application note

How to design gene disruption experiments using the Alt-R™ CRISPR-Cas9 System

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Abstract

Use of the CRISPR (clustered regularly interspaced short palindromic repeats) and associated Cas9 enzyme for genome editing has been a major technological breakthrough, making genome modification in cells or organisms faster, more efficient, and more robust than previous genome editing methods. The Alt-R™ CRISPR-Cas9 System is an optimized genome editing solution that outperforms other CRISPR approaches for producing on-target double-stranded DNA breaks. CRISPR-Cas9 genome editing requires 3 components to edit a genomic site. To target a specific sequence, researchers simply adjust a short 19–20 base protospacer element within the CRISPR RNA (crRNA). The crRNA combines with a transactivating CRISPR RNA (tracrRNA) to guide the Cas9 nuclease to the target site. The tracrRNA sequence and Cas9 nuclease do not change, but combine with the crRNA to form the active, site-specific editing complex. Here, we give an overview of how to design Alt-R CRISPR crRNA, focusing on gene disruption by non homologous end joining (NHEJ) and avoiding unintended, off-target editing.

Introduction

Numerous methods have been described for using the CRISPR-Cas9 system for genome editing. The native bacterial CRISPR system in *S. pyogenes* requires 2 short RNA molecules—a sequence-specific crRNA guide and a conserved tracrRNA. The crRNA and tracrRNA form a complex through short homologous regions, and subsequently guides and activates Cas9 to cleave double-stranded DNA targets (dsDNA) [1–3]. Double-stranded cleavage of the target DNA by Cas9 then activates the NHEJ system, or generates a potential insertion site for exogenous donor DNA by homology directed repair (HDR).

It has been shown that the crRNAs and tracrRNAs can also be fused into a chimeric single-guide RNA (sgRNA) [2,3]. This simplifies the introduction of the CRISPR RNAs and is regularly used, especially when using DNA expression constructs, such as gBlocks® Gene Fragments, or plasmid DNA. However, sgRNA may not be the most effective editing solution, and using dsDNA to express CRISPR components in the cell also allows that DNA to be incorporated into the host cell genome with potential for unwanted outcomes.

Another easy approach is to use RNA oligonucleotides to create the necessary crRNA and tracrRNA components. The Alt-R CRISPR-Cas9 System improves the efficiency of CRISPR genome editing performance through use of experimentally optimized Alt-R CRISPR crRNA and Alt-R CRISPR tracrRNA. We have found that chemically synthesizing the crRNA and tracrRNA as separate, RNA oligonucleotides, which are also shorter than the natural *S. pyogenes* RNAs, consistently improves on-target editing efficiency over other published methods. Here, we give an overview of how to design Alt-R CRISPR crRNA, focusing on gene disruption by NHEJ and avoidance of unintended, off-target editing.

