EditCo

XDel technology enables sustained protein depletion and simplified genotyping compared with conventional sgRNA designs

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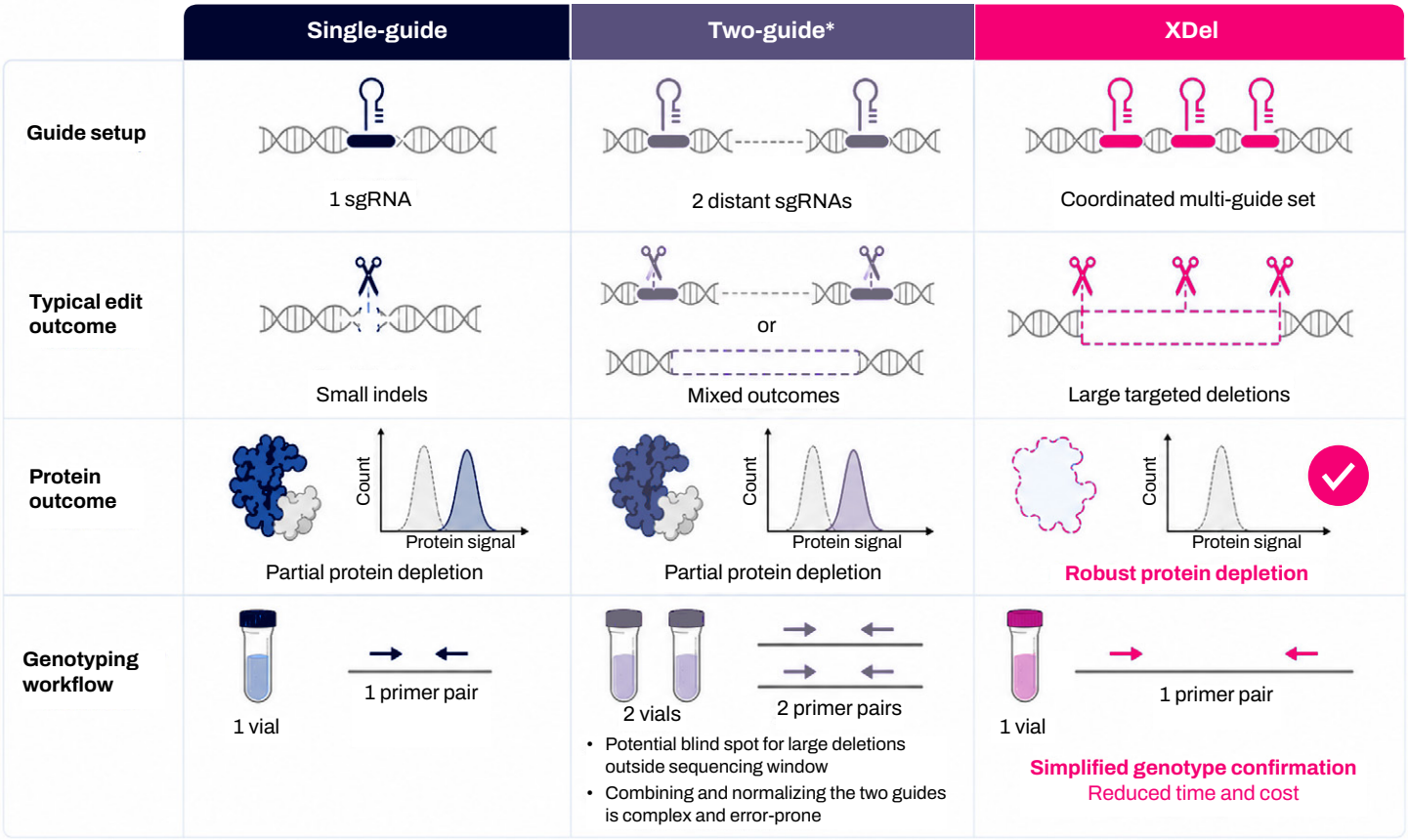
Key takeaways:

- XDel induced and maintained robust protein depletion across four membrane targets in human immortalized cells, with depletion sustained for 3 weeks post-transfection.
- NGS genotyping revealed stable indel profiles across multiple passages, with high protein depletion maintained despite the presence of in-frame deletions.
- Compared with single-guide and two-guide approaches, XDel led to greater protein depletion and larger fragment-deletion profiles while reducing setup complexity through its single-vial delivery.
- XDel also simplifies genotype confirmation by using a coordinated guide set in proximity, enabling NGS or Sanger genotyping with a single primer pair. In contrast, two-guide approaches with distant target sites require multiple primer pairs and sequencing reactions.
- Taken together, XDel is a high-quality, easy-to-use solution for reliable, reproducible CRISPR experiments.



