Advancing CAR T cell therapy with CTS TrueCut Cas9 Protein

Advancing cell therapy products to reach the clinic starts with the selection of the right reagents. Not only do the reagents need to be of sufficient quality to comply with rigorous regulatory standards, but they also need to be manufactured with scalable processes to meet future clinical demands. Under the Gibco™ Cell Therapy Systems™ (CTS™) brand, 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, all Gibco™ CTS™ products are manufactured in compliance with the 21 CFR Part 820 quality system regulation and/or are certified to ISO 13485 and ISO 9001. The adherence to stringent quality standards allows for a seamless transition from bench to clinic.

Chimeric antigen receptor (CAR) T cell therapy, first approved by the U.S. Food and Drug Administration (FDA) in 2017, is a rapidly growing field in cancer therapy and involves the isolation and activation of T cells from a patient's blood for *ex vivo* genetic modification. The engineered T cells are then infused back into the patient to enable T cell–mediated cytotoxicity as treatment. CRISPR-Cas9 is one of the most commonly leveraged nonviral editing tools for engineering T cells for therapeutic applications. Thermo Fisher now offers the Gibco™ CTS™ TrueCut™ Cas9 Protein for use as an ancillary material in cell and gene therapy applications.

We take a deeper look at the CTS TrueCut Cas9 Protein, from its detailed quality specifications to its performance in primary T cells. We assessed the performance of the CTS TrueCut Cas9 Protein (CTS Cas9) against our flagship, research-grade Invitrogen™ TrueCut™ Cas9 Protein v2 (RUO Cas9) to confirm that the manufacturing scale-up of the enzyme to GMP standards had no significant impact on the product performance.

CTS TrueCut Cas9 Protein—quality control and specifications

CTS TrueCut Cas9 Protein is a GMP-grade recombinant Cas9 protein manufactured in compliance with standards for *Ancillary Materials for Cell, Gene, and Tissue-Based Products,* including USP <1043>, Ph.Eur. 5.2.12, and ISO 20399-1, -2, -3, following the principles of 21 CFR Part 820 in an FDA-registered manufacturing site. In addition to having extensive traceability documentation, the product is also subjected to aseptic manufacturing, extensive safety testing, and sterile filling to eliminate potential contaminants that may affect the safety of cell-based therapies. CTS Cas9 is provided in large pack sizes (2.5 mg and 5.0 mg) at a high concentration (10 mg/mL) in a transfection-ready format for electroporation; its specifications are shown in Table 1.

Table 1. Representative quality specifications for the CTS TrueCut Cas9 Protein.

Assay	Specification
Purity by RP-HPLC	≥95.0%
Purity by SDS-PAGE	≥95.0%
Aggregates	≤5.0%
Concentration	10 mg/mL
Identity by HPLC-DAD	Conforms
Identity by SDS-PAGE	Conforms
pH	7.0–7.8
Activity (*in vitro*)	≥90% cleavage of a DNA reference
Residual DNase	Less than the limit of quantification (<LOQ)
Residual RNase	<LOQ
Residual host-cell protein	<LOQ
Residual host-cell DNA	<LOQ
Endotoxin	<10.0 EU/mg
Sterility	No growth
Mycoplasmas	Negative

Stability of the CTS Cas9 and RNP complex

The CTS Cas9 and the ribonucleoprotein (RNP) complex (i.e., the complex of Cas9 protein with guide RNA) were assessed for their stability under various conditions. First, the CTS TrueCut Cas9 Protein was serially diluted over a range of concentrations, and then subjected to five freeze-thaw cycles. Cleavage activity was then measured using an *in vitro* cleavage assay. No significant change to the cleavage activity was observed after five freeze-thaw cycles, compared to the control samples without any freeze-thaw cycles, as seen in Figure 1. Additionally, the stability of the RNP complex was assessed at various time points (0, 10 min, 1 hr, 4 hr, and 16 hr) to simulate normal use conditions. The RNP complex was serially diluted, and cleavage activity was measured using an *in vitro* cleavage assay. No significant impact to cleavage activity was observed at different time points, as seen in Figure 2.

