APPLICATION NOTE

Western blot validation of CRISPR-cas9-induced functional knockout using synthetic sgRNA molecules.

Western Blotting is an effective method to assess the accuracy and reliability of CRISPR targeting. Here, synthetic sgRNAs designed to precisely and efficiently generate functional protein knockouts were validated by western blot using premixed chemiluminescent substrates in three sensitivity levels ranging from mid-pico to low femto.

Abstract

Gene editing was revolutionized when the potential for CRISPR-Cas9 as a powerful tool for editing DNA was understood. Since then, the CRISPR-Cas system has been widely used to knock out genes and introduce disease-specific point mutations and large insertions or deletions (indels) in genetic sequences to further the pursuit of new cell and gene therapies. Precise targeting of the Cas protein (most commonly Cas9) to specific sites of the genome is achieved via recruitment by a single guide RNA (sgRNA) containing a 20-nucleotide complementary sequence to a specific genomic locus. The rational design of these sgRNA molecules is critical for precise DNA site selection, and without adequate strategies, their use can result in off-target effects. Thus, any well-designed CRISPR experiment must include a method to assess the accuracy and reliability of predicted gene editing results. Here, we demonstrate how Dharmacon™ Edit-R predesigned synthetic sgRNAs result in high efficacy knockout of target genes as validated by western blot using Revvity's Western Lightning™ ONE chemiluminescent substrates.

Introduction

The CRISPR-Cas system has been widely used to introduce specific editing events to create protein knockouts, disease-specific point mutations, and large indels1. Precise targeting of the Cas protein (most commonly Cas9) is achieved via recruitment by an sgRNA containing a 20-nucleotide complementary sequence to a specific genomic locus (Figure 1). Upon binding to the genomic target site, the Cas9 nuclease can initiate a cascade of events leading to a functional knockout by introducing a DNA double-strand break (DSB). This break is

repaired through various mechanisms, including the error-prone non-homologous end joining (NHEJ) repair pathway, which can result in indel mutations near the DSB. Indels will often introduce a frame shift mutation leading to nonsense translation, typically accompanied by the introduction of a premature stop codon thus truncating the protein. Truncated proteins are often unstable and result in the desired functional knockout. Here, using Revvity's chemiluminescent western blot reagents, we demonstrate functional knockout using Dharmacon™ Edit-R predesigned synthetic sgRNAs.

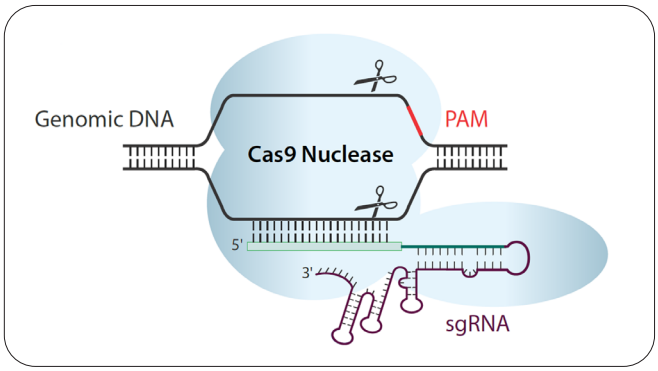

Figure 1: Single guide RNA (sgRNA) molecule binding the precise genomic target site.

Methods and results

Assessing functional knockout of Dharmacon™ Edit-R predesigned sgRNAs

Edit-R guides are designed with a proprietary algorithm to efficiently generate functional protein knockouts, rather than simply create an insertion or deletion. Additionally, chemical modifications are applied to all Edit-R synthetic guide RNA products to reduce degradation by nucleases and improve overall editing performance.

