

RESEARCH ARTICLE

Cell Culture and Tissue Engineering

Evaluation of gene editing in CHO cells using the Cas-CLOVER system

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Abstract

Recent advances in gene editing technologies have transformed the genetic engineering of Chinese hamster ovary (CHO) hosts, enabling the development of cell lines with improved stability and productivity. In this study, we employed the programmable nuclease (PN) Cas-CLOVER to precisely target the Glutamine synthetase (GS) locus in CHO cells. A total of 30 unique serum-free, suspension-adapted CHO-K1 candidate host cell lines were subjected to Cas-CLOVER-mediated gene editing, generating over one hundred potential GS knockout (GSKO) clones. A subset of the GSKO clones was subsequently validated using three orthogonal methods to confirm complete knockout of the GS gene in 98 clones. Randomly selected GSKO clones were utilized to produce standard monoclonal antibodies. The resulting pools demonstrated enhanced productivity, with up to a 14.5-fold increase in titer compared to their wild-type parental hosts. These findings highlight the potential of gene editing approaches to significantly improve recombinant protein production in CHO expression systems, offering valuable insights for biopharmaceutical manufacturing applications.

KEYWORDS

biopharmaceutical production, Cas-CLOVER, CHO cells, gene editing, glutamine synthetase knockout (GSKO)

1 | INTRODUCTION

The development of highly productive stable cell lines represents a significant investment of time and resources in biopharmaceutical

manufacturing, with outcomes heavily dependent on the quality of the Chinese Hamster Ovary (CHO) host used in cell line development (CLD) platforms. CHO cells have emerged as the predominant mammalian expression system for therapeutic protein production across the biopharmaceutical industry due to their adaptability to suspension culture, capacity for human-compatible

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post-translational modifications, and established regulatory acceptance.^{1,2}

Genome engineering technologies have expanded our ability to manipulate mammalian cells to achieve desired phenotypes. These technologies offer value in CLD processes, which traditionally require extensive screening of hundreds to thousands of clones to identify candidates with optimal expression profiles—a resource-intensive endeavor.³ Manipulation of the CHO host genome can influence numerous aspects of the CLD process, including selection efficiency, cellular productivity, and product quality attributes. These modifications can enhance pathways governing protein folding, secretion, glycosylation patterns, cell cycle regulation, and metabolic efficiency, ultimately affecting both titer and quality of the expressed therapeutic protein.^{4–6}

Genome editing in mammalian cells has historically relied on three main programmable nuclease platforms: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9 (CRISPR-Cas9).⁷ Both ZFNs and TALENs function through DNA-binding protein domains fused to the catalytic domain of FokI nuclease, which must dimerize to generate double-stranded breaks (DSBs) at targeted genomic loci.^{8,9} While effective, these systems require extensive protein engineering for each new genomic target, presenting significant technical barriers to widespread implementation.^{10,11} CRISPR-Cas9 has simplified mammalian genome editing, allowing for the rapid engineering of mammalian cell lines. Unlike protein-based DNA recognition in ZFNs and TALENs, CRISPR-Cas9 employs an often 20 base-pair guide RNA (gRNA) to direct the Cas9 nuclease to complementary DNA sequences adjacent to a protospacer adjacent motif (PAM) site, where it cleaves both DNA strands approximately 3 bp upstream of the PAM sequence on the target strand.^{12,13} This RNA-guided approach simplifies target design and implementation, requiring only the synthesis of new guide RNA sequences rather than complex protein engineering. CRISPR-Cas9 has made genome editing widely accessible, offering superior ease of use and cost-effectiveness compared to previous technologies.^{14–16}

While CRISPR-Cas9 offers simplicity in target design compared to ZFNs and TALENs, early implementations faced challenges with off-target effects, including unintended structural variants, insertions/deletions (indels), or mutations in non-targeted genomic regions.^{17,18} Subsequent engineering efforts have produced improved variants such as Cas9 nickases and high-fidelity Cas proteins (Cas12a-HiFi, Cas9-HiFi) as well as the development of better gRNA design algorithms, which have substantially reduced off-target activity while maintaining on-target efficiency.^{19–22}

Recently, hybrid nuclease systems have also emerged that combine the targeting precision of CRISPR with the specificity of dimeric nucleases. These include FokI-dCas9 (catalytically inactive Cas9 fused to the FokI catalytic domain) and Cas-CLOVER (dCas9 fused to the C-terminus of *Clostridium Clo051*, a type IIS endonuclease).^{23,24} FokI-dCas9 has demonstrated increased editing specificity, with studies reporting a 140-fold increase in on-target editing compared to wild-type Cas9 and approximately 1.3- to 8.8-fold improvement over Cas9

nickases.^{23,25} Similarly, Cas-CLOVER has shown high editing efficiency (~90%) in HEK-293 cells²⁶ and minimal off-target effects in T-cells (Madison, 2022), positioning it as a high-fidelity alternative for precise genome engineering. However, the application of Cas-CLOVER in CHO cells has not been thoroughly studied.

In this study, we present a comprehensive evaluation of Cas-CLOVER efficacy in 30 serum-free suspension-adapted CHO-K1 host cell lines, targeting Glutamine synthetase (GS) for knockout (KO). We successfully generated and confirmed gene-edited clonal cell lines within a six-week timeframe for 28 of the 30 cell lines, demonstrating the establishment of a rapid and efficient platform for gene editing in CHO cells. Furthermore, we developed a random integration platform using these GSKO cell lines that exhibits up to 14.5-fold improvement in protein titer compared to wild-type GS cells. The experimental workflow of this study is illustrated in Figure 1.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

All media and supplements for passage and culturing used an in-house proprietary media. Unless otherwise noted, cells were grown in suspension using vented cap shake flasks or vented cap shake tubes in a Kuhner Shaker ISF1-XC (Kuhner, Basel, Switzerland) at 37 °C, 5% CO₂, and 80% humidity, with shaking at 125 rpm (shake flasks) or 260 rpm (shake tubes) and 50 mm orbit. An adherent CHO-K1 cell line was adapted for suspension growth in animal derived component (ADC)-free media (unpublished data) to generate several ADC-free CHO-K1 host cell lines used in these studies. Cells were passaged every 3–4 days in proprietary media prior to transfection. On day –1, cells were plated at 1.25e5 vc/well in a 24-well plate (–wp). On day 0, cells were transfected with 0.5 µg Cas-CLOVER™ dimeric nuclease system (mRNA) (CCL-100, Hera Biolabs) using the Mirus mRNA transfection kit (MIR2250, Mirus Bio) according to the manufacturer's protocol. Approximately 6 h later, cells were again transfected with 0.5 µg of a 1:1 mixture of the left (5'-acatgttgacctttagaata-3') and right (5'-ccctgtgaaggaaatccgcat-3') sgRNA using the Mirus mRNA transfection kit (MIR2250, Mirus Bio). See supplementary Table 1 for full list of sgRNAs tested. The process was repeated every 3 days for a total of three transfections (Figure 2b).

2.2 | Construction of DNA transfection vectors

Plasmids containing either the light chain or heavy chain of a standard mAb and the wtSV40 promoter driving GS expression were previously generated in-house using restriction enzyme cloning. Plasmids containing the attSV40 promoter driving GS expression were generated by site-directed mutagenesis to delete 168 bp of the SV40 promoter containing two 72 bp repeats and a 21 bp repeat as previously described.²⁷ All final plasmid maps were sequence-verified by GENE-WIZ (Azenta Life Sciences).

