Genome editing

Exceptional editing specificity for therapeutics applications with CTS HiFi Cas9 Protein

Highlights

- CTS HiFi Cas9 Protein can reduce over 90% of off-target effects in primary T cells compared to CTS TrueCut Cas9 Protein (wild-type Cas9)
- CTS HiFi Cas9 Protein can maintain equivalent knockout and editing efficiencies compared to CTS TrueCut Cas9 Protein across several tested targets
- Increase in HDR % can be achieved when using CTS HiFi Cas9 Protein with CTS Xenon Genome Editing Buffer
- Consistent high performance was also achieved in stem cells with CTS HiFi Cas9 Protein

The CRISPR-Cas9 genome editing system has seen exponential growth in the last decade and is widely adopted in cell and gene therapy workflows. An ongoing risk associated with its use, particularly for therapeutics applications, as recognized by regulatory bodies like the FDA [1], is around the safety concerns of off-target cleavage events that can lead to loss of critical cell functions, activate oncogenes, or inactivate tumor suppressors. To address these safety risks, a high-fidelity Cas9 protein was developed to generate fewer off-target cleavage events. To facilitate its use for therapeutics applications, Thermo Fisher Scientific now offers the Gibco™ CTS™ HiFi Cas9 Protein—a GMP-grade high-fidelity Cas9 protein with exceptional editing specificity to support its use in cell and gene therapy development programs. The CTS HiFi Cas9 Protein is designed and manufactured in compliance with GMP principles to meet requirements of standards for ancillary materials for cell, gene, and tissue-based products in an FDA-registered manufacturing site. In addition to having extensive traceability documentation, the product is also subjected to extensive safety testing and sterile filling to eliminate potential contaminants that may affect the safety of cell-based therapies. For applications like CAR T cell therapy where high knock-in efficiency is required, the CTS HiFi Cas9 Protein and Gibco™ CTS™ Xenon™ Genome Editing (GE) Buffer Kit are also available.

We assessed the performance of the CTS HiFi Cas9 Protein against the Gibco™ CTS™ TrueCut™ Cas9 Protein and a GMP high-fidelity Cas9 protein from another supplier, in combination with a panel of sgRNAs targeting therapeutically relevant genes (*TRAC*, *CD52*, *TRBC*, and *B2M*), to compare genome-wide on-target and off-target activities. We also demonstrate a CAR T cell therapy workflow at scale with viral and nonviral donor DNA and at different electroporation scales. In each experiment, we demonstrate the exceptional performance of the CTS HiFi Cas9 Protein in all tested therapeutic cell types, including primary T cells and induced pluripotent stem cells (iPSCs).

CTS HiFi Cas9 Protein has exceptional editing specificity and off-target profile

To assess the off-target profile of each Cas9 protein, three gRNAs targeting therapeutic genes (*TRAC*, *CD52*, *TRBC*) were designed using the Invitrogen™ TrueDesign™ Genome Editor software and selected based on the *in silico* predicted level of off-target activity: low off-target activity (TRAC-1), medium off-target activity (CD52-3), and high off-target activity (TRBC-4). A genome-wide, unbiased *in cellulo* method like the Ion Torrent™ next-generation sequencing (NGS) target enriched GUIDE sequencing (TEG-seq) assay [2,3] was first used as a discovery tool for detecting off-target edits. Subsequently, three unique off-target events detected by the TEG-seq assay were selected and their presence confirmed using the targeted amplicon-seq validation (TAV-seq) method (TAV-seq data not shown).

As expected, both high-fidelity Cas9 proteins (CTS HiFi Cas9 Protein and supplier A's GMP high-fidelity Cas9 protein) generated fewer off-target cleavage events at all tested targets in primary T cells, compared to CTS TrueCut Cas9 Protein, a GMP wild-type Cas9 protein (Figure 1). This finding is expected, as wild-type Cas9 protein binds more strongly to the targeted DNA sequence and thus inherently generates more off-target cleavage events compared to its high-fidelity counterpart. When we compared the two high-fidelity Cas9 proteins, further reduction in off-target cleavage events was observed with CTS HiFi Cas9 Protein at all tested targets.

