HOW TO PERFORM SUCCESSFUL CRISPR EXPERIMENTS

A Step-by-Step Guide

SYNTHEGO

INTRODUCTION

CRISPR/Cas9 technology has revolutionized genome engineering. With this How To eBook, our goal is to set forth some best practices for using CRISPR so that researchers can utilize this powerful tool correctly and effectively.

We will provide a general guide to leading a successful CRISPR experiment and offer considerations regarding experimental design and recommendations on how to avoid common mistakes.

If you are still familiarizing yourself with what CRISPR is, how it works and what it can be used for, please refer to the CRISPR 101: Your Guide to Understanding CRISPR eBook, which can be found at synthego.com/resources.

SECTION 1

EXPERIMENTAL WORKFLOW

1. Design CRISPR Guide RNAs
2. Order Synthetic gRNAs
3. Deliver CRISPR Components
4. Analyze Gene Editing

INTRODUCTION

Using synthetic guide RNA (gRNA) can greatly simplify and expedite your experimental workflow. The workflow begins with the design and selection of CRISPR guide RNAs that target a region of interest in a genome.

Forming a ribonucleoprotein (RNP) complex with synthetic RNA and Cas9 nuclease protein is performed by co-transfecting all components with a transfection reagent. You may then observe phenotypes directly, or undertake a genetic screen to estimate gene editing efficiency prior to clonal cell line generation and characterization.

Delivery of RNPs means that CRISPR components exist only transiently inside the cell, limiting Cas9 and guide RNA expression – this allows for the highest levels of editing efficiency and greatly reduces the chances of possible off-target and toxic effects. Furthermore, the use of synthetic guide RNAs eliminates the risk of incorporating foreign DNA into the host genome, which can occur when using plasmid-based guides. Synthetic guide RNAs offer a superior alternative to in vitro transcribed (IVT) guide RNAs that are of variable quality and produce inconsistent editing results.

Please refer to Figure 1 for our recommended workflow on the next page.

DESIGN CRISPR GUIDE RNAs FOR KNOCKOUT

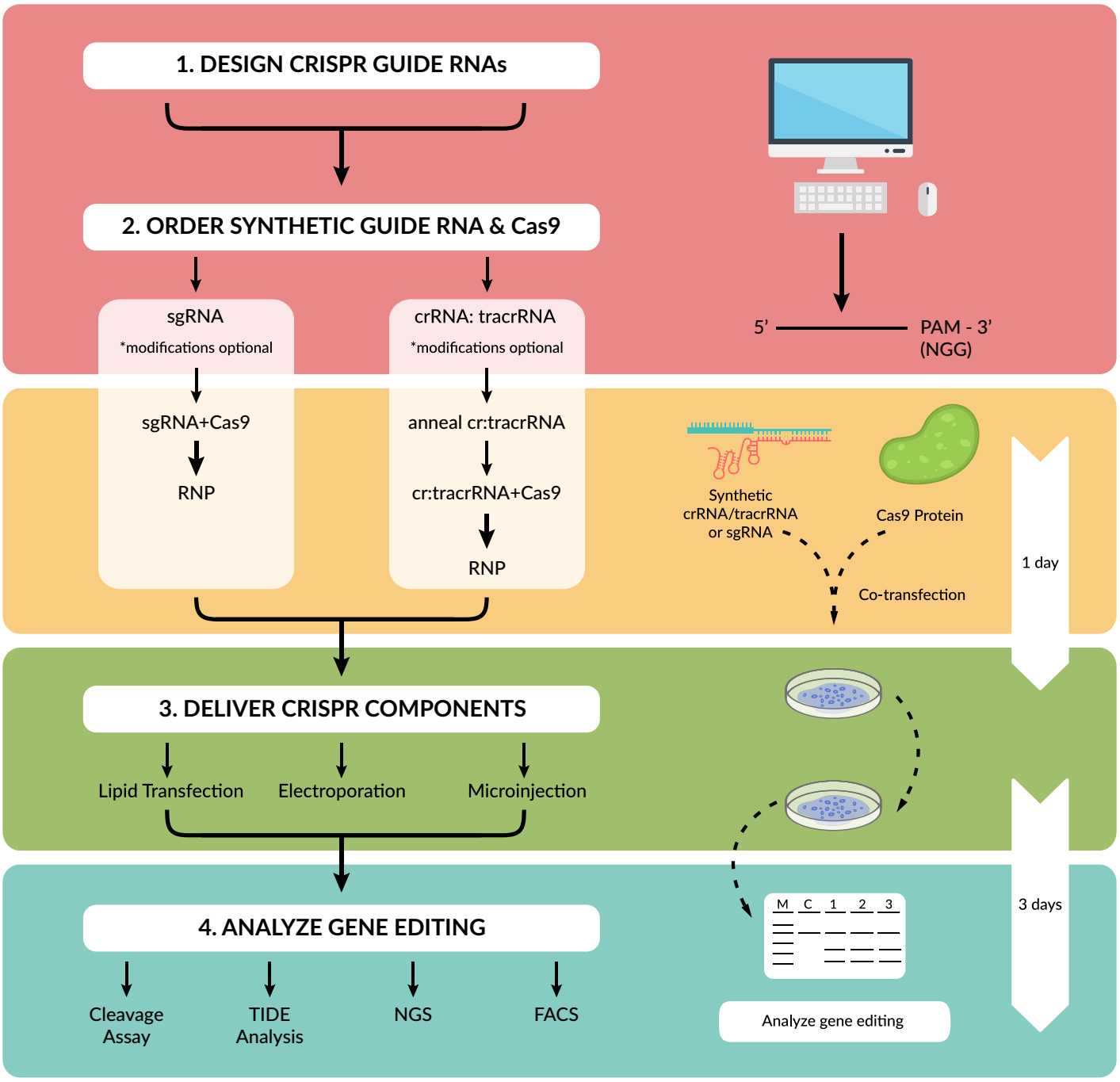
The first step in a CRISPR experiment is to identify your CRISPR target sequences and design guide RNAs. It is difficult to fully predict how well a guide RNA will work. We recommend that you try 3 or 4 different guides to find out which sequences have the best editing efficiencies.

