

Optimized CRISPR/Cas9 Gene Knockout: XDel technology maximizes on-target editing and minimizes off-target effects

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Key takeaways:

- EditCo Bio has developed a robust Cas9-mediated ex vivo gene editing method using unique XDel gRNA design technology that delivers guaranteed high editing efficiency without pre-screening guide RNA activity.
- The consistent base pair distance spanned by XDel multiple gRNAs enables the use of targeted next-generation amplicon sequencing as a standard quality control method for genotyping Cas9-edited single-cell clones and cell pools.
- High-throughput automation allowed editing of 768 samples from multiple immortalized and primary cell lines followed by NGS analysis of 4,816 high-quality libraries to compare the on- and off-target editing efficiency of XDel's multiple guide RNA editing strategy versus single-guide RNA editing.
- NGS results demonstrate that EditCo Bio's XDel CRISPR editing strategy consistently achieves high on-target editing efficiency for gene knockouts compared to using a single-guide RNA while simultaneously minimizing editing efficiency at known off-target sites.
- This simultaneous high on-target editing with low off-target effects is consistent across various cell types, including immortalized cells, iPSCs, and primary cell types, enabling robust and reliable editing for a wide range of applications.

Introduction

Predicting the editing efficiency of a single-guide RNA (sgRNA) is a complex challenge. Designing CRISPR knockout experiments is not straightforward due to the difficulty in predicting Cas9-editing outcomes since they are dependent on the cellular mechanisms employed for DNA double-strand break repair and vary widely by cell cycle and cell type. Due to this variability, achieving high on-target efficiency with a single sgRNA often requires pre-screening multiple guides in the target cell line, which adds significant upstream work. Because of this challenge, various guide RNA design tools are currently available to predict which sgRNAs may have higher knockout efficiency. Beyond on-target predictions, these design tools can also help minimize off-target effects by analyzing sequence similarity. However, *in silico* tools for predicting high knock-out efficiency are inherently limited by the cell models used to train their algorithms. To overcome the existing limitations of guide RNA (gRNA) design, EditCo Bio has developed an advanced editing strategy that ensures high on-target efficiency and gene knock-out while minimizing off-target effects. This approach eliminates the need for individual sgRNA screening and does not require increasing the amount of editing reagents – the latter being a tactic often used to increase on-target editing efficiency but includes the risk of significantly higher likelihood for unintended off-target edits. Furthermore, EditCo's novel strategy is effective across a wide range of cell types, including immortalized cells, iPSCs, and primary cell lines.

XDel CRISPR Design Strategy

EditCo's unique XDel approach uses a guide RNA design scheme that strategically places up to three guide RNAs in an early gene exon to work cooperatively to create targeted fragment deletions and increase editing efficiency (**Figure 1**). Using multiple sgRNAs enhances editing efficiency while eliminating the need to screen numerous single guides in the target cell line, which is typically required for individual sgRNAs in order to account for unpredictable double-strand break repair and resulting indels that may or may not affect gene expression.

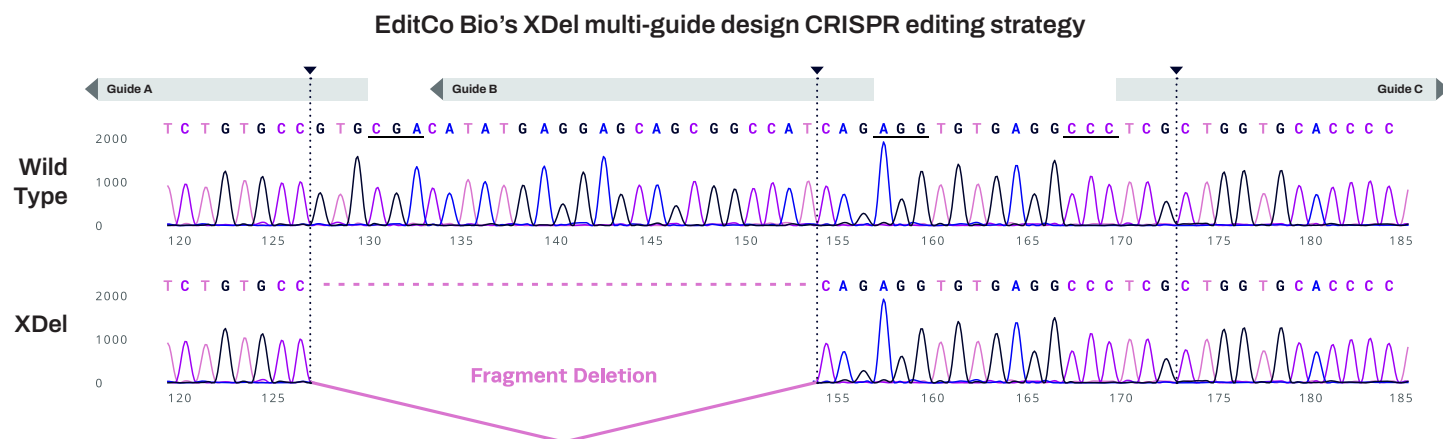


Figure 1: XDel guide RNA design promotes large fragment deletions by positioning up to three guide RNAs within an early gene exon. EditCo's XDel gRNA designs include up to 3 modified sgRNAs (grey bars) that target a single gene of interest. When co-transfected, the sgRNAs create concurrent double-stranded breaks (vertical dotted lines) at the targeted genomic locus inducing one or more 21+ bp fragment deletions.