With the low off-target activity gRNA (TRAC-1), CTS HiFi Cas9 Protein was able to eliminate all off-target edits compared to supplier A's GMP high-fidelity (HF) Cas9 (Figure 1, left), down to the limit of sequencing accuracy of approximately 0.1% (equivalent to 1 off-target read for 1,000 on-target reads). With CD52-3 (medium off-target activity), neither of the high-fidelity Cas9 proteins was able to eliminate the off-target edits, with nine off-target events detected for supplier A's GMP HF Cas9 and one detected for CTS HiFi Cas9 Protein (Figure 1, middle). In such cases, further bioinformatics analysis can be done to identify the locations of the off-target edits to help determine the risk level. For example, an off-target edit hitting a protein-coding region could be considered high-risk, especially for therapeutics applications.

Lastly, when using a high off-target activity gRNA (TRBC-4), we observed a significant improvement in the off-target profile with CTS HiFi Cas9 Protein, compared to supplier A's GMP high-fidelity Cas9 and CTS TrueCut Cas9 Protein, but none were able to eliminate all off-target edits (Figure 1, right).

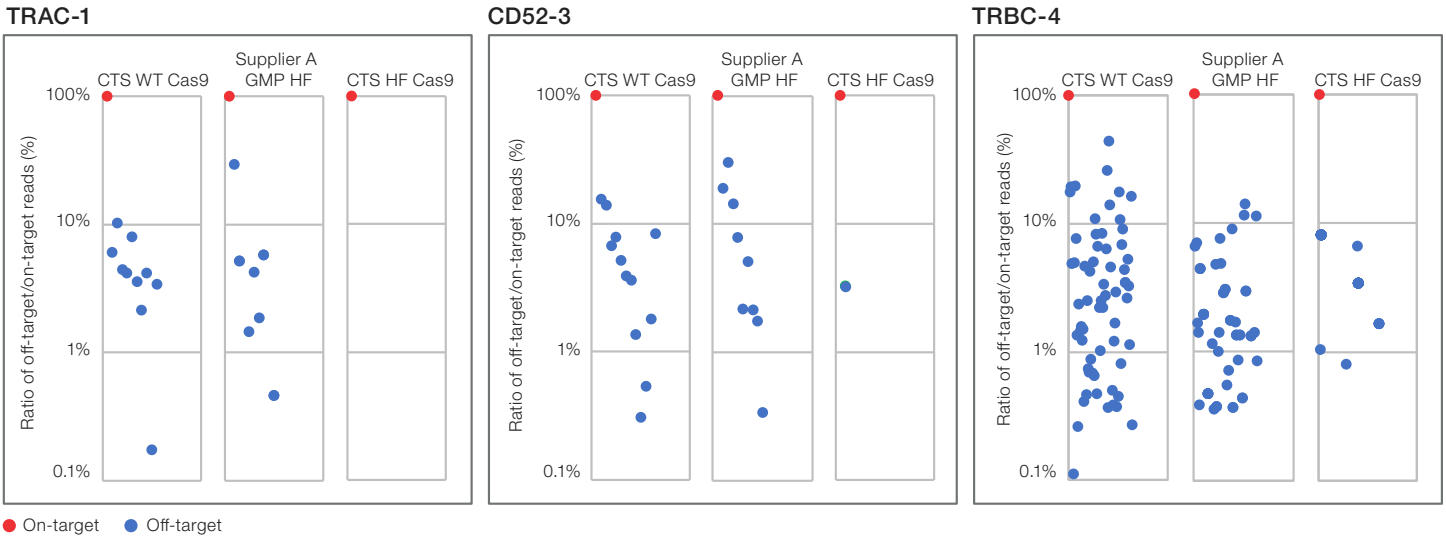

Figure 1. CTS HiFi Cas9 Protein (CTS HF Cas9) significantly reduced the occurrence of off-target effects in primary T cells. Three Cas9 proteins were mixed with three Invitrogen™ TrueGuide™ Synthetic sgRNAs to form nine complexes (RNP: 50 pmol sgRNA, 38 pmol Cas9, 50 pmol dsTag for 1.5 x 10^6 T cells in an Invitrogen™ Neon™ Transfection System (100 µL)*). Each RNP complex was delivered into primary T cells using the Neon Transfection System. Reads from on-target and off-target effects for each gRNA were obtained from an NGS TEG-seq assay (dot plots). Each off/on ratio (blue dot) was calculated based on the reads per million (RPM) for an individual off-target divided by the RPM for the corresponding on-target. The red dots represent the respective on-targets and is normalized to 100%. The x-axis is arbitrary. CTS WT Cas9 is CTS TrueCut Cas9 Protein.