Selecting a target site for gene disruption

In developing the Alt-R CRISPR-Cas9 system, we observed that using RNA oligonucleotides that are shorter than the native *S. pyogenes* CRISPR RNAs consistently performed well at most of the protospacer adjacent motif (PAM) sites tested (Figure 1). Along with improved efficiency, the system saves time by providing ready-to-use RNA reagents that reduce or eliminate activation of the cellular immune response observed with longer RNAs, such as *in vitro* transcribed sgRNA. The focus of this application note is to discuss crRNA design considerations for gene disruption/knockout. Many of the same design principles can also be applied to HDR and other applications, but there are differences.
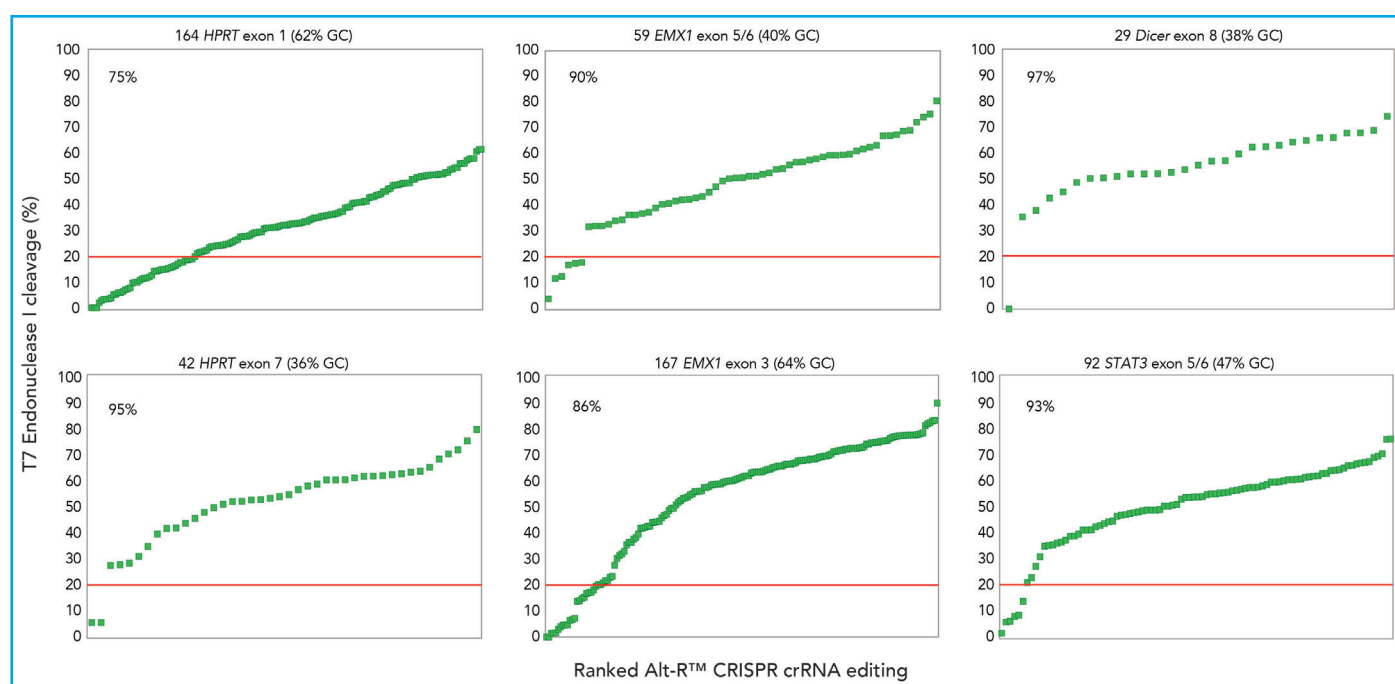

Figure 1. Alt-R™ CRISPR-Cas9 System functions well across many sites. Alt-R crRNAs were designed to 553 PAM adjacent sites in 4 distinct genes (*HPRT*, *EMX1*, *STAT3*, and *Dicer*). Complexes were reverse transfected using Lipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher) into a HEK293-Cas9 cell line that stably expresses *S. pyogenes* Cas9. Genomic DNA was isolated and editing measured by PCR amplification of target sites, followed by cleavage with T7EI mismatch endonuclease (New England Biolabs) and analysis using the Fragment Analyzer™ (Advanced Analytical).

Cleavage of genomic targets with the CRISPR-Cas9 system requires a PAM site at the 3' end of potential target sites. For *S. pyogenes* Cas9, the PAM sequence is NGG. The protospacer specifies the target sequence for Cas9, and is used by bacteria as a mechanism to remember and defend against foreign

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pathogens. Researchers can use this information to create synthetic RNA or DNA constructs with artificial protospacer elements that target Cas9 to sites with PAM sequences for genome editing in prokaryotic or eukaryotic organisms.

The strategy you choose for selecting PAM sites within your gene(s) of interest will depend on your overall goal. If your goal is the complete disruption of a target gene, one commonly used strategy is to select PAM sites located towards the 5' end of your gene (close to the ATG). NHEJ repair of double-stranded DNA frequently results in frame shift mutations and premature stop codons, so targeting 5' exons is more likely to disrupt all splice variants, making this first strategy a good general approach.