The XDel editing strategy increases the occurrence of larger fragment deletions within edited cells, regardless of cell type and targeted site, as observed using [EditCo's proprietary Next Generation Sequencing \(NGS\) analysis](#) of 1,249 total edited samples using XDel designs targeting 14 different genes in isolated clones from 15 different cell lines, including immortalized and primary iPSCs (**Figure 2**). Because the fragment deletions generated by the XDel editing strategy are larger compared to using a single guide RNA, there is a higher likelihood of gene disruption. Apart from high on-target editing efficiency, the XDel-designed guide RNAs are also ranked and subsequently selected for sequences predicted to have minimal off-target binding.

XDel technology results in robust and repeatable deletions across genes and cell lines

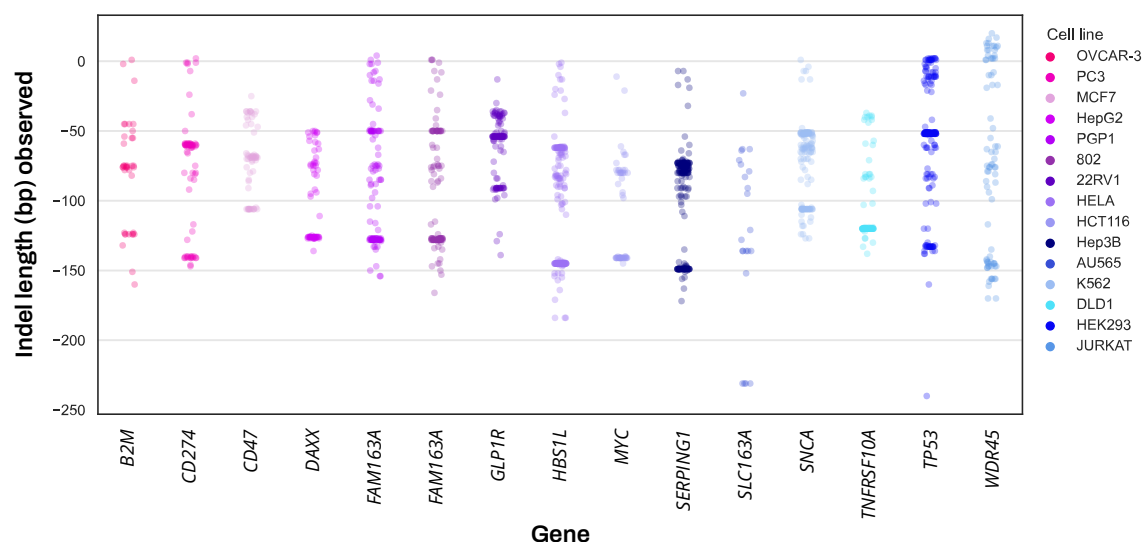


Figure 2: XDel's large fragment deletions span a distance that enables efficient genotyping through targeted next-generation amplicon sequencing. Dot plot of indel lengths (bp), including large fragment deletions, observed (y-axis) for 14 genes (x-axis) targeted by XDel guides across 1,249 total clonal samples isolated from 15 different cell lines (colors).

Identification of guide sequences and off-target loci

To increase the likelihood of generating detectable levels of off-target editing, target genes were intentionally selected with XDel designs containing individual sgRNA sequences that had been identified through internal and external data as having a high incidence of off-target editing. For this study, seven target genes (**Figure 3**) with XDel designs were selected because one or more of their individual sgRNAs previously demonstrated elevated/significant high off-target editing (data not shown).

In order to identify all off-target loci per gene in an unbiased manner and to assess the varying levels of off-target editing per locus, these guides were part of an internal GUIDE-seq¹ experiment performed in the HEK293 cell line. Cells were edited using the XDel multiple guide design versus each of the equivalent three single-guides from each XDel design. The off-target editing sites determined by GUIDE-seq were then further verified by performing Sanger sequencing followed by ICE analysis² (data not shown). In this investigation, we focused on the three off-target editing sites with the highest observed levels of editing activity for each single guide and each gene (**Figure 3**), as identified through GUIDE-seq data.

Off-target editing evaluation strategy for 3 off-target sites per single-guide compared to the XDel multi-guides at 7 target genes

