Application of gene editing technologies to improve biotherapeutic manufacturing

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1 Abstract

Gene editing tools such as the CRISPR-Cas9 system have gained traction over the last few years in the bioproduction space. They have been used to genetically enhance capabilities in industrially relevant host cells, including Chinese Hamster Ovary (CHO), and Human Embryonic Kidney (HEK) cells, amongst others. However, delivering gene editing projects in a successful and reliable manner remains a challenge due to the lack of well-defined pipelines and workflows.

We will present our well-defined workflows and established tools to isolate, identify, and characterize edited knock-out (KO) or knock-in (KI) clones in many different cell types. We will show a multiplex gene editing approach that was developed using CRISPR-Cas9 for R&D purposes, where four different loci were targeted simultaneously with high efficiency. Due to the risk of generating aberrant chromosomal rearrangements, Targeted Locus Amplification (TLA) was used to further validate, at the genetic level, the specificity of the edit.

Furthermore, we have integrated into our portfolio a highly precise gene editing CRISPR-based alternative technology. This technology enables us to successfully KO target gene(s) of interest with high efficiency (regardless of the gene copy number) and to generate KI edits of DNA fragments up to 13 kb (with and without the use of homology arms, HA), demonstrating the capability of the platform in integrating potentially complex gene structures.

2 Method

Revvity has access to a variety of gene editing technologies for the enhancement of expression platforms that can be used in research and manufacturing of biotherapeutics, such as:

Research cell line

CRISPR-Cas9:

- **Simple** reagent design; easy to implement
- **Fast** and **precise** editing performance
- Extremely **efficient** compared to other gene editing platforms

Commercial cell line

Recombinant adeno-associated virus (rAAV):

- Design of edits is **straightforward**
- **Does not integrate** in the host genome
- **Reliable** method for gene editing
- **Commercial rights granted** to edited cells
- **Less efficient** compared to CRISPR-based platforms

CRISPR-based alternative platform:

- Successfully **validated** in CHO cells
- **Less off-target edits** compared to CRISPR-Cas9
- **High editing efficiency** and comparable timeline to CRISPR-Cas9
- **Ready-to-market**: Commercial rights granted to edited cells

3 Case study 1: High efficiency multiplexed gene editing for bioproduction cells

I- Introduction

To improve the phenotype of bioproduction cell lines, it may be necessary to KO multiple genes. Our current workflow for the generation of a single gene KO, using CRISPR, enables us to develop edited cells in three to four months (**Fig. 1**). Therefore, we wanted to take advantage of our expertise and tools to target multiple loci, without impacting this timeline. As proof of concept, CRISPR-Cas9 was used to simultaneously KO four genes within the CHO genome. The four genes selected encode for impurities found in the supernatant of the CHOSOURCE™ GS KO cell line.

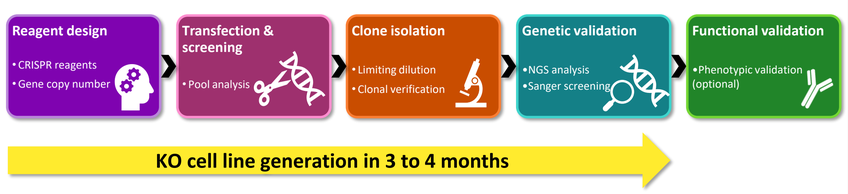

Fig. 1: Gene editing workflow using CRISPR platform.

II- Results

The first step of our process was to determine the target genes copy number in the CHOSOURCE™ GS KO cell line using droplet digital (dd)PCR. The analysis showed that each gene was found to have two copies in the CHOSOURCE™ GS KO cell line.

Next, the transfection protocol was optimised to allow the simultaneous targeting of the four genes and the editing efficiency on each locus was assessed at the pool stage, using an in-house algorithm (**Fig. 2**).

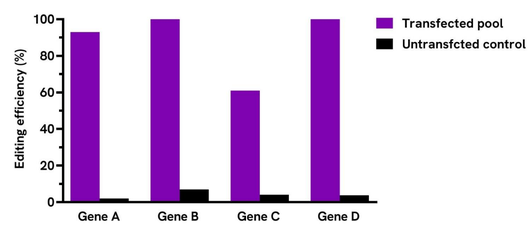

Fig. 2: Editing efficiency (%) obtained in four target loci in the transfected pool (purple). For each gene, a non-transfected control sample (black) was used for background analysis.

The transfected pool was subjected to limiting cell dilution to isolate and identify KO clones. A total of 298 clones were screened using an amplicon-based next-generation sequencing (NGS) approach, to identify KO clones carrying out-of-frame indels on both alleles of the four target genes. Seventeen putative KO clones were identified, and their genotype was further confirmed by analysing Sanger sequencing traces using an in-house TIDE-like algorithm. The data obtained for one KO clone is shown in **Table 1**.

Target gene	Copy number	Amplicon-based NGS sequencing		Sanger sequencing	
		Detected indels (bp)	%	Detected indels (bp)	%
Gene A	2	+1	97.7	+1	91.9
Gene B	2	NA	NA	-2	47.5
				-5	44.2
Gene C	2	-2	58.7	-2	48.8
		+1	40.4	+1	45.9
Gene D	2	+2	98.6	+2	94.0

Table 1: Data from amplicon-based NGS and Sanger sequencing in one KO clone.

With the double-strand breaks created following simultaneous gene targeting, there is a risk of unwanted chromosomal rearrangement. Thus, Targeted Locus Amplification (TLA) was performed to further validate the edited clones (**Table 2**).