XDel enables sustained protein depletion with a simplified genotyping workflow. High-level comparison of the advantages of the XDel approach versus conventional single-guide and two-guide CRISPR knockouts. *Note: Adding a third guide would increase workflow complexity further by introducing another vial that must be combined and normalized and would require a third primer pair and sequencing reaction.

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Introduction

Many commercial CRISPR-based editing systems use one to two guide RNAs to disrupt protein expression by generating insertions and deletions (indels) that shift the protein reading frame, often resulting in a non-functional protein through a frameshift mutation (**Figure 1**). However, the imprecise nature of DNA repair mechanisms can sometimes generate deletions whose lengths are multiples of three nucleotides, resulting in in-frame deletions. Such edits remove amino acids without disrupting the reading frame and may leave the protein partially or fully functional (**Figure 1**). When gene editing fails to completely eliminate target protein expression, confidence in the interpretation of downstream phenotypes diminishes.

To provide researchers with more efficient and reliable knockouts, EditCo's XDel technology is designed to generate larger deletions that result in high, sustained protein depletion. Edits generated using XDel's three-guide strategy demonstrated larger deletions associated with robust protein depletion, even when producing in-frame deletions as compared to those produced by standard single- and two-guide approaches.

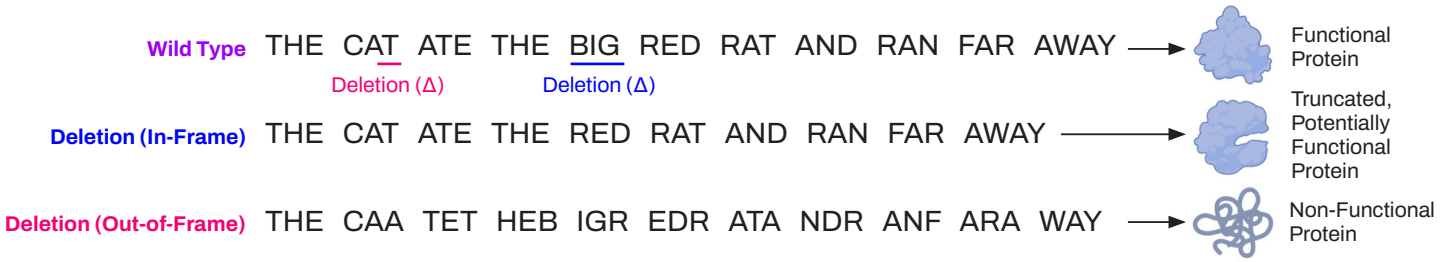


Figure 1. CRISPR deletion outcomes can preserve or disrupt the reading frame of the coding sequence. Ideally, CRISPR/Cas9 is used to generate out-of-frame deletions that disrupt the downstream gene sequence, resulting in loss of protein function. However, DNA repair following editing is imprecise and can generate in-frame deletions that preserve the reading frame, potentially producing a shortened protein with partial or residual function.

XDel technology overview

EditCo's unique XDel approach strategically uses up to three guide RNAs positioned close together in an early gene exon to work cooperatively to create large, targeted fragment deletions (**Figure 2**).