* In a comparative analysis of electroporation performance between the Neon NxT and the previous Neon systems, both systems demonstrated similar performance in terms of transfection and gene knockout efficiency; **click here** to see the full details.

CTS HiFi Cas9 Protein maintains high on-target activity in primary T cells

A high-fidelity Cas9 protein is designed to exhibit increased editing specificity, which typically results in a significant loss of activity due to a trade-off between activity and specificity. For therapeutics applications, it is critical to use a Cas9 nuclease that has an appropriate balance between high on-target activity and reduced off-target edits.

Here we demonstrate that CTS HiFi Cas9 Protein maintained equivalent knockout and editing efficiencies, as measured using flow cytometry and NGS, respectively, compared to CTS TrueCut Cas9 Protein across all tested targets (Figure 2). We observed that editing efficiency (indel% as measured by NGS) is lower than protein expression (knockout efficiency as measured by flow cytometry) across all targets. Since NGS is often limited to short reads (less than 300–500 bp, depending on the method), large genetic modifications are not observed in this analysis, thus resulting in lower measured indel% values. For comparative studies, we recommend either method. Overall, the comparable on-target activity suggested that CTS HiFi Cas9 Protein is preferred over CTS TrueCut Cas9 Protein in situations where high on-target activity and low off-target edits are desired.

Improved knock-in efficiency with CTS HiFi Cas9 and CTS Xenon GE Buffer

Gene knock-in is a common tool for gene modification for cell and gene therapy development, such as in a CAR T cell-generation workflow where a CAR donor is inserted. To drive knock-in efficiency, it is essential that the double-strand breaks (DSBs) generated by Cas9 be repaired by the homology-directed repair (HDR) pathway, as opposed to the more error-prone non-homologous end joining (NHEJ) repair pathway that can elicit a variety of small insertions and deletions (indels). Hence, the ability to promote HDR over NHEJ could provide better knock-in results.

Here we show significantly improved knock-in performance when using CTS HiFi Cas9 Protein with the CTS Xenon GE Buffer to knock in single-stranded oligodeoxynucleotide (ssODN) donor material. An ssODN was knocked in at two targeted gene loci (*TRAC* and *CD52*) in primary human T cells at a small electroporation scale. When using CTS Xenon GE Buffer, we observed up to a 3-fold increase in HDR% for TRAC-4 and CD52-5. Additionally, indel% was significantly reduced while overall editing efficiency was not significantly affected. Cell viability was maintained above 90% in all scenarios (Figure 3).

