



9 Top Tips for Success with CRISPR-Cas9

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CRISPR-Cas9 is a revolutionary genome and epigenomic editing resource that has rapidly displaced older genomic editing tools. Because this is such a new technology, many scientists and researchers don't quite understand its many intricacies. And, want to improve their knowledge of this novel tool that is making waves from lab to lab. If you fit in to that category or, would simply like to learn some tips and tricks for optimizing your CRISPR-based experiments, then this guide is for you.

Cells do not like double stranded breaks – at all

Quite simply, CRISPR-Cas9 DNA editing generates a DNA Double Stranded Break (DSB) guided by an RNA molecule that is complementary to its genomic target. However, DSBs are extremely cytotoxic and can lead to cell death if not repaired. There are two general ways in which the cell repairs a DSB: through non-homologous end-joining (NHEJ) or through homology-directed repair (HDR).^{1,2} NHEJ occurs when the two cleaved ends of the DSB are tethered back together, often resulting in random insertions or deletions (indels) on both strands of the DNA. Because of this, it is difficult to predict the exact sequence of the resulting NHEJ repair. Conversely, HDR is a much less random repair mechanism in which the homologous region to the DSB on the other chromosome is used as a repair template. The resulting sequence will be the exact sequence of the other chromosome. In the cell, both mechanisms are used to ensure genomic integrity, but CRISPR-Cas9 uses these methods to generate a gene edit of interest.¹

RNA components of Cas9 editing

Cas9 requires two RNA components to function properly: a structural component on the 3' end (tracrRNA) and a second component that directs Cas9 cleavage to its genomic target (crRNA) located on the 5' end. These can be combined to form a chimeric single-guide RNA (sgRNA).^{1,2,3} This section will focus on the design of the crRNA or, the portion of the sgRNA that directs Cas9 cleavage.

In general, at least 20 nucleotides of crRNA must be complementary to the target DNA strand with an obligate NGG Protospacer Adjacent Motif (PAM) sequence at the 3' end of the target strand (which is also the 3' end of the crRNA). The crRNA should be complementary to the target sequence. Nevertheless, there is some degree of tolerance between the complementary crRNA and target DNA.^{2,3} In fact, crRNA can bind to a target site with 3 or 4 base mismatches especially in sequences away from the PAM. Importantly, the NGG is not part of the 20-nucleotide homology. The target region should contain 50% GC content as too rich or poor GC content can disrupt crRNA targeting.³ Once the gRNA binds to the target sequence, a DSB is generated between the third and fourth nucleotide from the PAM (i.e. between bases 17 and 18 of the 20-base target sequence from 5' to 3' orientation).^{2,3} From here, the CRISPR-Cas9 cleavage event has taken place and the cell will attempt to repair the DSB using either the NHEJ or HDR repair pathways.



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Gene knockouts and deletions

Knockouts are arguably the easiest gene edit to generate using CRISPR-Cas9. Simply generate the gRNA, add Cas9, and cut! However, when characterizing the knockout, make sure the resulting indel is not in a multiple of 3 as that is considered an "in-frame" deletion or insertion and the gene may still function properly.

Deletions can be easily generated due to the robust nature of CRISPR-Cas9 editing. Cas9 can only bind one gRNA but a different Cas9 molecule can bind a different gRNA and target a different sequence. Because of this, deletions can easily be formed in a CRISPR-Cas9 experiment. Simply generate gRNAs both upstream and downstream from the desired deletion and include both in the experiment. Gene inversions and chromosomal relocation are also possible using this strategy.

Generating single nucleotide polymorphisms

To generate these types of edits we take advantage of the cell's innate HDR mechanisms. The procedure involves a Cas9 protein with a gRNA with a donor DNA template. When generating a SNP or conditional allele, a single-stranded DNA oligo will suffice as a DNA template donor. To direct proper DNA repair, homology arms of 50-80bp should be included in the DNA oligo.² The typical maximum length for standard ssDNA synthesis is 200bp, however there are companies that can generate up to 1.5kb of ssDNA if a larger knock-in is required. Ideally the cut site (between the third and fourth base from the PAM) is the location of the desired SNP but if not, the cut site should be no more than 10bp away from the bases to be edited. In the donor DNA template, it is extremely important to include some degree of variance to prevent the Cas9 from cutting previously edited DNA because, as long as the gRNA can recognize complementary DNA, Cas9 will continue to generate double stranded breaks.^{2,3} Therefore, it is important to try and locate the desired SNP as close to the PAM as possible to 1) disrupt the bases around the PAM to prevent gRNA annealing and enabling Cas9 cleavage and 2) to maximize the homology arms of the desired edit.