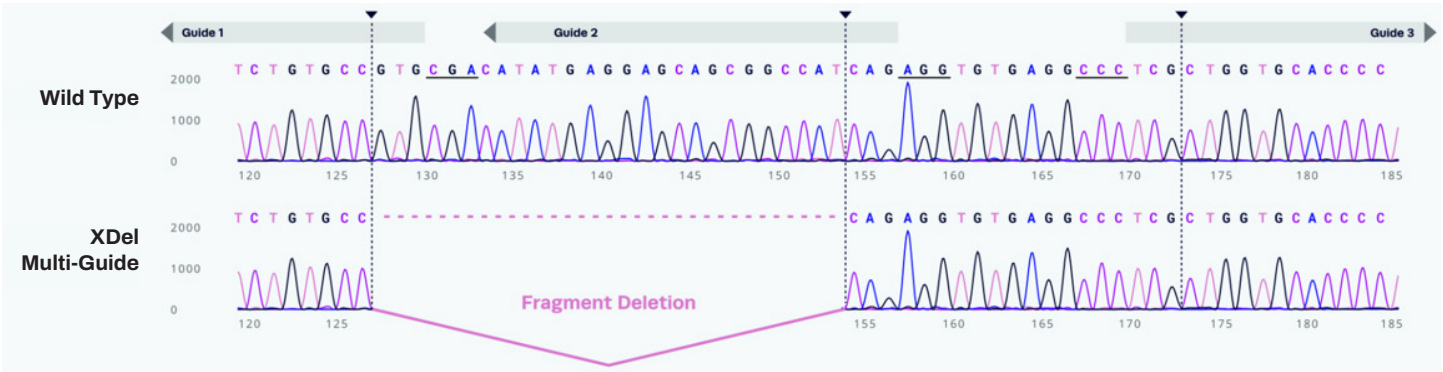
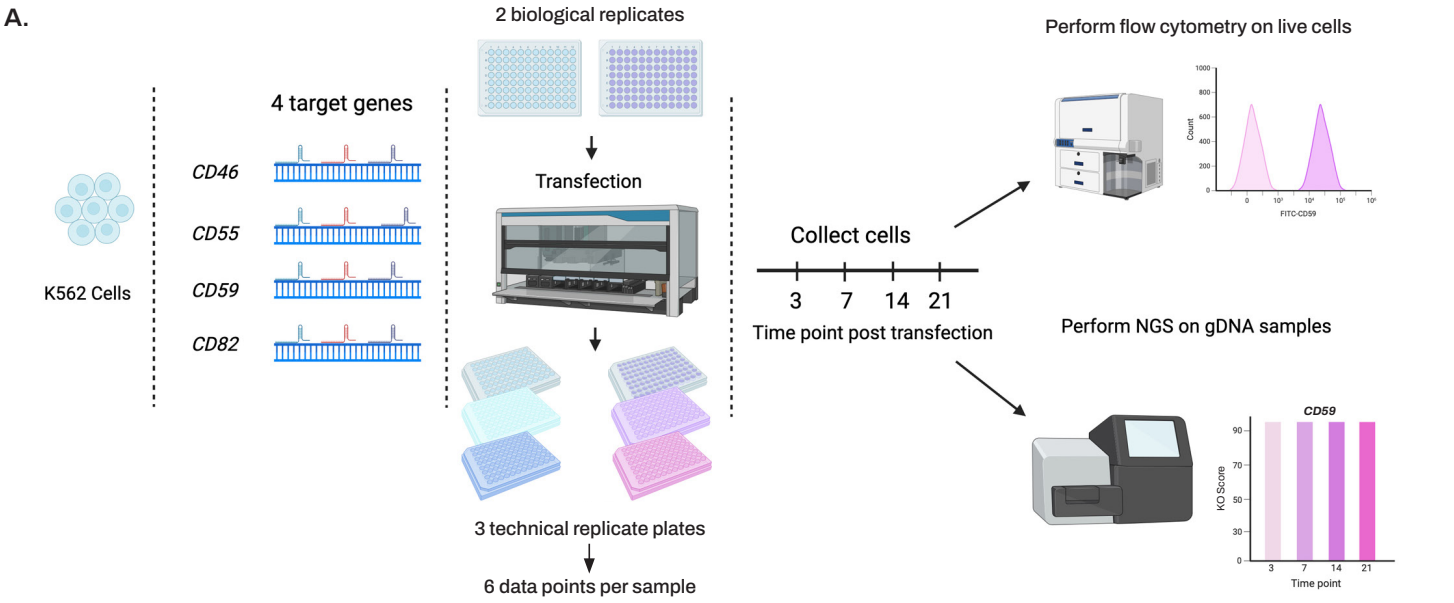


Figure 2. XDel's unique editing strategy generates large deletions, resulting in high editing efficiency and sustained protein depletion. XDel uses up to three modified sgRNAs (light gray) targeting a single early exon in the gene of interest. When co-transfected, these sgRNAs create concurrent double-stranded breaks (vertical dotted lines) at the targeted genomic locus and consequently induce one or more 21+ bp fragment deletions.

To demonstrate the efficiency and stability of the XDel editing technology, four unique surface membrane proteins were knocked out in human immortalized K562 cells using XDel designs, and flow cytometry was used to measure protein expression levels. Furthermore, next-generation sequencing (NGS) using EditCo's Eos NGS Library Preparation workcell and internal and proprietary NGS analysis tool was used to quantify editing efficiency (**Figure 3A**).

Flow cytometry revealed a high level of protein depletion (>90%) that persisted for up to 3 weeks post-transfection in the edited K562 cells across all four gene targets (**Figure 3B, C**). Cell viability remained high across all samples at the tested time points. Additionally, the biological and technical replicates showed highly consistent results, demonstrating the reproducibility of the XDel knockout strategy.