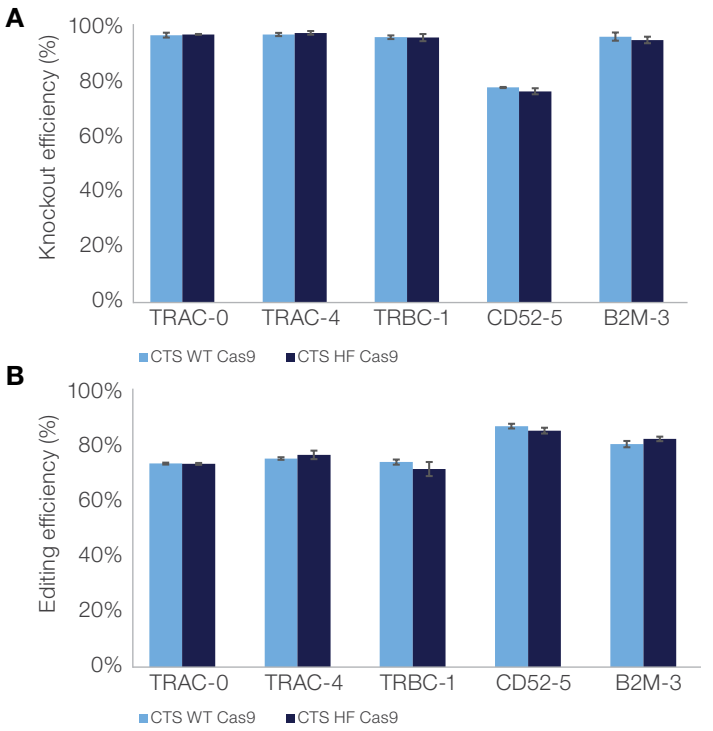

Figure 2. CTS HiFi Cas9 Protein (CTS HF Cas9) maintained high on-target activity in primary T cells comparable to its wild-type counterpart (CTS WT Cas9). RNP complexes: 12 pmol sgRNA and 9 pmol Cas9 for 0.2 x 10^6 T cells in the Neon Transfection System (10 µL).* **(A)** Comparable protein knockout efficiency (>95% for most targets), as measured using an Invitrogen™ Attune™ flow cytometer. **(B)** CTS HF Cas9 maintained the same level of editing efficiency as CTS WT Cas9, as measured using the Ion GeneStudio™ S5 System. All targets had >75% editing efficiency with NGS.

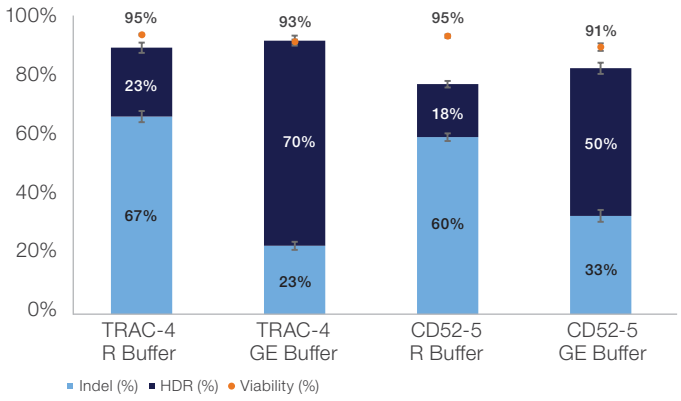

Figure 3. Improved HDR% for better knock-in efficiency when using CTS HiFi Cas9 Protein with CTS Xenon GE Buffer in primary T cells. An ssODN was used as a knock-in donor at two targeted gene loci (*TRAC* and *CD52*). The Neon Transfection System* was used with Neon R Buffer as the control. A 3-fold improvement in the HDR efficiency for TRAC-4 (from 24% to 71%) and a 2.8-fold improvement for CD52-5 (from 18% to 50%) were observed. Additionally, the indel% was significantly reduced while overall editing efficiency was not significantly affected. Cell viability was maintained above 90% in all scenarios.

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CTS HiFi Cas9 Protein can support a large donor knock-in workflow at various electroporation scales

Next we compared the use of CTS TrueCut Cas9 Protein and CTS HiFi Cas9 Protein at scale in a clinical workflow like the CAR T cell therapy workflow (Figure 4). For this workflow, the T cell receptor (TCR) is first knocked out, and then a large donor CAR construct (CD19) is knocked in at the *TRAC* locus. We first demonstrated this workflow using two donor DNA formats (nonviral dsDNA and viral AAV6 donor DNA) at research scale on the Invitrogen™ Neon™ NxT Electroporation System. Then we used the nonviral dsDNA donor on the Gibco™ CTS™ Xenon™ Electroporation System to demonstrate scale-up of the full nonviral workflow.

At day 14 (11 days post-electroporation), we saw minimal differences in cell viability, TCR knockout, and CAR knock-in between CTS TrueCut Cas9 Protein and CTS HiFi Cas9 Protein across all donor types and electroporation scales. We observed high TCR knockout and CAR knock-in for both donor types at a small, research scale. A similar result was observed when we scaled up the nonviral donor workflow to a large, clinical manufacturing volume with the CTS Xenon Electroporation System. This suggested that CTS HiFi Cas9 Protein and CTS TrueCut Cas9 Protein have comparable performance at scale and for different donor materials.