A second strategy is to target PAM sites coding key functional domains in the resulting protein. This second strategy also produces truncations, but disrupting the functional domain would mean that any gene products should be inactive. Depending on where the functional domains are located, this strategy may result in more truncation products being produced compared to targeting just the 5' end of the gene.

Once you decide on a location within your gene(s) that you wish to edit, you will want to search that region for all NGG sequences, and select one or more PAM sites for testing.

A key distinction between the NHEJ gene disruption approaches and making specific mutations using HDR is that HDR requires researchers to choose a PAM site that is as close as possible to the site they want to edit. If an NGG–PAM site is not available for HDR, it may be possible to use a Cas9 that recognizes a different PAM sequence (such as, a Cas9 from a different species of bacteria or an S. pyogenes Cas9 that has been engineered to recognize a different PAM sequence).

On-target performance

A perfect crRNA would only recognize 100% homologous sequences and facilitate cleavage at that specific site in every cell with the introduced CRISPR-Cas9 components. However, even crRNAs with perfect target sequence matches rarely show complete cutting of the targets in every cell. The observation that on-target editing varies when only the target site differs indicates the existence of site-specific factors that influence performance, but what these contextual features are and how they influence editing performance are not clear. There are multiple, open-source CRISPR design tools available that identify and attempt to rank potential target sites for CRISPR-Cas9; however, in our experience, we found that most of the tools do not consistently predict crRNA on-target performance.

Factors other than choice of target site also affect editing performance in more predictable ways. However, the importance of the on-target prediction is mitigated using the Alt-R CRISPR-Cas9 System, because the crRNA:tracrRNA complex used in this system is highly effective. For example, using the experimentally optimized Alt-R System, we routinely find that >80% of target sites work well—defined here as producing >20% on-target editing as determined by T7EI cleavage (Figure 1) or >40% on-target editing as verified by Sanger sequencing. Note that a large proportion of resulting NHEJ mutations are single-base alterations,

which are not detected by T7EI. Therefore, the T7EI assay is an indicator of editing performance, but it does underestimate the amount of on-target editing.

We recommend that you test 2–3 non-overlapping target sites to ensure that at least one will give >20% on-target editing, measured by the T7EI assay. This will provide a high likelihood of finding a crRNA with sufficient on-target performance for most applications.

Designing crRNA for gene disruption

Know your gene	Targeting an early 5' exon of your gene reduces the chances of functional truncation products.
	Targeting a specific functional domain may also be an effective approach.
	Ensure that targeted PAM sites do not affect other genes or regulatory elements that may overlap with your gene.
Check for off-target effects	Free, online tools such as the Broad Institute's CRISPR Design tool (http://crispr.mit.edu/) can help avoid unintended editing at off-target sites.
Select your CRISPR reagents	The highly effective Alt-R™ CRISPR-Cas9 System includes all of the core components you need for efficient genome editing. All of the components can be ordered at www.idtdna.com/CRISPR.

Off-target effects

Another concern when designing crRNAs is avoiding off-target effects. Off-target effects are caused when a crRNA has partial homology to unintended genomic sites that are sufficient to cause unwanted DNA cleavage by Cas9. Currently, we suggest screening target sites for off-target activity, beginning with the Broad Institute's CRISPR Design tool (http://crispr.mit.edu/).

This CRISPR tool allows users to enter 23–500 nucleotides of the target genomic region and compares the input region to one of the 16 genomes listed on the site. The tool identifies potential guide sequences from the user input, and compares potential guide sites to the whole genome for sites with partial homology. An example of the results for a 154 base region of the voltage gated calcium channel, *CACNA1A*, is shown in Figure 2. The upper part of the display has the search name and a schematic representation of the guide sequences, and their relative positions in the uploaded sequence. The first group of columns shows the ranked guide RNA (gRNA) sequences, with a color coded ranking of "high quality", "mid quality", and "low quality", and given a score of 0–100.