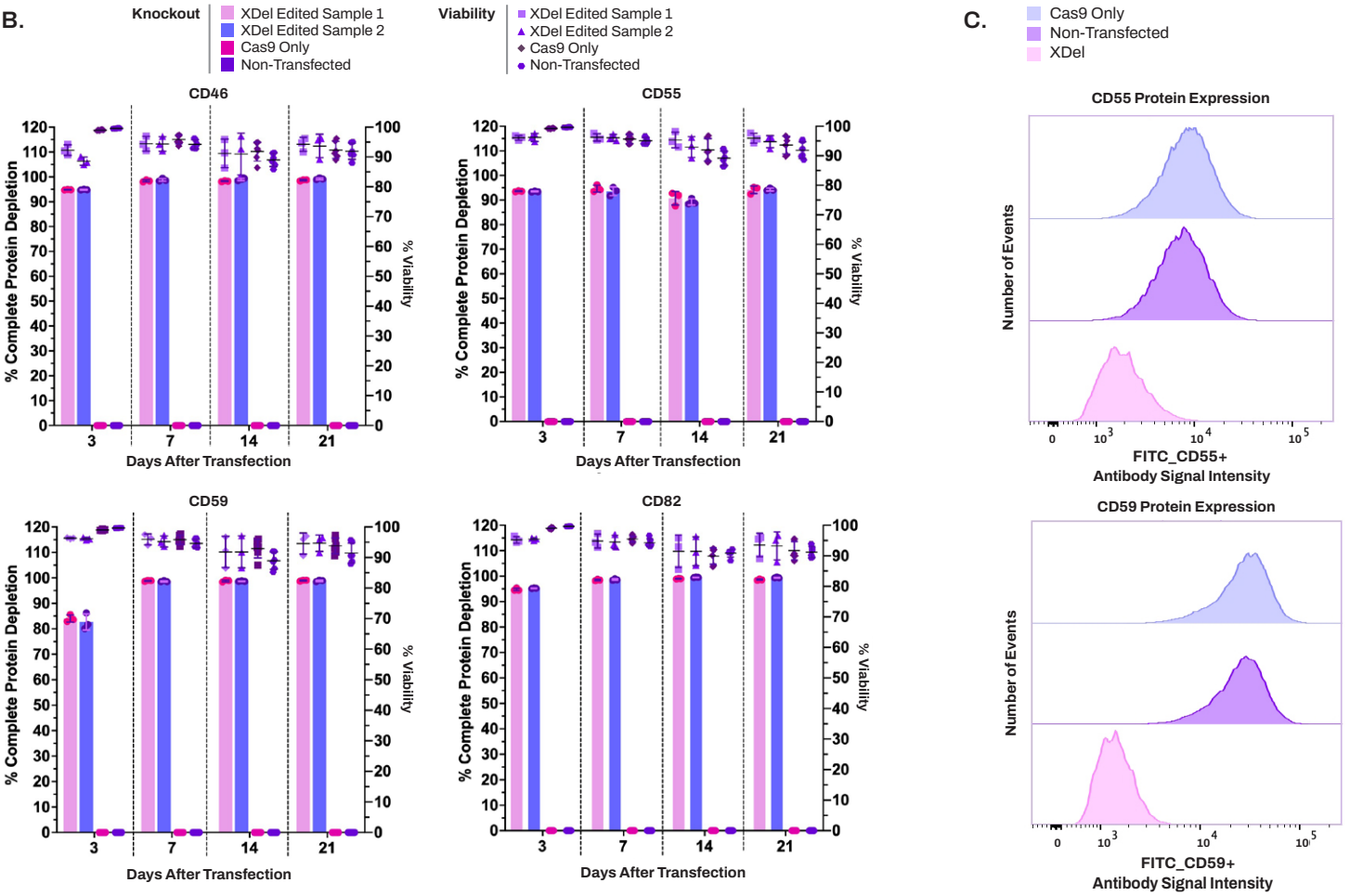


Figure 3. Time-course analysis through flow cytometry demonstrates a high level of membrane protein knockout that remains consistent over time. (A) Four membrane proteins were targeted for knock-out in K562 cells using XDel technology, following EditCo Bio's proprietary workflow. Transfection was performed in duplicate (n = 2), and each biological replicate was subsequently split into 3 technical replicates post-transfection (n = 6). Live cells were collected for flow cytometry analysis on days 3, 7, 14, and 21 post-electroporation. Antibodies against CD46, CD55, CD59, and CD82 were used to detect the expression of these surface membrane proteins on K562 cells at 4 specified time points on edited, Cas9-only, and non-transfected samples. Cell viability was measured using viability dye DRAQ7. *Figure created with BioRender.* (B) Bar plots showing high protein depletion and viability across two biological replicates for each of the four chosen gene targets. Tight error bars indicate high reproducibility of the XDel approach. (C) Representative flow cytometry histograms of CD55 and CD59 protein expression.

To determine the genotyping profile, NGS was performed on the *CD55*- and *CD59*-edited samples. For both targets, the observed indel lengths were ≥54 base pairs (bp) (**Figure 4A**), consistent with the large fragment deletions expected from XDel's multi-guide knockout strategy. The proportion of individual indels observed within the total population remained stable across multiple cell passages. Interestingly, despite the high proportion of in-frame deletions, which potentially could produce truncated proteins (**Figure 4B**), a complete loss of detectable protein expression was observed by flow cytometry and persisted over time (**Figure 3C**), suggesting that large in-frame fragment deletions through the XDel multi-guide knockout strategy are sufficient to cause sustained protein depletion.