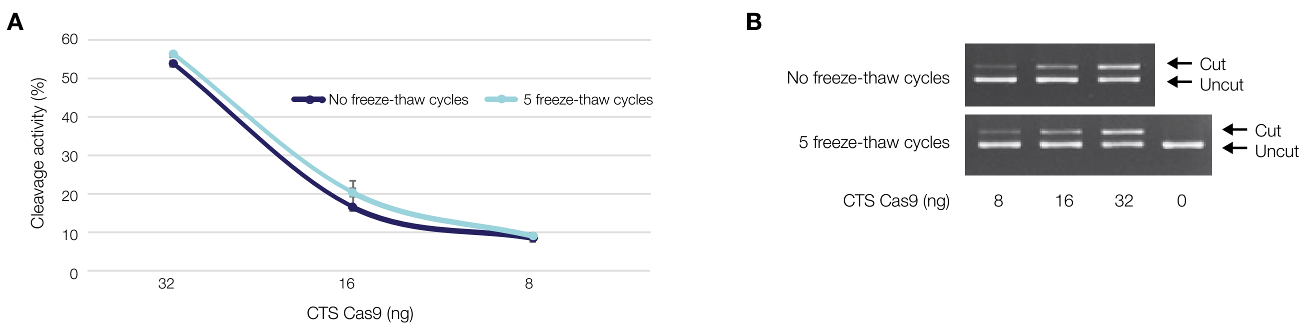


Figure 1. CTS Cas9 remained stable after five freeze-thaw cycles, as measured using an *in vitro* cleavage assay. (A) CTS Cas9 was serially diluted over a range of concentrations, and the cleavage activity was measured. The various amounts (8 ng, 16 ng, 32 ng, and 0 ng) of CTS Cas9 were incubated with excesses of gRNA (40 ng) targeting the *HPRT* gene, and a plasmid (300 ng) containing an *HPRT* sequence, for 10 minutes at 37°C. **(B)** The cleavage reactions containing uncut and cut plasmids were resolved on an agarose gel and quantitated using the Invitrogen™ iBright™ 1500 Imaging System. Reactions were performed in triplicate.

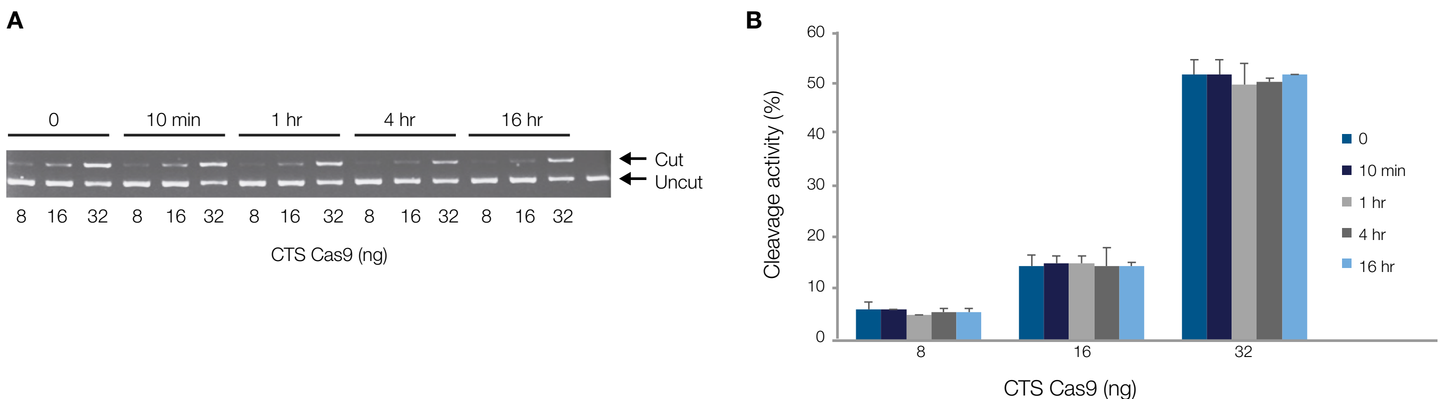


Figure 2. CTS Cas9–RNP complex maintained cleavage activity at room temperature over various time intervals. CTS Cas9 was serially diluted over a range of concentrations, and the cleavage activity was measured. The various amounts (8 ng, 16 ng, and 32 ng) of CTS Cas9 were mixed with an excess of gRNA (40 ng) targeting the *HPRT* gene, and incubated for different times from 10 minutes to 16 hours at room temperature. The samples were then incubated with an excess of plasmid (300 ng) containing an *HPRT* sequence, for 10 minutes at 37°C. **(A)** The cleavage reactions containing uncut and cut plasmids were resolved on an agarose gel and quantitated using an iBright Imaging System. **(B)** The cleavage activity plotted as a bar graph. Reactions were performed in triplicate.

Product consistency—CTS TrueCut Cas9 (CTS Cas9) vs. TrueCut Cas9 v2 (RUO Cas9)

As you transition from bench to clinic, it is important that your CTS TrueCut Cas9 (CTS Cas9) nuclease give you the same, consistent results as the TrueCut Cas9 v2 nuclease (RUO Cas9). To better assess the difference in activity between the CTS Cas9 and RUO Cas9, we leveraged an *in vitro* cleavage assay. The assay provided a direct side-by-side comparison of the proteins' cleavage activity without any cellular context. Both the CTS Cas9 and RUO Cas9 proteins were serially diluted for testing the cleavage activity. The *in vitro* assay results in Figure 3 showed that CTS Cas9 has cleavage activity comparable to that of the RUO Cas9 at all tested dosages.

Next, we wanted to confirm that the protein activity of CTS Cas9 observed *in vitro* is preserved when transferred into 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 RUO Cas9 using the Invitrogen™ Neon™ Transfection System (10 µL kit). Cell viability and cleavage activity were measured and assessed at the genetic level using next-generation sequencing.