In this study, three guide RNAs targeting genes encoding either HDAC2 or AKT1 proteins were used individually or pooled together to transfect U2OS cells that stably express Cas9. We then evaluated the gene knockout in each cell population by assessing the protein expression using western blot (Western Lightning ONE Pico), which showed that the sgRNAs, individually or pooled, reduced target protein levels by 80-99% compared to the non-targeted control (NTC) sgRNA (Figure 2). Pooling sgRNAs enables economical high-throughput screening across the human genome or gene families.

Notably, pooling our three sgRNAs also achieved a knockout efficiency comparable to the most active individual sgRNA for either target. Transfection of synthetic sgRNAs results in a heterogeneous population comprised of cells that have undergone editing; however, a small fraction of unedited cells is also expected. Thus, bulk protein expression in a population of edited cells assessed by appropriately sensitive western blot will show a very low but still detectable target band as our study shows, even with highly efficient guide RNAs.

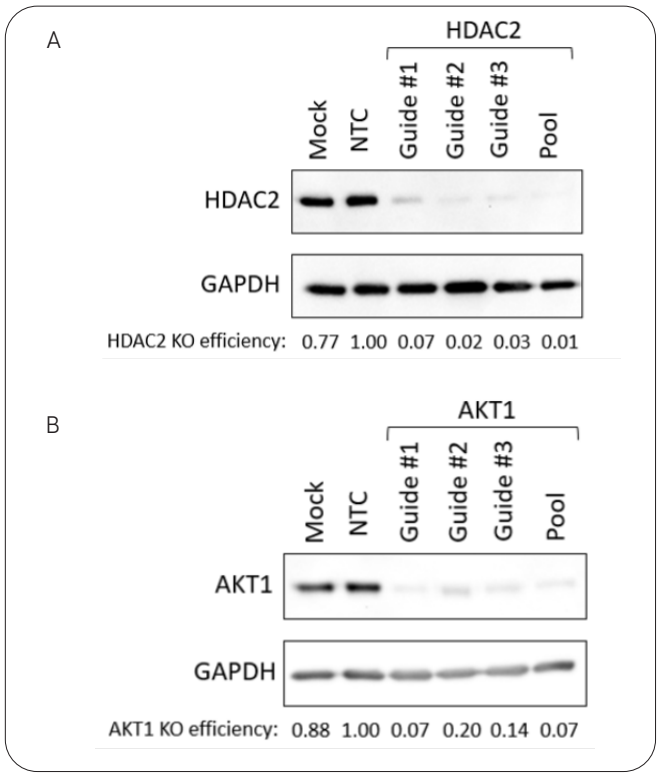

Figure 2: Western blot performed with Western Lightning ONE Pico chemiluminescent detection reagent shows 80-99% reduction of target protein levels in four cell populations, three transfected with individual sgRNAs and one pooled, compared to the non-targeted control (NTC) sgRNA.

Detection limits

To determine the detection limits of HDAC2 and AKT1 protein levels after functional knockout in a heterogenous cell population, we performed a Western blot utilizing three reagents with sensitivity levels ranging from high pico to low femto. Lysates from U2OS cells transfected with NTC or pooled sgRNAs targeting HDAC2 or AKT1 were serially diluted, used for Western blot, and detected with the Western Lightning ONE line of single-bottle

chemiluminescent substrates (Figure 3). Western Lighting ONE Femto Ultra, the most sensitive of the three chemiluminescent substrates, was able to detect trace bands in the lowest protein lysate concentrations (0.156 µg for HDAC2, Figure 3A, 0.078 µg for AKT1, Figure 3B) in unedited, NTC-transfected samples By contrast, in AKT1 or HDAC2 targeted cells, we could only detect a trace band in higher concentrated lysates (1.25 µg with HDAC2 pooled sgRNAs, Figure 3A, and 0.625 µg with AKT1 pooled sgRNAs, Figure 3B), representing an approximately 8-fold reduction in protein levels compared to NTC.