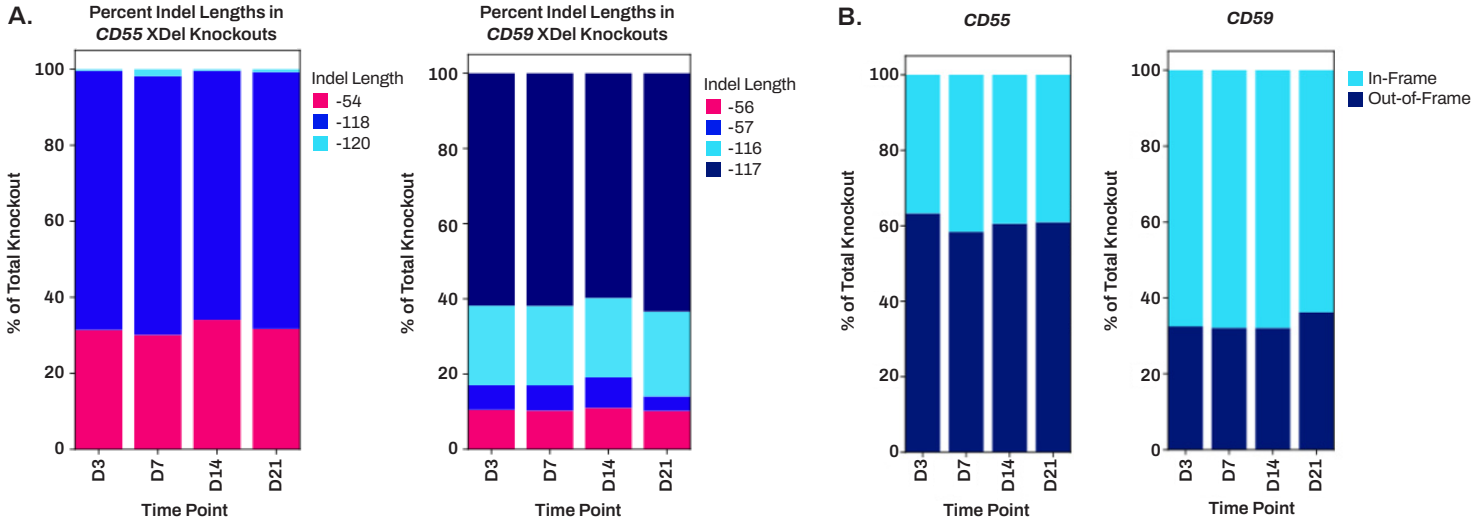


Figure 4. NGS genotyping revealed stable indel profiles across time points, with XDel achieving robust protein depletion despite a high proportion of in-frame deletions. First, cell pellets were collected at four post-transfection time points. Next, NGS libraries were prepared using EditCo's fully integrated automated platform. Sequencing was performed on an Illumina MiSeq, and analysis was performed with EditCo's proprietary software. (A) Indel length distributions were quantified and plotted as a percentage of the total indel population. (B) Indels were classified as in-frame or out-of-frame, with each category plotted as a percentage of the total indel population.

XDel multi-guide technology achieves a high level of editing efficiency, leading to robust protein depletion compared to Vendor X's single-guide RNAs

A common approach for CRISPR knockout generation is to use a single-guide RNA to introduce indels at a target locus. However, the single-guide editing strategy often produces smaller indels, which may result in only partial protein depletion. To evaluate whether XDel technology improves functional knockout performance, the editing efficiency of *CD46*-targeted XDel guides and individual Vendor X sgRNAs was compared. Flow cytometry was used to measure the targeted protein expression, and NGS was used for genotyping. XDel-treated cells showed high editing efficiency and a loss of detectable CD46 protein. In contrast, both Vendor X single-guide conditions resulted in low editing efficiency and only partial loss of CD46 expression (**Figure 5A**).

A closer look at the NGS genotyping data revealed that the XDel knockout generated fragment deletions ≥36 bp (**Figure 5B, C**), while Vendor X's single guides both resulted in indels that were ≤18 bp. Notably, although XDel generated a higher proportion of in-frame deletions than the single guides, a complete loss of protein expression was still achieved (**Figure 5A**).