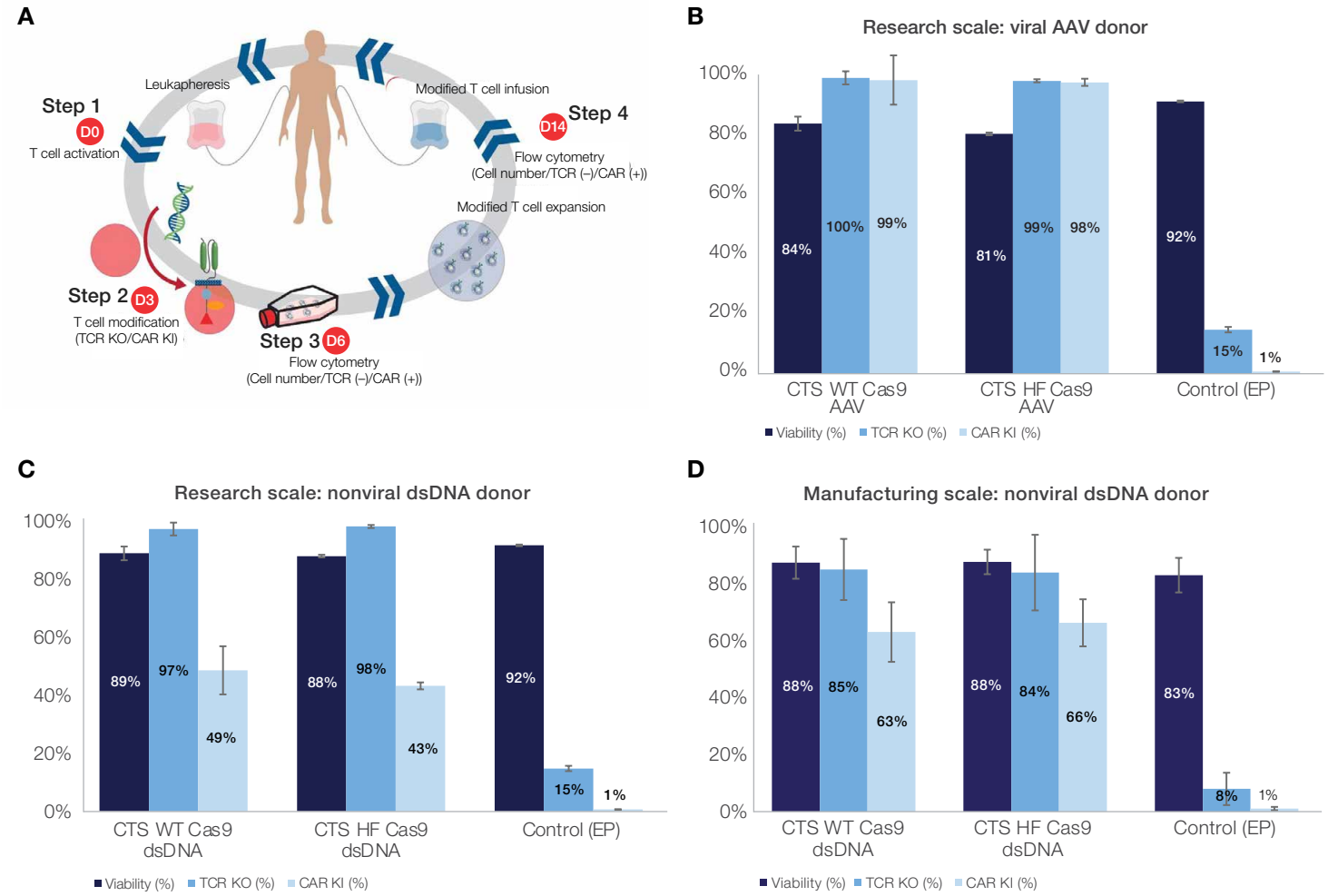

Figure 4. Efficiency of CTS HiFi Cas9 protein (CTS HF Cas9) comparable to that of CTS TrueCut Cas9 (CTS WT Cas9) for large donor (CD19) knock-in for CAR T cell generation. (A) There are 4 major steps in the CAR T cell generation workflow: step 1 (day 0): patient's PBMC activation; step 2 (day 3): T cell modification where electroporation is performed; step 3 (day 6): 3 days post-electroporation where critical parameters such as viability, TCR knockout, and CAR knock-in are measured; step 4 (day 14): 11 days post-electroporation when the expanded CAR T cells are ready for infusion. **(B–D)** Performance comparison of CTS HF Cas9 and CTS WT Cas9 Protein on day 14. In each workflow, the results (cell viability, T cell receptor knockout, CAR CD19 knock-in) as measured by flow cytometry are comparable, showing that the CTS HF Cas9 performs comparably to the CTS WT Cas9. **(B)** 10 µL Neon NxT electroporation with 7.5 pmol sgRNA, 7.5 pmol Cas9 for 2 x 10^5 T cells followed by adding 1 µL of AAV donor (titer: 6 x 10^{10}/µL) 5–15 minutes post-electroporation; **(C)** 10 µL Neon NxT electroporation with 7.5 pmol sgRNA, 7.5 pmol Cas9, and 0.5 pmol (2 µg) donor DNA for 2 x 10^5 T cells; and **(D)** 1 mL CTS Xenon electroporation with 930 pmol sgRNA, 730 pmol Cas9, and 50 pmol (240 µg) donor DNA for 5 x 10^7 T cells.