Large construct knock-in

For larger edits such as gene insertion or cassette insertion at a specific locus, plasmid DNA can be used as a donor template. In this case, homology arms should be at least 800bp in length and may be upwards of 2kb. Linear double-stranded DNA such as PCR fragments may also be used as a repair template, but you should bear in mind that linear fragments can randomly integrate into the genome in some organisms. Large knock-ins using CRISPR-Cas9 editing are extremely inefficient. In these instances, it may be useful to have a large sample size as only one or two cells may end up with the desired knock-in. Also, it is imperative to remove potential Cas9 cut sites in the knock-in construct as the gRNA-Cas9 complex will continue to generate DSBs as long as the target sequence remains. For larger knock-in cassettes, it may be useful to include an antibiotic resistance marker, fluorescent marker, or some other gene marker to identify cells that may have integrated the construct into their genome.



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CRISPR editing can generate multiple edits at once!

The underlying mechanism of all CRISPR-Cas9 events is that it cleaves DNA at a site as guided by an RNA molecule and then uses the native cellular machinery to generate the edit of interest. In one CRISPR experiment, there is the potential to create a range of useful edits in a gene. For example, there is a chance that in one cell or embryo the Cas9 DSB is repaired via NHEJ, generating indels which may result in a gene knockout. Whereas in another cell the DSB could be repaired via HDR using the DNA donor as a repair template resulting in a SNP at a gene of interest. In some instances, a biallelic edit can be generated where both chromosomes get the same knock-in or gene disruption, though these instances are rare.

Have a genotyping strategy before you begin

Believe it or not, generating CRISPR-editing events is very easy. Figuring out the resulting genotype on the other hand, can be much more labor-intensive. Here is a quick overview of some of the standard methods of genotyping:

- 1. PCR detection:** This method relies on amplifying a gene fragment from the genomic DNA of a sample and can be readily used to detect deletions or insertions.
- 2. Sanger sequencing:** To determine the exact sequence, the PCR fragments can be sequenced or cloned into a plasmid and then sequenced. The downside to Sanger sequencing a raw PCR product is that not much data can be obtained once the sequencing trace reaches the cut site as there is the chance of generating different reads from the two chromosomes. To discern the exact sequence, cloning is required which is extremely labor intensive and may not be suitable if screening many samples as only one genetic fragment from one chromosome can be cloned per colony.
- 3. Illumina next-generation sequencing:** This method is analogous to Sanger sequencing but offers much higher throughput as many thousands of individual reads per PCR fragment can be generated with the benefit of minimal labor. This also negates the need for cloning as each individual read is then pooled to provide a large dataset per sample submitted. A problem with this method is that only short fragments, less than 400bp, can be reliably sequenced and it may not be a cost-effective approach if you are only characterizing a handful of samples.
- 4. Restriction digest of a PCR product:** This can be used once an indel is characterized or if HDR is used containing a unique restriction site in the SNP. Simply amplify the fragment of purified genomic DNA and add the restriction enzyme. Make sure to check out the activity of the restriction enzyme in PCR buffer to avoid a PCR purification step.
- 5. Phenotype selection:** This can be an antibiotic resistance marker, production of a fluorescent protein, or introduction of a non-native gene to the cell. These are the easiest methods to detect the introduction of your gene of interest, but by far the least efficient. This type of selection should only be used when introducing a large knock-in or gene cassette.