Selecting any of the gRNA sequences will dynamically update the second and third groups of information, to the right of the list of guides. These information groups show the sequences of potential off-target sites, base positions of mismatches, and location in the genome.
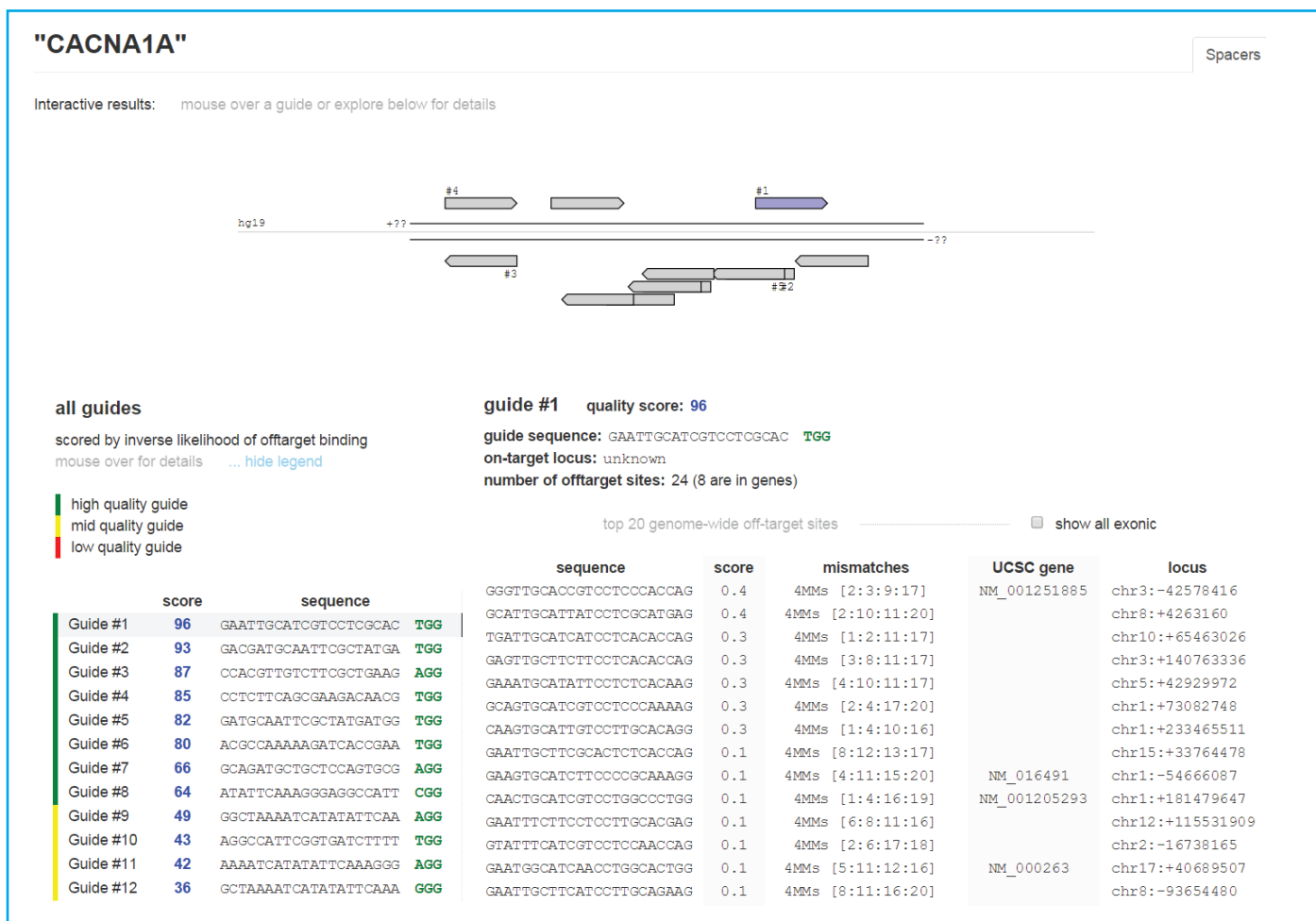
Figure 2. Guide sequence analysis for a 154 base region of *CACNA1A* using the CRISPR tool at http://crispr.mit.edu/. Output shows ranked gRNA sequences, with a color-coded ranking for quality, based on predicted off-target activity, potential off-target sites with locations, and potential genes that may be affected.