When designing for a complete knockout of a gene, it is best practice to choose targets within early coding regions of a common exon. When generating a knockout, it is preferred to introduce an indel as close to the 5′ end of the coding region as possible. This will have the highest likelihood of creating a nonsense mutation or loss-of-function frameshift. Regardless of application, guide RNAs with minimal off-target effects are preferred.

Synthego offers a CRISPR Design Tool for knockouts optimized for use with *Streptococcus pyogenes* Cas9. To generate guides, select your genome and gene of interest, and we'll provide recommended guides.

When you're ready, design your guide RNAs using the Synthego Design Tool at design.synthego.com.

Figure 1: Synthego's recommended CRISPR experimental workflow.



ORDER SYNTHETIC GUIDE RNAs

After designing your gRNA, you can order your CRISPR components online. As previously mentioned, it is important to consider what type of gRNA you wish to use and whether you should use chemical modifications or not. Please refer to **Choosing Your Components** to understand which components will be most suitable for your experiment. After ordering your CRISPR components you will need to anneal them to form a RNP. In the case of choosing cr:tracrRNA, there will be an extra annealing step between the crRNA and tracrRNA before moving forward with the experiment. In the case of ordering sgRNA there is only a single annealing step between sgRNA and Cas9 required before moving on.

DELIVER CRISPR COMPONENTS

Once an RNP is created, it can be delivered to the cells. Please see the section titled **Choosing Your Delivery Method** to understand which delivery technique will be most optimal for the cell type you work with. The most commonly used techniques include: lipid transfection, electroporation and microinjection. After transforming cells with CRISPR components using the appropriate method, they can be left to grow or divide. The cell confluency and other culture conditions will need to be optimized for the specific cell type you work with.

ANALYZE GENE EDITING

The final step after transforming cells with CRISPR components and allowing outgrowth is to analyze the CRISPR editing efficiency. We recommend the use of a number of commonly used techniques, including: cleavage assay, TIDE analysis, NGS, Site-Seq and FACS. A description of these techniques and specific recommendations are discussed in the section titled **Choose Your Analysis Method**.

SECTION 2

EXPERIMENTAL CONSIDERATIONS

1. Defining Your Goals
2. Choosing Your Components
   a. Cas9
   b. Guide RNA
3. Choosing Your Delivery Method
   a. Delivery Format
   b. Delivery Technique
4. Other Experimental Components to Consider
5. Choosing Your Analysis Method

INTRODUCTION

Before beginning your experiment, it is critical to understand the dynamics of your chosen cell type, delivery method, and CRISPR components. This section will focus on what to consider as you make decisions about these aspects of your CRISPR experiment. As you embark on your experiment please be advised that, in addition to taking into consideration our recommendations, some level of optimization may be required on your part. Please be aware that this guide is not intended to serve as a defined CRISPR protocol but rather a set of considerations, recommendations and best practices that can ensure your CRISPR experiments are successful. Additionally, it is assumed that the researcher possesses a working knowledge of molecular biology and cell culture techniques required for their specific cell type and application.

DEFINING YOUR GOALS

The CRISPR-Cas9 system is capable of generating a number of different types of genomic edits. Following a guide RNA/Cas9-mediated CRISPR double-stranded break (DSB), the cell can repair its DNA using either Non-Homologous End-Joining (NHEJ) or Homology-Directed Repair (HDR). To achieve a gene knockdown or to effect promoter elements, an Indel can be created through the NHEJ pathway. Conversely, a cell that is supplied with donor DNA can undergo HDR which can enable SNP (Single Nucleotide Polymorphism) editing, gene deletion, gene insertion, or promoter/enhancer activation or repression. For the purposes of this eBook, we will focus on using CRISPR to form Indels, and not HDR. CRISPR can also be used to silence genes (CRISPRi) or activate genes (CRISPRa), but these will not be discussed here.

Along with the type of gene edit you wish to create, your chosen cell type will also inform the components required to use in your CRISPR experiment. The following section will discuss how to choose the CRISPR components that can most effectively suit your experimental goals and highlights special considerations based on your desired gene edit and chosen cell type.

CHOOSING YOUR COMPONENTS

There are two components that comprise the CRISPR system: a nuclease (such as S.pyogenes Cas9, as will be discussed here) and a guide RNA (gRNA), typically delivered as a ribonucleoprotein (RNP) complex. These components take on a variety of forms that each have distinct advantages and recommended uses and should be chosen depending on the type of cell being engineered and the desired experimental outcome.

Cas9

Please refer to Table 2 below for a summary of suggested applications for several variants of *S. pyogenes* Cas9 and other nucleases.

Nuclease	Recommended Use
Cas9	Wild-type version of the Cas9 endonuclease that produces a DSB
Cas9 NLS, 2NLS	Contains SV40 nuclear localization signals that tag Cas9 for transport into the cell nucleus. Beneficial when eukaryotic cells are being edited. We recommend the 2NLS version which contains NLS signals on both the C and N terminal of the Cas9 protein.
Cas9 nickases	Contains a mutation (either D10A or H840A in S. pyogenes Cas9) that inactivates one of the two DNA strand cutting domains in Cas9, resulting in a nick to the target sequence, instead of a DSB. Useful for driving DSB repair to occur via HDR using donor DNA (instead of NHEJ).
dCas9	Known as “dead Cas9”, contains two mutations (D10A and H840A in *S. pyogenes* Cas9) that inactivates the DNA strand cutting activity of the Cas9 endonuclease. Useful for gene-silencing (CRISPRi) experiments.
Cpf1	Derived from *Prevotella* and *Francisella* Bacteria, Cpf1 is useful for recognizing AT rich sequences, and also generates a nick instead of a DSB.
C2c2	RNA-guided enzyme from *Leptotrichia shahii* capable of targeting and degrading RNA.

