to be optimized for each cell type to achieve best results. After editing is performed, the cells must be analyzed for knockout efficiency. The entire process of CRISPR design and optimization may extend experimental timelines by several weeks or months for each cell type, without any guarantee of success. This is a considerable barrier to many scientists, especially those who are new to genome editing.

At Synthego, we are dedicated to making CRISPR technology accessible to all researchers. Our full stack genome engineering solutions address and simplify each step of the CRISPR optimization process. By integrating engineering with biology, we have developed automated technology to make our products scalable and affordable.



Synthego's Engineered Cells Products

We have streamlined the editing step of the knockout experimental workflow by completely eliminating the need for scientists to optimize the transfection of CRISPR reagents themselves. Our newest product family, Engineered Cells, allows all researchers affordable access to state-of-the-art knockout cell pools and clonal cell lines.

Knockout Cell Pools

Synthego's Knockout Cell Pools take the complexity out of CRISPR with the first-ever solution that provides guaranteed results in more than 700 human cell lines. In as few as four weeks, your engineered cell pool arrives with a guaranteed 50% or greater protein knockout of your gene. Our knockout pools can achieve higher than 90% knockout frequency and enable you to directly use them in assays to study gene function or easily isolate clonal cell lines.

Knockout Cell Clones

Need a CRISPR knockout clonal cell line but don't have time to make it? Synthego has you covered. Our Knockout Cell Clones are 100% sequence verified to ensure total knockout of your target gene. You choose the genetic target and human cell line and we will get you the clone in as few as 8 weeks.

Advanced Cells

Some projects have specific needs that are not covered by our standard products. For ultimate customization and flexibility, Synthego offers Advanced Cells. We can generate the edit you need in the best cell type for your experiments. Whether you want to add a gene tag, introduce a SNP or a codon change, or edit a difficult cell line, our Advanced Cells can help. Let us create your custom engineered cells so you can focus on making discoveries.

CRISPR Editing, Synthego Style

The workflow for generating a knockout can be summarized in three steps: Design → Edit → Analyze (Fig 1). At Synthego, use cutting-edge bioinformatics technologies and innovative engineering tools to design and synthesize our guide RNAs, generate edits, and analyze the knockouts generated in our Engineered Cells.

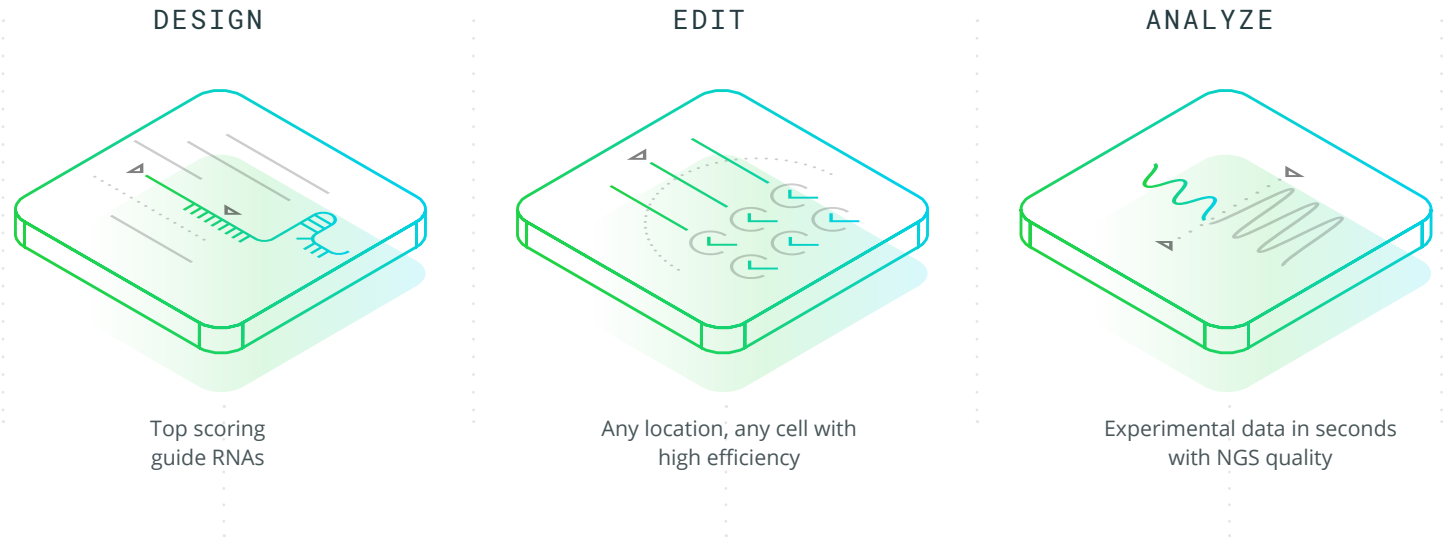


Figure 1. At Synthego, the workflow for generating a knockout comprises three steps: Design → Edit → Analyze. Synthego's Engineered Cells streamlined each step so that knockouts are generated in a fast, efficient, and cost-effective manner.