* In a comparative analysis of electroporation performance between the Neon NxT and the previous Neon systems, both systems demonstrated similar performance in terms of transfection and gene knockout efficiency; click here to see the full details.

Consistently high performance from CTS HiFi Cas9 Protein in iPSCs

The same comparative study was done with iPSCs where three gRNAs targeting the *TRAC*, *CD52*, and *TRBC* genes were selected based on the *in silico*–predicted level of off-target activity: low off-target activity (TRAC-1), medium off-target activity (CD52-3), and high off-target activity (TRBC-4). Similar to the performance in T cells, we observed that CTS HiFi Cas9 Protein more drastically reduced occurrence of off-target cleavage events than did CTS TrueCut Cas9 Protein and supplier A's GMP high-fidelity Cas9 protein (GMP HF protein) (Figure 5A).

The on-target activity was also evaluated (Figure 5B), and we found that the CTS HiFi Cas9 Protein performed comparably to CTS TrueCut Cas9 Protein at most targets (TRAC-4, TRBC-1, CD52-5), but differences were detected at some targets (TRAC-0, B2M-3). We attributed the differences at these targets to iPSCs being a more difficult cell type to transfect and iPSCs generally requiring more reagent payload (Cas9 and sgRNA) to achieve high editing activity. Additionally, performance (on- and off-target) also varies with target. As a result, we recommend optimizing your project with a higher payload dose when working with iPSCs (compared to the amount used for T cells) if the dose does not significantly affect cell viability.

