Design considerations for highly specific and efficient synthetic crRNA molecules

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Introduction

In order to understand the parameters affecting CRISPR-Cas9 gene editing efficiency, we systematically transfected synthetic CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) reagents targeting components of the proteasome into a reporter cell line in which knockout of proteasome function results in fluorescence of a ubiquitin-EGFP fusion protein that is normally degraded by the proteasome pathway. We evaluated the functionality of > 1100 crRNA sequences in this system; using these data, we developed and trained an algorithm to score crRNAs based on how likely they are to produce functional knockout of targeted genes. We further tested our algorithm by designing synthetic crRNAs to genes unrelated to the proteasome and examined their ability to knock out gene function using additional phenotypic assays. To augment our functionality algorithm, we developed an optimized alignment program to perform rapid, flexible, and complete specificity analysis of crRNAs, including detection of gapped alignments. We have combined this comprehensive specificity check with our functionality algorithm to select and score highly specific and functional crRNAs for any given gene target.

Off-target analysis should include gaps

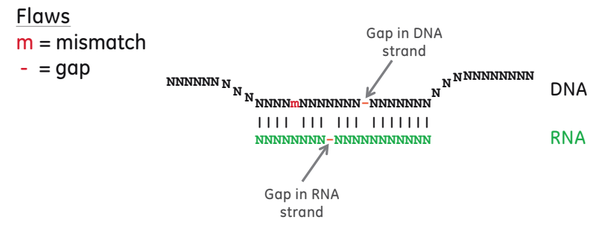


Figure 5. Potential off-target sites in the genome for any given crRNA include not just mismatches but gaps as well. Gaps can exist in the crRNA strand or in the DNA target strand. Many commonly used web-based crRNA specificity tools do not fully account for gaps when performing alignments.

Functional assay for crRNAs

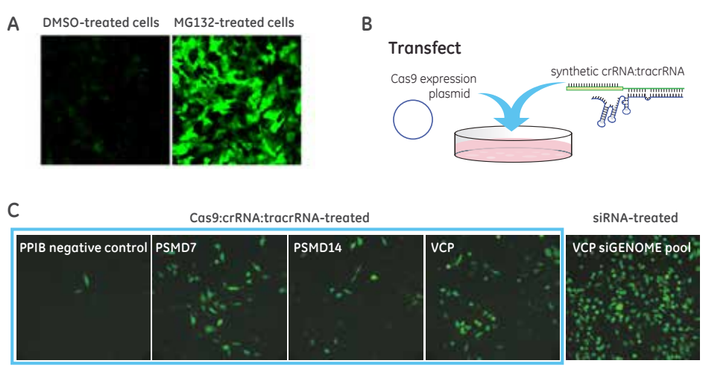


Figure 1. Recombinant U2OS cell line stably expressing a mutant human ubiquitin fused to EGFP. A Gly76Val mutation results in an uncleavable ubiquitin moiety fused to EGFP, which results in constitutive degradation of the protein and little detectable EGFP fluorescence. **(A)** Inhibition of proteasome activity with chemical treatment (MG132) results in EGFP fluorescence. **(B)** Schematic of a CRISPR-Cas9 experiment using synthetic crRNA and tracrRNA reagents. **(C)** Inhibition of proteasome activity with CRISPR-Cas9 or siRNAs targeting proteasome components results in EGFP fluorescence.