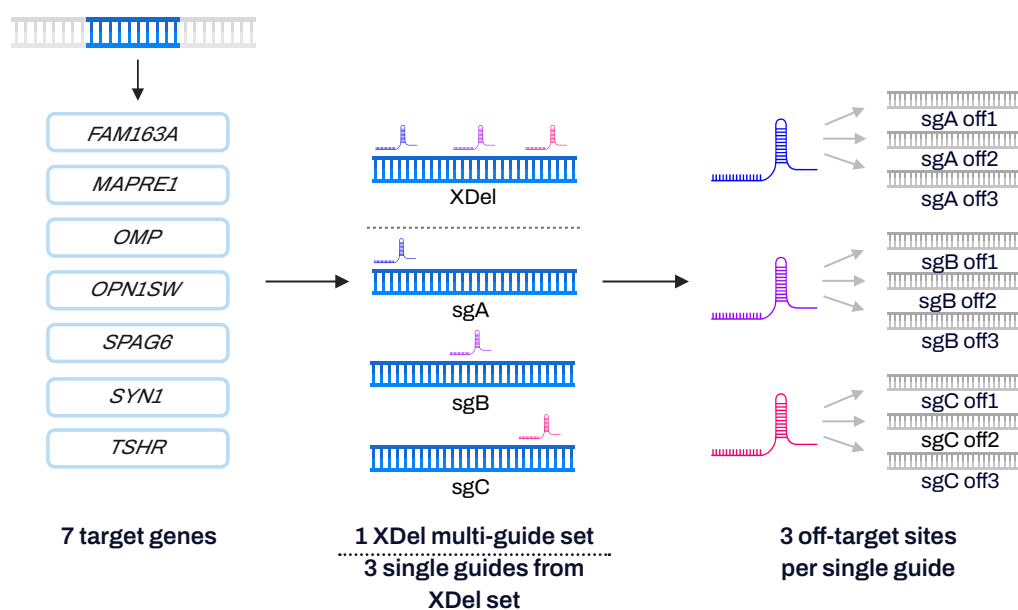


Figure 3: Experimental design for the comparison of XDel gRNA design vs individual single-guide on-target and off-target activity.

For a high-throughput on- and off-target editing and NGS analysis experiment, 7 target genes were selected for their guide RNA designs with known unusually high off-target editing. Editing of 6 different cell types was performed using both the XDel multiple guide sets and the equivalent single-guides from each XDel set. Amplicon sequencing was used to assess on-target and off-target editing efficiency. For each single-guide, off-target editing was assessed by sequencing 3 loci empirically determined to have the highest levels of off-target editing by a previous GUIDE-seq experiment. (Figure created by Biorender)

High-throughput editing using XDel versus single-guide

To assess XDel's on- and off-target editing efficacy in comparison to single-guide editing within the seven selected target genes, multiple cell lines and conditions were chosen for high-throughput electroporation, followed by fully automated library preparation for targeted next-generation sequencing and analysis. Cell cultures were transfected using Cas9 and sgRNA ribonucleoprotein complexes (RNPs) for both the XDel multi-guide set and each of the equivalent single-guides for the seven genes previously analyzed by GUIDE-seq (**Figure 4**) in a high-throughput manner using an automated liquid handler.

The cell lines assessed included two immortalized cell lines, HEK293 (also used for the GUIDE-seq experiment) and THP1, two iPSC lines, PGP1 and 802, and CD3+ T cells that were either resting or activated prior to transfection. In addition, to assess how RNP dosage affects both on- and off-target editing efficiency when using the XDel multi-guide versus single-guide strategy, transfections were performed in HEK293 using three different RNP concentrations (0.25X, 1X and 4X). Cells were transfected with single or XDel multiple guides in 96 well plates on a Hamilton instrument per triplicate. This workflow resulted in a total of **768 transfected cell samples, including both experimental and mock samples (those without RNP)** (**Figure 4**). All samples were harvested at 96 hours and lysed for genomic DNA as input into EditCo's automated NGS sequencing workflow.

High-throughput transfection and editing of 6 cell types by single-guide versus XDel multi-guide

