SYNTHEGO

SYNTHETIC GUIDE RNA FOR CRISPR GENOME EDITING

WHITE PAPER

INTRODUCTION

Any scientist who has utilized CRISPR-Cas9 genome editing understands that there are two essential parts of the technology's machinery: The Cas9 endonuclease protein that cuts DNA and guide RNA (gRNA) that directs Cas9 to its target sequence. Scientists using CRISPR-Cas9 also know that the gRNA is unique to each experiment, requiring that 17-20 nucleotides of its sequence be tailored to the specific locus of interest. Thus, every CRISPR experiment entails the sometimes tricky task of designing, synthesizing, and delivering one or more distinct gRNAs.

There are a number of in-house methods by which researchers prepare and deliver their own gRNA. Among the most common are the plasmid-based approach and the *in vitro* transcription (IVT) approach. In the plasmid-based approach, a recombinant plasmid is cloned to express the desired gRNA (either with or without Cas9 protein) upon transformation or transfection into the target cells. In the IVT approach, a DNA template for the gRNA is transcribed using an IVT kit, after which the gRNA is delivered to the cell by one of several possible methods.

In addition to these conventional methods of preparing gRNA in-house, another option is now becoming available to CRISPR researchers. Thanks to advances in RNA synthesis technology, gRNA can now be affordably commissioned and ordered from out-of-lab providers like Synthego. Synthetic gRNA offers some distinct advantages over IVT- and plasmid-based gRNA. In the following exploration of synthetic gRNA, we will focus on plasmid cloning and IVT as counterpoints to synthetic gRNA in order to show how this new option sizes up in the realm of CRISPR.

A CRISPR EXPERIMENT WITH PLASMID CLONING

A researcher that chooses to deliver gRNAs via a plasmid for their CRISPR experiment must first design the gRNA by identifying a string of 17-20 nt within the host organism's genome that will serve as the target sequence. Once the target sequence has been selected, it may be necessary to assemble this sequence together with additional components (such as a gRNA scaffold, promoter, terminator) depending on its destination expression vector. A wide variety of expression vectors are now available into which gRNA inserts can be cloned, and the complexity of the insert's design depends on the existing content of the vector being used. Care must also be taken to ensure that the vector selected is appropriate for the eventual host organism whose genome is to be edited.

Once an gRNA insert has been designed, it must be constructed using standard laboratory techniques such as PCR or acquired through a synthetic DNA synthesis provider. After the insert is obtained, it must be ligated into the plasmid and then be transformed into a microbial cloning strain. After this, transformed colonies must be screened via PCR and sequenced verified. Once a correct clone has been identified, it must be grown up at a larger scale so that plasmid DNA can be isolated and purified. Only after this is complete can a gRNA vector be transfected into cells to be edited via lipid transfection or electroporation. If Cas9 protein is not encoded in the plasmid, it must be provided separately as either purified nuclease or mRNA, so that the gRNA may form an effective ribonucleoprotein (RNP) complex inside the cell.

A CRISPR EXPERIMENT WITH IN VITRO TRANSCRIPTION

Like the plasmid cloning method, the IVT-based method of obtaining gRNA also requires design and construction of a DNA template, usually in the form of DNA oligonucleotides. The DNA template must contain the selected 17-20 nt target sequence and gRNA scaffold. If the target sequence does not begin with two Guanine nucleotides ("GG"), then these must be added immediately 5' to the target sequence. In addition, it must also be preceded by a promoter site that matches the type of RNA polymerase to be used during the transcription (most often a T7 promoter/polymerase). After the DNA template sequence is designed, a pair of DNA oligonucleotides containing a complementary region are synthesized.

Once DNA oligonucleotides are obtained, the DNA template must be generated. These must be annealed and then amplified using PCR and purified. The PCR product is then subjected to an RNA polymerase in order to generate the gRNA, and then purified. After gRNA has been transcribed and purified, it can be delivered to the cell in a number of different ways. The gRNA can be co-transfected into the target cell alongside Cas9 mRNA or, alternatively, be complexed with Cas9 protein outside of the cell and then delivered as an RNP through electroporation, lipid transfection, or microinjection.

