Designing Cas-CLOVER: A Dimeric RNA Guided Targeted Nuclease for Precision Gene-Editing

The purpose of this document is to teach Cas-CLOVER users how to design and order their own gRNA pairs for use with Cas-CLOVER. For new gene targets, we suggest three pairs per target. You can order mRNA for mammalian applications and DNA plasmids for yeast and plant applications through our website's Shop.

Please note that Demeetra provides gRNA pair design services but not synthesis which can be ordered from your favorite provider. We have listed suggestions below.

Introduction and Background

CRISPR-Cas9 System

Synthetic CRISPR single guide RNA (sgRNA) together with CRISPR-associated proteins (Cas) can be engineered to bind and cut target genomic sequences. Site-specific cleavage of the target DNA occurs at a location determined by the complementary base pairing of the gRNA and target DNA and a small motif, the protospacer adjacent motif (PAM) **(Figure 1)**. In the type II CRISPR-Cas9 system, the PAM sequence (NGG) is essential for DNA cleavage.

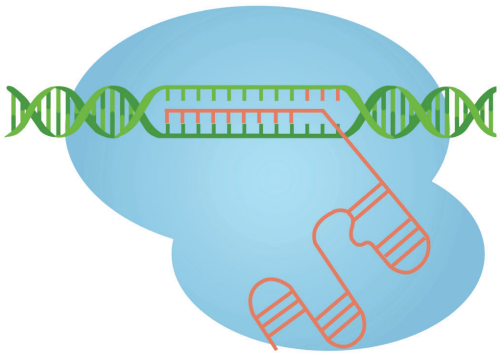

Figure 1: Mechanism of CRISPR-Cas9 Directed Mutagenesis
The synthetic gRNA interacts with the complementary sequence within the target region. Recruitment of the Cas9 endonuclease cleaves the DNA 5′ of the PAM. Creation of a double-stranded break results in DNA repair mechanism activation and repair of the break via non-homologous end joining (NHEJ) or homologous recombination (HR).

Cas-CLOVER's Dimeric Editing System with Dual gRNAs

Cas-CLOVER is a dimeric gene editing system that demonstrates a lack of off-targets associated with CRISPR-Cas9. Cas-CLOVER uses a catalytically inactive Cas fusion protein, or "dead Cas" (abbreviated as dCas), fused to the **dimeric** Clo051 endonuclease domain **(Figure 2)**.

Two gRNAs can be designed to target genes of interest to create double-stranded breaks, like other dimeric gene editing technologies (i.e. ZFN and TALEN). The Cas-CLOVER system requires a PAM sequence in each RNA. A flexible spacer range between the two guides of 15–30 nucleotides enables the dual-complex to function. The flexibility of Cas-CLOVER's guide RNA design allows the user to target any gene of interest.

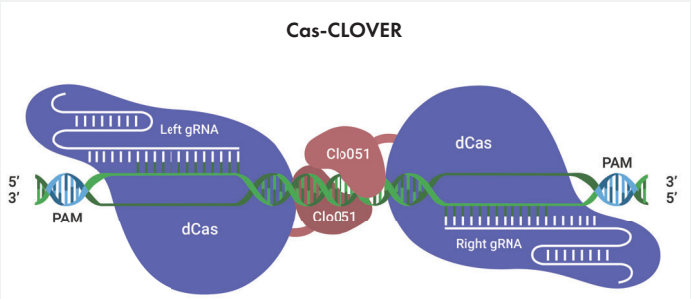

Figure 2: Mechanism of Cas-CLOVER Directed Mutagenesis
Dual synthetic gRNA/Cas-CLOVER complexes interact with the left and right complementary sequences within the targeted locus, which leads to dimerization of the Clo051 nuclease domains and cleavage of the targeted locus. Creation of a double-stranded break result in DNA repair mechanism activation and repair of the break via non-homologous end joining (NHEJ) or homologous recombination (HR).

Designing Guide-RNAs for Cas-CLOVER

A variety of open source gRNA design tools are available online for you: platforms such as CRISPR-MIT, E-CRISP, or CHOPCHOP, CRISPOR or ZiFit. The two guide-RNAs should be designed in the **PAMs-out orientation.**

When designing and ordering the left guide, the sequence should be copied from the bottom strand of DNA sequence, reading 5′ to 3′ orientation toward the PAM site. When designing and ordering the right guide, the sequence should be copied from the top strand, reading 5′ to 3′ toward a PAM site. It is important to choose left and right guides that are separated by a spacer region of 15–30 nucleotides **(Figure 3)**. The tools that accomodate gRNA designs for the Cas9 Nickase work well for Cas-CLOVER as well. The recruited Clo051 nuclease domains dimerize and introduce a double-stranded break in this spacer region between the two gRNA target sites.

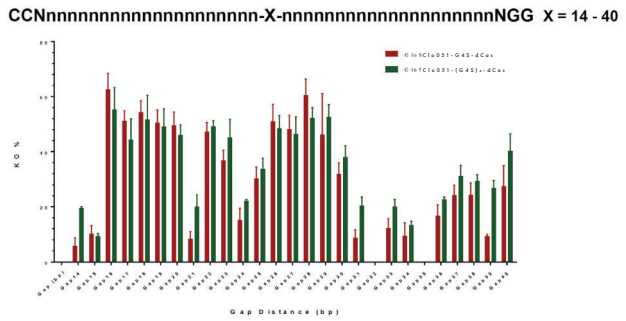

Figure 3: Cas-CLOVER KO Efficiency at Different Gap Lengths

Figure 4: Example Indicators with Different Gap Lengths

Demeetra only provides the Cas-CLOVER mRNA as a reagent and does not supply the gRNA synthesis. Please order synthesized gRNAs from your favorite provider. We recommend Synthego sgRNA or IDT Alt-R® Services. Please note: gRNAs should include on the first three, and the last three ribonucleotide bases, phosphorthioated/2′O-methyl modifications to enhance the stability and reduce immunogenicity of the gRNAs. Once both components are ordered use the delivery protocol we supply with your order.

Designing Guide-RNAs for Cas-CLOVER in Plasmid Format (Plant & Yeast Applications)

When using Cas-CLOVER in a plasmid based editing system, the two guide-RNAs should be designed in the PAMs-out orientation. Specifically, when designing and creating the left guide, the sequence matches the bottom strand of the chosen guide sequence, reading 5′ to 3′ orientation toward the PAM site. This 5′ to 3′ sequence should be cloned into the vector between an appropriate promoter for your system and a guide scaffold. When designing and creating the right guide, the sequence should match the top strand, reading 5′ to 3′ toward a PAM site. The 5′ to 3′ sequence for this guide should also be cloned into the vector between an appropriate promoter and a guide scaffold. There are various ways to create guides for cloning into vectors, which include synthesized fragments or annealing together two single stranded oligos to make a double stranded fragment. There are also several ways to clone the guides into vectors, depending on the user's personal preference.

It is important to choose left and right guides that are separated by a spacer region of 15–30 nucleotides.

Demeetra's Approach to Cas-CLOVER Licensing

By partnering with Demeetra, you get license rights to Cas-CLOVER for gene-editing, as well as full freedom to operate (FTO) in a single license for commercial use:

Evaluation License: Technology transfer with reagents and protocols for a set evaluation period. No risk. You just pay for the low cost of the reagents. Check out our online shop for more details.

Commercial License: Flexibility of a one-time fee-based license or an economical standard upfront/milestone/royalty structure.

Learn more about our research and how our team can help move your studies forward by contacting us today!

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