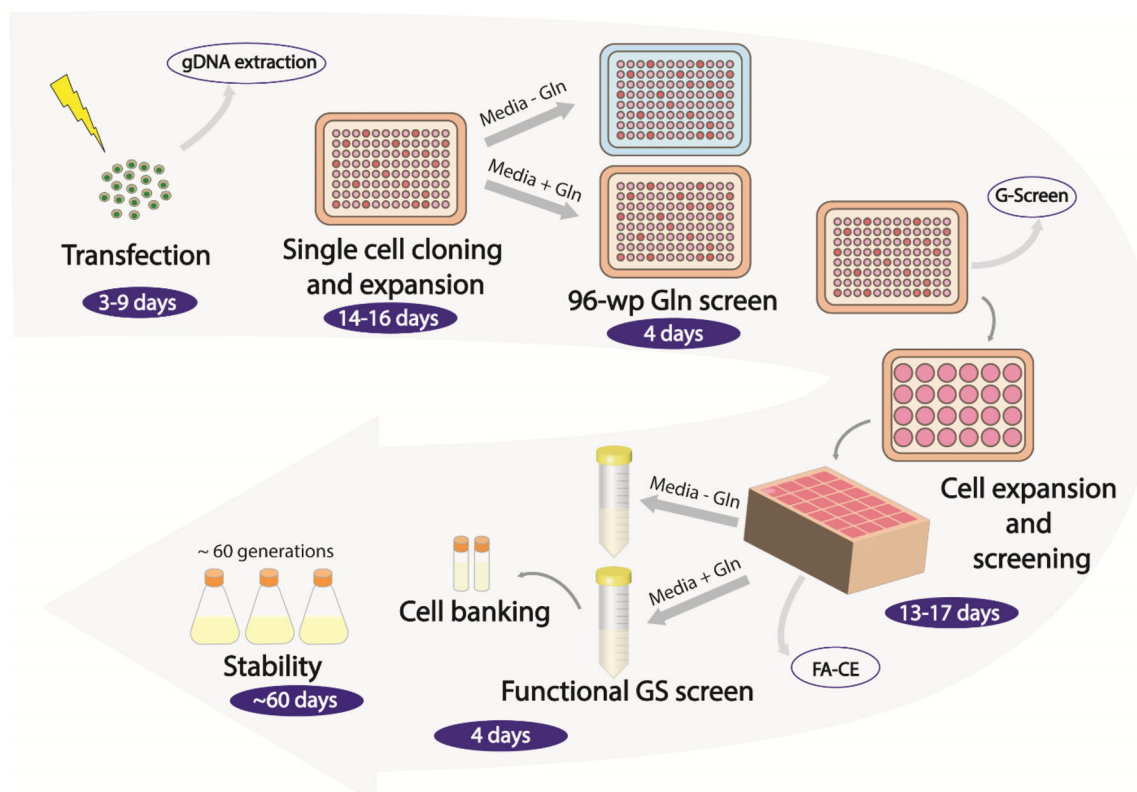


FIGURE 1 Experimental overview of gene editing and clone screening. After selection of gRNAs, cells are transfected with Cas-CLOVER and a single gRNA pair three times. Three days following the final transfection, edited pools are single cell sorted and subjected to a screen in 96-wps to determine glutamine auxotrophy. Glutamine auxotrophic clones are selected and sent for low resolution NGS (G-screen) while continuing expansion to 24-wps, 24-deep wps and spin tubes. Fragment analysis by capillary electrophoresis and a final glutamine auxotrophy screen under agitating conditions are performed to confirm gene editing at exon 5 of GS. Editing stability is confirmed in select clones over 60 generations.

2.3 | Transfection and selection of CHO pools for stable expression of a standard mAb

Internally derived GSwt clones or GSKO clones were co-transfected with 20 μ g plasmids containing either the heavy chain or light chain of a standard mAb and a wtSV40 promoter driving GS expression, or 20 μ g of the same heavy chain or light chain and an attenuated SV40 promoter (attSV40) driving GS expression using the Biorad electroporator at 250 V for 3 pulses. Pools were seeded at 1.0×10^6 vc/mL in CD CHO (Thermofisher Scientific, cat# 10743). A total of 1 day post-transfection, cells were seeded at 0.3×10^6 vc/mL in selection media containing either 50 μ M methionine sulfoximine (MSX) for wtSV40 pools, or 10 μ M MSX for attSV40 pools. Pools were passed every 3–4 days until they reached 90% viable with a doubling time <32 h. Upon completion of selection, cells were inoculated into spin tubes at 0.5×10^6 vc/mL for batch culture analysis. On day 7 of batch culture, conditioned media was harvested, clarified by centrifugation and analyzed for titer on the Octet RH16 using protein A biosensors (Octet RH16 – Sartorius).

2.4 | Single cell sorting and expansion of gene edited pools

On day 9 post-initial transfection, ~ 0.2 mL cell culture was counted using the ViCell XR and ~ 0.7 mL of cell culture from each well of a

24-wp was centrifuged at 900 rpm for 4 min at room temperature. Media was aspirated and cell pellets were washed $1 \times$ in cloning media (proprietary media supplemented with DMEM-F12, 8 mM L-glutamine and 20 mM HEPES). Cells were strained using a 0.4 μ m filter and centrifuged for 4 min at 900 rpm at room temperature. Media was aspirated and cell pellets were resuspended in 0.15 mL cloning media and targeted for live gate single cell sorting using the Automated Cell Deposition Unit (ACDU) into 96-wps using the BD Influx™ cell sorter. Live gate selection of cells was based on Boolean gates of viable cells (FSC-H and SSC-H) and doublet discrimination to identify live population for single cell plating via BD Influx ACDU to wells containing 100 μ L of cloning media. 96-wps were incubated at 37 °C for 15 days.

2.5 | T7EI mismatch assay

Genomic DNA from transfected cells was extracted from 100 μ L cell culture using the DNeasy Blood & Tissue Kit (Cat: 69506, Qiagen) according to the manufacturer's protocol. The mismatch assay was performed using the EnGen Mutation Detection Kit (Cat# E3321S, NEB) according to the manufacturer's instructions. Briefly, a region flanking GS exon 5 and the potential cut site was PCR amplified using Q5 high-fidelity polymerase master mix (Cat: NC0355755, NEB) and

