# Designing highly functional and specific guide RNAs for knockout and achieving precise knockin using the homology-directed repair pathway

Melissa L. Kelley, Emily M. Anderson, Shawn McClelland, Elena Maksimova, Tyler Reed, Steve Lenger, Žaklina Strezoska, Hidevaldo Machado, Eldon Chou, Maren Mayer, John Schiel and Anja van Brabant Smith
Dharmacon, part of GE Healthcare, 2650 Crescent Drive, Suite #100, Lafayette, CO 80026, US

## Introduction

The CRISPR-Cas9 system requires a Cas9 nuclease and two short RNAs, a crRNA and tracrRNA, to introduce double-strand breaks for functional protein disruption (knockout) or for insertion or replacement of genetic content, such as a selection marker (knockin). Our approach to better understand the parameters affecting CRISPR-Cas9 editing efficiency for functional gene knockout utilized synthetic crRNAs and tracrRNA, which can be chemically synthesized rapidly without the need for cloning and sequencing. We systematically transfected > 1100 synthetic crRNA:tracrRNAs into a GFP-reporter cell line to develop an algorithm to predict functional gene knockout. To minimize potential off-targets, we developed a rigorous specificity tool that is able to detect mismatches as well as gapped alignments that are typically missed using most existing specificity tools. Together, we are able to design highly functional and specific guide RNAs. In addition, we have expanded the use of synthetic crRNA:tracrRNA by demonstrating its utility in knockin applications using short single-stranded DNA as donor templates for small insertions, such as SNPs, or DNA vectors as donor templates for large insertions, such as a GFP reporter. Finally, we provide guidelines for design of these donor templates for optimal knockin efficiency.

## High-throughput assay for functional protein knockout

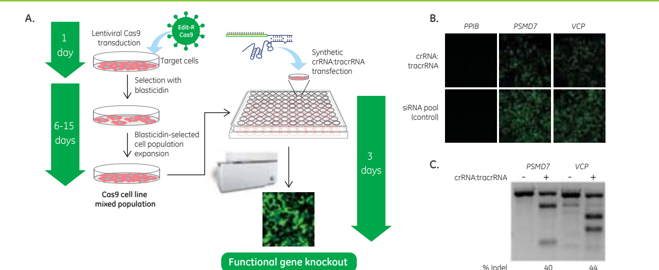



**Figure 1. A.** A stable Cas9-expressing recombinant U2OS cell line (Ubi[G76V]-EGFP U2OS) was generated and then transfected with synthetic crRNA:tracrRNAs (25 nM) targeting components of the proteasome. The recombinant U2OS cells constitutively express EGFP fused to a mutant ubiquitin (Gly76Val). When the proteasome is functioning, ubiquitin-EGFP is degraded (no signal), and when proteasome function is disrupted, ubiquitin-EGFP accumulates (GFP signal). **B.** Synthetic crRNAs targeting *PSMD7* and *VCP*, known components of the proteasome, show EGFP-positive cells indicating functional protein knockout. **C.** crRNAs targeting *PSMD7* and *VCP* resulted in high gene editing efficiencies estimated by a DNA mismatch detection assay using T7EI endonuclease.

## crRNA functionality is position and sequence dependent

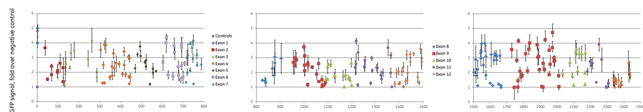



**Figure 2.** Ubiquitin[G76V]-EGFP U2OS cells stably expressing Cas9 were transfected with 266 synthetic crRNA:tracrRNA complexes targeting the coding region of the VCP gene. EGFP fluorescence was measured 72 hours post-transfection; an increase in EGFP fluorescence indicates functional knockout of the *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.

## crRNAs that have high functionality scores show high editing efficiency

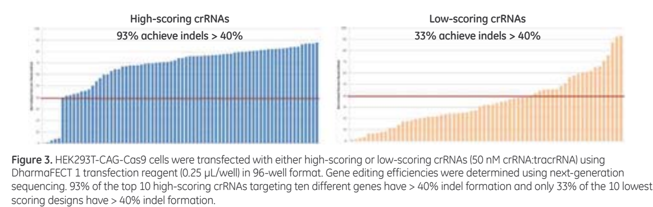
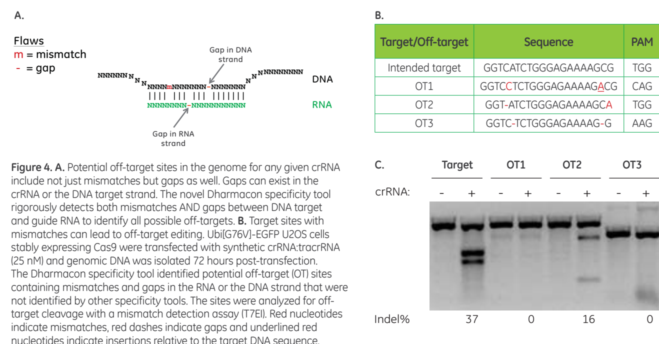



**Figure 3.** HEK293T-CAG-Cas9 cells were transfected with either high-scoring or low-scoring crRNAs (50 nM crRNA:tracrRNA) using DharmaFECT 1 transfection reagent (0.25 µL/well) in 96-well format. Gene editing efficiencies were determined using next-generation sequencing. 93% of the top 10 high-scoring crRNAs targeting ten different genes have > 40% indel formation and only 33% of the 10 lowest scoring designs have > 40% indel formation.