Table 1: Suggested Applications for Common CRISPR-associated Nucleases.

CHOOSING YOUR COMPONENTS (cont.)

GUIDE RNA

Guide RNAs contain the target sequence for the specific region of DNA that you wish to cut with Cas9 and perform CRISPR on. For recommendations on how to choose your guide RNA target sequences, please see the **Experimental Workflow** section below. Guide RNAs can be generated through several techniques: produced as part of a plasmid or vector via cloning; through a process known as *in vitro* transcription (IVT) or chemically synthesized as RNA oligonucleotides. Each of these gRNA formats present the researcher with certain benefits and constraints. For example, producing plasmid-based gRNAs is time-consuming and is impractical when generating a large number of guides. In addition, the use of plasmid gRNAs results in constant expression in the cell, leading to a higher level of off-target effects and the possibility that plasmid DNA can be incorporated into the host genome. Similarly, IVT-generated gRNAs are also time-consuming to produce and are not easily scalable beyond a few guides. Since the IVT process involves many steps, and relies on enzymes to transcribe RNA from DNA, the purity of the resulting gRNAs can be highly variable and their quality relies on the skill of the researcher. This variability can lead to inconsistencies in gene editing efficiency between replicates and unwanted off-target effects. In addition, IVT-derived gRNAs cannot be chemically modified - a significant disadvantage as cell types that are challenging to edit, such as stem cells, have been shown to require chemically-modified gRNAs for effective CRISPR editing (Hendel et al., 2015). Figure 2 highlights the variable purity seen in an IVT-derived gRNA, compared to the same gRNA that has been chemically synthesized.

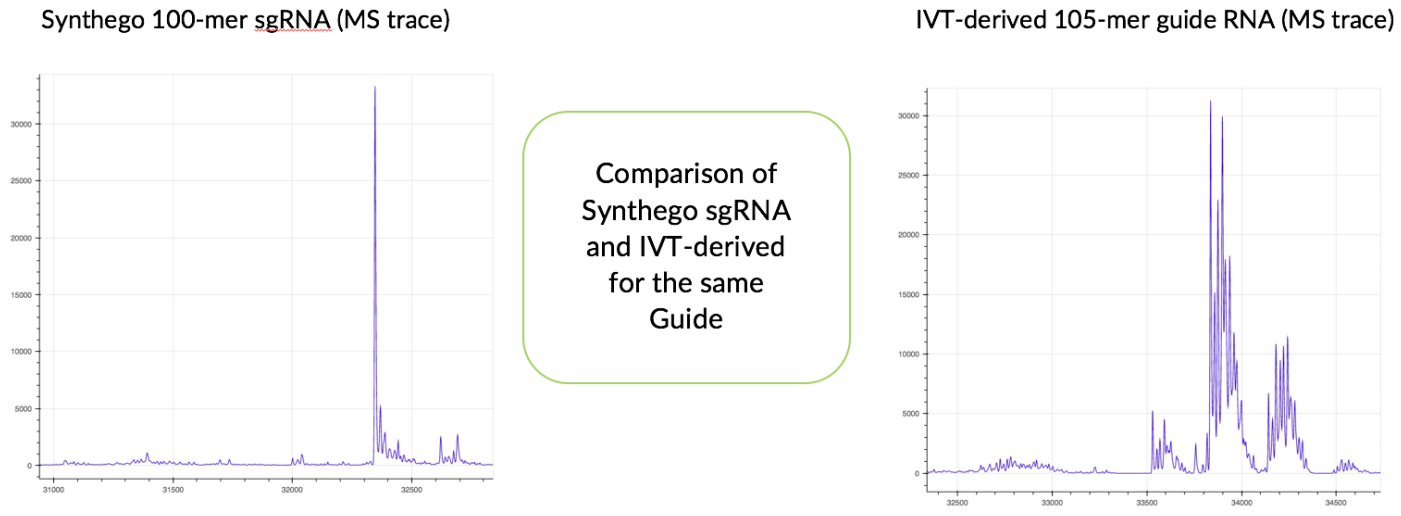


Figure 2. Mass Spectrometry traces demonstrating the greater homogeneity and purity of synthetic 100-mer sgRNA (left) compared to an IVT-derived sgRNA (right), for the same target and scaffold sequences. The IVT-derived guide is slightly longer due to the required additional transcription and terminator nucleotides.

CHOOSING YOUR COMPONENTS (cont.)

Synthetic gRNAs can be chemically synthesized as either two-part crRNAs and tracrRNAs (that require pre-annealing) or a hybrid single guide RNA (sgRNA). Chemically synthesized gRNAs provide the benefits of having a high level of purity and a low level of variability between batches. The image above highlights the superior purity of a synthetic gRNA over the same gRNA that was generated using IVT. The purity and consistency of synthetic gRNA enables a high-level of reproducibility between experimental CRISPR replicates. Furthermore synthetic gRNAs can be chemically modified, which is critical when editing particular cell types, such as stem cells (Hendel et al., 2015), or certain genomic targets that prove otherwise challenging to edit. Synthego offers 2'-O-methyl analogs and 3' phosphorothioate internucleotide linkages at the first three 5' and 3' terminal RNA residues. These modifications provide protection against exonuclease activity and immune responses. Table 3 highlights some commonly used cell types in which modified gRNAs are most effective for CRISPR.

We recommend the use of synthetic sgRNA over the annealed crRNA:tracrRNA duplex. Our own research, and that of our collaborators has shown that for the majority of targets, across many cell types, sgRNA provides superior editing efficiency compared to duplexed crRNA:tracrRNA. In addition, sgRNA requires no pre-annealing, which is an advantage when working with many guides. Annealing of the two-piece system is never 100% efficient which leads to inconsistencies in editing efficiency and experimental replicates. Furthermore, the tracrRNA can form tetramers with itself, which can lead to incomplete gRNAs, mitigating the dosage to a cell. For a comparison of the different methods used to create gRNAs, see Table 2.