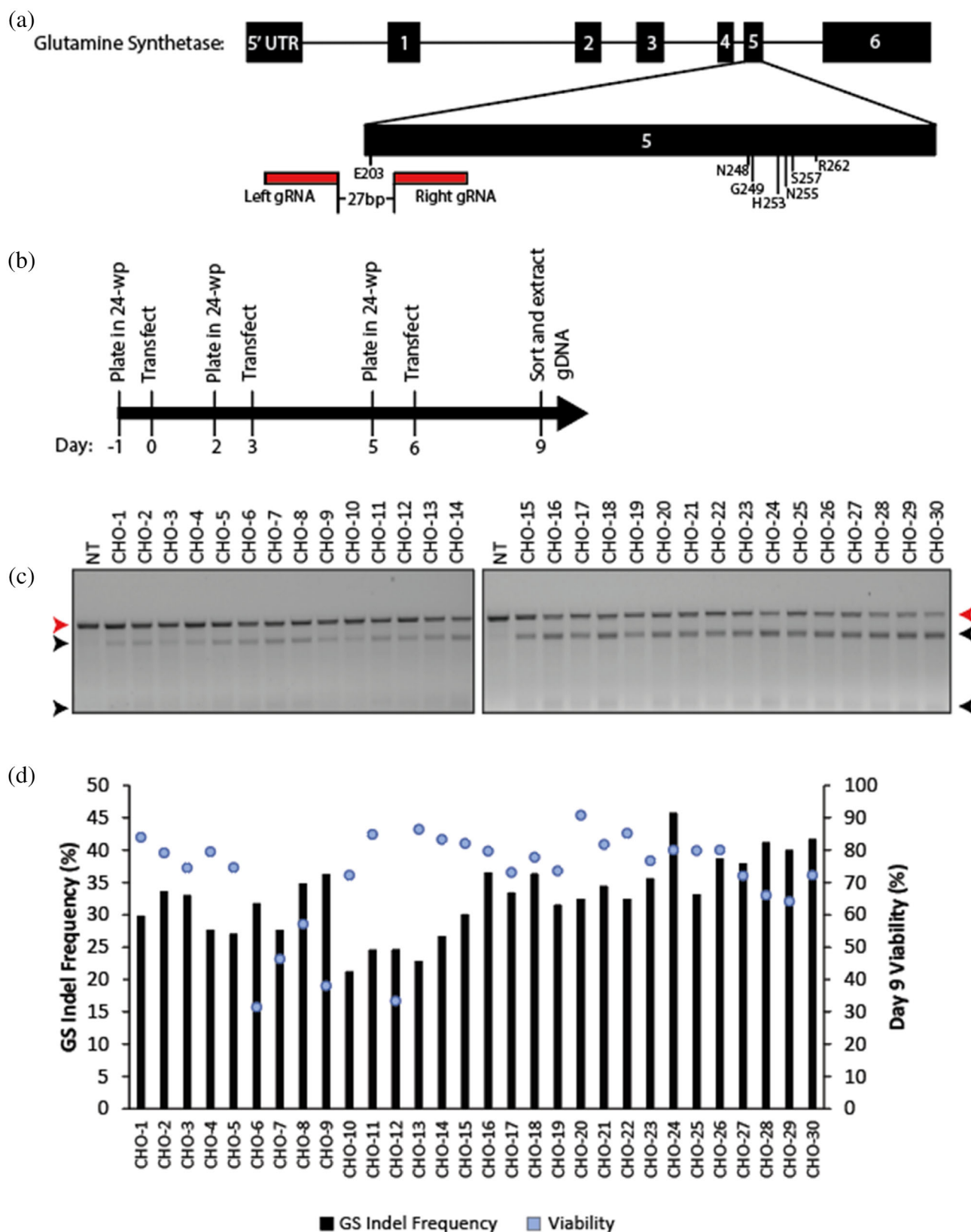


FIGURE 2 Cas-CLOVER gene editing design and implementation in 30 unique CHO-K1 parental cell lines. (a). Illustration of the Glutamine synthetase locus in CHO cells. Guide RNA pair 11 (red bars) was designed to target exon 5 of the GS. (b). Timeline of transfections with Cas-CLOVER and gRNA pairs. (c). Agarose gel depicting the indel frequency for each of the 30 unique CHO-K1 cell lines. Arrows highlight bands detected for uncut (red) and cut bands (black). (d). Densitometry analysis of indel frequency (black bars) depicted in c and viability (blue dots) of each parental cell line after gene editing and prior to single cell sorting.

TABLE 1 Table of primers.

Assay	Primers	Sequence (5'-3')	Amplicon (bp)
FA-CE	FA_F_seq	GCACATGGGGACTTTGGTTAAC	374
	FA_R_seq(6-FAM)	CCATGAGAATAAAGATGGC	
NGS	NGS_F_seq	GTGGTTCAGGGTAGAGGTAAGT	243
	NGS_R_seq	TGCACCATTCCAGTTCCAG	
T7EI	T7EI_F_seq	GCCCAGGTAAATGGCACTATTC	752
	T7EI_R_seq	TGACCTATGTCTCAAGTGCTTC	

primers as indicated in Table 1. PCR product was then denatured at 95 °C for 10 min and re-annealed by slowly cooling to room temperature to allow for heteroduplex formation. The annealed PCR product was digested with T7EI for 15 min at 37°C followed by a proteinase K treatment for 5 min. The digested product was run on a 2% agarose gel for 1 h in 1× TBE buffer and imaged on the Biorad UV imager. Indel frequency (cutting efficiency) was determined using densitometry with Biorad Imaging software. Final cutting efficiency was measured according to the following equation:

$$\text{Formula 1} := (\text{Cut Band 1} + \text{Cut Band 2}) / (\text{Cut Band 1} + \text{Cut Band 2} + \text{Uncut Band})$$

$$\% \text{Cutting Efficiency} := 100 \times (1 - \text{SQRT}(1 - \text{Value from Formula 1}))$$

2.6 | 96-wp glutamine screen

Glutamine screens began on day 15 post-sorting. Plates were imaged using the CellMetric whole well imager (Advanced Instruments) to identify wells with >10% cell confluence for selection using the Hamilton Vantage automated liquid handling system. Positive wells were split into two new 96-wp with the transfer of ~75 µL to each new well. Both plates were centrifuged at 900 rpm for 2 min at room temperature and media was aspirated and discarded. 200 µL fresh in-house proprietary media with 8 mM L-glutamine was added to each well in one plate and 200 µL media lacking L-glutamine was added to each corresponding well in the other plate. Plates were incubated for 20 min at 37 °C then imaged for confluence using the CellMetric whole well imager. Plates were subsequently incubated for 4 days at 37 °C and again imaged for confluence. The change in confluence over 4 days was determined and those clones with <2% change in confluence in media without glutamine were considered potentially positive for GSKO.

2.7 | Clone expansion

Positive GSKO clones from the glutamine dependency screen were expanded from 96-wps to 24-wps by adding 100 µL cell culture from each positive well in the 96-wp to 400 µL proprietary media in a 24-wp. After 4 days, each well with positive growth was expanded to

a 24-deep well plate (dwp) and incubated at 37 °C, 260 rpm. A total of 4 days after inoculation in the 24-dwp, 2 mL cells were expanded in 3 mL fresh media in 50 mL spin tubes and incubated at 37 °C, 260 rpm for 4 days. On day 4, each clone underwent a second screen for glutamine auxotrophy, and the positive clones were selected for banking.

2.8 | Next generation sequencing

Clones were sequenced by amplicon based NGS at two stages. First, wells identified as potentially positive from the glutamine screen were analyzed by low resolution NGS (G-screen) by transferring 100 µL cells from each well of the 96-wp containing glutamine from day 4 of the glutamine auxotroph screen (Gln screen) to a new 96-wp. Plates were centrifuged at 900 rpm for 4 min at room temperature; media was discarded, and cell pellets were frozen at –80 °C for G screening at GeneGoCell using proprietary G-screen services. Second, select gene edited clones passing the final spin tube glutamine auxotroph screen were sent for high resolution NGS via amplicon deep sequencing (G-AmpSM from GeneGoCell). In brief, 2.0×10^6 viable cells from 48 clones were centrifuged in 1.5 mL centrifuge tubes for 4 min at 900 rpm. Media was aspirated and pellets were stored at –80 °C until use. Analysis was performed at GeneGoCell, where two unique amplicons were generated per clone, each tagged with its own unique molecular identifier (UMI). Greater than 100,000 UMI reads were analyzed per sample for variant calling and frequency determination. Only clones with G-screen scores >30 passed quality control. Results were filtered in-house prior to analysis to eliminate variants with less than 10% frequency of detection and 197–199 bp variants which were determined to be an artifact of the variant calling algorithm.

2.9 | Fragment analysis by capillary electrophoresis

Genomic DNA from transfected clones was extracted from 96-wps using Molecular Research Center's DNAzol Direct (Cat: NC0109325, Fisher Scientific) according to the manufacturer's instructions. In brief, 100 µL of DNAzol Direct was added to each well of a 96-wp and pipetted up and down 3–5 times. Cells were then incubated for 15 min at room temperature for lysis. Resulting cell lysates were diluted 1:5 by transferring 20 µL of cell lysate to a new 96-wp containing 80 µL TE at room temperature. The diluted cell lysates were

stored at 4 °C until use. PCR amplification targeting a region flanking the potential indels was achieved using 2.5 µL of the diluted cell lysate, Q5 high-fidelity polymerase master mix (Cat:NC0355755, NEB), and the primers indicated in Table 1 according to the manufacturer's instructions. PCR products were diluted 1:3 in water and sent for fragment analysis by capillary electrophoresis at Eton biosciences or GENEWIZ (Azenta Life Sciences). The fluorescently labeled amplicon of 374 bp encompassing the indel range generated by PCR was run against a size standard using capillary electrophoresis to discriminate indel sizes down to a single base-pair for each allele. Fragment analysis data files were analyzed using Peakscanner v1.0 software to look at peak intensity and size. Peak analysis applied cutoffs to eliminate background; peaks less than 2000 RFU and smaller than 50 bp were eliminated.

3 | RESULTS

3.1 | Generation of GSKO clones using Cas-CLOVER

GSKO clones were generated using Cas-CLOVER mRNA and guide RNA pairs targeting exon 5 of the GS gene. The GS gene has been well characterized in CHO cells, with exon 5 established as the main target for GS knock-out (GSKO or GS^{-/-}) using ZFNs and CRISPR-Cas9 based approaches^{28–30} Several amino acids in exon 5 have recently been shown to be essential for GS activity in CHO cells²⁹ (Figure 2a). A total of 11 guide RNA (gRNA) pairs were therefore designed targeting exon 5 and evaluated for indel frequency in a CHO-K1 suspension cell line (Supplementary Figure 1a). Indel frequency ranged from 10.4% to 42.4% for the different gRNA pairs generated. A single guide RNA pair (gRNA pair 11) was selected. gRNA pair 11 contained a 27 base-pair (bp) spacer that flanked the 5' end of exon 5 in the GS gene (Figure 2a) to both eliminate an amino acid essential for GS enzymatic activity, a glutamic acid residue at position 203 (E203), and to cause a potential frameshift mutation in exon 5. Cells from 30 serum-free suspension CHO-K1 host cell lines were transfected with Cas-CLOVER mRNA and the selected guide RNA pair three times over a period of 9 days (Figure 2b). Genomic DNA was extracted on day 9 and cells were analyzed for GS indel frequency using an enzyme mismatch cleavage assay. Indels were detected at a frequency of 21% to 46% across the lineages (Figure 2c,d). Post-transfection viabilities ranged from 31% to 90% on day 9 prior to single cell cloning via flow cytometry (Figure 2d). Improved indel frequency of up to 72% was detected when using nucleofection on a subset of CHO hosts (Supplementary Figure 1b), potentially leading to a higher frequency of knock-out clonal cell lines.

