



## Targeted Knock-Ins in CleanCut GS CHO Cells: Advancing Programmable Landing Pad Methods for Next-Generation Cell Line Development

### Executive Summary

Cas-CLOVER enabled efficient targeted integration at the endogenous GAPDH locus in CleanCut GS CHO cells, generating a stable GFP-only population exceeding 20% without the use of selection. Targeted integrations at the Chr6-AttP and C12orf35 genomic safe harbor sites were likewise confirmed by sequencing, demonstrating that CleanCut GS cells support precise and programmable insertion across multiple genomic contexts. Together, these data establish CleanCut GS CHO as a strong foundation for designing recombinase- or integrase-based landing pads, whether single-copy, multi-copy, or orthogonal in architecture. By enabling controlled, site-specific integration, Cas-CLOVER knock-ins reduce variability associated with random integration, improve expression predictability, and decrease screening burden. These results highlight the platform's relevance for next-generation CHO engineering, including site-specific expression systems, host optimization strategies, and more streamlined CLD workflows for biomanufacturing.

### Background & Rationale

Stable expression systems are central to cell line development (CLD), enabling consistent protein production and reproducibility across clones and manufacturing lots. CHO systems underpin commercial biologics manufacturing, where cell-to-cell variability and unpredictable expression can lengthen development timelines and complicate scale-up.

The GS double-knockout (GS-DKO) platform, CleanCut GS CHO, increases selection stringency and enriches for high producers under glutamine-free and MSX selection. This has become a widely adopted foundation for high-yield CHO CLD.

Concurrent industry needs include programmable landing pad systems that enable controlled gene integration, predictable single-copy insertion, elimination of positional effects, simplified clone selection, and clear regulatory expectations.

Cas-CLOVER, a next-generation dimeric nuclease, provides significantly improved specificity over CRISPR/Cas9 and is an attractive tool for precise genome engineering in CHO (Madison et al., 2022).

### CleanCut GS CHO Engineering Overview

CleanCut GS CHO cells were engineered using Cas-CLOVER, a high-fidelity, dimeric RNA-guided nuclease. The system requires paired gRNAs, producing double-stranded breaks only when both guides bind correctly, thereby reducing off-target risk.

Mechanistic advantages include:

- High on-target efficiency (Andorfer et al., 2024)
- Near-zero off-target indels
- No monomeric nicking
- Compatibility with HDR-mediated knock-ins
- Favorable IP positioning for commercial bioprocessing use

CleanCut GS represents a GS5/GS1 double knockout CHO-K1-derived host, validated for strong selection performance and stable expression.

## Methods Summary

### GAPDH Knock-In

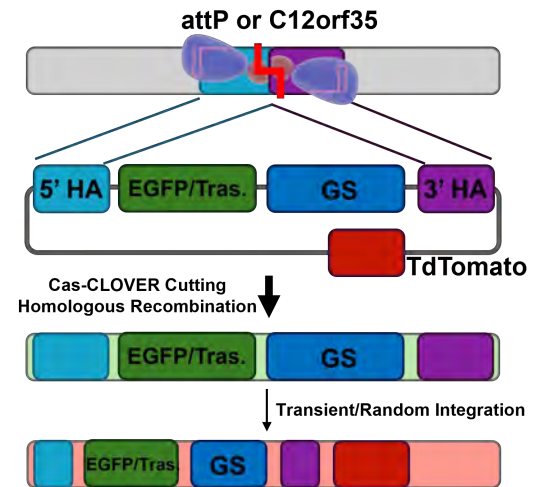
A promoterless GFP cassette was integrated at the GAPDH start codon. GFP indicated HDR-mediated knock-in, while tdTomato tracked backbone presence. Cells were evaluated by flow cytometry over time. PCR and nanopore sequencing confirmed targeted integration.

### Chr6-AttP & C12orf35 Knock-Ins

Two genomic safe harbor sites, Chr6-AttP and C12orf35, were targeted using donor plasmids with ~700 bp homology arms. The construct encoded GFP-GS and trastuzumab for functional selection.

### Figure 1. Overview of Cas-CLOVER-Mediated Knock-In Workflow in CleanCut GS CHO Cells

Cas-CLOVER and a donor vector are co-transfected into CleanCut GS CHO cells, where paired gRNAs direct Cas-CLOVER binding to the genomic target site. The Clo051 nuclease domain generates a double-strand break with 4-bp overhangs, enabling homology-directed repair using donor-provided arms. Successful HDR produces a GFP-only signal, confirming site-specific knock-in. Cells retaining tdTomato or showing mixed fluorescence represent backbone-containing or non-HDR events.