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Off-target effects

CRISPR is not a perfect genome editing tool. As mentioned previously, there can be variations in the binding of the crRNA and the genomic target sequence allowing for the crRNA to anneal to an off-target sequence and generate a DSB. However, advances have been made to mitigate off-target effects that may arise from a CRISPR-Cas9 editing event. For one, genome sequencing libraries and online tools can help in designing gRNAs that have reduced off-target sites in whatever genome you are attempting to edit. A gRNA design tool that I frequently use is CRISPOR server (<http://crispor.tefor.net/>) which evaluates target activity as well as potential off-target sites. Alternatively, the CCTop server (<https://crispr.cos.uni-heidelberg.de/is>) is useful for determining on-target sites, while the Cas-OFFinder server (<http://www.rgenome.net/cas-offinder/>) is good at finding potential off targets. Most model organisms have their genome entirely sequenced therefore gRNA development can be readily streamlined to find potential off-target loci. As off-target effect analysis becomes more mainstream, our understanding of which gRNAs to choose becomes clearer.

Another way to diminish off-target effects is by modifying the Cas9 protein so that it cuts less. There are two main variants of Cas9 that have been developed, where the DNA binding ability of the protein is substantially reduced.² The logic here is that there will be less off-target edits if the Cas9 protein cannot physically bind to the DNA, which is true. The payoff with these modified Cas9s is that the crRNA can no longer have any mismatch with the target sequence. Cas9 variants with reduced DNA binding can be purchased under the names Cas9-HF1, eCas9, and Alt-R Cas9.

A final variant of Cas9 that reduces off-target effects are variants that merely nick the DNA instead of generating a DSB.² Nicking the DNA prevents NHEJ from occurring and creates breaks that can be repaired using HDR. This gives a window of opportunity to provide a donor DNA template at the nick site to integrate the edit of interest into the genome. Off-target effects of nicking Cas9 are almost completely mitigated because DSBs are not generated and any off-target sites will be minimized by repairing the nicks. However, gene knockouts are much rarer with nicking Cas9s and repair via HDR is also reduced as it is much easier for the cell to repair a nick in the DNA than to invest the energy for HDR.

Modern CRISPR adaptations

Many groups are utilizing a catalytically dead form of Cas9 (dCas9) to generate epigenetic edits.^{1,4} The principals of CRISPR-Cas9 remain the same: targeting Cas9 using gRNA to a specific region on the genome, with the only variant being that the Cas9's active residues (an aspartate and a histidine) are substituted with alanine, rendering it catalytically inactive. Groups have used dCas9 to modulate gene expression when transcriptional activators or repressors are fused to it.⁴ dCas9 has also been used for epigenetic modification in terms of histone modification or DNA methylation.⁴ Cytidine deaminases can also be fused to dCas9 to generate site specific base substitution without generating a DSB. Green fluorescent protein can be fused to dCas9 to track and image a specific site on the chromosome. Finally, there are homologous proteins to Cas9 such as Cpf1 (Cas12a) that can also generate DSBs in genomic DNA, except generating staggered ends. Cpf1 also recognizes a different PAM sequence, NTTT, which expands the editing capabilities in the genome.⁵ Additionally, Cas13 can edit RNA instead of DNA which may be of benefit for further therapeutic development as no edits to DNA are made.⁶



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References

1. Doudna, J. A., & Charpentier, E. (2014). The new frontier of genome engineering with CRISPR-Cas9. *Science*. doi:10.1126/science.1258096
2. Wang, W., La Russa, M., & Qi, L. S. (2016). CRISPR/Cas9 in Genome editing and beyond. *Annual Review of Biochemistry*. doi:10.1146/annurev-biochem-060815-014607
3. Wiles, M. V., Win, W., Cheng, A. W., & Wang, H. (2018). CRISPR-Cas9-mediated genome editing and guide RNA design. *Mammalian Genome*. doi:10.1007/s00335-015-9565-z
4. Dominguez, A. A., Lim, W. A., Qi, L. S. (2016). Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nature Reviews Molecular Cell Biology*. doi:10.1038/nrm.2015.2
5. Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., Volz, S. E., Joung, J., van der Oost, J., Regev, A., Koonin, E. V., Zhang, F. (2015). Cpf1 is a single RNA-guided Endonuclease of a Class 2 CRISPR-Cas system. *Cell*. doi:10.1016/j.cell.2015.09.038
6. Cox, D. B., Gootenberg, J. S., Abudayyeh, O. O., Franklin, B., Kellner, M. J., Joung, J., & Zhang, F. (2017). RNA editing with CRISPR-Cas13. *Science*. doi:10.1126/science.aaq0180