To increase the selection of bi-allelic GSKO clonal cell lines, an initial glutamine dependency screen for potential GSKO clones was employed. Because GSKO clones always showed reduced growth in media lacking glutamine supplementation, potential GSKO clones would display limited growth over 4 days in a 96-wp (Figure 3a,b). To determine the level of growth over 4 days, cells were imaged for

confluence on the day of plating and 4 days later. Figure 3b depicts a cell confluence assay for a single clone; cell confluence increases when plated in media containing glutamine, while the confluence for the same clone is relatively unchanged in media lacking glutamine, indicating a potential GSKO clone. Imaging to detect a change in confluence over 4 days was repeated for 2065 clones that showed normal growth (expansion) in media with glutamine. From these, 751 clones also demonstrated lack of growth and/or cell death (<2% change in confluence) in glutamine-free media while maintaining positive growth in media containing glutamine (Figure 3c). Up to 28 clones from each of the GSwT parental hosts were selected at random for further evaluation, resulting in 547 total glutamine auxotrophic clones. (Figure 3d).

3.2 | Evaluation of GSKO clones generated using Cas-CLOVER

Potential GSKO clones identified from the initial 96-wp glutamine screen were sent for high-throughput resolution genotyping via targeted next generation sequencing (G-screen) to determine the size and location of possible indels in the GS locus. Of the 547 potential GSKO clones, sequence variants were detected in amplicons from 432 clones at any frequency. Further analysis was performed on 260 clones with high frequencies (>10%) of sequence variants detected by low resolution NGS. Clones were evaluated for indel frequency, size, location, and type. We found that 78% of variants caused by Cas-CLOVER were deletions and 1.4% were insertions. Complex indels (deletion followed by insertion, or vice versa) comprised 12.5% of all variants while single nucleotide substitutions or mutations made up the other 8.1% (Figure 4a). Next, we analyzed the ploidy and zygosity of the amplified locus. Of the 260 clones analyzed, one to two variants (likely diploid with homozygous or heterozygous deletions) were detected 94.2% of the time, with only 5.8% of clones containing 3 variants (possible triploids) (Figure 4b). Clones with 4 or more variants detected by NGS were likely not of monoclonal origin and eliminated from the study. Of the 15 clones with 3 variants, only 5 contained indels (data not shown). The remaining variants detected were substitutions, indicating a low number of possible triploid cells at the GS locus. Finally, we determined Cas-CLOVER causes roughly an equal number of 1n, 2n, or 3n bp indels in the GS exon 5 locus allowing for approximately 65% of clones to have potential frameshift mutations (number of base pairs in indel divisible by 1 or 2, but not 3) (Figure 4c). Complex indels (deletion followed by insertion or insertion followed by deletion) were treated as standard indels in the analysis with the net indel size noted (either 1n, 2n or 3n bp deletion or insertion). For example, if 3 bp were deleted and 1 bp was inserted, the net indel would be 2 bp. Indels generated by Cas-CLOVER gene editing ranged from +13 bp to -128 bp with many different sizes of deletions detected. The most common deletions were -12 bp and -26 bp at a 3.7% and 3.3% frequency, respectively (Figure 4d). This contrasts with the often small (1–2 bp) insertions or deletions induced by other PNs.^{31–34} The precise location of the cut site and indels generated

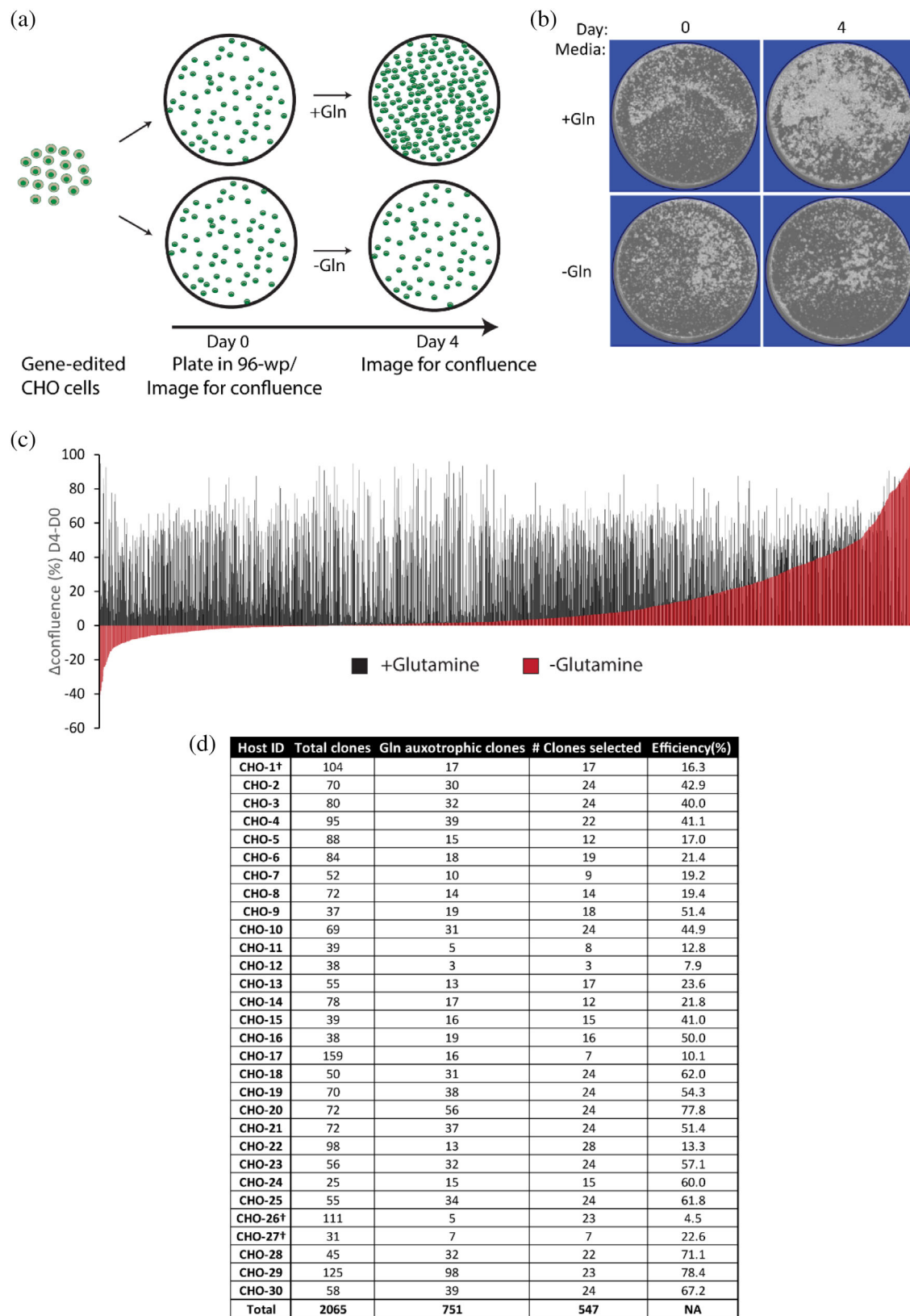


FIGURE 3 96-wp screen for glutamine auxotrophs. (a). Illustration outlining confluency screen for glutamine auxotrophs. (b). Example images of cell confluency on day 0 (left) and day 4 (right) in media with (top) and without (bottom) glutamine. (c). Change in confluence over 4 days in media with (black bars) and without (red bars) glutamine. Cells with <2% growth in media without glutamine were selected for further examination. (d). Table outlining the numbers of total clones and percentage of GS gene edited clones (Gln auxotrophs) from each host. †Cells transfected 1X and single cell sorted on day 3.