## Results

### 1. GAPDH Targeted Knock-In Efficiency

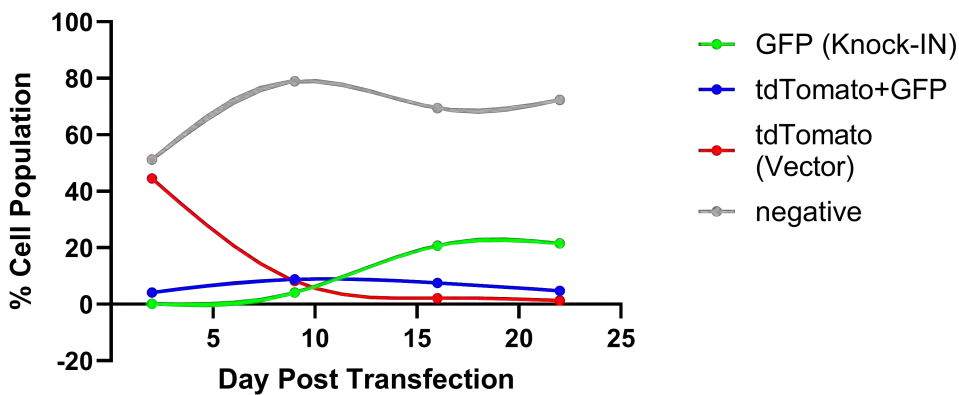
#### Emergence of Stable GFP+ Knock-In Population

- Day 2: Mixed GFP+/RFP+ population
- Day 9: Clear GFP-only knock-in population appears
- Day 16: >20% stable GFP-only population without selection

Vector controls lost tdTomato over time but never formed GFP-only populations, supporting HDR specificity.

#### Temporal Emergence of GFP Knock-In Population Measured by Flow Cytometry

#### GAPDH Promoterless-GFP Knock-IN



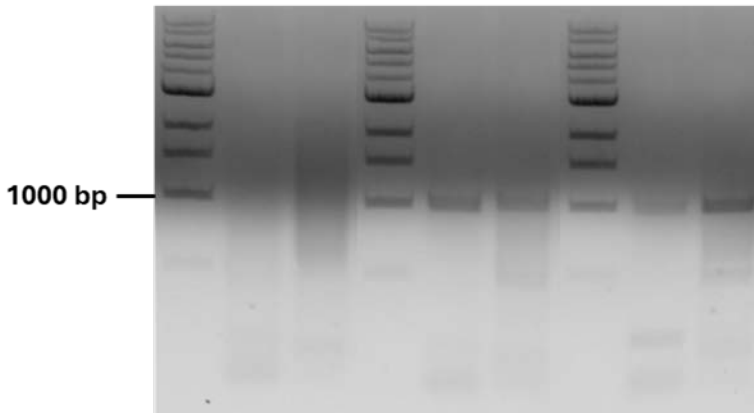
**Figure 2:** Flow cytometry analysis shows the change in fluorescence-based population distribution over the course of knock-in establishment. On day 1, the majority of cells are tdTomato-positive or fluorescence-negative. Prior to day 10, a small but distinct GFP-positive population begins to emerge. By days 15–20, GFP-positive cells represent the dominant fluorescent population, approaching and exceeding 20% of total cells, indicating successful and sustained HDR-mediated knockin.

### 2. PCR & Sequencing Confirmation

Before interpreting fluorescence-based knock-in readouts, it was essential to confirm that GFP-positive cells reflected true HDR-mediated insertion rather than transient expression or backbone retention. PCR and sequencing were therefore used to validate correct junction formation and confirm precise targeting at the intended genomic loci.

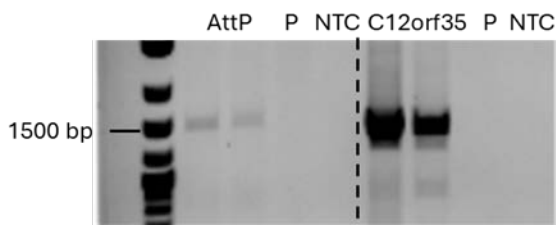
## PCR Confirmation of GFP Knock-In at the GAPDH Locus

Plasmid only control    Cas-CLOVER + guide pair 1    Cas-CLOVER + guide pair 2



**Figure 3:** For each sample, junction PCRs were performed to validate both the upstream and downstream GFP/GAPDH insertion boundaries. A 1 kb DNA ladder was used to separate PCR amplicons for three conditions: plasmid-only control (left), CasCLOVER + guide pair 1 (middle), and CasCLOVER + guide pair 2 (right). For each sample, lanes were run in the following order: upstream junction PCR (950 bp), downstream junction PCR (1000 bp).