Note that sequence mismatches closer to the PAM sequence have a more negative impact on Cas9 cleavage than mismatches further away in the protospacer sequence. Therefore, off-target sites with mismatches near the PAM sequence are less likely to have an unwanted editing event than those with mismatches more distal from the PAM [4]. The output also displays information for genes that have been identified at potential off-target sites.

Progress has been made in developing methods for detecting off-target activity, such as the GUIDE-seq method, which uses incorporation of artificial sequences at double-stranded break sites and subsequent NGS mapping of these sites [5]. However, even with sophisticated methods, it is still impossible to know with absolute certainty that all off-target events have been identified. Confidence in experimental outcomes can be supported by performing negative controls, observing the same phenotype when targeting different PAM sites in the same gene region, and a careful design strategy.

Protospacer length, and off-target activity

It has been reported that using shorter protospacer motifs in the crRNA design can reduce off-target effects [6]. Therefore, we examined crRNAs with shorter guide sequences to see what effect they had on performance. Our data show that protospacer sequences shorter than 19 nt severely compromised on-target performance, becoming more variable when the spacer was 18 nt, and consistently producing very low numbers of editing events when the protospacer was only 17 nt (Figure 3). Therefore, we currently do not offer Alt-R CRISPR crRNAs that target less than 19 nt.
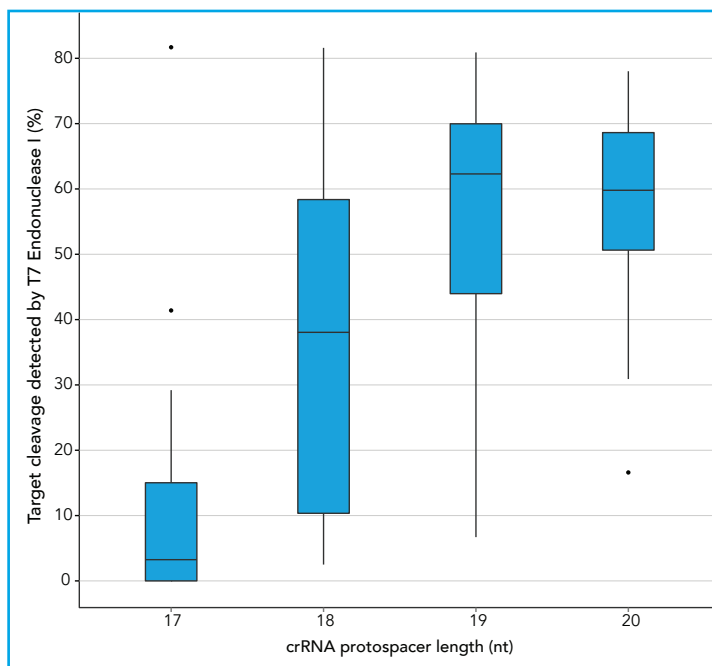

Figure 3. 19–20 nt protospacer element provides optimal genome editing. crRNAs with varying protospacer element lengths (17–20 nt) were designed to 12 distinct HPRT target sites. crRNA:tracrRNA complexes were reverse transfected using Lipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher) into a HEK293 cell line stably expressing *S. pyogenes* Cas9. Genomic DNA was isolated. CRISPR-Cas9 genome editing was measured by PCR amplification of target sites, cleavage with T7EI mismatch endonuclease (New England Biolabs), and analyzed using the Fragment Analyzer™ (Advanced Analytical). At all but one of the 12 target sites, crRNAs with 19 and 20 nt protospacer elements produced the greatest amount of genomic editing. Each of the 12 data points for each category represent the average of 3 biological replicates, with the exception of one data point in the 19 nt category , which was composed of 2 biological replicates.