Targeted loci	Breakpoint reads	Indels (bp)	Breakpoints in locus (%)	WT reads (%)	Amplicon-NGS detection	Sanger sequencing detection
Gene A	2	-457	25.0	×	×	×
		+1	75.0	×	✓	✓
Gene B	2	-5	48.0	×	N/A	✓
		-2	52.0	×	N/A	✓
Gene C	2	-2	48.0	×	✓	✓
		+1	52.0	×	✓	✓
Gene D	2	+2	60.0	×	✓	✓
		-300	40.0	×	×	×

×; No detection, ✓; Detection

Table 2: Events detected by TLA in each of the targeted loci, in one KO clone.

Data acquired from TLA confirmed the genetic validation obtained by amplicon-based NGS and, Sanger sequencing analysis using an internal TIDE-like algorithm. In addition, TLA-NGS analysis identified a large deletion which could not be detected by previous methods. Most importantly, TLA confirmed absence of complex chromosomal rearrangements between the simultaneously targeted loci, which further validates the robustness of the multiplexed gene editing approach used.

Finally, mass spectrometry analysis was performed for functional validation in the parental line and two edited clones. The target genes were shown to be inactivated in the KO clones (**Table 3**).

Target gene	Protein in parental cell line (%)	Protein in KO cell line (%)	
		Clone 1	Clone 2
Gene A	0.21	0	0
Gene B	0.46	0	0
Gene C	0.30	0	0
Gene D	0.2	0	0

Table 3: Mass spectrometry results indicating the percentage of protein detected in parental and edited cells for the four genes targeted.

4 Case study 2: CRISPR-based alternative technology for CHO cell genome editing

I- Evaluation of CRISPR-based alternative technology for KO

Genes with copy number ranging from one to three (**Fig. 3A**) were selected to assess performance of the CRISPR-based alternative technology. High editing efficiency was achieved with at least one design tested for each of the genes selected (**Fig. 3B**). Performance comparison between CRISPR-Cas9 and CRISPR-based alternative technology showed both technologies perform similarly, when studying four individual genes (all with copy number of 2) (**Fig. 3C**).

A

Target gene	Copy number
Gene E	1
Gene F	2
Gene G	2
Gene H	3

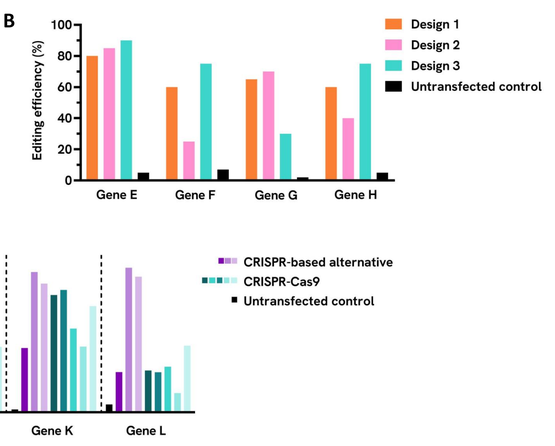

Fig. 3: Evaluation of a CRISPR-based alternative technology for the generation of KO CHO cells. A: Selection of genes with different copy number. B: Editing efficiency (%) obtained for each target gene, using different designs, in the transfected pools. C: Editing efficiency (%) obtained for each target gene, using various designs, in transfected pools using either CRISPR-based alternative or CRISPR-Cas9 reagents.

II- Evaluation of CRISPR-based alternative technology for KI

CRISPR-based alternative technology was tested with CHOSOURCE™ GS KO cell line to KI DNA fragments of varying sizes (from 1.5 kb to 13 kb) using either Homology Directed Repair (HDR) (**Fig. 4A**) or Non-Homologous End Joining (NHEJ) (**Fig. 4C**). For each method, several clones were successfully isolated and shown to have on-target integration (**Fig. 4B and 4D**), indicating CRISPR-based alternative technology as a tool for successfully generating KI clones.

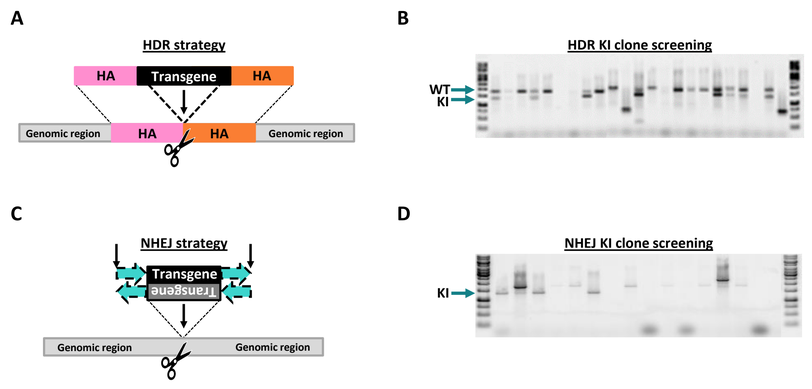

Fig. 4: CRISPR-based alternative technology for KI of DNA of various sizes. A: HDR strategy used for KI of 1.5 kb insert. B: Clone screening PCR showing presence of KI band. C: NHEJ strategy used for large DNA cassette (13 kb) KI, using CRISPR-based alternative technology. D: Clone screening PCR showing presence of KI band.

5 Conclusion

- **Gene editing can be used to generate bioproduction cell lines with improved phenotype(s).**
- **Revvity's gene editing expertise and tools were successfully used, and a robust pipeline developed, for multiplexed gene editing where four genes were simultaneously targeted using CRISPR-Cas9. Genetic validation of the editing for the different target genes was performed using a variety of methods, including confirmation of the KO for the target gene at protein level.**
- **The CRISPR-based alternative technology exhibits comparable performance to CRISPR-Cas9 for generating KO and KI. This technology has the benefit of achieving high editing efficiency with comparable timelines to CRISPR-Cas9.**