Hera BioLabs | White Paper

The Unmatched Precision of Cas-CLOVER™ Editing Allows Stacking of Error-Free Gene Edits in CHO for Continuous Improvement of Host Genetics

Elimination of the Glutamine Synthetase Pseudogene in GS (-/-) CHO to Create a GS Double Knockout

Utilization of Chinese Hamster Ovary (CHO) cells for the manufacture of recombinant protein therapeutics continues to fuel the global clinical pipeline.[1] Use of CHO offers several advantages over other cell lines such as high protein expression as well as providing a human-like glycosylation pattern.[2] Modification of the host CHO cell lines through the introduction of specific genetic changes intended to drive performance provides an avenue to enhance clonal selection, antibody production, and antibody potency and function. Removal of the GS gene allows for the selection of clones in glutamine-free conditions and results in a much more efficient timeline to produce stable antibody-secreting cell lines without the need for additional costs from the utilization of selection antibiotics.[3,4] Inactivation of the *FUT8* gene by sequential homologous recombination in CHO enables the production of antibodies completely lacking fucose, leading to dramatic increases in antibody-dependent cell mediated cytotoxicity.[5] These early examples of host engineering in CHO are now commonplace among CDMOs, and additional genetic improvements introduced into CHO hosts may expand or alter the competitive landscape.

The Cas-CLOVER™ Site-Specific Gene Editing System Incorporates Quality-By-Design

As the understanding of CHO biology continues to evolve through the identification and subsequent engineering of novel targets, so do the methods available to introduce genetic changes. The CRISPR-Cas9 system emerged in recent years as a simple, efficient, and scalable approach to introducing changes within the mammalian genome.[6] However, one significant on-going limitation of the CRISPR-Cas9 system is a tendency to create off-target mutations. Since the system only exhibits a single layer of specificity through the complementary binding of a single guide RNA, Cas9 endonuclease-mediated cutting is known to introduce mutations at sites where varying levels of homology are present with the guide RNA.[7] Indeed, multiple studies concluded that Cas9 introduces off-target mutations at sites that differ by as many as 5 nucleotides from on-target sites in human cells.[8–10] The off-target cutting rate is reported to be as high as 13%.[11]

Functional consequences of this documented off-target mutagenesis can be catastrophic as disruption of normal cellular function and induction of toxicity can occur.[12] Further, unwanted chromosomal rearrangements such as deletions, inversions, and translocations are reported.[13–17] Proprietary CHO host cell lines represent significant investments and are the foundation of therapeutic mAb manufacturing. Employing promiscuous CRISPR-based methods with known off-target activity poses significant risks to CHO genetics and can induce unwanted or unintended cell line instability and performance.

Given the potential for CRISPR-Cas-mediated off-target mutations, enhanced quality approaches would ideally include additional mechanistic safeguards to increase the specificity of the core editing system. The Cas-CLOVER editing technology embraces this quality-by-design approach for precise cell line

engineering using a "two-factor authentication" to achieve the desired genetic editing activity. This high-fidelity system consists of an inactive RNA-binding Cas protein (dCas) fused to a nuclease from a Clostridium Clo051 type II restriction endonuclease.[18] This fused dCas-Clo051 has an absolute requirement for dimerization to be enzymatically active. Site-specific activation of Clo051 is achieved using a pair of offset, but physically proximal, gRNAs complementary to opposite strands of the target site.[19] Dimerization of the Clo051 nuclease domain occurs only through the RNA-guided recognition of the two proximal sequences bound by gRNAs. Monomeric Cas-CLOVER does not carry the inherent off-target nicking or double strand break (DSB) risks that CRISPR-based approaches possess, as the binding of a gRNA is insufficient to induce Clo051 endonuclease activity.[18]

High Specificity and Precision of Stacked Genetic Edits in CHO Utilizing Cas-CLOVER

The Cas-CLOVER system was utilized to inactivate the glutamine synthetase (GS) gene by introducing a deletion in exon 6 on chromosome 5 by using the GS5 guide pair (GP) (Figure 1, red).