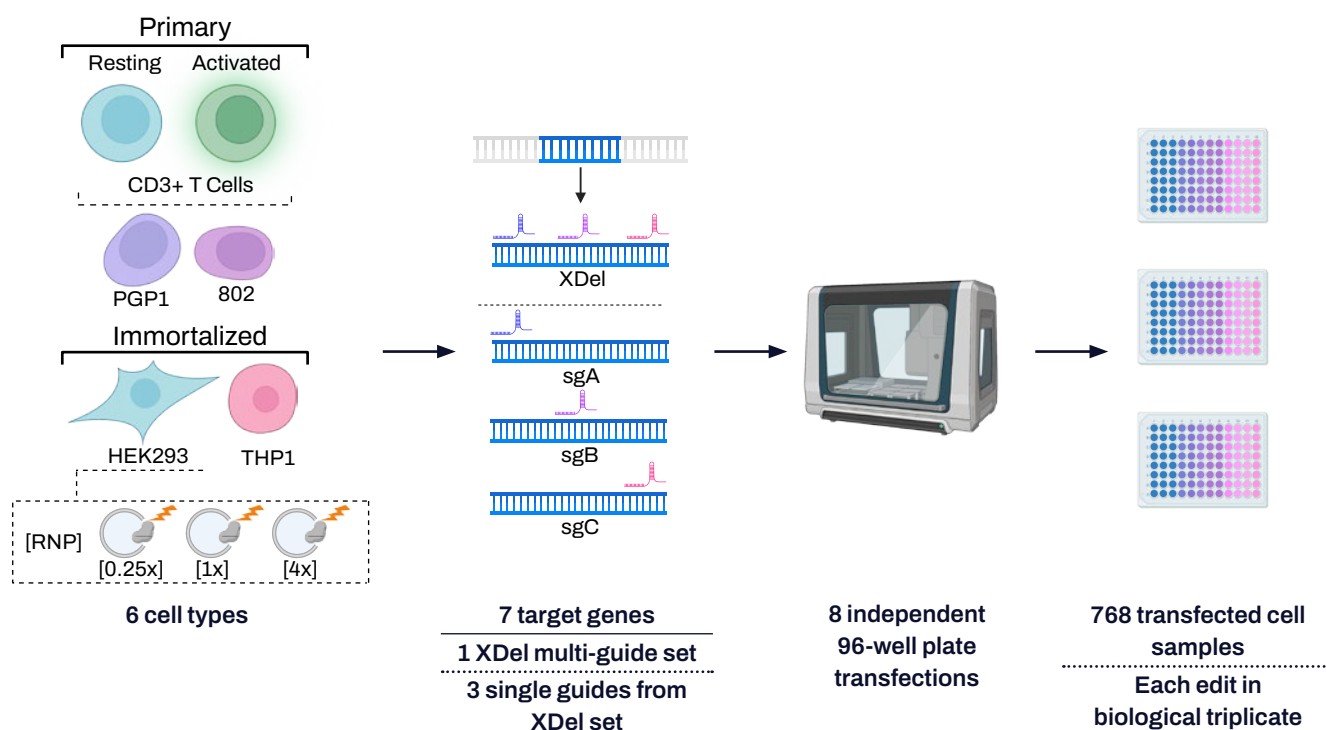


Figure 4: XDel versus single-guide on- and off-target edited sample generation. Genome editing was performed in six different cell lines, including both immortalized and primary types, with an additional RNP titration conducted in the HEK293 cell line. The target genes were edited with the XDel multiple gRNA set or the equivalent single-gRNAs in a high-throughput manner using a Hamilton liquid handler. All transfections were performed in triplicate leading to over 700 individual transfected cell samples. (Figure created by Biorender)

Automated NGS library preparation and sequencing analysis of on- and off-target editing sites

Amplicon sequencing primers were designed for the seven target genes to assess the on-target editing efficiency for XDel-edited samples versus samples edited with the equivalent single-guides per each target gene. Primers were also designed for the three previously identified most highly edited off-target loci for each single-guide as observed by GUIDE-seq. Thus, each of the seven target genes had a total of ten amplicon primer designs to cover the one on-target editing site and nine off-target editing loci. Since multiple amplicons were amplified in each sample, **4,816 individually barcoded NGS libraries were generated** from the 768 transfected cell samples using [EditCo's Eos](#) NGS Library Preparation workcell (**Figure 5**). This resulted in an average PCR success rate of 99.4% across all libraries. The on- and off-target editing efficiencies across all samples were then assessed using [EditCo Bio's internal and proprietary NGS analysis tool](#).

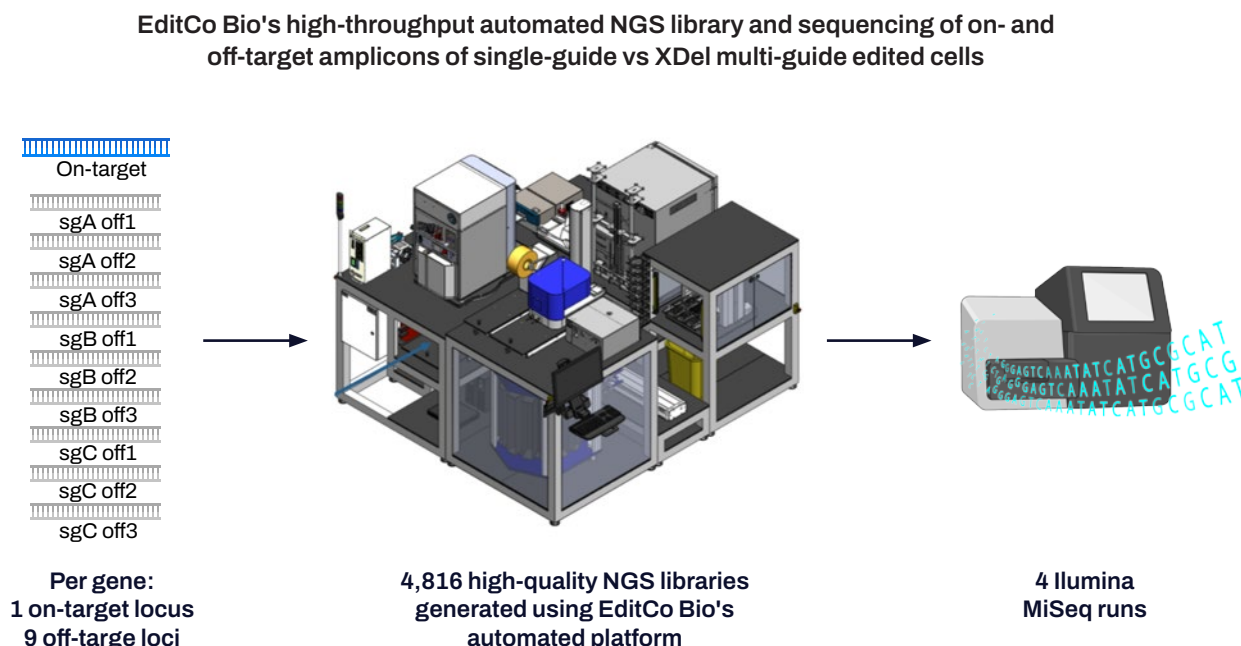


Figure 5: Next-generation amplicon sequencing and analysis workflow to assess on- and off-target edited samples. The genomic DNA of the samples was extracted and used as input in EditCo Bio's automated NGS library preparation workcell. For each target gene, amplicons were generated to analyze on-target and off-target editing at 9 loci per target, which were previously identified as high off-target sites by GUIDE-seq. Over 4,800 NGS amplicon libraries were sequenced on 4 illumina MiSeq runs and assessed with EditCo Bio's editing analysis pipeline. (Figure created by Biorender)