## PCR Confirmation of GFP/Trastuzumab at AttP and C12orf35 Safe Harbor Sites

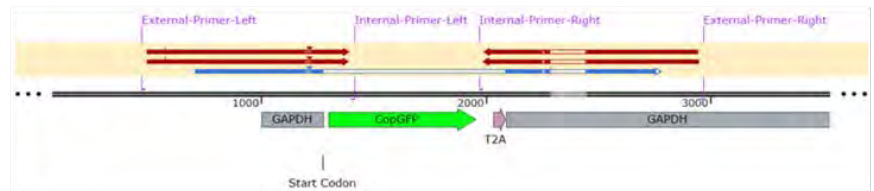


**Figure 4:** Knock-in at the AttP and C12orf35 sites were identified using an external primer paired with an internal primer to confirm the left homology arm junction (~1500 bp). All primer sets were validated using plasmid and no-template- controls (NTC) to rule out non-specific amplification and contamination from any reagents. gDNA from plasmid-only transfected cells (P) was also tested to confirm that observed amplicons were specific to Cas-CLOVER-mediated knock-in.

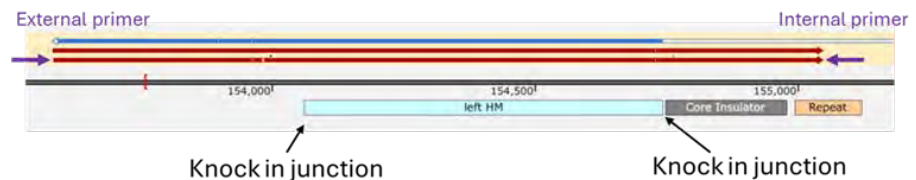
## Sequencing Confirmation of Targeted Insertions at GAPDH, AttP, and C12orf35

**Figure 5:** Correct targeted knock-ins were verified by nanopore sequencing of both left and right junctions of the GFP/GAPDH integration (A), as well as sequencing of the left homology arm junctions at the AttP and C12orf35 loci (B), confirming precise HDR-mediated insertion at all three sites.

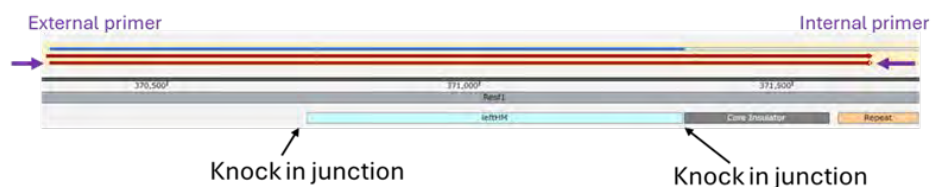
### A) GAPDH



### B) AttP



### C12orf35



— PCR amplicon of CHO-WT  
— PCR amplicon from Knock-in CHO cells

Together, the PCR assays produced clear knock-in junction fragments ~1,000 bp for GAPDH and ~1,500 bp for the AttP and C12orf35 safe harbor loci, while controls showed no detectable amplicons. Nanopore sequencing further validated these findings by confirming precise HDR-mediated insertions at all targeted sites.

## Discussion: Implications for Landing Pad Engineering

These results show that combining Cas-CLOVER editing in CleanCut GS CHO cells support efficient HDR knock-ins at multiple loci including safe harbor sites. This enables construction of programmable landing pads, including single-locus, multi-arm, and orthogonal recombinase sites.

Compared with random integration, targeted knock-ins offer greater predictability, reduced clone screening, enhanced stability, and clearer regulatory pathways.

## Applications for CLD & Biomanufacturing

- High-consistency clone development
- Reduced screening burden
- Predictable expression and improved stability
- Faster CLD timelines
- Improved product quality and robustness

## Conclusion

CleanCut GS CHO cells demonstrate strong compatibility with Cas-CLOVER-mediated targeted knock-ins. GAPDH knock-ins reached >20% stable GFP-only populations without selection, while safe harbor integrations at Atp and C12orf35 were confirmed via sequencing.

These findings support the deployment of programmable landing pads in CHO, enabling more predictable, high-efficiency CLD workflows and next-generation biologics manufacturing.

## References

Andorfer, P., Kahlig, C.-I., Pakusic, D., et al. (2024). Cas-CLOVER-mediated knockout of STAT1: A novel approach to engineer packaging HEK-293 cell lines used for rAAV production. *Biotechnology Journal*. <https://doi.org/10.1002/biot.202400415>

Madison, B.B., Patil, D., Richter, M., et al. (2022). Cas-CLOVER is a novel high-fidelity nuclease for safe and robust generation of TSCM-enriched allogeneic CAR-T cells. *Molecular Therapy: Nucleic Acids*. <https://doi.org/10.1016/j.omtn.2022.06.003>