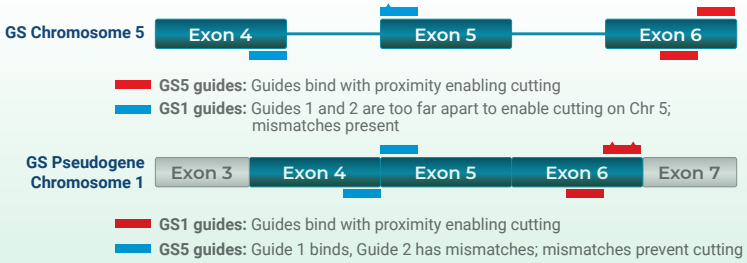

Figure 1. Cas-CLOVER gRNA Designs. Schematic showing guide RNA strategies specific to chromosome 1 and 5. Cutting should only occur when guide pairs identify target sequences near one another. Editing should not occur when either guide pair binds in the absence of sufficient proximity (chromosome 1 guides on chromosome 5) or in close proximity but contains mismatches in complementary binding chromosome 5 guides on chromosome 1.

The CHO transfection pool was evaluated for editing efficiency of knockout clones isolated from the pool. A high performing knockout clone, dubbed 7G2, was shown to be highly dependent on glutamine supplementation in culture as the cell line failed to expand upon withdrawal of glutamine from the culture medium (data not shown). Further, sequencing was performed in the 7G2 clone and large targeted deletions on chromosome 5 were identified (Figure 2). Studies published after the establishment of the 7G2 clone indicated that an alternate and truncated GS pseudogene on chromosome 1 with high homology to GS gene on chromosome 5 may provide some supplemental GS activity to the full-length.[20] This pseudogene located on chromosome 1 may contribute to residual cellular durability in the absence of supplemented glutamine and the selection procedure could

Figure 2. Chromosome 1 and 5 Edit Status in 7G2. The single knockout clone 7G2 subjected to chromosome 5 guide pair-mediated chromosome 5 editing showed large deletions in chromosome 5 (red box) but not in the highly homologous GS pseudogene sequence on chromosome 1.

therefore be improved by elimination of the GS pseudogene. This gene located on chromosome 1 was deemed as a target for subsequent stacked editing via Cas-CLOVER.

As shown in Figure 1, the GS gene (chromosome 5) and the GS pseudogene (chromosome 1) have largely identical exonic sequences. To delete GS pseudogene sequences in the 7G2 line, one gRNA was designed to sequences identical on chromosomes 1 and 5. The second gRNA was designed in adjacent sequences in the pseudogene, but across an intron boundary on chromosome 5 (Figure 1, blue). This guide pair is expected to recognize sequences on chromosome 5 and chromosome 1, however, due to the lack of proximity of binding of the guide pairs, editing is not expected to occur on chromosome 5. Likewise, the GS pseudogene was not expected to carry any mutations from the previous GS gene editing using the chromosome 5 guide pair as guide 2 contains two mismatches in the gRNA's base pairing, which prevents cutting. These versatile guide designs emphasize the key differences of Cas-CLOVER's inherent added specificity as compared to CRISPR.

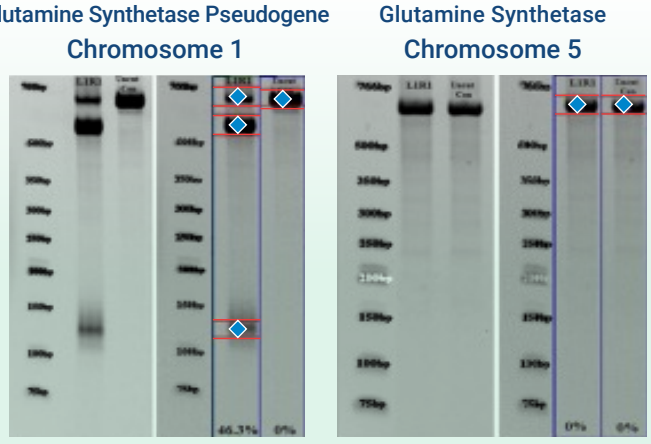

Figure 3. T7 Endonuclease Assay for Estimating the Percent of Glutamine Synthetase Pseudogene and Gene Sequences Using Cas-Clover. CHO clones were screened for the degree of editing. As expected, cleavage products (blue diamonds) were identified in clones transfected with the GS1 guide pair on chromosome 1 (left panel) while no cutting was demonstrated in chromosome 5 (right panel).