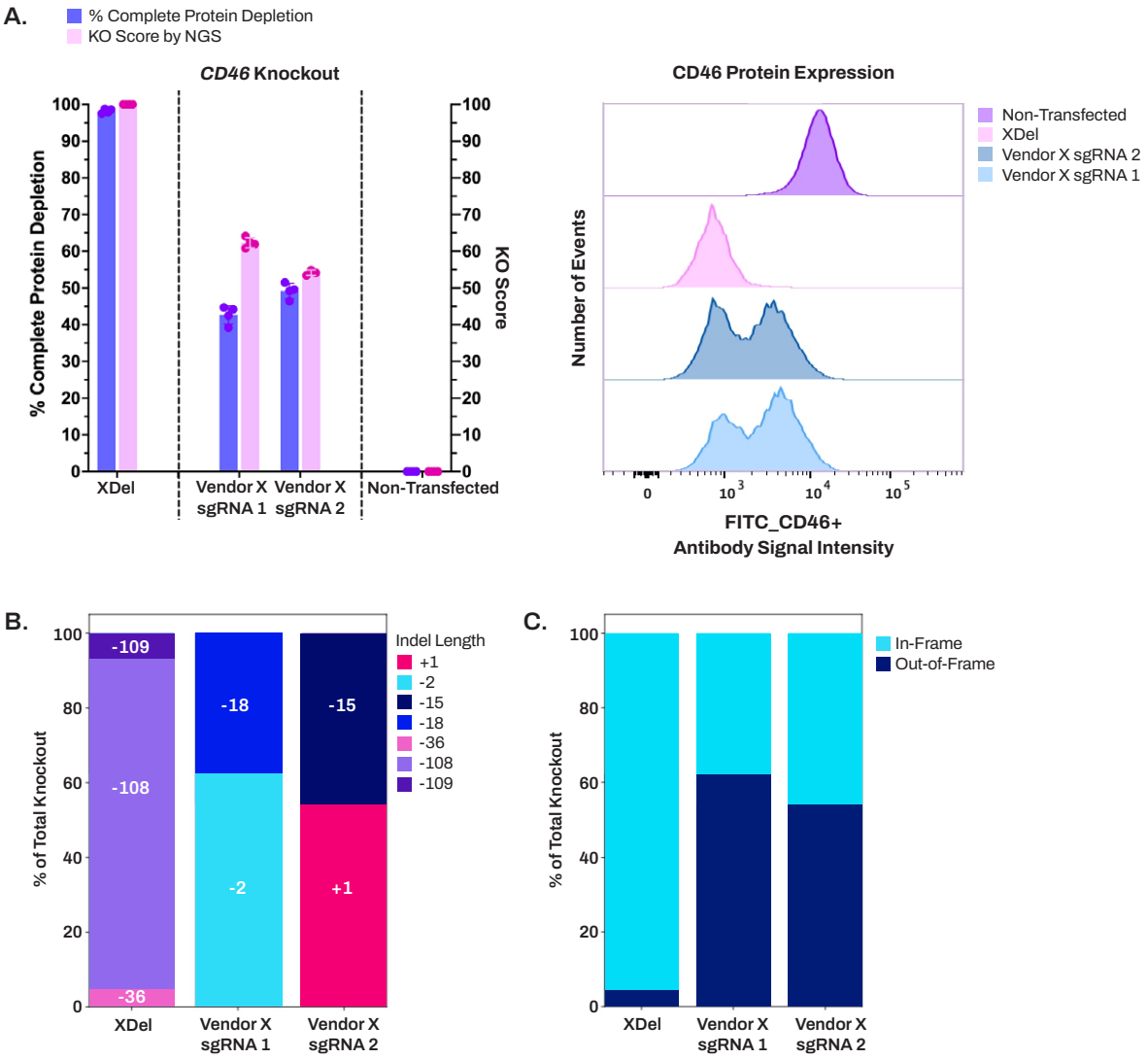


Figure 5. XDel achieves robust protein depletion compared with a single-guide approach. Editing efficiency comparison between XDel technology and single guides from another vendor (Vendor X) targeting *CD46*. Transfection was performed in duplicate (n = 2). Each biological replicate was split into 2 technical replicates (n = 4). Live cells were collected for flow cytometry analysis on day 7 post-electroporation. An antibody against CD46 was used to detect CD46 membrane protein expression on K562 cells in edited and non-transfected samples. Cell viability was measured using viability dye DRAQ7. Cell pellets were collected on day 7 post-transfection for NGS sequencing. Library preparation was performed using EditCo's fully integrated and automated platform, and the sequencing was performed on an Illumina MiSeq. (A, left) Protein depletion was assessed via flow cytometry, and editing efficiency (KO score) was determined by NGS genotyping using EditCo's proprietary software. (A, right) Representative flow cytometry histograms for each knockout condition show that only partial loss of CD46 resulted from Vendor X's sgRNA. (B) Indel length distributions were quantified and plotted as a percentage of the total indel population. (C) Indels were classified as in-frame or out-of-frame, with each category plotted as a percentage of the total indel population.

XDel technology outperforms Vendor X's two-guide combination and streamlines genotyping confirmation

When the single-guide strategy produces inadequate knockouts, a two-guide approach can be attempted. Thus, XDel technology was compared with Vendor X's two-guide combination. Although Vendor X's two-guide combo achieved up to 70% loss of protein expression, XDel showed a significantly higher level of protein depletion (95%; unpaired t-test, *p < 0.05) (**Figure 6A**). Flow cytometry histograms revealed a complete loss of CD46 expression generated by XDel, while Vendor X's two-guide combo resulted in only a partial loss of CD46.