RNP concentration effect on on- and off-target editing efficiency

An additional advantage to XDel gRNA design is that, with this approach, the same total RNP concentration used for a single-guide RNA editing experiment is used for the XDel multiplexed guides, whereby each individual XDel guide has one-third of the concentration as a traditional single-guide. To test whether this decrease in individual guide concentration within the XDel strategy results in lower off-target editing while maintaining a high on-target level as compared to the single-guide approach, a high-throughput editing followed by NGS analysis experiment was designed. In order to determine whether this difference in concentration of the individual guides within the XDel approach affected levels of on- and off-target editing efficiency as compared to the equivalent three single-guides (A, B, and C), HEK293 cells were transfected with the XDel multi-guide vs the equivalent single-guide RNAs at three different RNP concentrations: 0.25X, 1X (the industry standard) and 4X.

It was observed that even at 0.25X levels of RNP, XDel multi-guide had a significantly higher average on-target editing efficiency (independent t test, $p < .001$) as compared to single-guides across the 7 on-target genes tested. In addition, as RNP levels increased, XDel multi-guide average on-target editing efficiency remained highly consistent (89.88%-93.73%). In comparison, single-guide RNAs had a much higher level of variability of the average on-target editing efficiency across the RNP concentrations (52.57%-91.45%). Furthermore, even at 1X RNP concentrations, on-target editing efficiencies of less than 40% were observed for several single-guide RNAs (**Figure 6**).

Average percent on-target editing efficiency by XDel Multi-guide Knockouts (XDel) vs Single-guide Knockouts (SGKO)

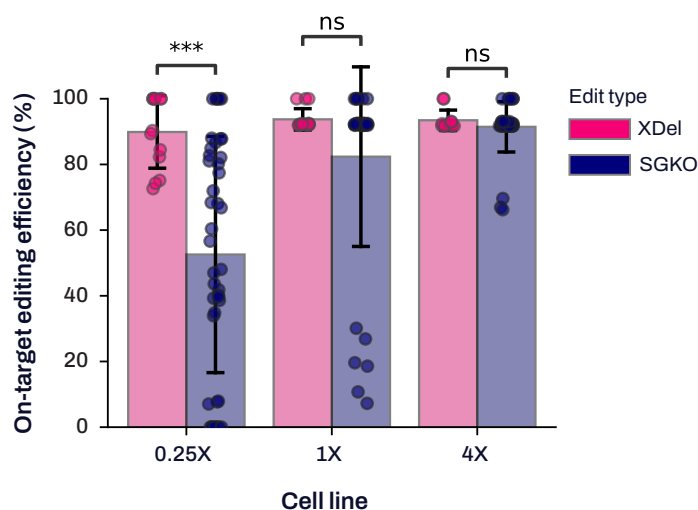
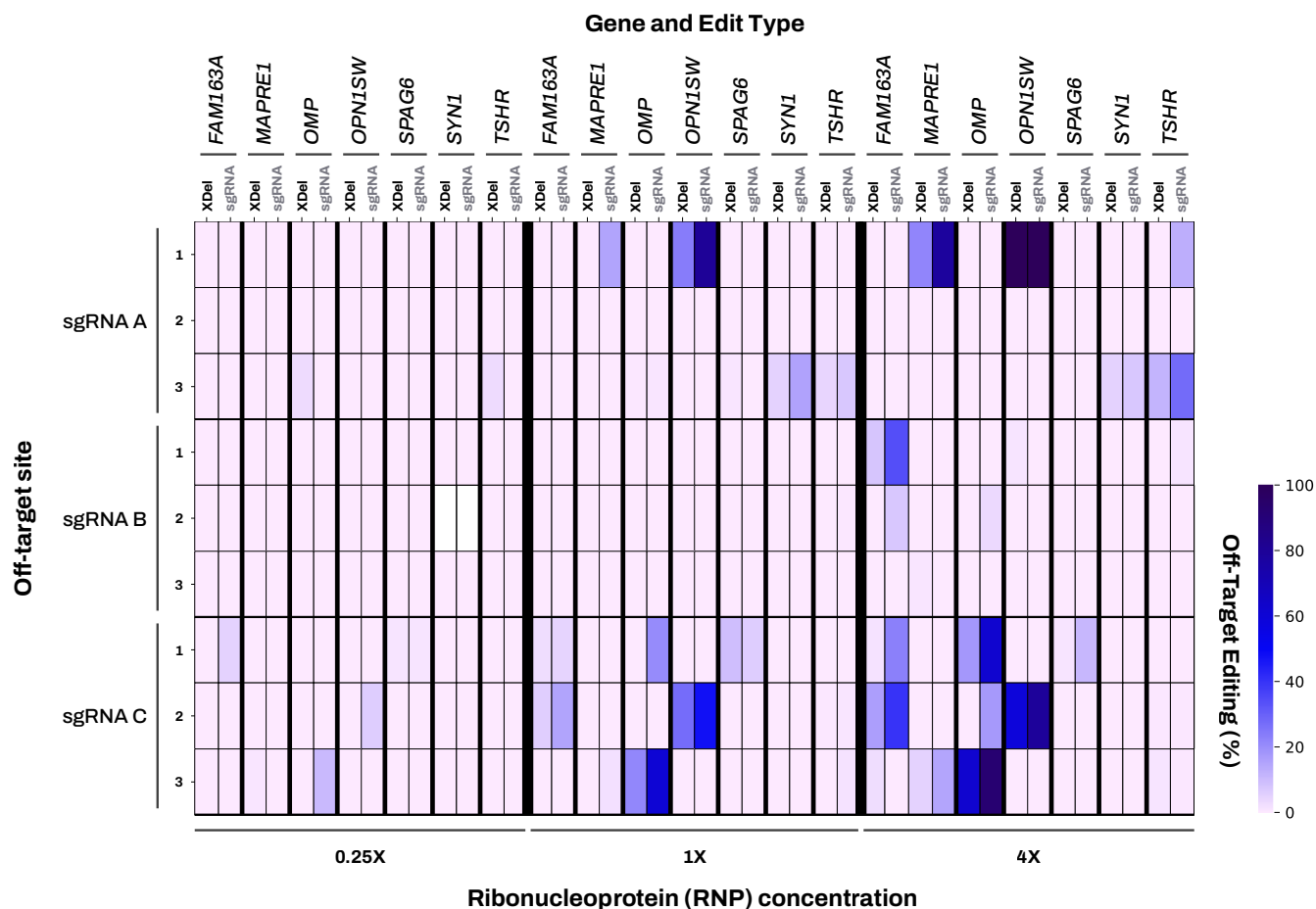