Conclusion

The Alt-R CRISPR-Cas9 System makes it easy to conduct genome editing experiments. Due to the excellent performance of the system, ordering 2–3 crRNAs to target different PAM sites in your gene of interest will give you a high likelihood of success. Once you have generated your designs, simply enter the 19 or 20 nt protospacer domain for each crRNA directly into the CRISPR oligo entry tool at www.idtdna.com/site/order/oligoentry/index/crispr. The crRNA protospacer bases must be entered as the 19–20 bases, upstream of the PAM site, in the forward orientation as shown in Figure 4. The ordering system will automatically append a 16 nt tracr interaction domain to the final sequence for manufacturing of the complete crRNA. High quality manufacturing and standard desalting will ensure >80% full length crRNAs as verified by ESI-MS, our standard QC method.

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Genomic target sequence          5'...CGAAATCGATCGATCGATCGATCGTGGATCGATC...3'
Correct protospacer sequence          5' ATCGATCGATCGATCGATCG 3'

Common errors:
Target sequence plus NGG               ATCGATCGATCGATCGATCGTGG
Reverse complement of target           CGATCGATCGATCGATCGAT
Reverse of target                      GCTAGCTAGCTAGCTAGCTA
Complement of target                   TAGCTAGCTAGCTAGCTAGC
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Figure 4. How to enter your crRNA target sequence. Because the crRNA recognizes and binds 20 bases on the DNA strand opposite from the NGG sequence of the PAM site, order your crRNA by entering the 20 bases upstream of the PAM site, in the forward orientation as shown. If you are pasting your CRISPR target site from an online design tool, make sure you verify the correct strand orientation. Do not include the PAM site. Common incorrect design examples are shown in red.

Finally, in order to perform your CRISPR-Cas9 genome editing experiment, you will also need to select a source for expressing Cas9 in your cells. IDT offers the Alt-R S.p. Cas9 Expression Plasmid (Cat # 1072566) that is 7.3 kb in size. The Alt-R S.p. Cas9 Expression Plasmid does not contain a eukaryotic selection marker-however, the smaller size of this plasmid is designed to improve transfection efficiency. The Alt-R CRISPR crRNA and tracrRNA are also compatible with *S. Pyogenes* Cas9 from other sources, including stably transfected Cas9 cell lines, Cas9 mRNA, or recombinant Cas9 protein. Instructions for using the Alt-R CRISPR-Cas9 System with the associated expression plasmid can be found in the user guide at www.idtdna.com/CRISPR.

Materials and methods

Transfection of HEK293-Cas9 cells with CRISPR RNA: Alt-R CRISPR crRNA and tracrRNA were resuspended to a 100 µM concentration in Nuclease-Free IDTE buffer, pH 7.5 (1X TE solution; Cat # 11-01-02-02). The crRNA and tracrRNA were then annealed by heating to 95°C and cooling to room temperature, at a 3 µM working concentration. In each well of a 96-well plate, 1.5 µL of the 3 µM crRNA:tracrRNA mix (30 nmol of each tracrRNA and crRNA) was added to 0.75 µL ofLipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher) and 47.75 µL of Opti-MEM® Media (Thermo Fisher). 100 µL of suspended HEK293-Cas9 cells that stably express *S. pyogenes* Cas9 were added to each well at ~75% confluency to complete reverse transfection. The plate containing the transfection complexes and cells was placed in a tissue culture incubator (37°C, 5% CO2) for 48 hr.

Mutation detection by T7EI mismatch endonuclease: DNA from CRISPR-Cas9 edited cells was isolated by adding 50 µL of QuickExtract™ DNA Extraction Solution (Epicentere) and transferred to PCR plates. DNA was vortexed and heated to 65°C for 15 min in a thermal cycler, then vortexed and heated to 95°C for 15 min in a thermal cycler. Following heating, the DNA was diluted with 100 µL of Nuclease-Free Water (Cat # 11-04-02-01). CRISPR target sites were then amplified with KAPA HiFi HotStart PCR Kit (Kapa Biosystems). Heteroduplexes were formed by denaturing at 95°C and cooling slowly to room temperature. Quantification of CRISPR editing was completed by visualizing T7EI cleavage products, and results were quantified using a Fragment Analyzer™ system with the Mutation Discovery Kit (Advanced Analytical).

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