Complete off-target analysis is important for crRNA specificity

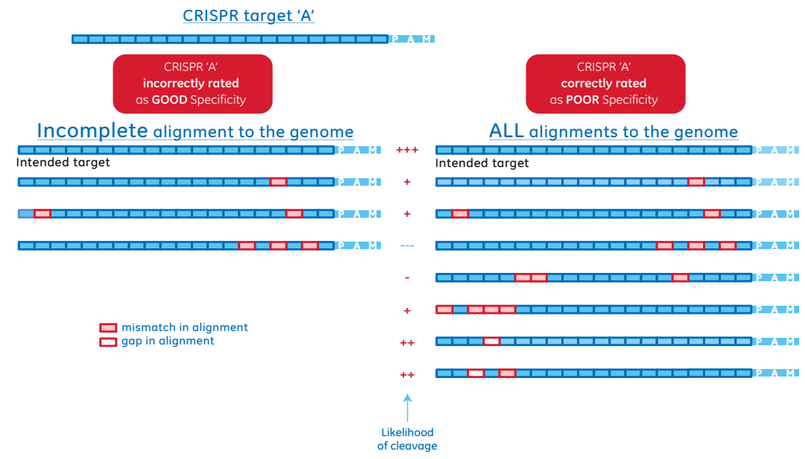


Figure 6. Schematic demonstrating the effect of incomplete alignment during off-target analysis for a crRNA.

crRNA functionality is position- and sequence-dependent

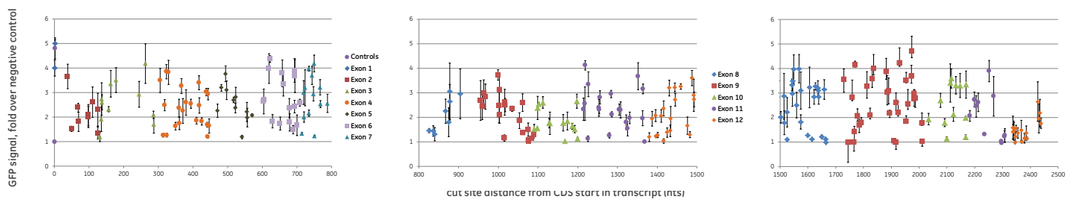
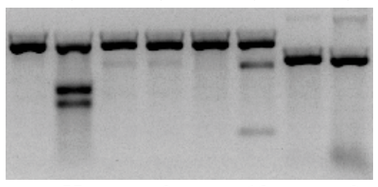


Figure 2. Ubiquitin[G76V]-EGFP U2OS cells stably expressing Cas9 were transfected with 266 synthetic crRNA:tracrRNA complexes targeting the coding region of the VCP gene. EGFP fluorescence was measured 72 hours post- transfection; an increase in EGFP fluorescence indicates functional knockout of the VCP gene resulting in disruption of proteasome function. crRNAs in different exons are indicated by the different colors. The data indicate that crRNAs vary in their ability to cause functional gene disruption.

crRNA: - + - + - + - +



Indel%	37	0	16	0
	Target	OT1	OT2	OT3

Target/Off-target	Sequence	PAM
Intended target	GGTCATCTGGGAGAAAAGCG	TGG
OT1	GGTCCTCTGGGAGAAAAGACG	CAG
OT2	GGT-ATCTGGGAGAAAAGCA	TGG
OT3	GGTC-TCTGGGAGAAAAG-G	AAG

Figure 7. Ubi[G76V]-EGFP U2OS cells stably expressing Cas9 were transfected with 25 nM synthetic crRNA:tracrRNA and genomic DNA was isolated 72 hours after transfection. Potential off-target (OT) sites containing mismatches as well as gaps in the RNA or the DNA strand were analyzed for off-target cleavage with a mismatch detection assay (using T7EI endonuclease). Red nucleotides indicate mismatches, red dashes indicate gaps, and underlined red nucleotides indicate insertions relative to the target DNA sequence. The off-targets were identified using the Dharmacon specificity tool and are not identified by other common web-based crRNA specificity tools.