Figure 6: XDel multi-guide RNA editing efficiency remains high at decreased RNP concentration compared to single-guide RNA. Bar plot of average on-target editing efficiency (y-axis) of XDel multi-guide (pink) vs single-guide (dark blue) across 7 genes at increasing concentrations of RNP (0.25X, 1X, 4X) in HEK293 immortalized cells (x-axis). Stars indicate statistical significance, independent t test (***)= $p < 0.001$, ns= not significant).

Next, comparison of average off-target editing levels revealed that both XDel multi-guide and single-guide RNAs exhibited increased off-target effects as RNP concentration increased (**Figure 7A**). However, the off-target editing efficiency increases at a higher rate in single-guide versus the XDel multi-guide approach, as further evidenced by the off-target locus 3 of the *OMP* gene for single-guide RNA C (10.29% at 0.25X RNP, 92.72% at 4X RNP) vs XDel multi-guide (0% at 0.25X RNP, 63.42% at 4X RNP) (**Figure 7B**). Thus, while the XDel guide design method keeps the overall RNP concentration unchanged, each individual guide has a lower relative concentration than single-guide RNAs, thereby reducing off-target editing at specific loci. Furthermore, the three guides in the XDel multi-guide approach work synergistically at the target site, ensuring higher and more consistent on-target editing and gene knockout, even at low RNP concentrations.

7A. Off-target editing remains low by XDel multi-guide vs single-guide at increasing RNP concentrations



7B.

Off-target editing remains lower at off-target locus 3 of the *OMP* target gene by XDel multi-guide vs single-guide RNA at increasing RNP concentrations

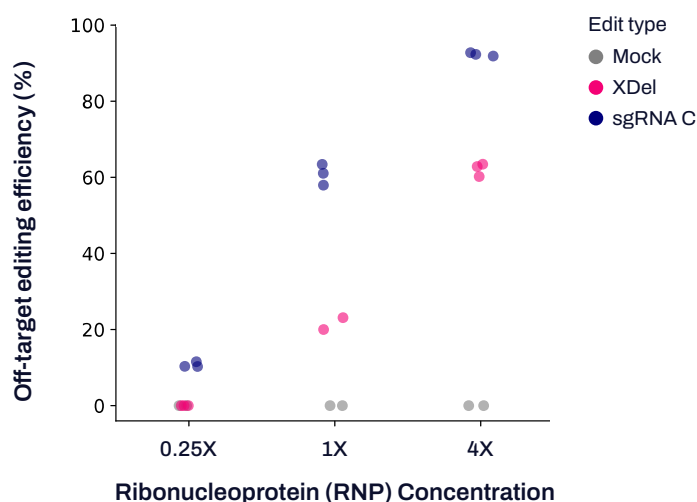


Figure 7: XDel multi-guide RNA editing efficiency remains low at off-target sites at increased RNP concentration compared to single-guide RNA.