Design

We design our guide RNA sequences using the Synthego Design Tool. Guide sequences are selected based on maximal homology to the primary and alternative transcripts, a high predicted on-target score, and a very low probability of off-target effects. We then synthesize the guides as chemically modified single guide RNAs (sgRNAs) through a proprietary synthetic process. We have found that guides delivered to cells in an RNA format are superior to those in a DNA format (plasmids) and that single guide RNAs are more reliable than their two-piece counterparts (cr:tracr). The synthetic manufacturing process generates guides of higher purity than those produced by in vitro transcription, and also enables chemical modifications at specific nucleotide residues that protect the RNA from intracellular degridation[3]. The combination of robust guide design, single guide RNA format, and synthetic production results in the highest likelihood of generating successful knockouts.

Edit

To generate the edit, we deliver the CRISPR-Cas9 machinery into cells as ribonucleoprotein (RNP) complexes, with synthetic sgRNA already complexed withCas9. This format allows for maximal editing efficiency while also reducing off-target effects (i.e., there is not risk of unwanted DNA insertions as with plasmid-based editing)[3,4] .

We test several guides at the same time and assess knockout score (KO score), the proportion of sequences lead to a putative KO out of all sequences in a CRISPR-edited pool, as a metric of knockout efficiency. The cell population with the highest KO score is then expanded for further quality control, including a freeze-thaw viability check, mycoplasma testing, and another confirmation of the knockout-inducing indels.

To identify the best editing parameters, we assess over 200 conditions to optimize every cell line we edit. We have developed a high-throughput automated platform

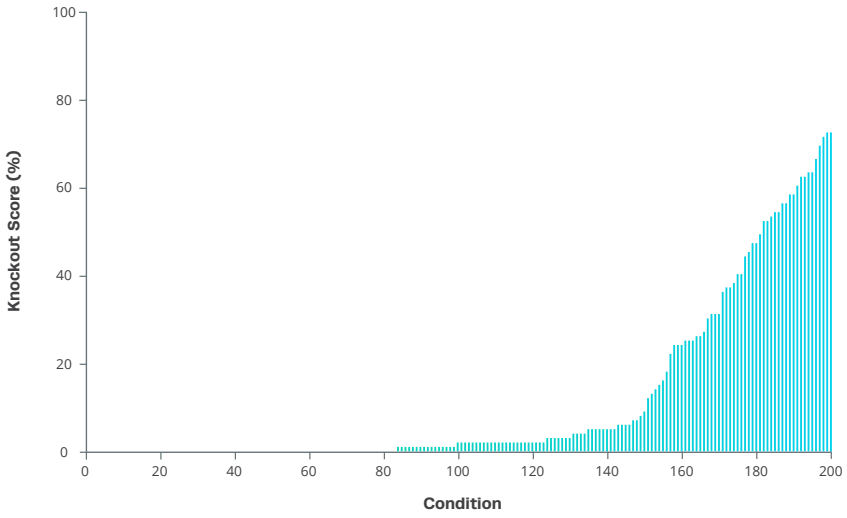


Figure 2. The KO score (proportion of indels that cause a putative knockout) for 200 different conditions in difficult-to-edit THP-1 cells. The KO score was improved from 5% (standard protocol) to 72% after Synthego's CRISPR knockout optimization.

that enables us to perform this optimization in a very short time frame, often within a few days. This optimization process drastically improves the KO score that can be achieved even in cell lines that are traditionally considered difficult to transfect.

For example, we were able improve the KO score in experiments using the notoriously difficult THP-1 cells, from ~5% using the standard protocol to 72% post-Synthego optimization (Fig 2). This high knockout frequency is extremely powerful because it allows either rapid isolation of clonal cell lines, or direct use in many types of assays, which was previously impossible with other CRISPR methods.