A CRISPR EXPERIMENT WITH SYNTHETIC RNA

An alternative to generating gRNA in house is to custom order synthetic gRNA from an out-of-lab provider like Synthego. Researchers who choose this route need only identify the 17-20 nt target sequence, not including the PAM, and the providers handle the remainder of the labor involved in its design and synthesis. The chemical synthesis method of obtaining gRNA is uniquely capable of incorporating site-specific chemical modifications to constituent nucleotides, and therefore researchers whose cell lines require such modifications can also indicate this upon placing their order. Once a target is identified and an order is placed, synthetic gRNA is supplied to the researcher within a few days. Synthetic gRNA can be ordered in the form of separate crRNA and tracrRNA fragments that the researcher must anneal together before use, or as one seamless single guide RNA (sgRNA) molecule. However, while both options are available, in general sgRNA is associated with a higher level of editing efficiency. Although the annealing rate of the cr/tracrRNA fragments can reach up to 90% efficiency, tracrRNA fragments tend to form tetramers due to their inherent secondary structure that bind to Cas9 without crRNA, reducing the number of functional RNPs delivered to the cell.

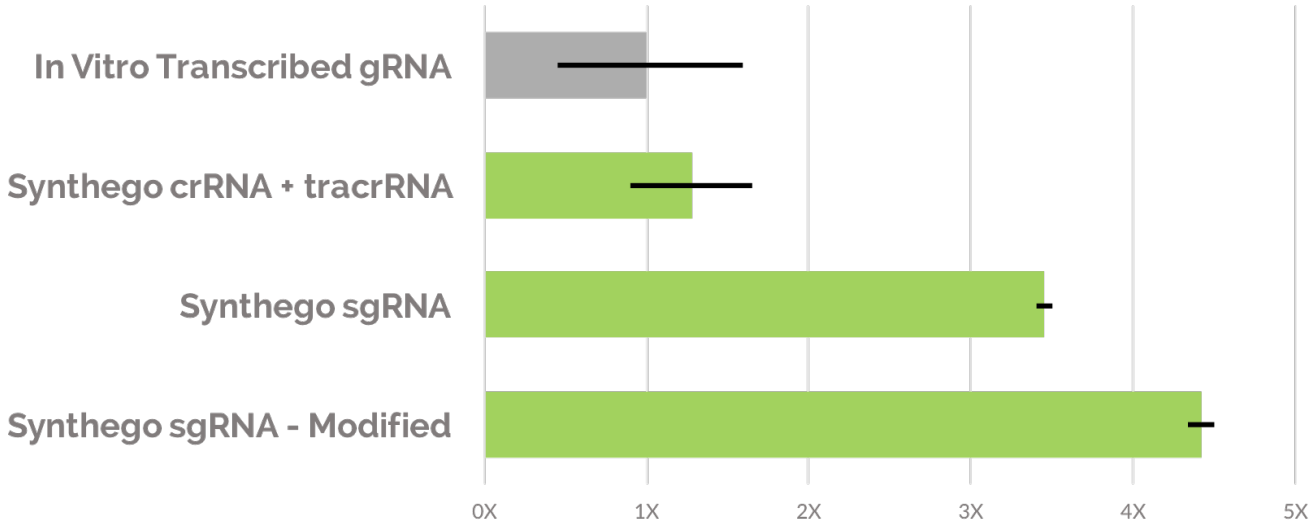
Regardless of whether a two-part crRNA/tracrRNA or one-part sgRNA system is used, synthetic gRNA may be delivered to cells by one of the two routes: by co-transfecting it alongside Cas9 mRNA or by complexing it with Cas9 protein outside of the cell and then delivering it in the form of RNPs through electroporation, lipid transfection, or microinjection.

	Synthetic Guide RNA	Plasmid	IVT
Process	1. Choose target sequence 2. Order synthetic RNA	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

EDITING EFFICIENCY

Compared to the plasmid cloning and IVT methods of preparing gRNA, synthetic gRNA can produce measurably higher editing efficiencies, and a greater level of consistency between replicates in CRISPR experiments. Users of Synthego's synthetic gRNA have reported dramatic improvements in editing efficiency compared to their previous methods of using plasmids of IVT-generated gRNAs. In some cases, they have increased their editing efficiency to upwards of 90% in a wide range of cell types.

The chart below compares average (solid bars) editing efficiencies and their standard deviation (black lines) between 3 replicates of IVT vs. different formats of synthetic gRNA on a challenging genomic target in HEK293T cells. Industry partner validated data.