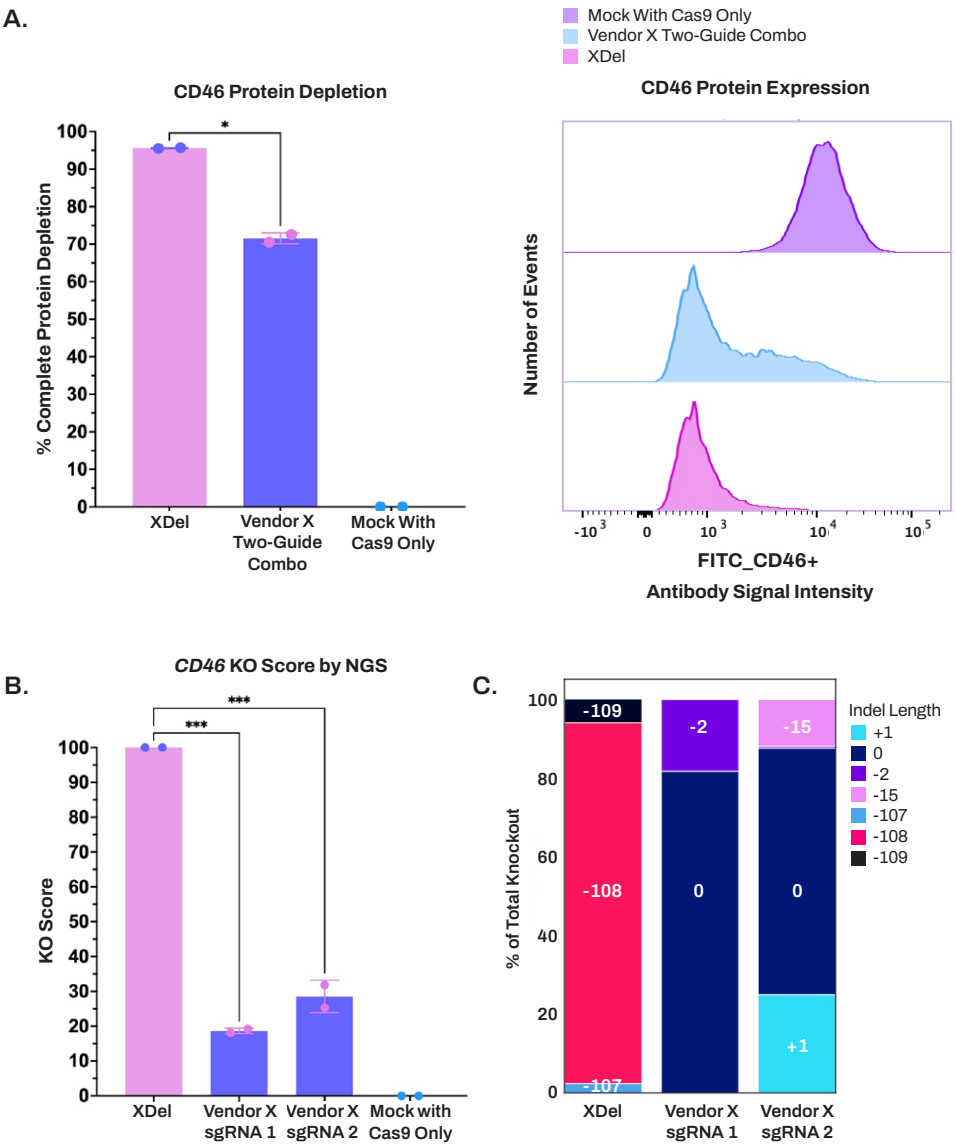


Figure 6. XDel also outperforms the two-guide approach, resulting in a more robust protein depletion. Transfection was performed in duplicate (n = 2). Live cells were collected for flow cytometry analysis on day 7 post-electroporation. An antibody against CD46 was used to detect protein expression on K562 cells in edited and non-transfected samples. Cell viability was measured using viability dye DRAQ7 (data not shown). Cell pellets were collected on day 7 post-transfection for NGS sequencing. Library preparation and NGS sequencing were performed as previously described. One primer pair was used to sequence the XDel replicates, whereas the Vendor X two-guide combo replicates required two primer pairs due to the long distance between the two guides. (A, left) Protein depletion was assessed via flow cytometry. Statistical significance determined by unpaired t-test (*p < 0.05). (A, right) Representative flow cytometry histograms. (B) NGS genotyping also demonstrates that XDel achieved a significantly higher KO score. Statistical significance determined by one-way ANOVA (***p < 0.001). (C) Indel length distributions were quantified and plotted as a percentage of the total indel population.