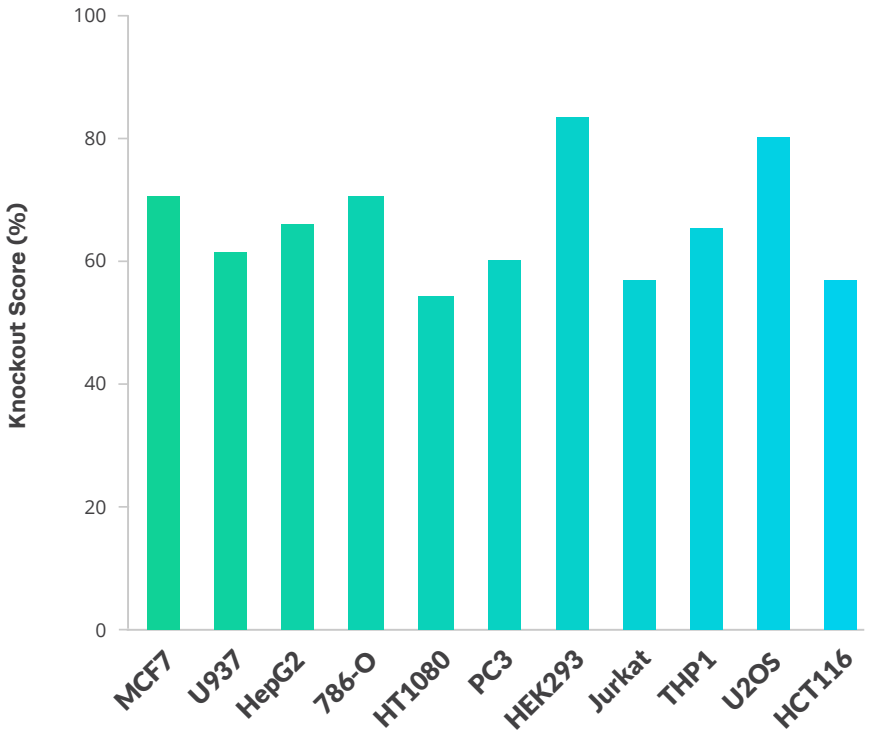


Figure 3. The KO score for 12 different cell lines following Synthego's CRISPR optimization process. Using standard conditions, the KO score for some cell lines was as low as 5% and on average was 35%. After optimization, the KO score averaged 79.5%.

We have made similar improvements in other cell lines, a subset of which are shown in Figure 3). The extensive optimization we are able to conduct in a very short time period enables us to generate highly efficient KO cell pools and clones in a cost- and time-effective manner.

Analyze

To analyze editing efficiency and KO score, we have developed a free bioinformatics tool that enables fast and reliable detection of indels resulting from CRISPR-Cas9 editing. Once cells have undergone editing, we extract genomic DNA and PCR-amplify the edited region. Sanger sequencing of the resulting PCR product is then performed and the data are analyzed using our Inference of CRISPR editing (ICE) software.

By comparing the control and edited sequencing data, ICE calculates the percentage of DNA that has been edited (ICE Score), the frequencies of each indel type, and the KO score. This methodology is much more precise and informative than enzymatic methods such as T7E1/Surveyor and easier, less time-consuming, and more affordable than next-generation sequencing.

Expansion and Quality Control

As mentioned above, multiple guides are designed and tested when creating a Synthego Engineered Cell product. From the ICE analysis, we determine the guide that generated the best editing efficiency and KO score and expand this population of cells. Once we have reached several million cells, we re-test the edited region via Sanger sequencing and ICE analysis to ensure that edited cells are viable and propagate normally. For clonal projects, editing metrics are checked multiple times once clones are derived. Multiple vials of cells are frozen and wee thaw one vial to test post-thaw viability and evaluate mycoplasma contamination prior to shipping cells to customers. Frozen cells are shipped on dry ice in special containers to prevent cells from thawing during shipping.

Conclusion

CRISPR technology has opened the door to a multitude of advancements in functional genomics and a variety of other fields. While CRISPR is a simpler technique than previous genome engineering technologies, time-consuming design and optimization steps still present a roadblock for many researchers. Those who want to incorporate CRISPR into their research but do not have time to fully evaluate guide designs and transfection conditions have to settle for poor editing and knockout efficiencies, which ultimately lead to subpar results.

Synthego Engineered Cells provide all researchers with easy, affordable, and reliable access to genome engineering. Researchers can now take full advantage of CRISPR without needing to edit cells themselves.

All Engineered Cells come with quality guarantees, allowing scientists to focus on generating results and publishing, rather than on developing the cells required for their experiments.

Visit **Synthego.com/EC** to learn more.

REFERENCES

1 Jinek M., et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012;337(6096):816-21.DOI:10.1126/science.1225829.

2 Hsu P., et al. Development and Applications of CRISPR-Cas9 for Genome Engineering 2014;157(6):1262-1278. DOI:10.1016/j.cell.2014.05.010.

3 Hendel A., et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nature Biotechnology 2015;33:985-989.

4 Farboud, B., et al. Enhanced Genome Editing with Cas9 Ribonucleoprotein in Diverse Cells and Organisms. Journal of Visualized Experiments: JoVE. 2018;135. DOI:10.3791/57350.