We recommend the use of synthetic sgRNA in CRISPR experiments because it offers the best combination of editing efficiency, consistency, speed, ease of use and allows the possibility of chemical modifications.

CHOOSING YOUR COMPONENTS (cont.)

	Synthetic Guide RNA	Plasmid	IVT
Process	**sgRNA** 1. Choose target sequence 2. Order synthetic RNA **crRNA + tracrRNA** 1. Choose target sequence 2. Order Synthetic RNA 3. Anneal crRNA + tracrRNA	1. Choose target sequence 2. Design/order DNA primers 3. PCR insert 4. Ligate into plasmid 5. Transform into cells 6. Screen cells 7. Sequence verify plasmid 8. Purify plasmid DNA	1. Choose target sequence 2. Design/order DNA primers 3. Assemble guide by PCR 4. Perform IVT 5. Purify guide RNA
Time to Transfection	Ready for transfection	7-14 days	1-3 days
Transfection Labor Time	Minimal	Days of lab work	Full day of lab work
Off-target Effects	Lowest	Variable	Variable
Efficiency	Up to 90% efficiency	Variable	Variable
Consistency	Highest	Variable	Variable

Table 2: Comparative advantages of synthetic sgRNA over two-piece synthetic crRNA:tracrRNA, IVT-derived guide RNA and plasmids.

CHOOSING YOUR DELIVERY METHOD

There are several ways to deliver CRISPR components to a cell, and these are dependent upon the types of CRISPR components you choose to use and if the cell type you are using is best transformed using electroporation, lipid transfection or microinjection.

DELIVERY FORMAT

We recommend the ribonucleoprotein (RNP) format of CRISPR components because we have found, and others have shown, that it is the most efficient way to deliver CRISPR components into the cell. A recent study by Liang and coworkers in the Journal of Biotechnology demonstrated that RNP delivery provides more control, higher editing efficiency and fewer potential off-target effects in a variety of cell types when compared to other delivery methods, such as plasmid-based components. The RNP approach allows the CRISPR components to exist transiently inside the cell, limiting Cas9 and gRNA expression – and this reduces the chances of off-target effects and possible toxicity. In addition, the RNP format helps to protect the gRNA from degradation. They are easily formed at room temperature and can be refrigerated for several weeks for later use. Figure 3 shows the different formats of CRISPR components that can be delivered into a cell.

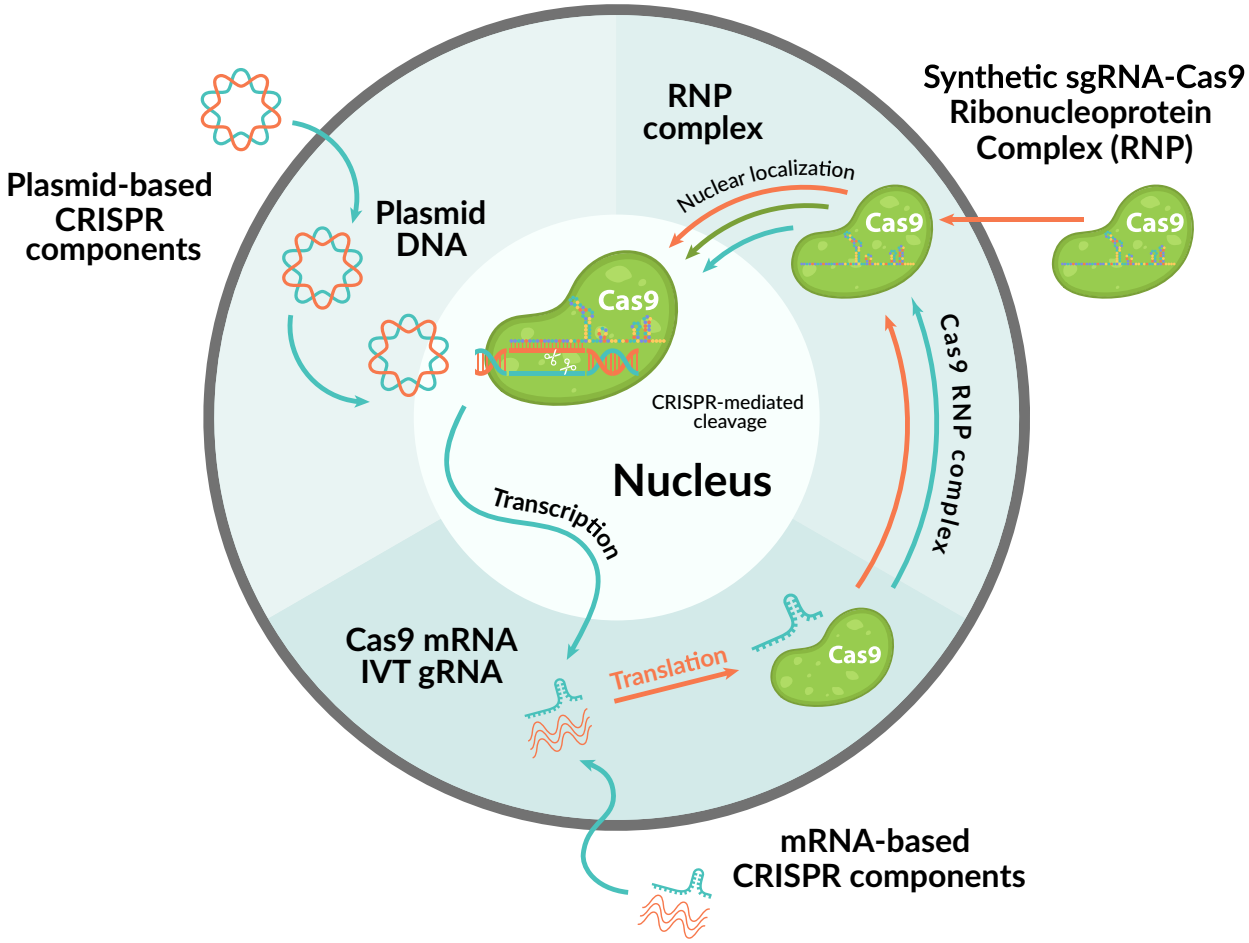


Figure 3: Delivery formats of CRISPR components to the cell.