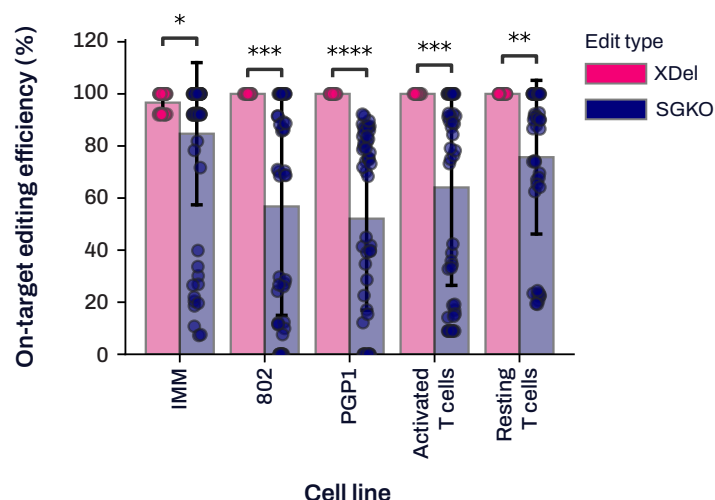
(A) Heatmap of average levels of off-target editing efficiency of XDel multiple sgRNA design vs sgRNA A (first three rows), B (rows four through six), and C (last three rows) for all tested genes across three increasing RNP concentrations (0.25X, 1X, 4X, demarcated by thick vertical black bars) in HEK293 immortalized cells (x-axis) at 3 off-target sites per sgRNA (y-axis). White indicates insufficient data available. (B) Dot plot of off-target editing efficiency (y-axis) at the empirically selected *OMP* gene off-target locus 3 of single-guide RNA C in HEK293 cells transfected with mock (grey) vs XDel multi-guide (pink) and single-guide RNA C (dark blue) at increasing concentrations of RNP (0.25X, 1X, 4X) (x-axis). Data show three biological replicates.

On- and off-target editing performance of XDel vs single-guide designs remains consistent across cell types

To verify that the high on-target and low off-target editing efficiency by XDel multiple guide design versus a single-guide RNA approach is repeatable, editing efficiencies were compared across all 6 cell types including immortalized (IMM) cell lines (THP1 and HEK293), two induced pluripotent stem (iPS) cell lines (802 and PGP1), and CD3+ T cells that were either resting or activated prior to transfection. Indeed, the average on-target editing efficiency of XDel vs single-guide RNA was found to be significantly higher across all cell types tested (independent t test, IMM $p=0.023$, 802 $p<0.001$, PGP1 $p<0.0001$, activated T cells $p<0.001$, resting T cells $p=0.0024$) (**Figure 8A**). Taking the *OPN1SW* gene in THP1 cells as example, as observed previously (**Figure 6**), single-guide RNA A was found to have inconsistent on-target editing efficiency (range = 71.67-100%) as compared to XDel multi-guide and single-guides B and C (all replicates = 100%) (**Figure 8B**). Unlike the XDel multi-guide approach, which requires no optimization, this further emphasizes the importance of conducting additional pre-screening for single-guide RNAs in the cell line of interest.

8A.

Average percent on-target editing efficiency by XDel Multi-guide Knockouts (XDel) vs Single-guide Knockouts (SGKO)



8B.

On-target editing efficiency remains consistently high with XDel guide design at the *OPN1SW* on-target gene in THP1 cells

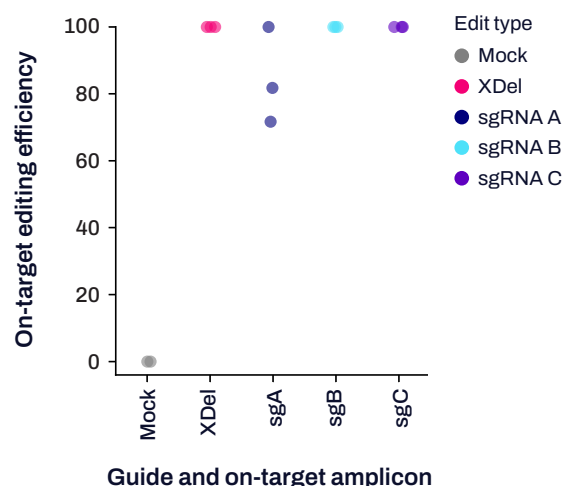
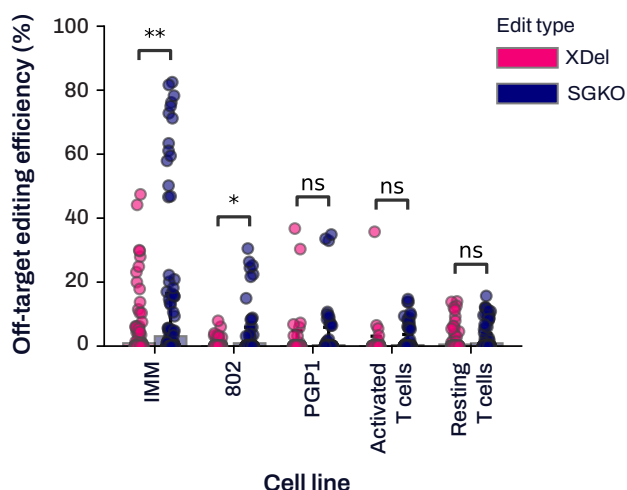


Figure 8: XDel gRNA results in significantly high consistent on-target editing efficiency compared to single-guide RNAs across multiple cell types. (A) Bar chart of average on-target editing efficiency (y-axis) of XDel guide design (pink) vs individual sgRNA (dark blue) across 7 genes in 6 cell types (Immortalized includes HEK293 and THP1 cells, x-axis). Stars indicate statistical significance, independent t test (*= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$). (B) Dot plot of on-target editing efficiency (y-axis) observed at the on-target *OPN1SW* gene locus in THP1 cells transfected with mock (grey) versus XDel design (pink) and single-guide A (dark blue), B (light blue), and C (purple) RNAs (x-axis). Data show three biological replicates.