The NGS genotyping showed significantly higher knockout (KO) scores (one-way ANOVA, ***p < 0.001) (**Figure 6B**) and larger indel fragments (≥107 bp) (**Figure 6C**) for the XDel-edited sample as compared to the Vendor X two-guide combo. It is worth noting that performing NGS genotyping for the Vendor X two-guide combo setup adds complexity and cost to the genotyping workflow, as the significant distance between the two guides along the genomic sequence requires two primer pairs and separate sequencing reactions to target each sgRNA-edited site (**Figure 7**). This potentially creates the discrepancy observed between the KO score and protein depletion in Vendor X's two-guide combo due to the occurrence of fragment deletions outside the NGS sequencing window of the two primer pairs.

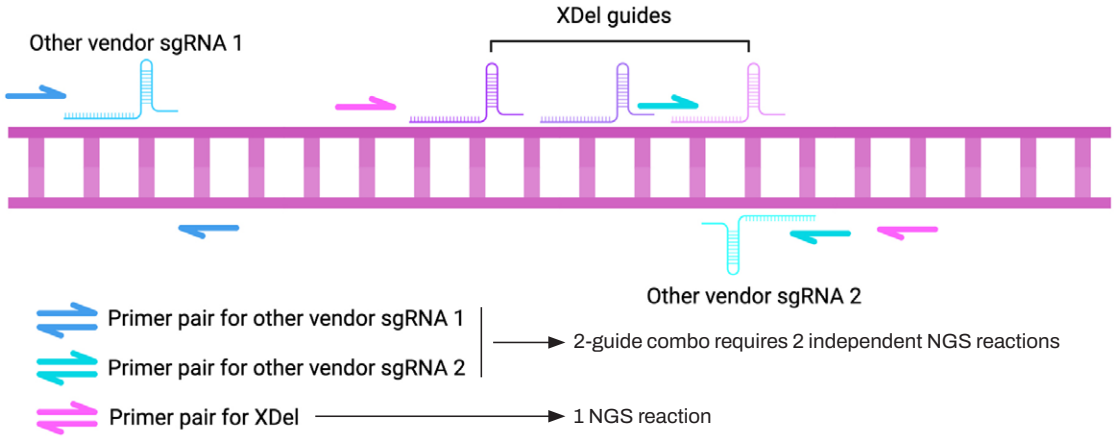


Figure 7. XDel's multiple guides are strategically positioned to enable simpler genotyping. Since XDel's three guides are in proximity, genotyping can be performed using a single primer pair. In contrast, separate primer pairs are required when using the traditional multi-guide strategy. *Figure created with BioRender.*

XDel technology reduces the time, cost, and complexity of knockout workflows

The complexity of any experimental workflow is an important consideration for discovery teams operating under tight deadlines and resource constraints. XDel provides workflow advantages beyond robust protein depletion by reducing the complexity of both transfection setup and genotyping confirmation.

Two- and three-guide workflows using separately supplied sgRNAs require resuspending, normalizing, and combining guides from multiple vials when performing transfection. This introduces additional handling steps that could potentially lead to a setup error. In contrast, the XDel strategy eliminates this error-prone workflow since the three guides are delivered as a coordinated set in a single vial. Moreover, this unique design simplifies the genotyping process, as only a single primer pair is used for either Sanger or NGS sequencing to determine the genotyping profile, given the proximity of the three XDel guides. (**Figures 2 and 7**). In contrast, using separate guides positioned far apart requires multiple primer pairs and separate sequencing reactions. As a result, performing genotyping confirmation becomes more complex and requires additional time and cost.

Another advantage of XDel technology is the reduced risk of missing larger deletion events that fall outside a sequencing window, a potential limitation when distant guide sites are analyzed independently. In addition, XDel's multi-guide architecture reduces the dependence on any single guide-binding site, providing built-in redundancy if a sequence variation, such as a single-nucleotide polymorphism, affects guide affinity at one of the target sites.

Table 1. Workflow advantages of EditCo's XDel approach

Workflow feature	XDel	Vendor X two- and three-guide designs
Guide format	One XDel guide set	Separate sgRNAs
Transfection setup	One guide vial	Multiple guide vials
Guide normalization	Simplified	Requires resuspension and normalization for each guide
Primer pairs	One primer pair	Multiple primer pairs
Sequencing reactions	One reaction	Multiple reactions
Genotype interpretation	Compact target region	Separate analysis of distant guide sites with potential blind spots

Conclusion

In this CRISPR knockout study, EditCo Bio's XDel technology generated large-fragment deletions associated with a sustained loss of detectable protein expression in several targets, as measured by flow cytometry. Compared with Vendor X single guides or a two-guide combination, XDel produced larger fragment deletions, resulting in complete loss of protein expression. Furthermore, compared with Vendor X's two-guide design, XDel reduced genotyping complexity by requiring only a single NGS primer pair and a single sequencing reaction. Together, these results demonstrate that XDel provides a streamlined and effective CRISPR knockout strategy for applications such as antibody validation that require a clear linkage between genotype and protein-level loss of function.