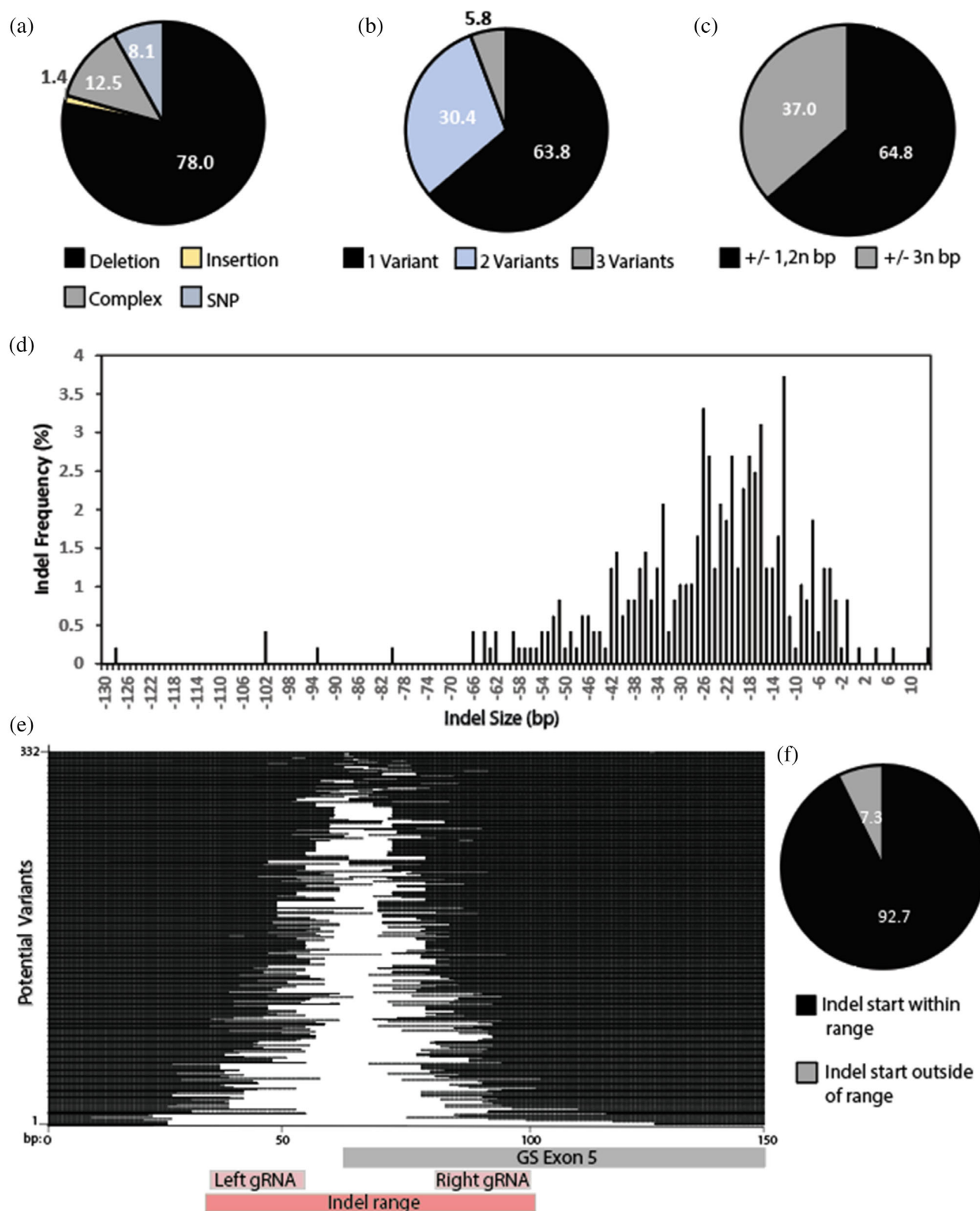


FIGURE 4 Next generation sequencing analysis of indels caused by Cas-CLOVER gene editing. Low resolution NGS data was analyzed for indel type, frequency and location: (a) Percentage of variants detected that were deletions, insertions, complex or single nucleotide polymorphisms (SNPs). (b) Percentage of clones with 1, 2, or 3 variants detected in exon 5. (c) Percentage of variants with the potential to cause frameshifts (1 or 2n). (d) Distribution of indel sizes across all clones. (e) Location of variants detected in each amplicon by NGS relative to the start of exon 5 and the gRNA pair. The indel range comprises both gRNAs and their respective PAM sites (73 bp). (f) Comparison of the number of indels that start within versus outside the indel range.

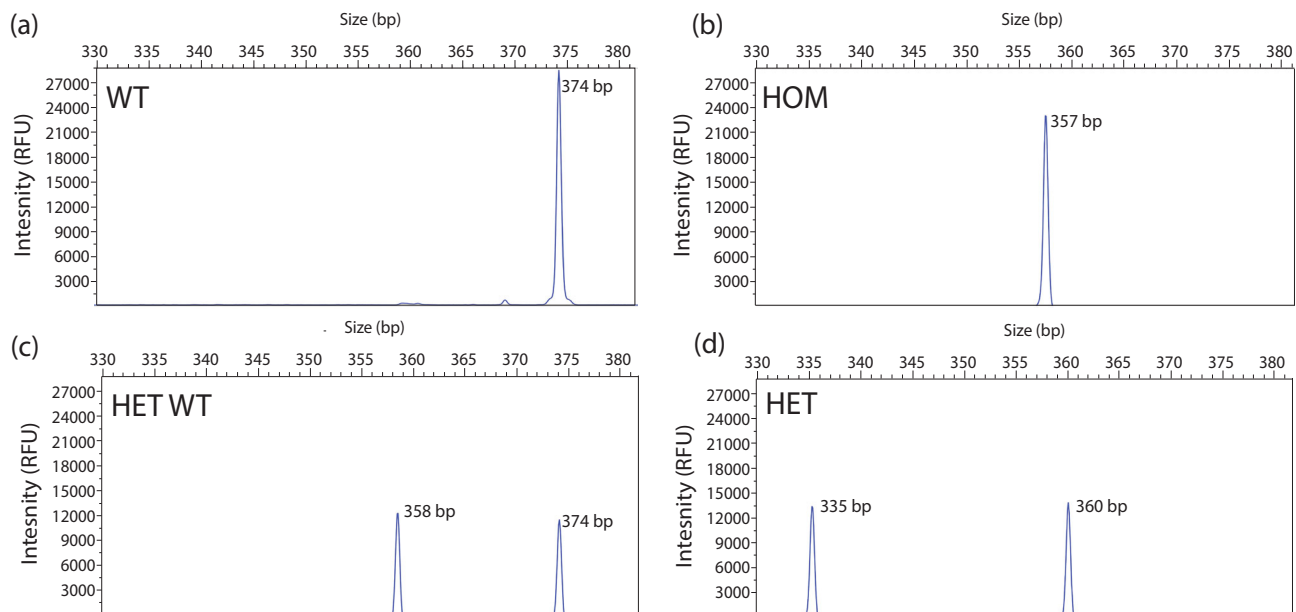


FIGURE 5 Fragment analysis by capillary electrophoresis diagrams of clones with four distinct genotypes: (a) Wildtype (WT) genotype: Unedited clone with two amplicons in GS of the same size (374 bp). (b) Homozygous deletions (HOM) genotype: Edited clone with two smaller amplicons of the same size (357 bp) in the GS locus. (c) Heterozygous wild-type (HET WT) genotype: Edited clone with one amplicon of the same size as WT and one amplicon that is smaller in the GS locus. (d) Heterozygous double deletion (HET) genotype: Edited clone with two amplicons that are smaller than WT.

when using Cas-CLOVER has yet to be demonstrated. We found that 92.7% of indels started within the 73 bp range (indel range) comprised of the two sgRNAs and their respective PAMs (Figure 4e,f) indicating a high likelihood of cleavage in this range.

Targeted low resolution NGS allows for rapid identification of potential knock-out clones based on the frequency of the sequence variant detected. The higher the frequency of detection, the higher the likelihood of a clone being a true variant. To confirm candidate clones for GS exon 5 locus editing found by G-screen, we selected 100 clones for further analysis using fragment analysis by capillary electrophoresis. Using fragment analysis by capillary electrophoresis, we can discern four main genotypes for clones based on the size and fluorescent intensity of the peaks generated: wild-type (no indel detected), heterozygous wild-type (a single WT allele and an allele with an indel), homozygous insertion/deletion (same indel on each allele), heterozygous insertion/deletion (different size indels on each allele) (Figure 5a). A fifth, triploid genotype was only detected in two of the clones (Supplementary Figure 2). Three additional clones containing 3 variants each by G-screen, all appeared to be diploid by fragment analysis with one peak correlating to the size of one variant detected by G-screen (Supplementary Figure 2b), while the other peak correlated to the sum of two variant sizes detected by G-screen. Thus, it is possible those samples with 3 variants detected by G-screen had two variants on one allele and a third variant on a second allele. From this we can conclude that the likelihood of duplication events of GS during gene editing is minimal.