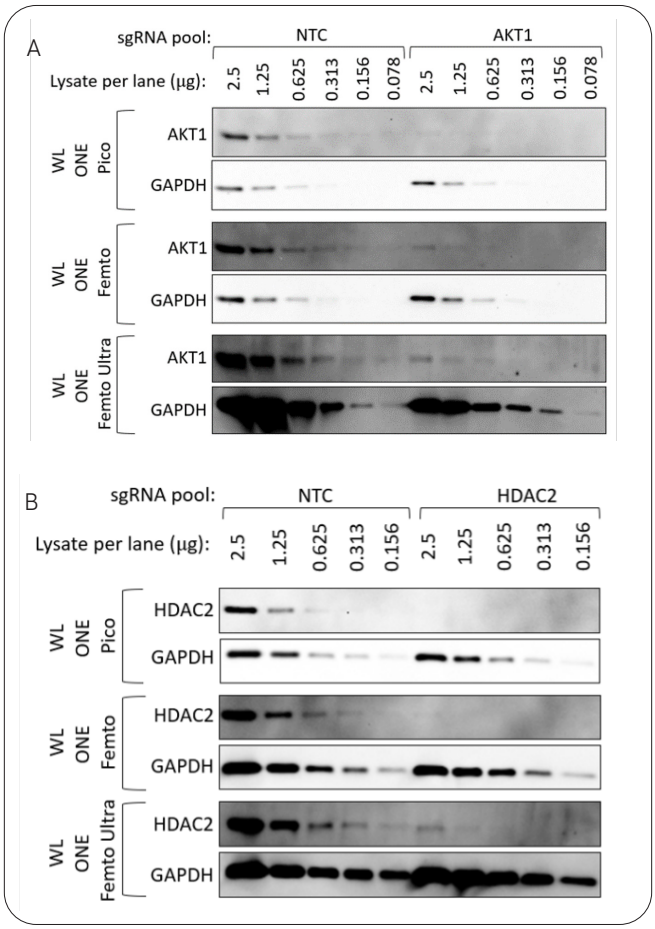

Figure 3: Single guide RNA (sgRNA) molecule binding the precise genomic target site.

Discussion

The promise of CRISPR-Cas9 systems to deliver precise genomic alterations for therapeutic applications remains a driving force for gene editing research. However, many challenges remain, including the risk of non-specific DNA editing. With effective strategies, sgRNA design can be optimized to minimize the occurrences of off-target cutting.

Here, we demonstrated the efficiency of functional knockout achieved via lipid transfection of Dharmacon™ predesigned Edit-R synthetic sgRNAs. We show that both individual guide RNAs as well as pooled sgRNAs achieved high levels of functional knockout in a heterogeneous population of cells, demonstrated by 80-99% reduced protein levels. While target protein levels were dramatically reduced by transfection of individual or pooled sgRNAs, trace protein levels were still detectable. This remaining protein fraction in these lysates can be attributed to either cells that did not uptake lipid/sgRNA micelles, or cells that did not undergo an out-of-frame NHEJ editing event. Delivery of pooled sgRNAs is a useful method for rapid assessment of gene function in a mixed cell population, and clonal isolation would only be needed if assays require complete loss of protein expression.

Materials

Reagents:

Antibodies
Anti-Rabbit AKT1 antibody, polyclonal (Cell Signaling #2938)
Anti-Mouse HDAC2 antibody, monoclonal (BioLegend 680102)
Anti-Mouse GAPDH antibody, monoclonal (Invitrogen AM4300)
Goat α-Mouse HRP-conjugated secondary (Thermo 31431)
Goat α-rabbit HRP-conjugated secondary (Thermo A16096)
Western blot reagents
NuPAGE 4-12% Bis-Tris, 1.5 mm, Mini Protein Gel, 15-well gradient gel (Invitrogen NP0336BOX)
NuPAGE MOPS SDS Running Buffer, 20X (Invitrogen NP0001)
NuPAGE Sample Reducing Agent, 10X (Invitrogen NP0004)
NuPAGE Antioxidant (Invitrogen NP0005)
NuPAGE LDS Sample Buffer, 4X (Invitrogen NP0007)
NuPAGE Transfer Buffer, 20X (Invitrogen NP0006)
Nitrocellulose membranes (Thermo 88024)
Western Blotting Filter (Thermo 84783)
Pierce BCA Protein Assay Kit (Thermo 23227)
SuperBlock T20(PBS) Blocking Buffer (Thermo 37516)
10X Phosphate buffered saline (HyClone SH30258.02)
Western Lightning ONE Pico (Revvity)
Western Lightning ONE Femto (Revvity)
Western Lightning ONE Femto Ultra (Revvity)