CHOOSING YOUR DELIVERY METHOD (cont.)

DELIVERY TECHNIQUE

The most appropriate delivery technique for CRISPR components depends upon the cell type you are using. There are three primary methods for delivering CRISPR components into cells: lipid-based transfection, electroporation and microinjection. Please see Table 3 for a list of recommended delivery methods for commonly used cell types. Detailed protocols for several delivery methods can be found on the Synthego Website on the Resources Page.

Cell Type	Lipid Transfection[1]	Electroporation[2]	Microinjection	Chemical Modifications Recommended?
HEK 293/T	X			May Provide Benefit
U2	X			May Provide Benefit
CHO-K1	X			May Provide Benefit
Stem Cells (iPSC, hPSC)		X		Yes
HeLa		X		May Provide Benefit
K562		X		Yes
A549		X		Yes
Jurkat		X		May Provide Benefit
Prokaryotes		X		Yes
Yeast		X		Yes
Plant (e.g. protoplasts)		X		May Provide Benefit
Embryo (Mouse, Zebrafish, *C. elegans*, etc.)			X	May Provide Benefit

[1] e.g., Lipofectamine etc.
[2] e.g., Thermo Neon, Lonza Nucleofection etc.

Figure 3: Delivery formats of CRISPR components to the cell.

CHOOSING YOUR DELIVERY METHOD (cont.)

For commonly used adherent cell lines, such as HEK293/T, U20S and CHO, lipid-based transfection reagents, such as those utilizing Lipofectamine™, are most optimal for delivery of CRISPR components. Generally speaking, the use of optimized lipid nanoparticles for transfection is very cost effective and gentler on cells than electroporation. Additionally, lipid-based transfection uses liquid reagents, making it more amenable to transformation scalability. We recommend the use of the Thermo Fisher CRISPRMAX™ kit, which has been optimized for delivery of RNP-format CRISPR components into cells (Yu et al., 2016).

While lipid-based transfections can be used for a wide-variety of cell types, they can perform poorly or be completely ineffective for challenging to transform cell lines. In particular, suspension cells such as K562, Jurkat, plant and primary cells, including T-cells and stem cells, may not transform well using lipid-based transfection. In addition, prokaryotic and yeast cells cannot be transformed this way. For these cell types, electroporation is recommended. Several electroporation systems exist, and each has its benefits. For example, the Lonza Nucleofector™ system utilizes a combination of cell-type specific solutions and electroporation cuvettes to achieve maximum transformation rates. In addition, Lonza provides cell-type specific transformation protocols for over 600 cell types (including primary cells and stem cells), and the system can be scaled to various reaction volumes - utilizing 100µl cuvettes, 20µl electroporation strips or 96-well plates. Another popular electroporation system is the Thermo Neon™ system, which utilizes a 10µl volume electroporation “tip”, which generates a more uniform electric field, and allows samples to be directly, and sterilely, transferred directly into tissue culture vessels, such as 96-well plates. For electroporation, we recommend the use of either the Lonza Nucleofector™ system or the Thermo Neon™ system - and leave it up to the CRISPR researcher to decide which system may function best for their particular cell type.

For delivery of CRISPR components directly into embryonic-stage organisms, microinjection is the preferred method. This includes the use of CRISPR to generate transgenic mice, zebrafish or *C. elegans*. In this format, CRISPR components should be delivered using the RNP format in an appropriate microinjection buffer, and injected directly into the embryonic cell. Sometimes, a dye can be used to visualize the microinjection mixture.

Given these recommendations, it may still be the case that experimental optimization will need to be performed in order to determine the best transformation method for CRISPR components into your cell type. With more and more types of cells being edited using CRISPR every day, this is to be expected.

OTHER EXPERIMENTAL COMPONENTS TO CONSIDER

It is also important to consider the use of controls for your CRISPR experiment. Controls will vary based upon the type of cells being edited.

For CRISPR editing of human cell lines, a popular positive control is to edit the hypoxanthine phosphoribosyltransferase (*HPRT*) locus. This gene encodes the HPRT protein that catalyzes the conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate in the non-essential purine salvage pathway. Cells that are *HPRT*+ are sensitive to 6-thioguanine (6-TG), which can be converted to the nucleotide form by *HPRT* and incorporated into DNA by DNA polymerase, killing cells by a process involving post-replicative mismatch repair (Liao et al., 2015) Cells that have formed an Indel in the *HPRT* locus via CRISPR-Cas9 mediated editing are thus capable of growing in media supplemented with 6-TG and can be selected for as a positive control. A typical negative control is to form and include a guide RNA that targets a sequence not found on the host's genome. Both of these controls can also be assessed by utilizing a genetic assay, as described in the next section.

Another example of a control system for CRISPR-edited cells is to start with a Green Fluorescent Protein (GFP)-expressing cell line and attempt to form an Indel to remove function or, conversely, start with a cell line with non-functional GFP and attempt to repair the GFP reading frame using HDR. Both of these have the added advantage of relying on an indirect, non-genetic screen in order to assess the viability of CRISPR-mediated genome cleavage.

For microinjected embryos, a negative control for CRISPR components may only contain microinjection buffer and no RNP, and a positive control may include a guide RNA that is known from previous experiments to edit efficiently and/or yield a particular phenotype.

