Analysis of the performance of GMP-manufactured Cas9 nucleases for cell therapy applications

Chimeric antigen receptor (CAR) T cell therapy, first approved by the US Food and Drug Administration (FDA) in 2017, is a rapidly growing field in cancer therapy that involves the isolation and activation of T cells from a patient's blood for *ex vivo* genetic modification. One of the most widely used nonviral editing tools for this application is CRISPR-Cas9. CRISPR-Cas9 is an RNA-guided DNA nuclease system comprising a guide RNA (gRNA) and a Cas9 nuclease. The Cas9 nuclease plays an important role in the overall performance of the tool and is a critical component for therapy development. Despite the vital role Cas9 nuclease plays in the CAR T cell therapy workflow, the ability to obtain a reliable supply of high-quality and high-performing GMP-manufactured Cas9 has been a challenge.

Thermo Fisher Scientific offers a broad array of high-quality products specifically designed for use in cell therapy research applications. From media, reagents, growth factors, and enzymes to selection beads and devices, Gibco™ Cell Therapy Systems™ (CTS™) products are manufactured in compliance with 21 CFR Part 820 Quality System regulatory standards and/or are certified to ISO 13485 and ISO 9001. The adherence to these quality regulations enables a seamless transition from bench to clinical settings. To address the need for a robust supply of high-quality and high-performing GMP-manufactured Cas9 nuclease, Thermo Fisher now offers the Gibco™ CTS™ TrueCut™ Cas9 Protein with consistent high-quality performance and supply to support cell and gene therapy development programs.

We performed a side-by-side performance comparison of the CTS TrueCut Cas9 (CTS Cas9) against a GMP-manufactured Cas9 from another supplier (Supplier A Cas9) in increasingly complex, more physiologically relevant model systems. In each experiment, the performance of the CTS Cas9 consistently matched or exceeded the performance of Supplier A Cas9, demonstrating the utility of the CTS TrueCut Cas9 Protein for cell- and gene therapy–based applications.

Performance comparison: CTS Cas9 vs. Supplier A Cas9

In vitro cleavage assay

To compare the performance of the CTS Cas9 and Supplier A Cas9, we first used the *in vitro* cleavage assay. This assay provides an easy way to perform a direct side-by-side comparison of the proteins' cleavage activity without any interference from the cellular context (e.g., toxicity and other cellular conditions) or transfection efficiency. For a more in-depth comparison of the two nucleases, both CTS Cas9 and Supplier A Cas9 were diluted to nonsaturating concentrations and assessed for cleavage activity. As shown in Figure 1, both Cas9 proteins demonstrated similar cleavage activity at a high (or saturated) dose of 80 ng. However, the difference in cleavage activity between the two proteins started to become more significant at lower doses. The cleavage activity of the CTS Cas9 is significantly higher than that of Supplier A Cas9 at lower doses, between 48 ng and 16 ng. As shown in Figure 1, 48 ng of Supplier A Cas9 is required to achieve 35% cleavage activity, compared to only 16 ng of CTS Cas9. These data suggest that CTS Cas9 offers higher cleavage activity compared to Supplier A Cas9.