The chromosome 1 guide pair was synthesized and CHO cell pools were subsequently generated by transfection using the parental GS chromosome 5 null 7G2 line. The resulting CHO pools were evaluated for the location and extent of editing on chromosome 5 and chromosome 1 via the T7 Endonuclease Assay (Figure 3). In the GS pseudogene (chromosome 1), a high degree (46.3%) of cutting was observed using the chromosome 1 guide pair while no cutting was observed at the GS gene on chromosome 5. The absence of editing on chromosome 5 using the chromosome 1 guide pair highlights the enhanced dual specificity of the Cas-CLOVER system. Editing will not occur when there is sufficient binding of one guide that is not in proximity to the second guide, as is the case with the guides previously used on chromosome 5. Further, editing activity is highly unlikely when one of the guide pairs binds in proximity but does not exhibit 100% complementary binding, as is the case with the chromosome 5 guide pair on chromosome 1. In the context of CRISPR-Cas9, off-target edits on chromosome 1 or 5 would certainly be expected to occur in either of these scenarios, not to mention the potential for cutting at sites with partial homology to the GS locus elsewhere in the genome.

Single cell clones transfected with the chromosome 1 guide pair were isolated from the transfection pools and further propagated for clonal analysis. Sequencing was performed in these single celled clones and large targeted deletions on chromosome 1 introduced by Cas-CLOVER targeting of the GS pseudogene were identified. In total, 83 clones were sent for sequencing based on PCR screening results. 80 out of the originally submitted 83 clones (96.3%) were sequence confirmed for targeted chromosome 1 editing. None of chromosome 1 edited clones examined contained genetic changes at the identical GS gene sequence on chromosome 5 (Figure 4).

Figure 4. Chromosome 1 and 5 Edit Status in Isolated Clones. Multiple single cell double knockout clones were recovered and found to have large targeted deletions (red boxes) on chromosome 1 following Cas-CLOVER targeting of the GS pseudogene. None of the recovered chromosome 1 GS pseudogene knockout clones had any genetic changes at the identical GS gene sequences on chromosome 5.

To assess the functional consequence of this additional GS pseudogene knockout on chromosome 1, the growth kinetics of double knockout (DKO) clones were determined in glutamine free medium. 12 of the 80 sequence-confirmed clones were chosen for growth assessments and cell banks were generated. Selected clones exhibited robust growth kinetics in chemically defined media containing 8 mM glutamine that was indistinguishable from the parental 7G2 single knockout line (data not shown). As expected, when glutamine was withdrawn, the viability of the clones decreased significantly over the 6-day investigational window. Further, these DKO CHO clones exhibited elevated sensitivity to glutamine withdrawal compared to the 7G2 control (data not shown). This finding suggests that the GS DKO CHO clones generated by Cas-CLOVER-mediated stacked editing of the truncated GS pseudogene on chromosome 1 may offer higher selection pressure workflows than the original clone. As a result, when utilizing expression vectors that restore GS activity, these CHO clones may translate to more rapid timelines to develop antibody expressing lines. Further, these data and the absence of off-target edits in highly homologous sequences highlights the fidelity of the Cas-CLOVER gene editing system in that a two-factor authentication is required for gene editing activities (Figure 5). Based on these like sequences, undesired edits would have been observed using CRISPR-Cas9.