If the cells being edited are not amenable to the usage of controls such as these, or too precious to run controls on (e.g., primary cells, stem cells), it is possible to conduct an *in vitro* cutting assay with the guide RNA. In this scenario, a guide RNA is duplexed with Cas9 nuclease *in vitro* to form an RNP, which is then incubated for a short period of time with a fragment of DNA that corresponds to the target sequence on the guide RNA. Cleavage efficiency can then be determined by gel electrophoresis. However, it is important to note that *in vitro* cleavage of DNA cannot be used as a predictor of in vivo cleavage activity of a guide RNA.

CHOOSING YOUR ANALYSIS METHOD

Following transformation of CRISPR components into your cell type, and subsequent outgrowth or cell division of cell cultures or embryos, it is is critical to genotype cells in order to determine the efficiency of the CRISPR events and identify CRISPR-edited clones for isolation and propagation. Typically, a genetic analysis is performed on a subset of transformed cells in order to assess CRISPR-Cas9 editing efficiency and to identify cultures that contain edited cells. Here we will discuss several types of genetic analysis, in addition to more indirect methods, such as protein detection. For CRISPR-edited embryos, a phenotypic readout may be utilized in conjunction with a genetic analysis.

In general, there are two ways to consider CRISPR genotyping methods. These can be classified as low vs. high throughput, and biased vs. unbiased analysis methods. Throughput reflects the ease, speed and suitability of a technique for CRISPR genotyping. Either type of detection method may be unbiased or biased in nature. If a sequencing approach is used, these labels indicate whether the sequencing is targeted to specific regions in the genome based on an algorithmic prediction or not. Specifically, unbiased assays search for evidence of CRISPR editing across the entire genome. In a biased assay, only bioinformatically predicted regions are analyzed (e.g., predicted on- and off-targeted regions). Table 4 below summarizes CRISPR genotyping methods.

Scope	Low Throughput	High Throughput
Biased	Surveyor / T7E1 mismatch cleavage assay Sanger sequencing / TIDE analysis	Targeted deep amplicon sequencing Site-Seq
Unbiased	None Available	Whole genome sequencing Guide-Seq Digenome-Seq Circle-Seq BLESS

Table 4. An overview of CRISPR genotyping techniques for validating genome editing outcomes.

CHOOSING YOUR ANALYSIS METHOD (cont.)

A common and relatively easy method for analyzing CRISPR editing is the use of mismatch cleavage assays that rely on T7 endonuclease I (T7E1). These endonucleases cleave double-stranded DNA wherever there are mismatches, which are formed following CRISPR-Cas9-mediated DSBs and subsequent Indel formation. First, the targeted region is amplified using Polymerase Chain Reaction (PCR) and the resulting amplicons are incubated with T7E1. Fragments are then analyzed by gel electrophoresis and band intensity measured using gel analysis software to determine the percentage of cleaved/uncleaved DNA. This can give a rough estimation of editing efficiency and demonstrate that CRISPR-Cas9 mediated cleavage has taken place. However, this assay provides no sequence information and is not considered to be very sensitive; the limit of detection is likely close to 5% (Fu et al. 2013, Vouillot et al. 2015). When performing this assay, it is critical to include a PCR amplicon on the gel that has not been incubated with T7E1. The T7E1 assay is considered a biased assay since they rely on simple visualization of gel fragments and are not truly quantitative.

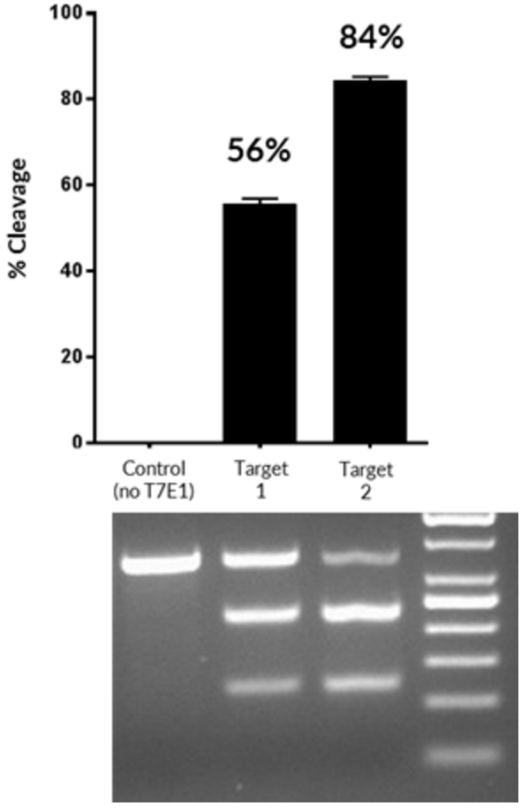


Figure 4: Typical T7E1 cleavage assay result of two CRISPR genome edited targets, amplified by PCR, on an agarose gel. Mismatches (formation of Indels) are identified by the presence of smaller bands which indicate cleavage by the T7E1 enzyme of the PCR product. Cleaved PCR products are analyzed using gel-band intensity software to calculate the percentage of the total PCR product that has been cleaved (% Indel formation).

CHOOSING YOUR ANALYSIS METHOD (cont.)

In conjunction with a T7E1 cleavage assay, a Tracking of Indels by Decomposition (TIDE) sequencing analysis can also be performed on the PCR amplicons of the edited region. Critically, a PCR amplicon of the same region from unedited cells (not given CRISPR components) must also be obtained. Both PCR amplicons are sequenced using traditional Sanger sequencing, and the resulting DNA sequencing chromatogram files are uploaded to the online TIDE webtool (Brinkman et al., 2014). The webtool utilize an algorithm that accurately reconstructs the spectrum of Indels from the sequence traces, which will contain mixed peaks due to the formation of Indels at the editing site. The web tool then reports the identity of the mutations and their frequencies in a graphical and tabular output. Like the T7E1 assay, TIDE analysis is a rough estimate of the true CRISPR editing efficiency of a cell population, and may under or overestimate actual editing efficiency. In addition, can be performed in most laboratories without access to special equipment, since Sanger sequencing is very inexpensive and can be outsourced overnight. However, Sanger sequencing has a lower detection limit of 50-20% (although this has been improved in some studies) (Davidson et al., 2012, Tsiatis et al., 2010). PCR of edited regions and subsequent sequencing and TIDE analysis are considered biased approaches because only regions targeted for editing are analyzed.