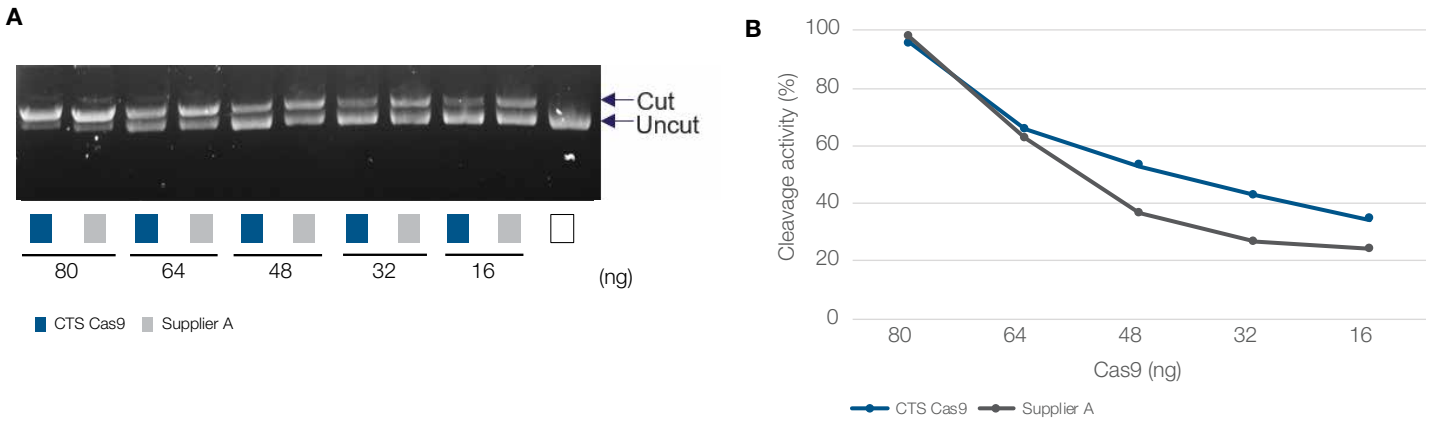

Figure 1. Higher cleavage activity with CTS Cas9 than with Supplier A Cas9, at lower protein doses. Different amounts of Cas9 were incubated with the same amount of excess (40 ng) gRNA targeting the *HPRT* gene, and a plasmid (300 ng) containing an *HPRT* sequence, for 10 minutes at 37°C. **(A)** The cleavage reactions containing uncut and cut plasmid were resolved on an agarose gel and quantitated using the Invitrogen™ iBright™ FL1500 Imaging System. Reactions were performed in triplicate. At saturating conditions, CTS Cas9 and Supplier A Cas9 demonstrated similar cleavage activity. **(B)** As the samples were diluted, a difference between the two nucleases became more apparent—at 48 ng, CTS Cas9 achieved a cleavage efficiency of 53%, compared to 36.5% achieved by Supplier A CTS.

Cellular assays

Next, we wanted to confirm that the activity of CTS Cas9 observed *in vitro* is preserved when transferred to a cellular environment. Primary T cells were selected, as they offer the appropriate cellular context needed for CAR T cell therapy development. Seven gRNAs targeting four CAR T cell–related therapeutic genes—*TRAC*, *TRBC*, *PD1*, and *CD52*—were used in this experiment. The primary T cells were isolated and activated, then edited with CTS Cas9 and Supplier A Cas9 using the Invitrogen™ Neon™ Transfection System (10 µL kit).

To assess the performance of the Cas9 nucleases in a cellular environment, we first performed a head-to-head comparison between the CTS Cas9 and Supplier A Cas9 using flow cytometry to assess the T cell receptor (TCR) knockout (KO) at the protein level. We performed a functional analysis to assess how efficiently each Cas9 was able to knock out the gene of interest. Four gRNAs targeting both the alpha and beta T cell receptor (*TRAC* and *TRBC*) genes were selected, and the results can be seen in Figure 2. Figure 2A shows representative data of the editing efficiency achieved by the two Cas9 proteins. CTS Cas9 achieved over 88.7% TCR KO, compared to Supplier A Cas9, which achieved 61.7% TCR KO.

We then expanded the gRNA panel to assess the genotyping changes using next-generation sequencing (NGS)-based targeted amplicon-seq validation (TAV) to count the average indel (KO) percentage at four targets—*TRAC-0*, *TRAC-4*, *TRAC-6*, and *TRBC-1*. On average, CTS Cas9 achieved over 20% higher efficiency of knocking out both *TRAC-0* and *TRBC-1* compared to Supplier A Cas9, as seen in Figure 2B. No significant differences in KO efficiency were observed with *TRAC-4* and *TRAC-6*. This result suggests that when testing the nucleases at equal doses, the CTS Cas9 was able to knock out the gene of interest more efficiently at varying targets compared to Supplier A Cas9.

To further determine Cas9 editing efficiency at the genomic level, a broader set of gRNAs were selected and assessed using NGS-based TAV. Three additional gRNAs targeting the *CD52* and *PD1* genes (*CD52-5*, *CD52-6*, and *PD1-2*) were added to the panel, and the editing efficiency (indel) of each protein was measured using NGS-based TAV. As seen in Figure 3A, CTS Cas9 achieved higher editing efficiency with three (*TRAC-0*, *TRBC-1*, and *CD52-5*) of the seven gRNAs, compared to Supplier A Cas9. The other four gRNAs (*TRAC-4*, *TRAC-6*, *CD52-6*, and *PD1-2*) all achieved a high editing efficiency of over 80%, with no significant differences between the two Cas9 proteins. We hypothesized that the amount of reagents used in the reaction (including the amount of Cas9 proteins) may be in a saturated condition, which could have led to achieving the maximum editing efficiency for both Cas9 proteins.