Machine learning for algorithm development

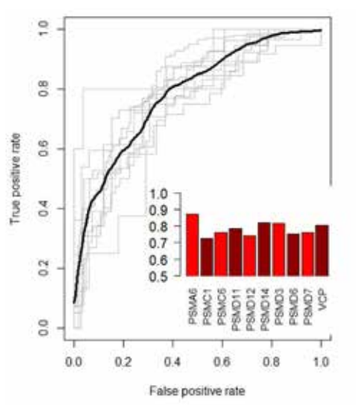
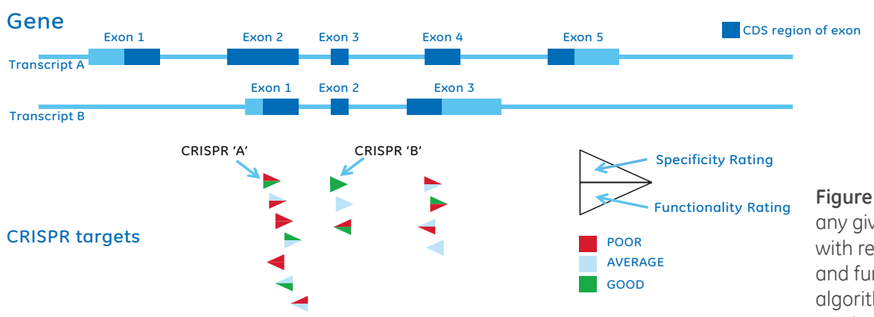


Figure 3. A training set consisting of 10 genes and 1115 crRNA target sites was used to train our functionality algorithm. Features examined include nucleotide composition, nearest neighbor effects, PAM sequence, position in exons, and distance from the start codon. Receiver Operating Characteristic (ROC) shows good fit of training set data. The ROC measures the area under the curve of True Positive Rate vs False Positive Rate. The ROC for our test set data is 0.78.

The optimal crRNAs balance functionality and specificity



CRISPR target 'A' – May function, but poor specificity
CRISPR target 'B' – OPTIMAL PICK, scores well in both functionality & specificity

Figure 8. Schematic of how any given crRNA may differ with regard to its specificity and functionality. Our algorithm balances these two attributes to pick the crRNAs predicted to have the highest specificity and functionality.

Functionality of algorithm-designed crRNAs in other assays

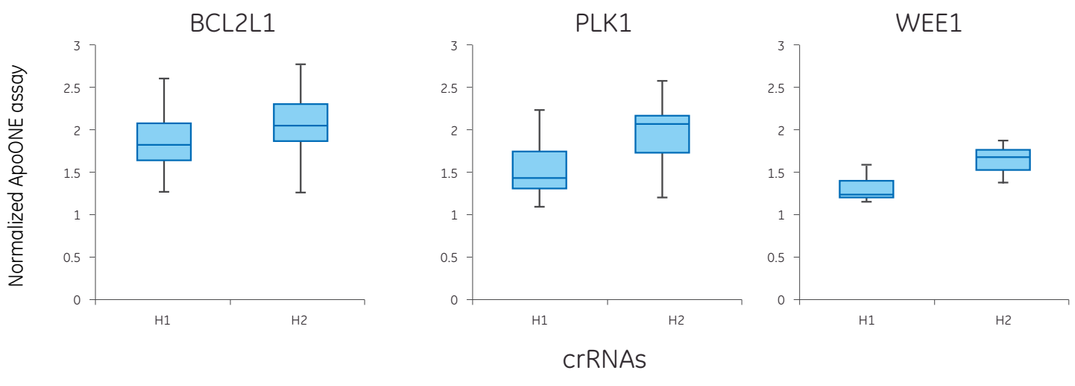


Figure 4. Box plot representation of the functionality of crRNAs targeting *BCL2L1*, *PLK1* or *WEE1* as determined by the ApoONE homogeneous assay (Promega) 48 hours after transfection. For the box plots, crRNAs were divided into bottom half (H1) and top half (H2) based on their functionality score. The medians as well as the distribution of data between the lower and upper quartile demonstrate that high-scoring crRNAs have increased functionality.

Conclusions

- crRNAs vary in specificity and in efficiency for creating functional gene knockouts
- We used a high-throughput fluorescence assay to develop and train an algorithm to score crRNAs for likelihood of producing functional gene knockouts
- We developed an optimized alignment program to perform rapid and complete specificity analysis of crRNAs
- We demonstrate that targets with gaps in either the RNA or DNA strand can be cleaved and are therefore important to identify during specificity checking

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