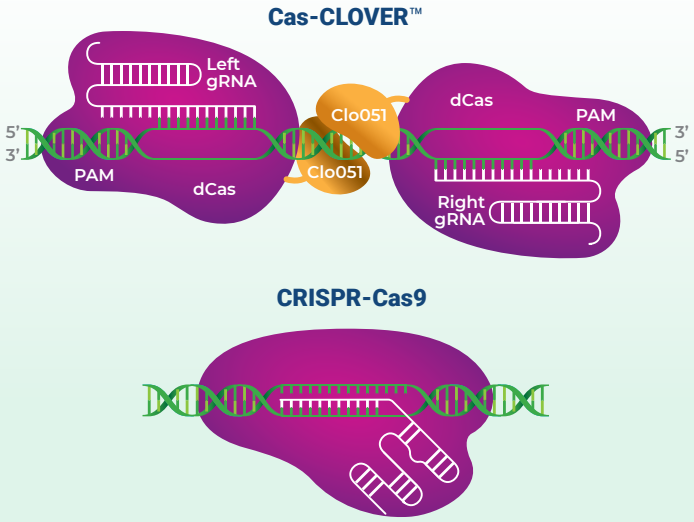

Figure 5. The Dual Specificity of Cas-CLOVER Represents Quality-by-Design in Gene Editing. Functionality of Cas-CLOVER requires the dimerization of the fused dCas-Clo051. Site-specific activation of Clo051 is achieved using a pair of offset and physically proximal gRNAs complementary to opposite strands of the target site. CRISPR-Cas9, on the other hand, utilizes a guide RNA as the only layer of specificity, which is the source of the inherent off-target nicking or DSB risks.

Summary and Conclusions

Here, the accuracy, versatility and precision of the dual specific Cas-CLOVER system is demonstrated in two different approaches at highly homologous sequences. In targeting chromosome 5, gRNAs had partial mismatches to chromosome 1, and these mismatches were sufficient to prevent efficient cutting at chromosome 1. In targeting chromosome 1, one of the gRNAs had 100% homology to sequences on chromosome 1 and 5 and cutting at chromosome 5 was not observed because the second gRNA was not adjacent, preventing Clo051 dimerization, which is absolutely required for activity. Cas-CLOVER is a robust editing strategy with built-in safeguards to prevent and overcome the off-target mutagenesis observed from Cas-based systems that do not incorporate multi-factor quality attributes.

Utilization of the Cas-CLOVER editing system to create genetic edits in CHO is as straightforward as CRISPR-Cas-dependent protocols. However, the quality of the edited CHO cell pools is expected to be significantly enhanced due to the diminished probability of off-target edits. This will translate to lower final costs compared to CRISPR-Cas-based technologies as the screening requirement for selected clones in high value applications will be significantly reduced. As shown here, this technology can be utilized to create stacked edits within existing producer cell lines as novel scientific discoveries are made. Thus, implementation of the Cas-CLOVER editing system represents a quality-by-design approach as this dual specific system is ideally suited for the use in cell lines with elite genetics such as CHO to induce precise genomic changes without the concern of unintended off-target edits. Sanofi recently reported a *FUT8* knockout campaign in CHO using Cas-CLOVER, highlighting that extensive characterization of more than 5000 off-target sites identified zero mutations.[21]

The utility of Cas-CLOVER is not limited to cell line engineering applications for recombinant protein production. Off-target mutations are a significant concern in all therapeutic and industrial gene editing applications, most notably cell and gene therapy due to enhanced risk of tumorigenesis from unwanted chromosomal rearrangements such as deletions, inversions, and translocations.[13–16] Cas-CLOVER's editing specificity may enable significant advances in these fields, notably the generation of stable allogeneic human T cells, a major step toward off-the-shelf CAR-T therapies.[18] Data generated in multiple systems and cell types demonstrates that the Cas-CLOVER retains the scalability, simplicity, and speed of CRISPR-Cas9, while dramatically reducing the well-documented unintended effects from off-target cutting observed with CRISPR. Therefore, the Cas-CLOVER™ Site-Specific Gene Editing System presents an opportunity to further advance precision cell line engineering, not only in protein-based therapeutics, but in all gene editing applications.

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