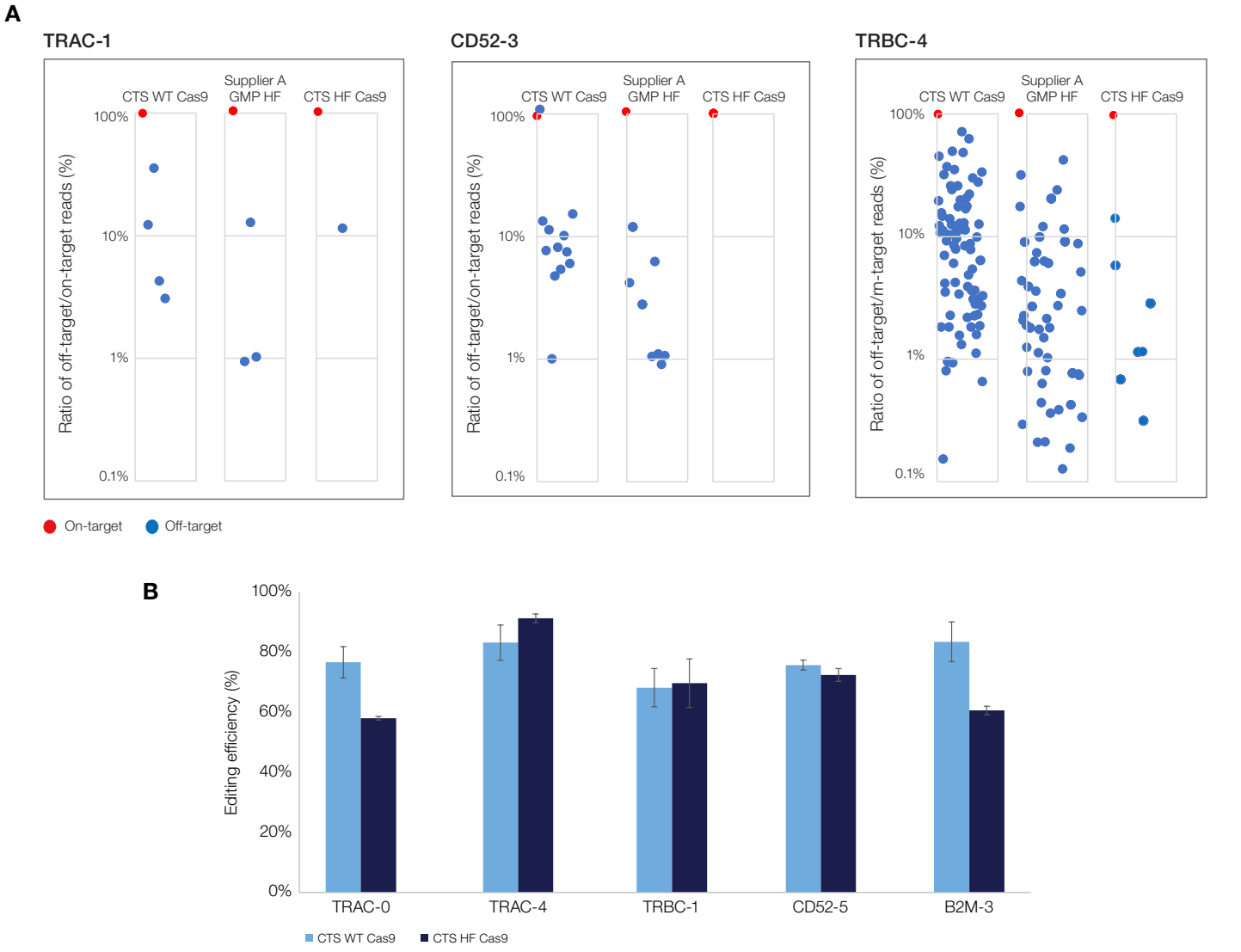

Figure 5. CTS HiFi Cas9 Protein (CTS HF Cas9) significantly reduces off-target edits in iPSCs. (A) Nine RNP complexes were made using the three Cas9 proteins of interest (38 pmol) and the same sgRNAs used for T cell testing (50 pmol), with 50 pmol dsTag. Each RNP complex was delivered into iPSCs using the Neon Transfection System.* Ratio of NGS reads for off-target relative to on-target reads. Each CTS HF Cas9 graph shows fewer off-target edits (blue dots) and with generally lower ratios (representing lower probability of occurrence). **(B)** On-target activity (indel%) of CTS HF Cas9 in iPSCs may not always be as consistent as we saw in T cells. RNPs with 9 pmol Cas9 and 12 pmol sgRNA were transfected using the Neon NxT Electroporation System (10 µL) into 2 x 10^5 iPSCs and cultured for 3 days. Indel% was measured using the Ion GeneStudio S5 NGS System. CTS WT Cas9 is CTS TrueCut Cas9 Protein.

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Conclusion

Regulatory bodies recognize that, in the development of cell and gene therapy products, there are risks from off-target cleavage events; the use of a high-fidelity Cas9 nuclease can help address those safety concerns. Here we have highlighted the exceptional off-target profile of the CTS HiFi Cas9 Protein compared to CTS TrueCut Cas9 Protein and another supplier's GMP high-fidelity Cas9. We also demonstrated equivalent on-target activity of CTS HiFi Cas9 Protein and CTS TrueCut Cas9 Protein in primary T cells and iPSCs. This confirmed that CTS HiFi Cas9 protein can minimize off-target events while maintaining high on-target editing activity. Additionally, when used with CTS Xenon Genome Editing Buffer, we saw improved knock-in efficiency (represented by an increase in HDR%). Finally, we highlighted the exceptional results when using CTS HiFi Cas9 Protein in a CAR T cell generation workflow at scale. With CTS HiFi Cas9 Protein, you can now accelerate your cell and gene therapy development more confidently and at the scale you need.

References

1. U.S. Food and Drug Administration/Center for Biologics Evaluation and Research. (2022) *Human Gene Therapy Products Incorporating Human Genome Editing.*
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3. Tang PZ et al. (2023) Target-seq: single workflow for detection of genome integration site, DNA translocation and off-target events. *BioTechniques* 74(5), 211–224.

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