Cell culture & transfection reagents
DMEM High Glucose (Cytiva SH30081.01)
Fetal Bovine Serum (HyClone SH30071)
Minimum Essential Medium – reduced serum (MEM-RS) (Cytiva SH30564.01)
Dharmacon DharmaFect DUO (Revvity T-2010-03)
Guide RNAs
Edit-R Human AKT1 synthetic guide RNA #1 SG-003000-01
Edit-R Human AKT1 synthetic guide RNA #2 SG-003000-02
Edit-R Human AKT1 synthetic guide RNA #3 SG-003000-03
Edit-R Human HDAC2 synthetic guide RNA #1 SG-003495-01
Edit-R Human HDAC2 synthetic guide RNA #2 SG-003495-02
Edit-R Human HDAC2 synthetic guide RNA #3 SG-003495-03

Lipid transfection

U2OS cells stably expressing Cas9 were seeded into 6-well plates at a density of 1.67 × 105 cells per well one day prior to transfection. On the day of transfection, 6 µL of DharmaFECT DUO was added to 194 µL MEM-RS medium. Liposome complexes were allowed to form undisturbed for five minutes at room temperature, at which point transfection mixture was added to individual or pooled sgRNAs diluted in MEM-RS (sgRNA final concentration, 25 nM per well). Transfection mixture was incubated undisturbed for an additional twenty minutes at room temperature before diluting with growth medium (DMEM High Glucose + 10% FBS) and adding to cells. Cells were cultured for an additional 72 hours and harvested for Western blot analysis.

Western blot

72 hours post-sgRNA transfection, U2OS cells cultured in 6-well plates were lysed in protein lysis buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.4, 1 mM EDTA, 1% Triton X-100) supplemented with 1:100 Halt protease and phosphatase inhibitor cocktail (Thermo 78440). Protein concentration was determined by BCA assay and lysates were diluted in protein lysis buffer containing 1X NuPAGE LDS Sample Buffer and 1X NuPAGE Sample Reducing Agent. Samples were boiled for 10 minutes and loaded onto a NuPAGE 4-12% gradient Bis-Tris gel and run in 1X MOPS buffer supplemented with NuPAGE antioxidant at 185V for 1 hour.

Gels were transferred to a nitrocellulose membrane in 1X NuPage Transfer Buffer at 250 mA for 2 hours on ice. Transfer quality was confirmed by Ponceau S staining. Ponceau S was washed off membrane with 1X PBS supplemented with 0.05% Tween 20 (PBS-T) and membranes were blocked in SuperBlock blocking reagent for 30 minutes at room temperature. Blots were incubated in primary antibody overnight at 4 °C (1:1000 HDAC2 or AKT1) or for 1 hour at room temperature (1:20,000 GAPDH). Blots were then washed four times in PBS-T to remove excess primary antibody and incubated in secondary antibody (1:20,000 anti-rabbit or anti-mouse HRP) for one hour at room temperature. Blots were then washed four times in PBS-T, incubated in Western Lightning ONE Pico, Femto, or Femto-Ultra chemiluminescent reagents and developed on an iBright 1500FL digital imager (Thermo).

Acknowledgments

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References

1. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013 Nov;8(11):2281-2308.

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