There are a number of reasons why the use of synthetic gRNA results in a dramatically improved cutting rate across multiples cell types. First, compared to other methods, this approach allows for lower variability and greater control over the volume of CRISPR complexes being delivered to the cell. Second, the use of plasmids in particular prolongs the expression of the CRISPR machinery inside of the cell and can lead to problems with toxicity. Comparatively, synthetic gRNA delivered in the form of RNPs exists only transiently inside of the cell, degrading after its function has been carried out. This characteristic also explains why RNPs produce lower levels of off-target effects. Persistence of CRISPR complexes in the cell can lead to such off-target cutting and problematic alterations in the host genome.

In addition, while synthetic gRNA RNPs represent a wholly DNA-free approach, the same cannot be said of the plasmid method. Even IVT-made gRNA can be plagued by DNA contamination. Thus, both the plasmid and IVT methods run the risk of allowing foreign DNA to integrate into the host genome—a possibility that can be particularly problematic for CRISPR applications in human medicine and food crop engineering. No such risk is associated with the use of synthetic gRNA. Furthermore, synthetic gRNAs can be chemically modified, which is critical when editing particular cell types, such as stem cells, 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 intracellular immune responses. It should be noted that these chemical modifications are possible only if you use synthetic gRNA.

EDITING EFFICIENCY (CONTINUED)

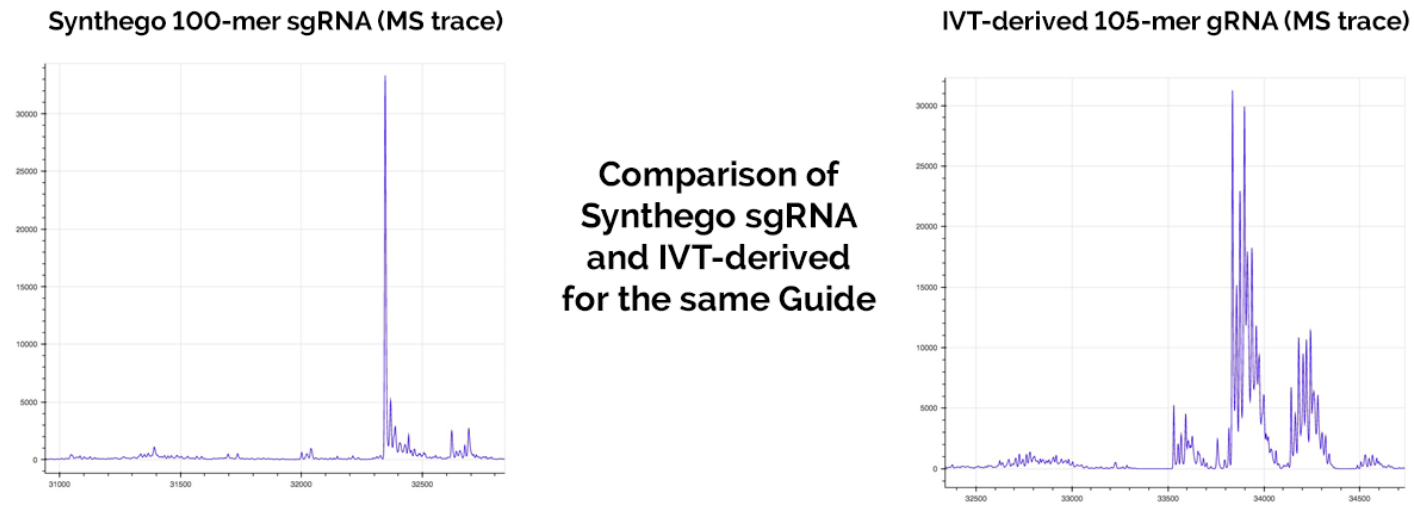
Yet another advantage of using synthetic gRNA over plasmid- and IVT- based approaches.

Finally and most importantly, the greatest leap in editing efficiency over other methods is achieved when synthetic gRNA is deployed in the form of an RNP complex. The benefits of synthetic gRNA are less significant when the RNP form is not used. Liang et al. reported editing rates of 42% in Jurkat T cells and 32% in human iPSCs when utilizing Cas9 mRNA and gRNA—a measurably lower success rate than the 94% and 87% rates respectively achieved through the use of RNPs. The lower editing efficiencies associated with co-transfection of RNAs compared to the RNP form of delivery can be explained by the fact that Cas9 protects gRNA from degradation and this benefit is foregone when the two components are delivered separately. It is worthy to note that the formation of RNPs is also possible for researchers who use IVT to generate gRNA. However, the lower purity and quality of IVT-made gRNA can drag down the effectiveness of RNP delivery, as will be discussed shortly in more detail.