A total of 48 clones from 28 of the 30 hosts were selected at random for confirmation of gene editing using high resolution NGS via amplicon deep sequencing (G-AmpSM from GeneGoCell). Approximately 97% of the indel sizes were within the margin of error (1 bp) between

fragment analysis and high resolution NGS (Table 2). Discrepancies between FA-CE and NGS may not have been detected due to the size of the indel (for example, clone 075) or poor amplification by PCR. Specific genotypes for each clone can be found in Supplementary Figure 3.

All 48 GSKO candidate clones were concomitantly subjected to a final screen for GS functionality. Each clone was inoculated in media with or without glutamine supplementation, and viability was measured after 4 days. All clones demonstrated glutamine auxotrophic behavior in media lacking glutamine, which is indicative of a functionally inactive GS gene (Figure 6a). The two clones that were heterozygous wild-type by NGS demonstrated a decrease in viability in media without glutamine, confirming that one copy of the GS gene may yield an auxotrophic phenotype, as previously observed.²⁸ Cell viability was comparable between heterozygous wild-type and complete knockout clones. Indel analysis was limited to the GS exon 5 locus, leaving potential modifications at other GS loci unexamined. To verify the glutamine auxotrophic phenotype of these edited clones, six clones were grown in media with or without glutamine over a period of 9 days. All six clones remained highly viable (>98% viability) over 9 days in media containing glutamine (Figure 6b), while demonstrating a decrease in viability in media lacking glutamine (Figure 6c).

3.3 | Stability of GSKO clones over several generations

Clonal populations of CHO cells can demonstrate a high level of genome instability during cell divisions leading to a low level of

TABLE 2 Comparison of Indel sizes and frequencies detected by FA-CE to Hi Resolution NGS.

Clone	Fragment Analysis by Capillary Electrophoresis Indel size (+/- 1 bp)				NGS(Hi-Res) Indel size (bp)				Genotype
	Variant 1	Frequency 1	Variant 2	Frequency 2	Variant 1	Frequency 1	Variant 2	Frequency 2	
Clone 001	-33 bp	0.452	-13 bp	0.548	-33 bp	0.497	-13 bp	0.504	HET
Clone 007	-16 bp	1			-16 bp	0.998			HOM
Clone 010	-85 bp	1			-128 bp	0.990			HOM
Clone 015	-20 bp	0.538	0 bp	0.462	-20 bp	0.551			HET WT
Clone 016	-38 bp	1			-39 bp	0.997			HOM
Clone 018	-12 bp	1			-12 bp	0.998			HOM
Clone 021	-25 bp	0.507	-2 bp	0.493	-25 bp	0.499	-2 bp	0.509	HET
Clone 025	-21 bp	0.517	-7 bp	0.483	-41 bp	0.507	-7 bp	0.510	HET
Clone 027	0 bp	1			-17 bp	0.534			HET WT
Clone 029	-25 bp	1			-25 bp	0.938			HOM
Clone 038	-22 bp	0.443	-96 bp	0.557	-22 bp	0.996			HET
Clone 040	-8 bp	1			-8 bp	0.996			HOM
Clone 044	-45 bp	1			-45 bp	0.994			HOM
Clone 048	-39 bp	0.444	-20 bp	0.556	-39 bp	0.499	-19 bp	0.497	HET
Clone 052	-50 bp	0.505	-19 bp	0.495	-50 bp	0.505	-19 bp	0.493	HET
Clone 054	-27 bp	1			-27 bp	0.998			HOM
Clone 058	-23 bp	1			-23 bp	0.953			HOM
Clone 060	-23 bp	1			-23 bp	0.994			HOM
Clone 066	-16 bp	1			-16 bp	0.997			HOM
Clone 069	-40 bp	0.490	-38 bp	0.510	-40 bp	0.505	-37 bp	0.474	HET
Clone 075	-12 bp	0.652	+213 bp	0.348	-12 bp	0.996			HET
Clone 076	-35 bp	0.542	-18 bp	0.458	-35 bp	0.518	-18 bp	0.498	HET
Clone 078	-54 bp	0.484	-25 bp	0.516	-54 bp	0.452	-25 bp	0.541	HET
Clone 084	-46 bp	0.571	-3 bp	0.429	-46 bp	0.572	-3 bp	0.478	HET
Clone 090	-42 bp	0.492	-12 bp	0.508	-42 bp	0.578	-12 bp	0.444	HET
Clone 092	-33 bp	0.524	-26 bp	0.476	-33 bp	0.527	-26 bp	0.486	HET
Clone 098	-27 bp	1			-27 bp	0.996			HOM
Clone 102	-36 bp	0.530	-17 bp	0.470	-36 bp	0.514	-17 bp	0.500	HET
Clone 112	-39 bp	0.533	-25 bp	0.467	-39 bp	0.514	-26 bp	0.482	HET
Clone 113	-51 bp	0.527	-24 bp	0.473	-51 bp	0.494	-24 bp	0.522	HET
Clone 115	-21 bp	1			-21 bp	0.999			HOM
Clone 117	-28 bp	1			-28 bp	0.997			HOM
Clone 118	-42 bp	1			-42 bp	0.998			HOM
Clone 128	-35 bp	1			-35 bp	0.991			HOM
Clone 132	-19 bp	0.488	-16 bp	0.512	-19 bp	0.509	-16 bp	0.499	HET
Clone 134	-49 bp	0.510	-22 bp	0.490	-48 bp	0.428	-22 bp	0.543	HET
Clone 136	-30 bp	1			-30 bp	0.984			HOM
Clone 139	-18 bp	0.512	-12 bp	0.488	-18 bp	0.497	-12 bp	0.504	HET
Clone 141	-38 bp	0.554	-9 bp	0.446	-38 bp	0.507	-9 bp	0.498	HET
Clone 142	-69 bp	0.539	-26 bp	0.461	-68 bp	0.412	-26 bp	0.573	HET
Clone 144	-55 bp	1			-55 bp	0.998			HOM
Clone 145	-103 bp	1			-102 bp	0.991			HOM
Clone 153	-23 bp	0.553	-17 bp	0.447	-23 bp	0.527	-17 bp	0.480	HET
Clone 159	-35 bp	1			-35 bp	0.995			HOM

TABLE 2 (Continued)

Clone	Fragment Analysis by Capillary Electrophoresis Indel size (+/- 1 bp)				NGS(Hi-Res) Indel size (bp)				Genotype
	Variant 1	Frequency 1	Variant 2	Frequency 2	Variant 1	Frequency 1	Variant 2	Frequency 2	
Clone 160	-18 bp	1			-18 bp	0.999			HOM
Clone 166	-19 bp	1			-19 bp	0.988			HOM