## Comprehensive identification of mismatches & gaps is important for crRNA specificity

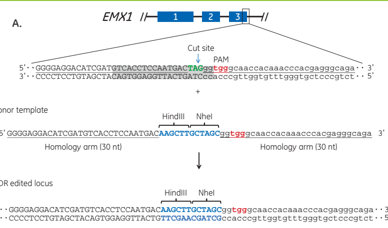



B.

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

**Figure 4. A.** 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 or the DNA target strand. The novel Dharmacon specificity tool rigorously detects both mismatches AND gaps between DNA target and guide RNA to identify all possible off-targets. **B.** Target sites with mismatches can lead to off-target editing. Ubi[G76V]-EGFP U2OS cells stably expressing Cas9 were transfected with synthetic crRNA:tracrRNA (25 nM) and genomic DNA was isolated 72 hours post-transfection. The Dharmacon specificity tool identified potential off-target (OT) sites containing mismatches and gaps in the RNA or the DNA strand that were not identified by other specificity tools. The sites were analyzed for off-target cleavage with a mismatch detection assay (T7EI). Red nucleotides indicate mismatches, red dashes indicate gaps and underlined red nucleotides indicate insertions relative to the target DNA sequence.

## Using a single-stranded DNA donor template & HDR for knockin of a small insertion



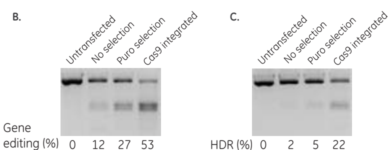




**Figure 5. A.** Schematic of the *EMX1* locus. Exons (blue boxes), crRNA guide sequence (gray highlighted), stop codon (green), PAM (red), cut site (blue arrow) and inserted sequence containing restriction sites (blue) are shown. Analysis of indel mutations using a mismatch detection assay **(B)** or an RFLP assay to detect insertion of the restriction sites **(C)** in untransfected and transfected cells (with or without antibiotic selection or using a Cas9-integrated cell line). Co-TfX = co-transfection.

## Optimization of homology arm length & concentration for a single-stranded DNA donor for HDR

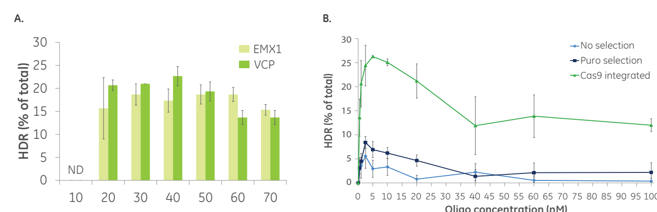



**Figure 6.** A Cas9-integrated U2OS cell line was transfected with either EMX1-crRNA:tracrRNA or VCP-crRNA:tracrRNA and a ssDNA donor using DharmaFECT Duo transfection reagent. **A.** Different ssDNA donors were used, each with increasing homology arm length (10-70 nt). A RFLP assay was performed to determine HDR knockin efficiency. Optimal arm length: 30-40 nt. **B.** Increasing concentrations of a ssDNA donor with 30 nt homology arms were assessed for HDR knockin efficiency using the RFLP assay. Optimal donor DNA oligo concentration: 2.5-10 nM. Data presented are from three independent transfections.

## Insertion of fluorescent reporter on N-terminus of *ACTB* using HDR

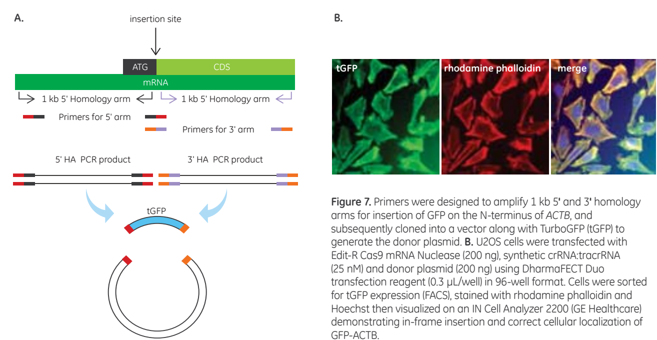



**Figure 7.** Primers were designed to amplify 1 kb 5' and 3' homology arms for insertion of GFP on the N-terminus of *ACTB*, and subsequently cloned into a vector along with TurboGFP (tGFP) to generate the donor plasmid. **B.** U2OS cells were transfected with Edit-R Cas9 mRNA Nuclease (200 ng), synthetic crRNA:tracrRNA (25 nM) and donor plasmid (200 ng) using DharmaFECT Duo transfection reagent (0.3 µL/well) in 96-well format. Cells were sorted for tGFP expression (FACS), stained with rhodamine phalloidin and Hoechst then visualized on an IN Cell Analyzer 2200 (GE Healthcare) demonstrating in-frame insertion and correct cellular localization of GFP-ACTB.

## Conclusions

Edit-R predesigned crRNA reagents offer improved function and specificity

- The Edit-R CRISPR algorithm was trained on functional gene knockout data and can be applied across genome-wide guide RNA designs
- The Edit-R specificity tool detects gaps and mismatches to avoid potential off-targets for increased specificity

Precise genomic insertions can be readily achieved through HDR using Edit-R gene editing reagents and a ssDNA donor oligo for short insertions (< 50 nt) or a donor plasmid for larger insertions

**gelifesciences.com/dharmacon**

GE, imagination at work and GE monogram are trademarks of General Electric Company. Dharmacon is a trademark of GE Healthcare companies. All other trademarks are the property of General Electric Company or one of its subsidiaries. ©2016 General Electric Company—All rights reserved. Version published January 2016. GE Healthcare UK Limited, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK