# The CHOlympian™ Platform Combines Transposase-Mediated Stable Integration and Cas-CLOVER Host Modification to Deliver Highly Productive CHO Cell Lines for Biotherapeutic Manufacturing

**Valeriya Steffey**[1], Daniel Machado[1], Abigail Ross[1], George Loughrin[1], Cameron McHargue[1], Frank Chen[1] Shilpa Sampathi[1], Shameem Hassan[1], Chris Brenzel[1]

1. Hera BioLabs, Inc. Lexington, KY Contact: cbrenzel@herabiolabs.com

Hera BioLabs

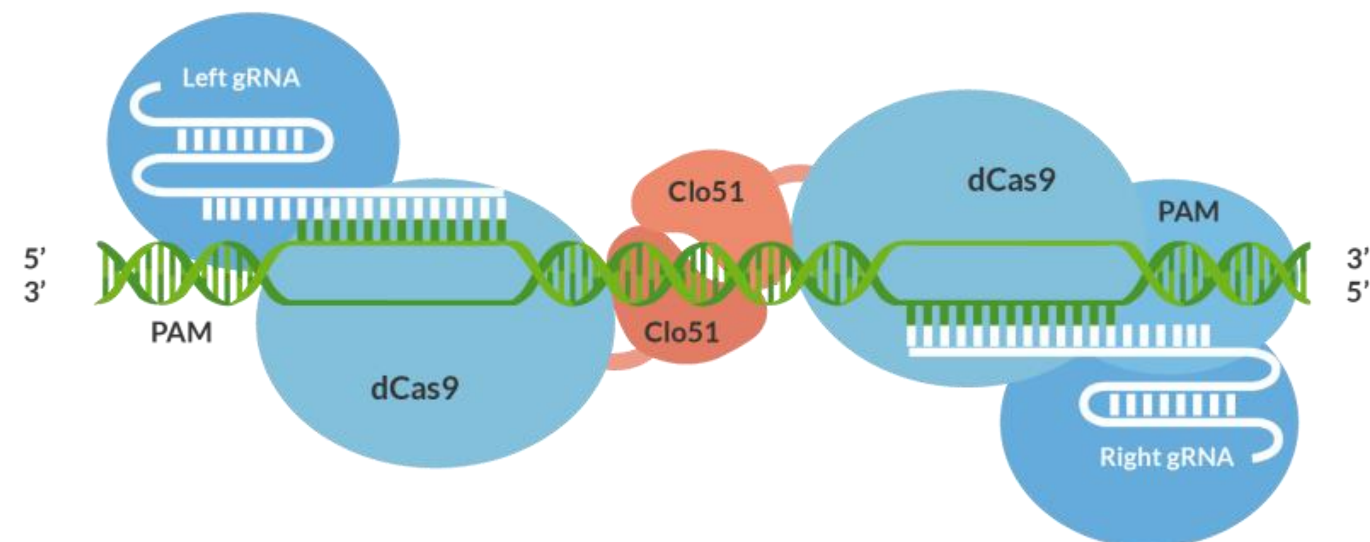
## Abstract

Chinese Hamster Ovary (CHO) cells continue to be a cornerstone in biopharmaceutical manufacturing, and improvements in CHO workflows continue to increase speed and decrease cost of goods. The CHOlympian™ platform leverages leading technologies to provide high yielding, stable cell lines rapidly. Biopharmaceutical-encoding vectors are integrated into transcriptionally active chromatin using the piggyBac® transposase system. In addition, Hera's proprietary gene editing technology, Cas-CLOVER[1], can be utilized to introduce targeted mutations in the host genome with unmatched efficiency and specificity as well as knock-in genes of interest to improve host performance.

To launch the CHOlympian™ platform, we first leveraged Cas-CLOVER to develop a novel suspension CHO K1 cell line with glutamine synthetase (GS) genes knocked out. The widely used GS knockout strategy allows amplification of genes of interest when introduced on the same plasmid construct used for GS rescue. Approximately 35% of GS knockout candidates had both alleles of the GS gene inactivated by Cas-CLOVER. With the stable GS knockout CHO cell line established, we initiated antibody production efforts for rituximab[2], a well-studied IgG1, as a test case. Constructs encoding rituximab heavy and light chains with a GS marker were stably integrated using the piggyBac® transposase system. The Solentim VIPS™ was utilized for automated single cell cloning and visual reporting consistent with regulatory compliance and quality processes.

Preliminary clones exhibited titers approaching 1000 mg/L of rituximab, successfully demonstrating antibody production in a multi-well system suitable for preliminary clone validation, further optimization, and scale-up. The CHOlympian™ platform is a new offering leveraging state-of-the-art molecular tools for cell line development and host modification.

## Cas-CLOVER™ – Enhanced Specificity via Two Guide RNAs




The Cas-CLOVER nuclease technology utilizes two guide RNAs and the proprietary dCas9-Clo051 nuclease system to improve specificity over traditional CRISPR methods relying on a single guide RNA. This method is highly efficient at introducing custom, site-specific genetic modifications at both genetic alleles and is demonstrated to exhibit lower off-target activity than CRISPR[1]. Cas-CLOVER is an essential tool to introduce additional future genetic advantages to our CHOlympian™ platform without introducing unwanted mutations at off-target sites.

## GS Knockout Using Cas-CLOVER

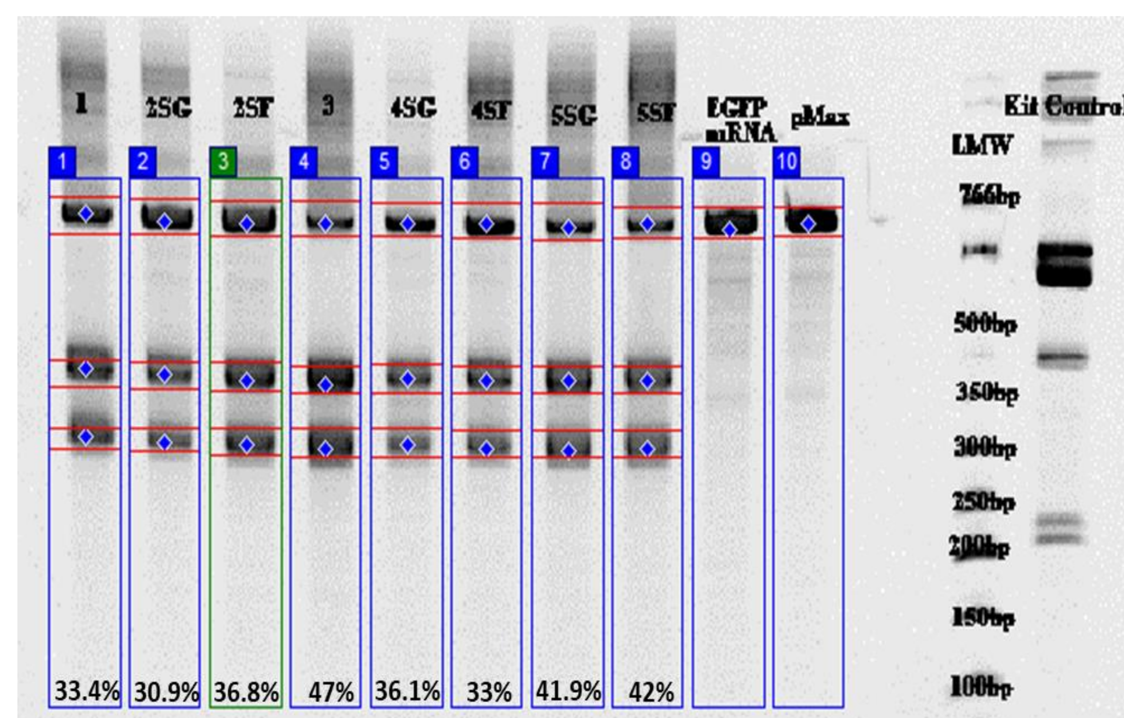



**Figure 1. *In vitro* Cas-CLOVER gRNA pair evaluation using an EnGen assay.** High on-target nuclease activity to glutamine synthetase gene sequences was observed. Cas-CLOVER *in vitro* cutting efficiency was calculated to be between 30-47% across the evaluated guide RNA pairs.

**Figure 2. Nucleofection of CHO cells.** Nucleofection efficiency of gRNA and Cas-CLOVER mRNA was estimated at 95%. EGFP mRNA (Trilink) was used as a transfection control.

**Figure 3. Guide RNA strategy and characterization of mutants. Top:** Illustrates left and right pair binding sites of gRNAs to the CHO GS gene. **Bottom:** Sequence alignments following Cas-CLOVER mediated targeting of GS in CHO pools (**A**) and individual clones (**B**). The mutation strategy was designed to create a substantial deletion and frameshift in exon 6; 13, 22 and 25 bp deletions are shown. Large deletions are routine to introduce using Cas-CLOVER.

## CHO-GS-KO Cell Line Phenotyping

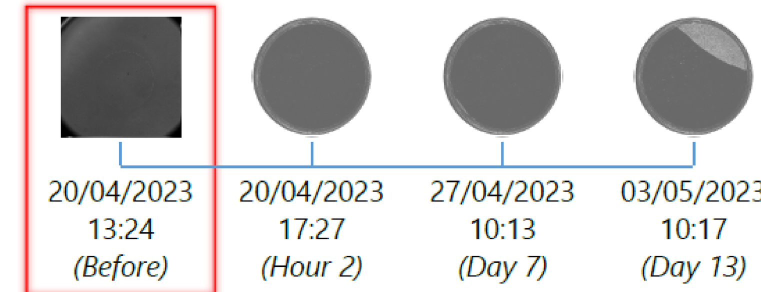



**Figure 4. CHO-GS-KO monoclonality by VIPS.**
A sequence confirmed pool of GS KO clones was subjected to single cell cloning (SCC) using VIPS and incubated at 37°C under conditions of 8% $CO_2$ and 80-90% humidity. On Day 13, clones were analyzed and select clones were expanded for future work.

| Clone ID | Viability≥90% | Time recovery(less than 3 days) | DT(hrs) |
|---|---|---|---|
| 9E11 | + | + | 17.21 |
| 8H7 | + | + | 19.87 |
| 6E7 | + | + | 20.08 |
| 7G2 | + | + | 19.46 |

**Figure 6. Growth parameters of CHO-GS-KO candidates.**
Viability, time after thawing and doubling time of final CHO-GS-KO cell lines in CD FortiCHO media for 4 passages BID.

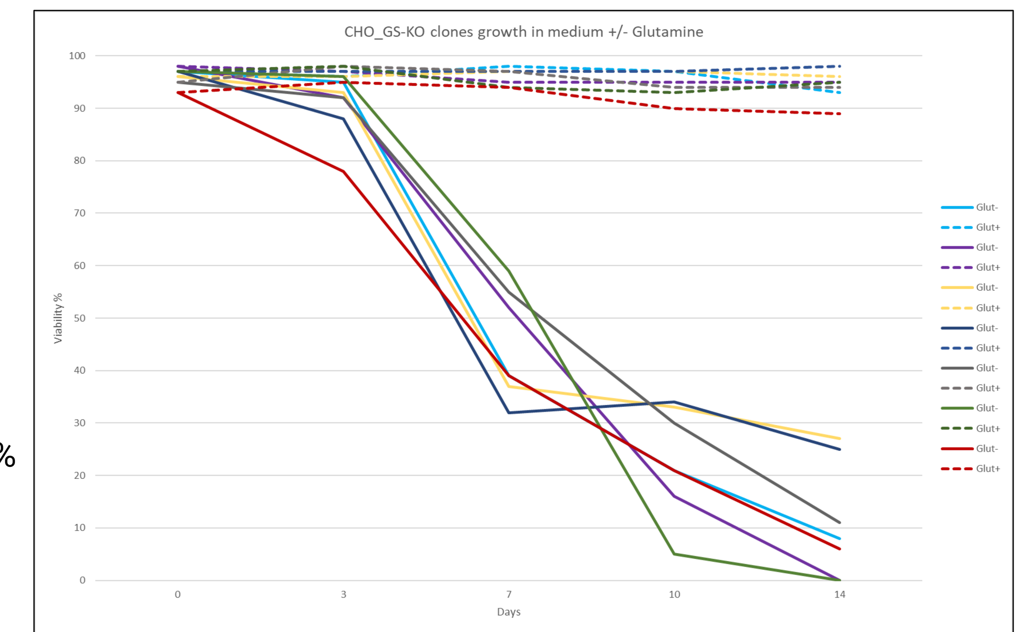


**Figure 5. Sensitivity of CHO-GS-KO clones to glutamine.**
As expected, CHO-GS-KO cell lines show consistently decreased viability in medium without glutamine

## Rituximab Expression with piggyBac®-mediated Integration

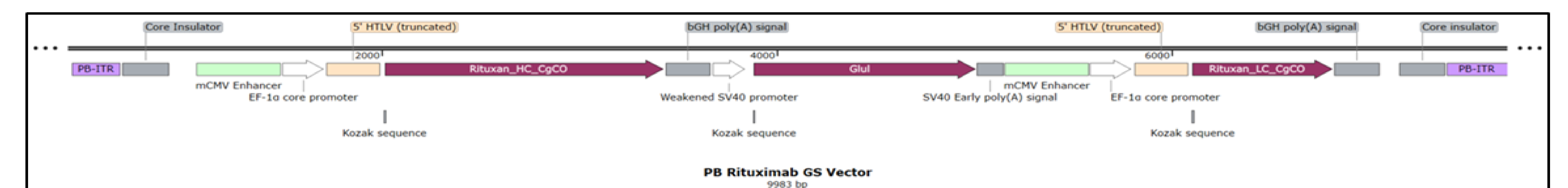


**Figure 7. PiggyBac transposon encoding rituximab heavy and light chains and GS selection cassette.**
Both heavy and light antibody chains are driven by CMV enhancer EF-1α promoter 5'HTLV (EFH). Glutamine synthetase is restored with the addition of GluI gene driven by an SV40 promoter. All components lie within piggyBac ITRs.

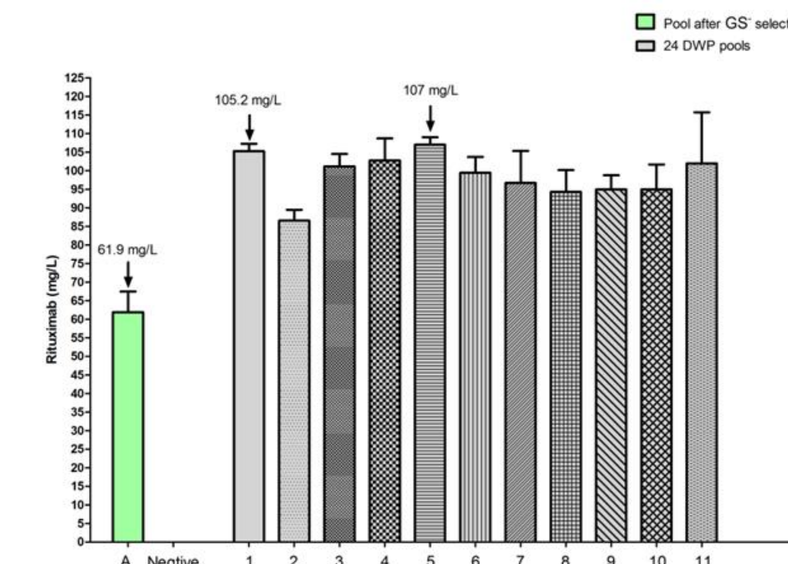



**Figure 8. Early ELISA to screen CHO pools.**
Supernatants were collected 96 hrs after nucleofection and used as a negative control. A pool was selected in medium without glutamine for 2 weeks and the anti-rituxmab titer was measured by ELISA. Cells were transferred to 24 well plates at 1X10^6 cells/mL. At Day 5 of culturing two pools showed titers >100 mg/L.

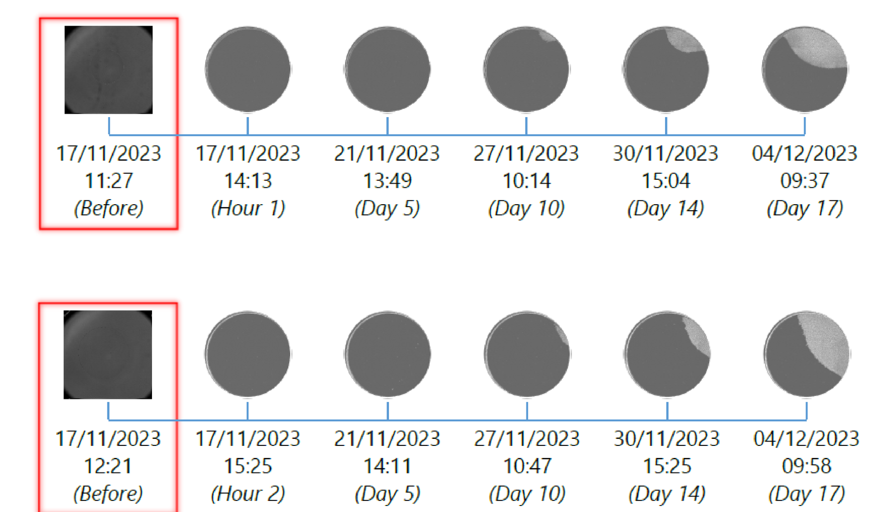



**Figure 9. Monoclonality of CHO-GS-KO/rituximab clones.**
Single cell clones were derived from the rituximab clone pool with the highest titer using VIPS. Clones were incubated at 37°C under conditions of 8% $CO_2$ and 80-90% humidity to assess monoclonality. On Day 17, clonality was analyzed by VIPS, and titers were assessed by ELISA. Select clones were expanded for further characterization based on clonality and titer.

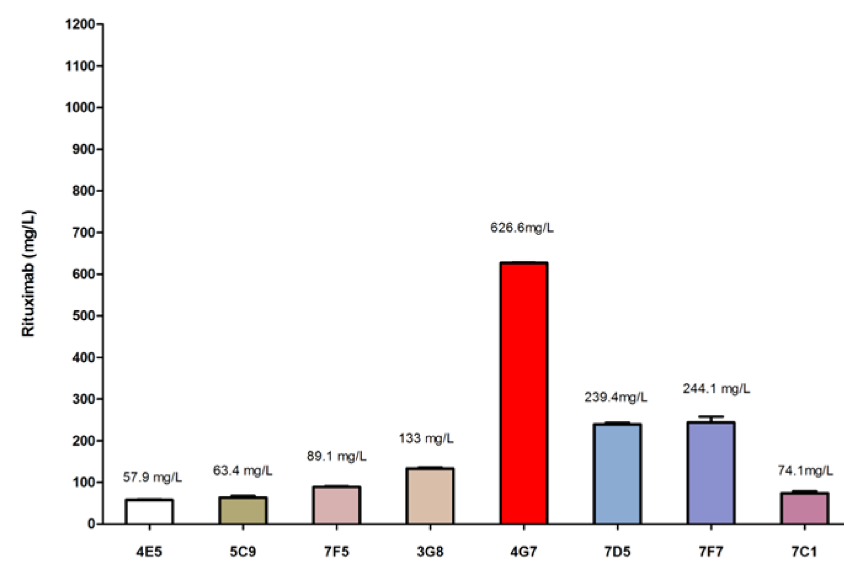



**Figure 10. Rituximab production by 8 clones.**
Titers were assessed on day 3 after transferring from 3X96 well plates into 24DWPs. Clone 4G7 showed the highest titer of 626 mg/L.