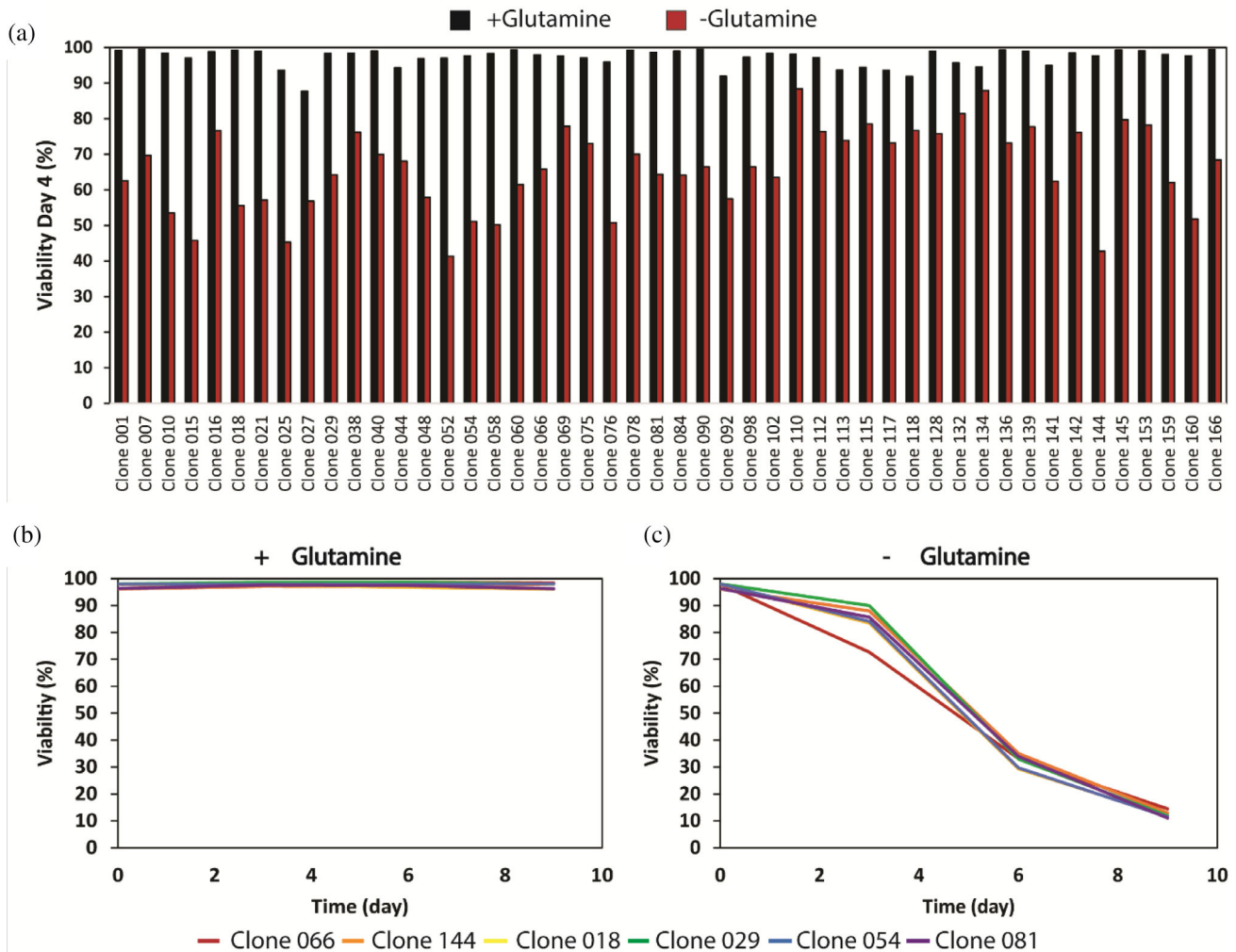


FIGURE 6 Cas-CLOVER gene editing of GS exon 5 generates functional knock-out of the GS gene. (a) 48 gene edited clones were inoculated in media with and without 8 mM L-glutamine supplementation and grown for 4 days with shaking. All clones demonstrated a viability loss in media without glutamine supplementation. Six edited clones were inoculated in media with (b) and without (c) glutamine supplementation and passed for 9 days. All six clones maintained growth and viability in media supplemented with glutamine.

genomic homogeneity at the time of cell banking.^{35–38} To determine the stability of the GS indels within cells, three clones were analyzed for indel size in the GS exon 5 for over ~60 generations using FA-CE. To assess the potential impact of subcloning on the knockout phenotype, a single subclone from clone 2 (clone 2b) was analyzed. Over the 63 days in culture, the size of the indels on each allele from each of the three clones remained the same. Furthermore, the indel sizes from subclone 2b remained the same as clone 2 indicating this region of the genome and the indels generated

remain stable in progeny from the original clones and subclones over many generations (Table 3).

3.4 | Productivity improvement in the GSKO platform

GSKO cell lines are often generated in the pharmaceutical industry to improve the productivity of CHO cells producing protein biologics

Clone	Indel size (bp)					
	Variant 1			Variant 2		
	Day 0	Day 34	Day 63	Day 0	Day 34	Day 63
Clone 1	−14 bp	−14 bp	−14 bp	−3 bp	−3 bp	−3 bp
Clone 2	−24 bp	−24 bp	−24 bp	−2 bp	−2 bp	−2 bp
Clone 2b	−24 bp	−24 bp	−24 bp	−2 bp	−2 bp	−2 bp

TABLE 3 Indels detected by FA-CE over ~60 generations.

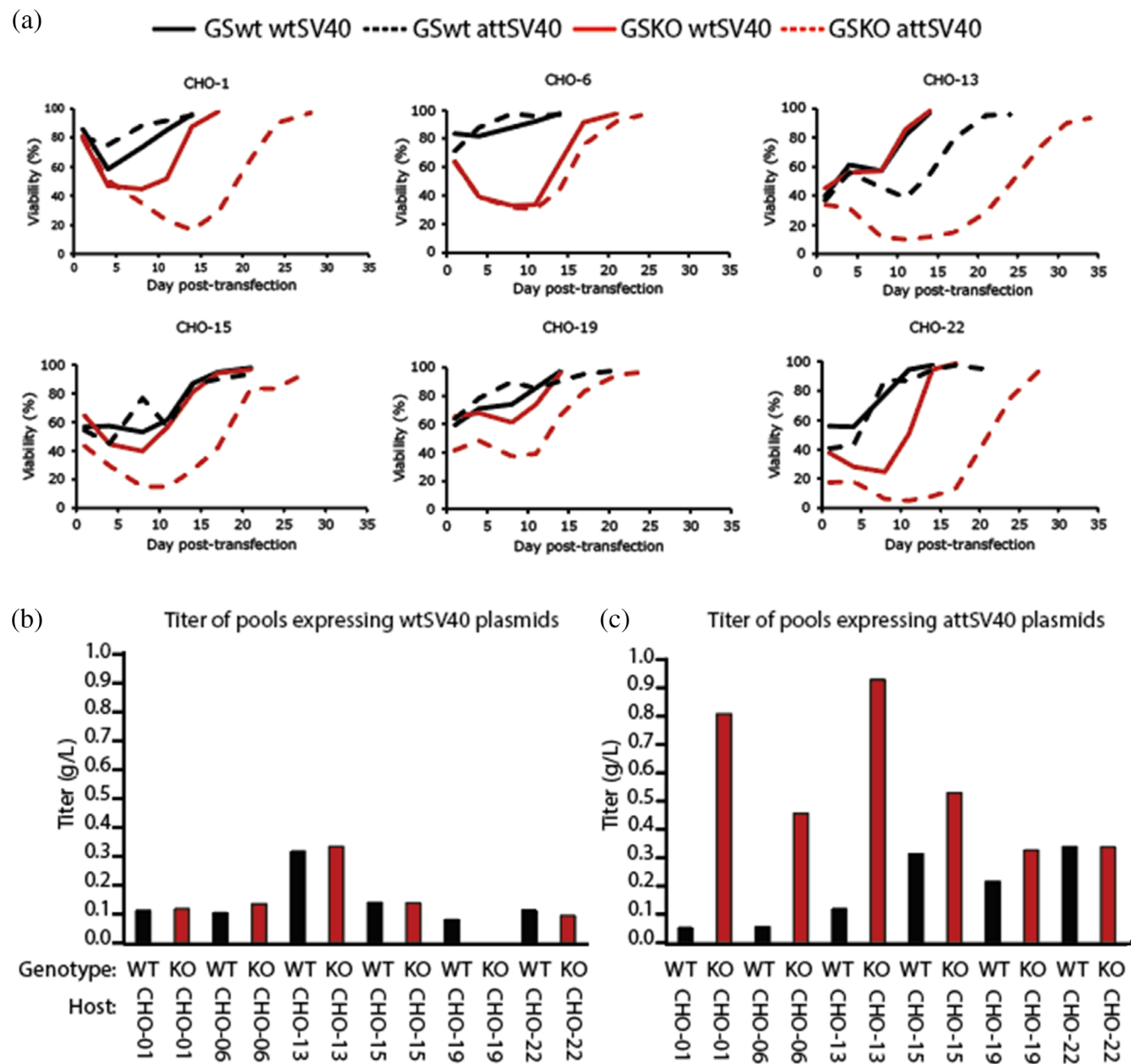


FIGURE 7 Selection and productivity of GSwT and GSKO cell lines expressing a standard mAb. Stable pools expressing a standard mAb were generated in 6 GSwT cell lines and their respective GSKO progeny by co-transfecting each cell line with plasmids containing either the light chain or heavy chain and a wtSV40 promoter driving GS or an attenuated SV40 (attSV40) promoter driving GS. Pools expressing wtSV40 plasmids were selected at 50 uM MSX and 10 uM MSX for those expressing attSV40 plasmids. (a) Viability profiles for pools generated from 6 parental CHO cells lines. Black line: GSwT CHO cell + wtSV40 promoter; Black dotted line: GSwT CHO cells + attSV40 promoter; Red line: GSKO cell line + wtSV40 promoter; Red dotted line: GSKO cell line + attSV40 promoter. (b) Titer of pools expressing wtSV40 plasmids (Black: GSwT pools; Red: GSKO pools). Titer for CHO-19 KO pool below detectable limits. (c) Titers of pools expressing attSV40 plasmids (Black: GSwT pools; Red: GSKO pools).