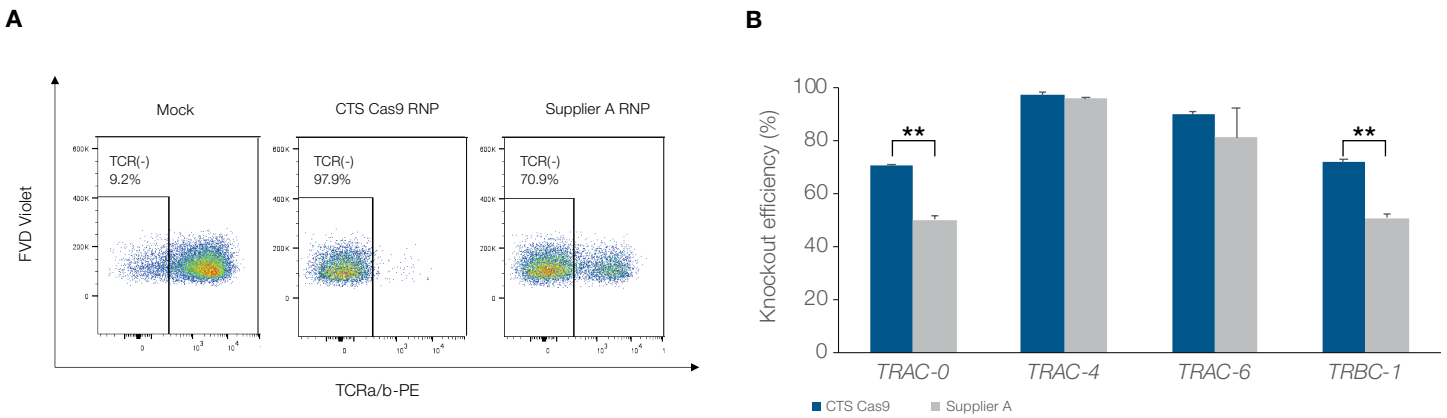

Figure 2. Higher TCR knockout efficiency with CTS Cas9 than with Supplier A Cas9. Each Cas9 (7.5 pmol) and Invitrogen™ TrueGuide™ Synthetic sgRNA (7.5 pmol) targeting alpha and beta T cell receptor gene (*TRAC* and *TRBC*) regions were mixed to create Cas9-RNP complexes. Each Cas9-RNP complex was used to transfect 500,000 T cells using the Neon Transfection System (Cat. No. MPK5000). Cells were harvested after 72 hours and stained with Invitrogen™ eBioscience™ Fixable Viability Dye (FVD) Violet (Cat. No. 65-0863-14) and eBioscience™ anti-TCR a/b antibodies (Cat. No. 12-9986-42), then analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer and also genotyped using NGS-based TAV. **(A)** An example of flow cytometry data for TCR KO efficiency. CTS Cas9 achieved over 88.7% KO efficiency compared to Supplier A Cas9, which achieved 61.7% KO efficiency. **(B)** Average KO efficiency from NGS-based TAV. CTS Cas9 achieved higher average KO efficiency compared to Supplier A Cas9 at various targets. All reactions were performed in triplicate (** $P < 0.01$).

To further explore the potential performance differences between the two Cas9 proteins, we performed a dose-dependent study with serial dilutions from the original amount of Cas9 used in previous studies (7.5 pmol; dilution from 1 to 1/16). Both Cas9 proteins were serially diluted, and the cleavage activity was measured, and a functional assay was performed in primary T cells on *TRAC-4*, *TRAC-6*, *CD52-6*, and *PD1-2*. Data shown in Figure 3B demonstrated that CTS Cas9 achieved higher editing efficiency compared to Supplier A Cas9 for all four gRNAs as the Cas9 dilutions fell out of saturating levels. In each case, Supplier A Cas9 lost editing efficiency at a lower dilution factor (i.e., at a higher concentration) compared to CTS Cas9. The difference in performance between the two Cas9 proteins was most evident with *TRAC-6*. At the 1/4 dilution, Supplier A Cas9 had 40% editing efficiency, compared to 60% efficiency with CTS Cas9. Overall, CTS Cas9 had higher cleavage activity than Supplier A Cas9 at different dilutions across all targets.