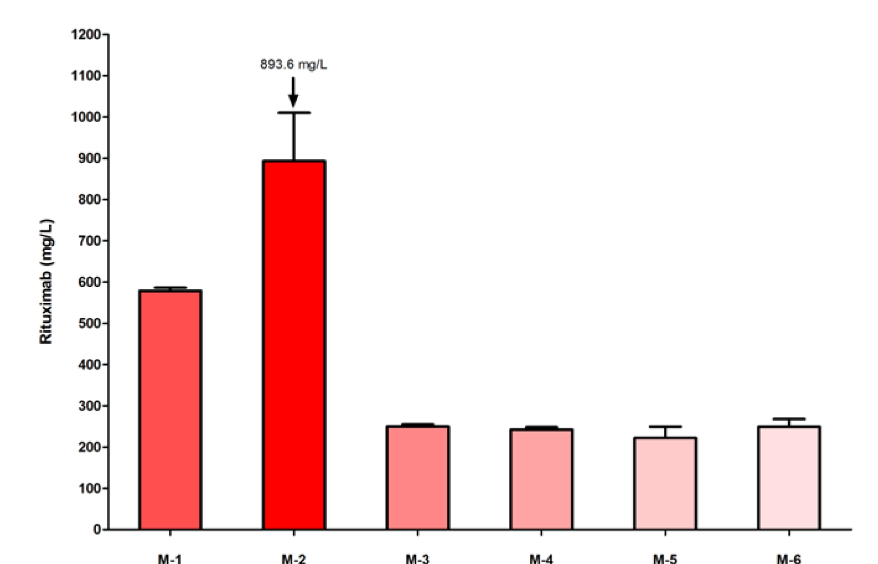



**Figure 11. Evaluation of media composition.**
Clone 4G7 was cultured in six distinct media to assess growth characteristics. Cells were seeded at Seeding Density 1X10^6 cells/mL density and grown for two weeks. Media 2 enhanced Rituximab production.

## Conclusions & Future Research

Hera established the CHOlympian™ CHO-K1-GS cell line and demonstrated proof of concept for antibody production. PiggyBac is used to introduce expression constructs and cells are selected using glutamine. Additional designer and proprietary CHO hosts can easily be created using the highly efficient and site-specific Cas-CLOVER gene editing tools.

Hera is currently optimizing and expanding the CHOlympian™ offering to knock out GS pseudogenes, build a library of expression vector cis elements, demonstrate scalability of growth parameters and create additional hosts for glycoengineering and improved performance.

## References

1. Madison, B. B., Patil, D., Richter, M., Li, X., Tong, M., Cranert, S., Wang, X., Martin, R., Xi, H., Tan, Y., Weiss, L., Marquez, K., Coronella, J., Shedlock, D. J., & Ostertag, E. M. (2022). **Cas-CLOVER is a novel high-fidelity nuclease for safe and robust generation of $T_{SCM}$-enriched allogeneic CAR-T cells.** *Molecular therapy. Nucleic acids, 29*, 979–995. https://doi.org/10.1016/j.omtn.2022.06.003

2. Mellahi, K., Brochu, D., Gilbert, M., Perrier, M., Ansorge, S., Durocher, Y., & Henry, O. (2019). **Process intensification for the production of rituximab by an inducible CHO cell line.** *Bioprocess and biosystems engineering, 42*(5), 711–725. https://doi.org/10.1007/s00449-019-02075-z