through various platforms (random integration, transposase-based, etc.). Initial studies performed on GSKO cells demonstrated only a 1.8-fold increase in bulk pool titer compared to the wild-type CHO host.²⁸ Further studies revealed that reducing exogenous GS expression from the vector construct was necessary. The elimination of endogenous GS function alone was insufficient to counteract the GS expressed by the transfected plasmid, which was under the control of a wild-type SV40 promoter.²⁷ Consistent with this analysis, cell lines producing monoclonal antibodies generated by random integration often maintain tens to hundreds of copies of the integrated plasmid containing the GS cassette (data not shown). With many copies of GS integrated stably into the genome under control of a wild-type SV40 promoter, GSKO cell lines producing monoclonal antibodies may not demonstrate an improvement in titer over the wt cell lines from which they were derived. To test this, we generated pools stably expressing a standard mAb in 6 internally derived CHO GSWt clonal hosts and a single GSKO progeny from each. We found that under selection at 50 μ M MSX, GSWt pools and GSKO pools completed selection on the same day or within 7 days of each other (Figure 7a – solid black line: GSWt pools; solid red line: GSKO pools). For these pools, titers from the GSKO-derived pools were often very similar to the GSWt parental they were derived from (Figure 7b). Due to the high number of copies of GS stably integrated into the genome in this random integration platform, we hypothesized that attenuation of the exogenously expressed GS may also improve the titer of the knockouts compared to the wild-type cells in our cell lines. Fan et al. (2013) demonstrated an ~61% decrease in expression of GS when using an attenuated version of the SV40 promoter compared to the wt SV40 promoter. The same six GSWt parental cells and GSKO progeny were co-transfected with plasmids encoding for the same light chain and heavy chain as used previously, but with an attenuated SV40 promoter (attSV40) driving GS expression. AttSV40 pools were selected at 10 μ M MSX compared to 50 μ M MSX for wtSV40 pools to account for the decreased expression of GS. We found an increase in both the stringency of selection for GSKO pools at 10 μ M MSX and time to completion of selection using this system (Figure 7a – dotted black line: GSWt pools; dotted red line: GSKO pools). Pools stably expressing a standard mAb and an attenuated GS demonstrated between 0 and 14.5-fold increase in titer in GSKO cell lines compared to the GSWt parental from which they were derived (Figure 7c). While titers for GSKO pools expressing attSV40 promoters often increased, one host saw no increase in expression indicating the impact of attenuating exogenously expressed GS on titer may be host specific.

4 | DISCUSSION

4.1 | Generation and validation of GSKO CHO cell lines

Recent advances in genome engineering technologies have provided researchers with different methodologies to efficiently generate genetic knockouts and knock-ins with minimal off-target effects. In

this study, we employed the novel Cas9-based system, Cas-CLOVER, to rapidly generate over 100 potential GSKO CHO cell lines in approximately 6 weeks. To facilitate high-throughput screening and validation of these engineered cell lines, we implemented parallel analytical approaches in 96-well plate formats, including low-resolution NGS and fragment analysis by capillary electrophoresis (FA-CE) to identify and verify indels at the GS locus for 98 clones. Functional validation through glutamine dependency screening confirmed successful GS knockout clones, with bi-allelic edits verified in 46 of 48 randomly selected clones by high-resolution NGS.

4.2 | Indel analysis and off-target effects

The sizes of indels generated by Cas-CLOVER included larger deletions than typically seen in CRISPR-Cas9 gene editing, however indel size patterns vary across cell types and experimental systems. Madison et al. (2022) characterized indel patterns across three sites in resting pan-T cells, observing 1–40 bp deletions with peaks at 2–6 bp and 12–14 bp.²⁴ In contrast, studies in banana yielded larger deletions (17–90 bp).³⁹ Unlike Madison's results, CHO cells at the GS locus showed fewer small deletions (<6 bp) but frequent 12 bp deletions—suggesting both cell-type specificity and potential conservation of certain deletion sizes across systems.

DSBs induced by Cas-CLOVER may generate larger 5' or 3' overhangs than Cas9's predominantly blunt-ended breaks,³⁴ resembling other dimerizing nucleases such as ZFN's FokI. FokI cleaves asymmetrically—9 bp from its recognition site on the sense strand and 13 bp on the antisense strand—creating 4 bp overhangs.⁴⁰ Similarly, FokI-dCas9 hybrids cut centrally between gRNA binding sites, with spacer requirements of ~15 bp or 25 bp.²⁵ These extended overhangs likely favor non-canonical NHEJ pathways, consistent with >90% of deletions in the GS gene generated by Cas-CLOVER exceeding 4 nucleotides.⁴¹ These larger deletions may provide distinct advantages for generating functional knockouts. While Cas9's 1–2 bp deletions typically require homozygous frameshift mutations, Cas-CLOVER's larger asymmetric deletions can disrupt gene function in heterozygous clones by preventing homologous recombination between alleles. This permits selection of non-frameshift deletions that remove functionally essential amino acids while maintaining a knockout phenotype for enzymes. NGS analysis revealed that approximately 50% of Cas-CLOVER-mediated indels at the GS locus manifested as homozygous deletions; however, the frequency of true homozygous deletions requires further validation. Of particular interest, heterozygous wild-type clones identified through amplicon-based NGS and FA-CE exhibited glutamine auxotrophy, suggesting potential modifications at unexplored GS gene loci, haploinsufficiency or unintended off-target effects. While amplicon-based NGS and FA-CE effectively identify small indels within amplicons, they exhibit limitations in detecting certain genetic modifications, including large insertions/deletions, chromosomal rearrangements, translocations, and inversions.^{34,42–44} Inversions or translocations within the GS locus could produce the intended auxotrophic phenotype while appearing

wild-type in small amplicons, potentially explaining glutamine auxotrophy in clones with seemingly intact alleles. These structural variants would escape detection by amplicon-based sequencing methods. Although findings by Madison et al. (2022) demonstrated significantly reduced frequencies of structural variants in T-cells when utilizing Cas-CLOVER compared to Cas9 and TALENs, additional research is warranted to elucidate the impact of off-target effects and to quantify the prevalence of structural variants both proximal to the target site and throughout the genome. While genome-wide off-target analysis was outside this study's scope, it would be a valuable future direction to determine the prevalence of off-targets in CHO cells using Cas-CLOVER.

4.3 | Variability in productivity among CHO cell lines and future directions

The observed variability in productivity among different CHO-K1 host cell lines following GS knockout underscores the complex interplay between genetic background and protein expression capabilities. While all 30 parental CHO-K1 lines shared common ancestry, their divergent serum-free adaptation strategies (data not shown) and potential genetic drift may have contributed to the heterogeneous responses to GS deletion. This variability was demonstrated by enhancements in productivity (ranging from 0-fold to 14.5-fold increases in titer) depending on the host cell line used.

These findings highlight the importance of comprehensive host cell line screening when establishing production platforms, as the genetic context significantly influences the resulting phenotype.³ The differences in productivity between similar CHO-K1 variants suggest that additional, uncharacterized genetic or epigenetic factors may play crucial roles in determining a cell line's capacity for recombinant protein expression.^{37,45} Future research should explore the relationship between cell line ploidy, growth characteristics, and productivity, while optimizing vector components including promoters, enhancers, signal peptides, and topologies. Multi-omics analysis of high-performing GSKO clones, combined with advanced tools like Cas-CLOVER for host genome engineering, could reveal key molecular factors for designing more efficient CHO expression systems.

AUTHOR CONTRIBUTIONS

T. McLamarrah, V. Cairns and C. DeMaria conceived and planned the study. T. McLamarrah, E. Aral, M. Hoffman, Kate Selvitelli, J. Vitko, T. King, J. Tedstone, L. Stephen, M.J. Sebastião, J.M. Escandell and M.M. Dias executed the experiments. T. McLamarrah and J. Scarcelli contributed to the interpretation of the results. T. McLamarrah, V. Cairns and J. Scarcelli wrote and revised the manuscript. The authors acknowledge the use of Concierge to edit portions of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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