Furthermore, the average off-target editing efficiency of XDel multi-guide vs single-guide RNA was found to be significantly lower (independent t test) in immortalized cell lines ($p=.0045$) and 802 iPSCs ($p=.0175$) and remained extremely low across all other tested cell lines using both approaches (XDel range = 0.09-1.41%, single-guide range = 0.30-4.58%) (**Figure 9A**) suggesting that off-target levels also depend on cell type. Also consistent with previous observations (**Figure 7B**), when off-target editing is observed, it is higher by the individual single-guide RNA as compared to its XDel multi-guide counterpart, such as at off-target site 1 of single-guide A (XDel = 0%, single-guide A = 59.47-72.79%) and off-target site 2 of single-guide C (XDel = 5.41-47.42%, single-guide C = 74.98-78.27%) of the *OPN1SW* off-target sites in THP1 cells (**Figure 9B**). Thus, the high on-target editing efficiency and low off-target editing efficiency of the XDel multi-guide strategy remains consistent across cell types.

9A.

Average percent off-target editing efficiency by XDel Multi-guide Knockouts (XDel) vs Single-guide Knockouts (SGKO)



9B.

Off-target editing efficiency remains low with XDel Multi-guide design at all off-target sites tested of the *OPN1SW* gene in THP1 cells

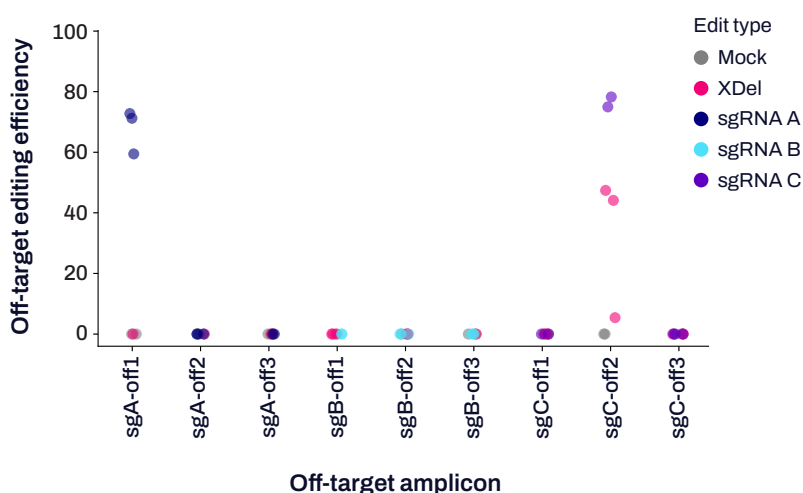


Figure 9: XDel gRNA results in significantly low off-target editing efficiency compared to single-guide RNAs across multiple cell types. (A) Bar chart of average off-target editing efficiency (y-axis) of XDel design (pink) vs individual sgRNA (dark blue) across 63 off-target sites in 6 cell types (Immortalized includes HEK293 and THP1 cells, x-axis). Stars indicate statistical significance, independent t test (*= $p<0.05$, **= $p<0.01$, ns= not significant). (B) Dot plot of off-target editing efficiency (y-axis) observed at 9 off-target *OPN1SW* sites (x-axis) in THP1 cells transfected with mock (grey) vs XDel design (pink) and single-guide A (dark blue, 3 off-target sites), B (light blue, 3 off-target sites), and C (purple, 3 off-target sites) RNAs. Data show three biological replicates.

Conclusion

The XDel guide RNA design technology achieves significantly higher on-target editing than single-guide RNAs, even at reduced RNP levels. This high on-target consistency was observed across immortalized, iPSCs and primary cell types. Thus, EditCo's XDel designs overcome the necessity of pre-screening multiple single-guide RNAs in the target cell line, resulting in rapid, robust, and reliable gene knockout. XDel's improved editing efficiency is driven by its unique design, which enables coordinated fragment deletions for reliable gene disruption. Additionally, by using lower individual sgRNA concentrations, XDel significantly reduces off-target editing compared to single-guide RNAs, regardless of RNP concentration or cell type. Lower off-target editing efficiency by XDel reduces the burden of extensive off-target screening for potential downstream deleterious effects. These findings were confirmed through EditCo's internal automation and NGS analysis pipeline, which leverages high-throughput capabilities to provide a comprehensive assessment of editing performance. In total, 768 edited samples resulted in targeted sequencing of 4,618 high-quality NGS libraries allowing for rapid assessment of on- and off-target editing efficiencies. Overall, EditCo's XDel gRNA design technology provides a more efficient and precise approach to on-target gene editing and knockout with minimal off-target effects, compared to the single-guide RNA strategy.

Citations

1. Tsai, S., Zheng, Z., Nguyen, N. *et al.* GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol* 33, 187–197 (2015). <https://doi.org/10.1038/nbt.3117>
2. David Conant, Tim Hsiao, Nicholas Rossi *et al.* Inference of CRISPR Edits from Sanger Trace Data. *The CRISPR Journal* 2022 5:1, 123-130, <https://doi.org/10.1089/crispr.2021.0113>