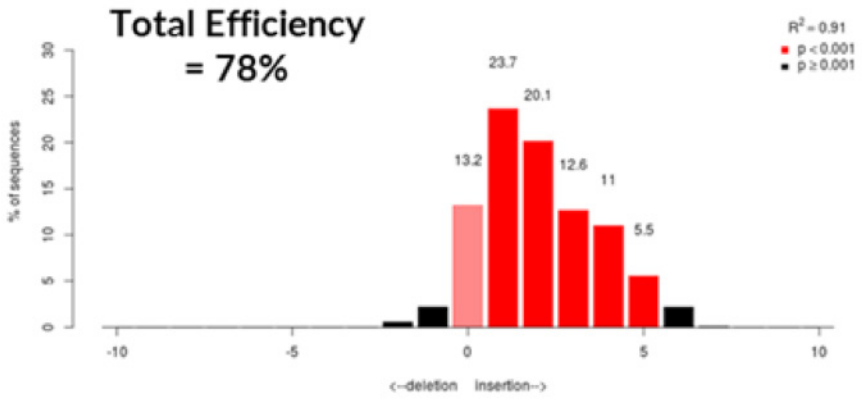


Figure 5: Typical TIDE analysis of a CRISPR-edited genomic region. The edited region was amplified using PCR and sequenced. TIDE analysis was then used to determine the percentage of Indel formation after deconvoluting mixed peaks in the DNA chromatograms, compared to a wild-type sequence.

A high throughput, and more accurate method for analyzing CRISPR editing is the use of Next-Generation Sequencing (NGS). The primary advantage of NGS is that it provides a picture of not only on-target cleavage but also of off-target effects throughout the genome. However, if an unbiased approach such as Whole genome sequencing (WGS) is employed by comparing pre-editing sequencing with CRISPR-edited sequence, it is challenging to deconvolute what (if any) mutations besides those targeted by CRISPR are truly off-target effects or baseline mutations unless the model is genotyped before and after CRISPR editing. A more practical, but biased approach is to perform NGS on targeted amplicons throughout the genome that may have a high probability for off-target effects. In addition, target amplicon deep sequencing is much less expensive than WGS.

CHOOSING YOUR ANALYSIS METHOD (cont.)

An alternative to NGS are unbiased cell-based analyses such as Guide-Seq (Tsai et al., 2015). Guide-Seq is a genome-wide method of identifying DSBs by sequencing and can detect sites with cleavage activity as low as 0.1%. Guide-seq relies on erroneous NHEJ-mediated DNA repair to capture co-introduced blunt-ended double stranded oligonucleotides (dsODNs) at CRISPR/Cas9-induced breakpoints within the genome. These oligonucleotides display a high frequency of insertion at DSBs caused by Cas9 and in effect tag the edited loci for subsequent amplification and deep sequencing.

An effective combination of the aforementioned methods is Site-Seq. This is a biased, high throughput technique used for assessing and validating the size, frequency and identities of CRISPR mutations at on- and off-target sites. By combining targeted amplicon sequencing with bioinformatic prediction, Site-Seq provides a quantitative report of guide cutting efficiency and specificity, and the size, frequency and distribution of indel mutations within the sample population. Site-seq is offered as a service by Desktop Genetics. Figure 6 below shows an example of a Site-seq analysis and the reports generated when using this service.