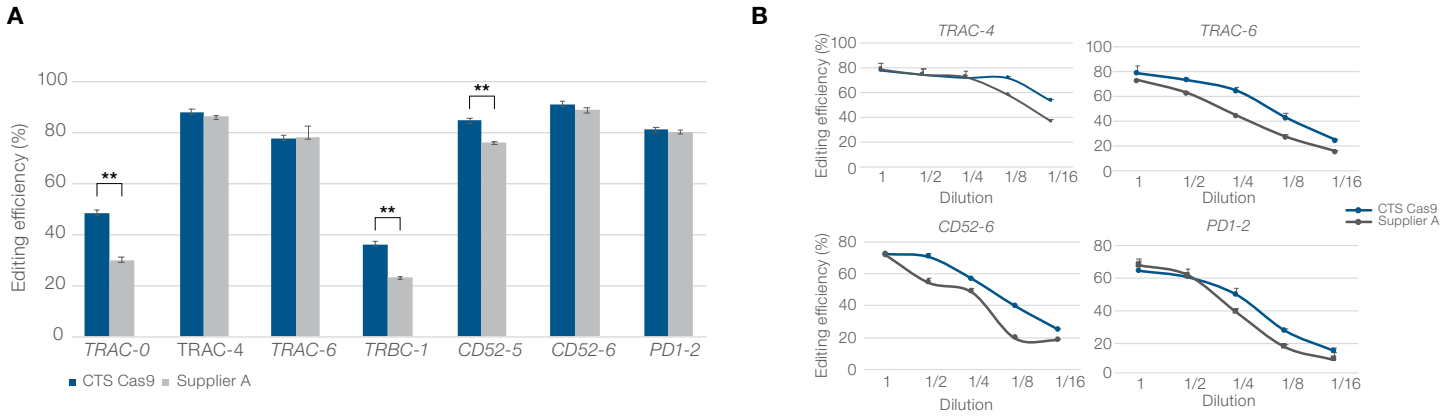

Figure 3. Higher editing efficiency with CTS Cas9 than with Supplier A Cas9 in primary T cells. (A) CTS Cas9 or Supplier A Cas9 (7.5 pmol) was mixed with 7.5 pmol of seven corresponding TrueGuide Synthetic sgRNAs and transfected into T cells using the Neon Transfection System at a 10 µL scale. Three days after transfection, the cells were lysed and amplified using a pair of primers flanking cleavage sites. The amplicons were barcoded and sequenced using an Ion Torrent™ NGS system and analyzed using an editing efficiency analysis tool developed in-house. Significant differences in performance were observed on three targets (*TRAC-0*, *TRBC-1*, and *CD52-5*). **(B)** CTS Cas9 and Supplier A Cas9 were serially diluted from 1X (7.5 pmol) to 1/16X (0.47 pmol). The diluted Cas9 proteins were mixed with 7.5 pmol of corresponding TrueGuide Synthetic sgRNA (*TRAC-4*, *TRAC-6*, *PD1-2*, and *CD52-6*) and transfected into T cells using the Neon Transfection System, and analyzed after 72 hours. The results suggested that CTS Cas9 has comparable or higher editing activity compared to Supplier A Cas9. Reactions were performed in triplicate (** $P < 0.01$).

Conclusions

The ability to obtain a reliable supply of high-quality and high-performing GMP-manufactured Cas9 has been a challenge. In this work, we demonstrated side-by-side comparisons of the performance of CTS TrueCut Cas9 Protein (CTS Cas9) and another supplier's GMP-manufactured Cas9 protein (Supplier A Cas9). In each of the model systems, CTS Cas9 demonstrated superior performance in comparison to Supplier A Cas9. At saturating concentrations of Cas9, CTS Cas9 consistently matched, if not outperformed, Supplier A Cas9. Interestingly, a difference in performance between CTS Cas9 and Supplier A Cas9 became apparent as we diluted the Cas9 in the genome editing reaction. This suggests CTS Cas9 can achieve more robust editing. With the launch of the CTS TrueCut Cas9 Protein, you can accelerate your therapeutics more confidently knowing that Thermo Fisher can supply a high-quality product at the scale you need for cell and gene therapy development.

Learn more at **thermofisher.com/ctscas9**