CONSISTENT OUTPUTS

Another point of difference between methods of sourcing gRNA is in the level of consistency in the outcomes they produce. Such variable consistency can be attributed to the very different degrees of purity that are achieved when using IVT-derived gRNA versus chemically synthesized synthetic gRNA. Mass spectrometry of IVT-made gRNA reveals that gRNA samples generated by this method are cluttered with unwanted fragments, partial transcripts, and byproducts, sometimes to such an extent that the existence of the desired gRNA is almost impossible to detect. The low purity of IVT-made gRNA samples owes to multiple factors, including DNA contamination by the transcript template and errors made by PCR and IVT enzymes. This can lead to the production of gRNAs with subpar fidelity to the original sequence and partial transcripts, resulting in off-target effects and highly variable editing efficiencies when these samples are deployed to a host cell. In addition, IVT-derived guides are susceptible to RNAse degradation, since they must be prepared in a clean, RNAse-free environments.

Synthetic gRNA offers a solution to the problems associated with low-purity gRNA made by IVT. With synthetic gRNA samples, there is virtually no variability in the RNA sequence produced. This is because the process of creating synthetic gRNA eliminates both human and IVT error. Synthego's automated platform for synthetic gRNA production allows every molecule to be made with the same high quality and same level of precision with almost no variation—a condition that is impossible to achieve when it comes to IVT-made gRNA. Gene editing assays that use synthetic gRNA have been shown to produce much more consistent results compared to IVT-made gRNA. Furthermore, as was previously discussed, the use of synthetic gRNA leads to significantly higher editing efficiencies and lower off-target effects. Thus, synthetic gRNA leads to superior gene editing performance in terms of both precision and accuracy. In other words, synthetic gRNA is better able to cut only the desired target at a higher rate and to maintain that high rate of correct cutting across multiple experimental replicates.



SPEED

Each of the three approaches to creating gRNA that we've focused on here (plasmid cloning, IVT, and synthetic gRNA) is associated with a very different time requirement. To clone a recombinant plasmid that will express gRNA involves a great many different steps and reactions, including amplification, digestion, ligation, transformation, screening, and purification. In total, the time committed to preparing a CRISPR expression vector typically ranges from 1-2 weeks before the plasmid can even be introduced to the host cell.

IVT production of gRNA, on the other hand, requires only 1 day to achieve, but that includes one full day of involved labor on the part of the researcher. If oligonucleotides must be ordered for the preparation of the DNA template, then this timeframe can be extended out by multiple additional days. Nonetheless, IVT is generally a much more expedient method of preparing gRNA compared to plasmid cloning and, if used in conjunction with an RNP mode of delivery, usually results in somewhat improved editing efficiencies.

However, the time required to prepare a CRISPR experiment that relies on synthetic gRNA is negligible on the part of the researcher: they need only select the target sequence from the host genome and place a corresponding order. The time elapsed between the placement of an order and the delivery of gRNA ranges from 3-5 days. While delivery time can be slightly longer than the time investment for IVT, it's important to note that these days are labor-free and thus, with proper planning, synthetic gRNA can easily be the most efficient route of acquiring gRNA. Indeed, early users of Synthego's synthetic gRNA have reported that their laboratory workflow and experimental capacity has been completely transformed by the convenience and efficiency offered by synthetic gRNA.

Finally, scalability is another factor that is relevant to the speed of a laboratory's experimental capacity. Unlike the plasmid- and IVT-based approaches, Synthego's platform for generating synthetic gRNA is scalable. Synthego is capable of generating small volumes of gRNA ranging from single targets to entire libraries, the latter of which would be prohibitively time consuming and expensive if attempted through other methods. Many of the more advanced applications emerging from CRISPR, such as multiplex engineering and CRISPR screens, demand the level of efficiency and scalability that is furnished by synthetic gRNA. It is for these reasons that more and more laboratories around the world are transitioning to the use of synthetic gRNA to become full-fledged contributors to the CRISPR revolution.

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.