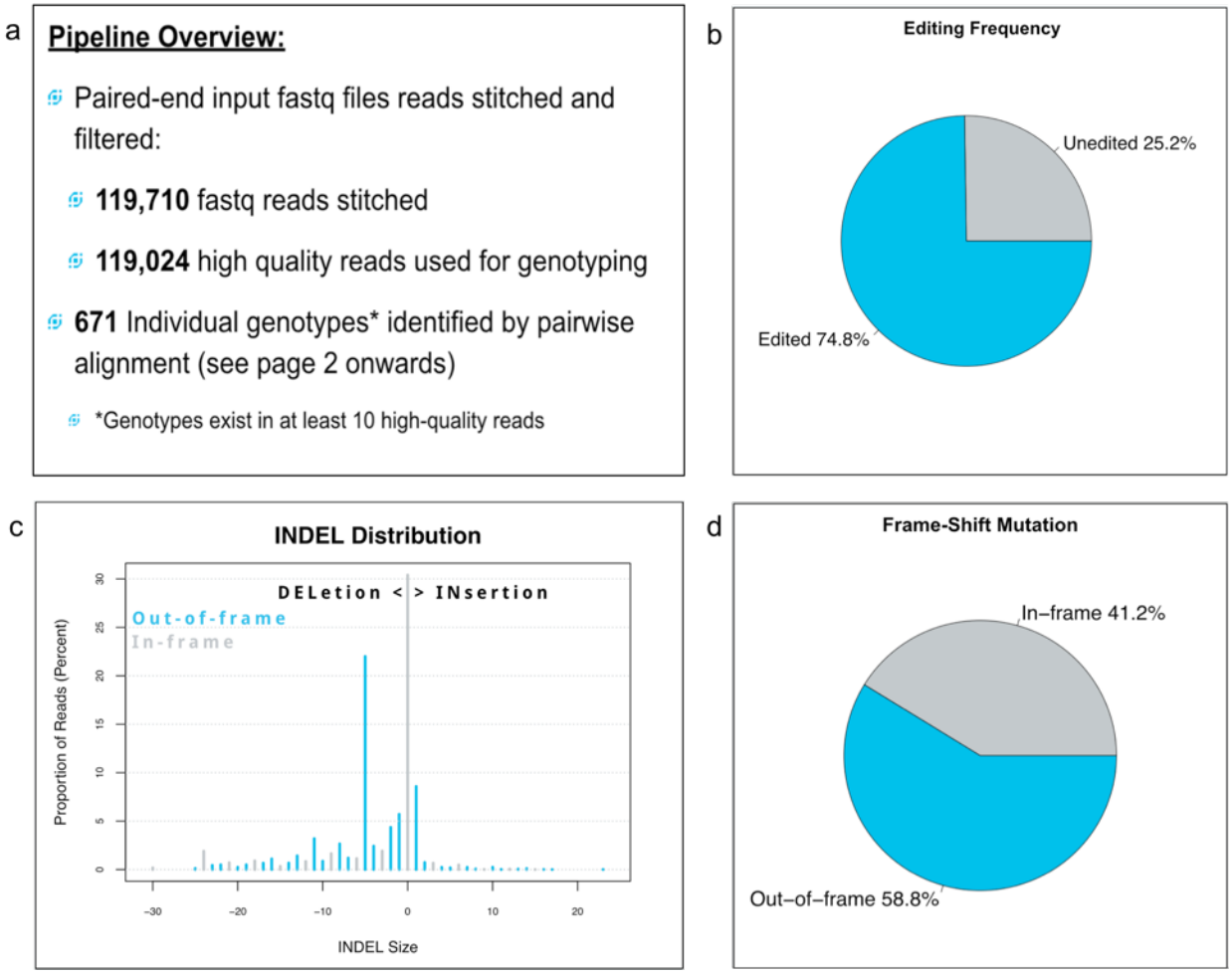


Figure 6. An example of a Site-Seq Guide Characterization report.

CHOOSING YOUR ANALYSIS METHOD (cont.)

If the gene target that is being edited using CRISPR produces a cell surface receptor or exterior facing membrane protein, then fluorescence-activated cell sorting (FACS) can be used to sort cell populations that have been edited or have not been edited. An example of this is shown in Figure 7 below. Although this method can quite accurately report the percentage of the cell population that have a successful CRISPR edit, they also provide no sequence information regarding the edit itself or off-target effects.

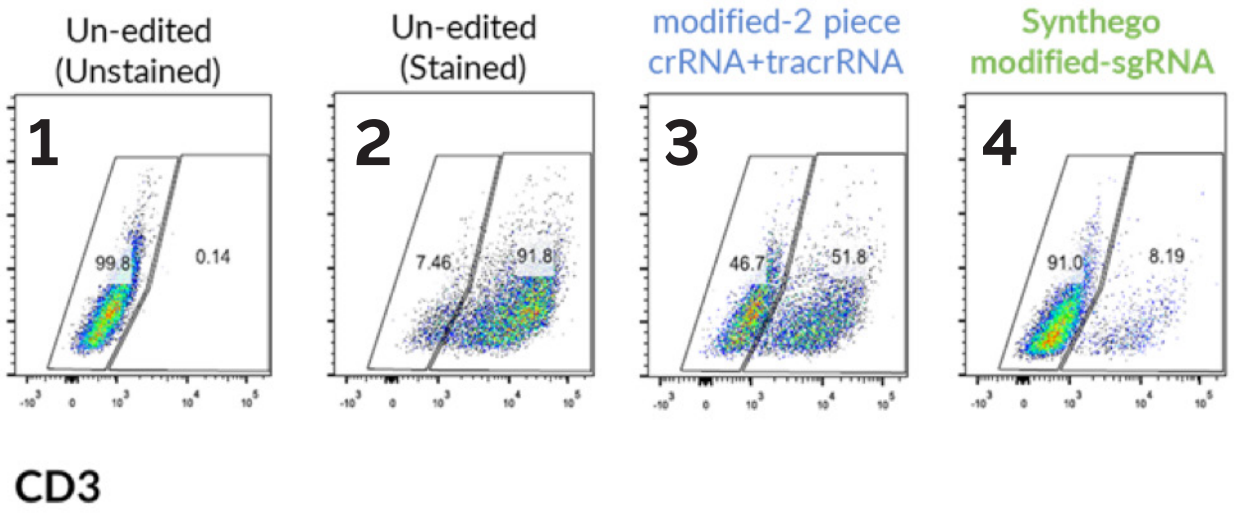


Figure 7: FACS analysis of CD34+ cells edited using CRISPR. Using CRISPR, the CD3 cell surface receptor gene was knocked out, using either a chemically modified 2-piece crRNA:tracrRNA or a chemically modified single sgRNA. Using the crRNA:tracrRNA pair (box3), 46.7% of cells showed knockdown of the CD3 receptor, while with the Synthego sgRNA (box4), 91% of cells showed knockdown of CD3. Box 1 shows un-edited cells that have not been stained. Box 2 shows un-edited cells that have been stained for CD3.

CONCLUSION

We are confident that, if followed, the approaches proposed in this eBook will lead to the completion of a successful CRISPR experiment. Most critically, we believe it is important to understand the specific CRISPR components, delivery technique and analysis method that best suit your goals and the particular cell type you work with. We anticipate that your experiments will run smoothly and encourage you to contact us with any questions you may have.

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THANKS FOR READING

ABOUT SYNTHEGO

Founded by former SpaceX engineers, Synthego is a leading provider of genome engineering solutions. The company's flagship product, CRISPRevolution, is a portfolio of synthetic RNA designed for CRISPR genome editing and research.

Synthego's vision is to bring precision and automation to genome engineering, enabling rapid and cost-effective research with consistent results for every scientist.

Headquartered in Silicon Valley, California, Synthego customers include leading institutions around the world.