Figure 4A shows that there was high (>90%) cell viability post-transfection for both CTS Cas9 and RUO Cas9 proteins across all targets. This result showed that the CTS Cas9 had low toxicity to cells and was comparable to RUO Cas9. Additionally, no significant difference in cleavage efficiency was observed between the CTS Cas9 and RUO Cas9 (Figure 4B) across all target loci. The experimental results confirmed that CTS Cas9 maintains high editing efficiency and low cell toxicity comparable to RUO Cas9 across all target loci in primary T cells. The results from the cellular assays are consistent with the activity measured using *in vitro* assays.

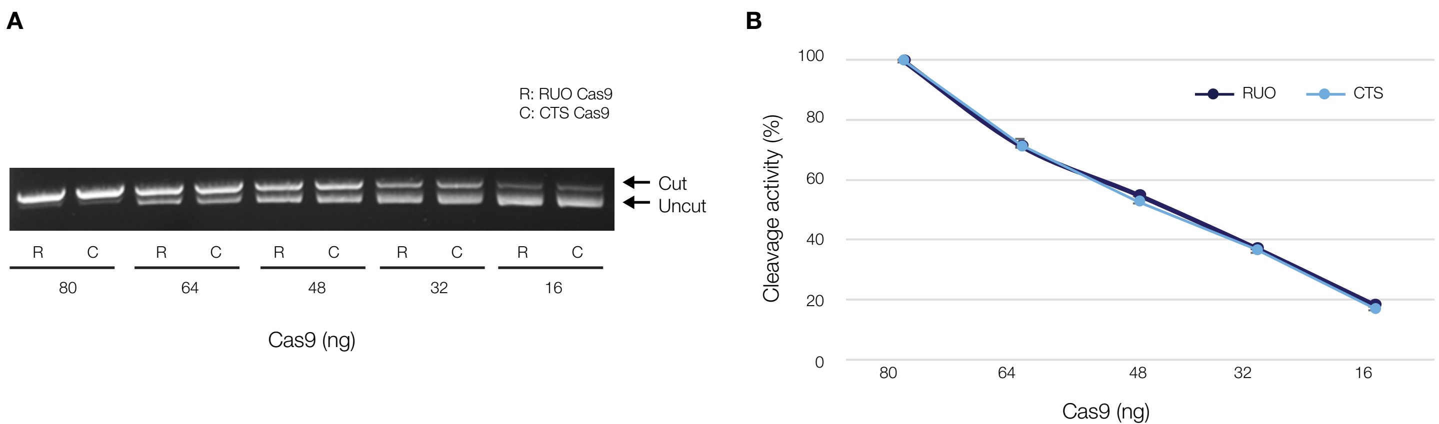


Figure 3. Comparable *in vitro* cleavage activity between CTS Cas9 and RUO Cas9. RUO Cas9 and CTS Cas9 were serially diluted (80 ng to 16 ng), and the cleavage activity was measured. Different amounts of RUO Cas9 (R) and CTS Cas9 (C) were mixed with excesses of gRNA (40 ng) targeting the *HPRT* gene, and a template plasmid (300 ng) containing an *HPRT* sequence, for 10 minutes at 37°C. **(A)** The cleavage reactions containing uncut and cut plasmids were resolved on an agarose gel and quantitated using an iBright Imaging System. **(B)** The cleavage activity plotted as a graph. Reactions were performed in triplicate.

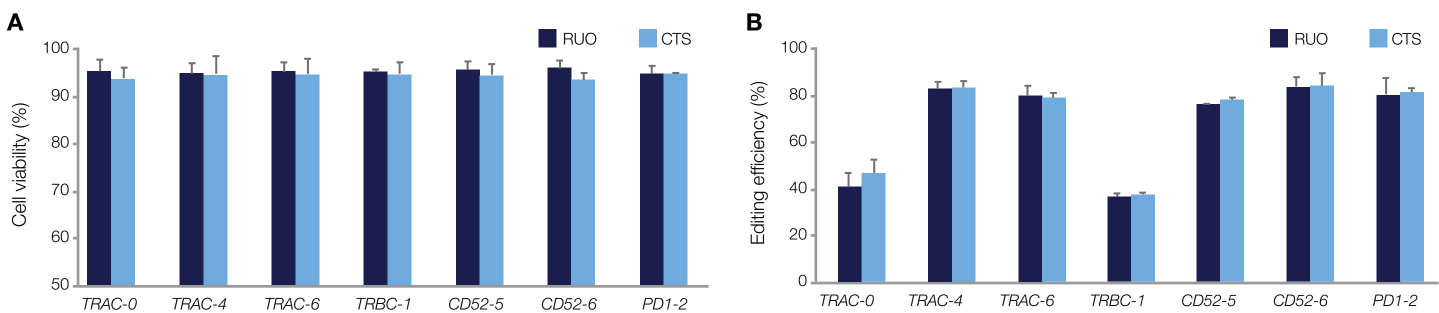


Figure 4. Comparable cell viability and editing efficiency between CTS Cas9 and RUO Cas9 in T cells. CTS Cas9 and RUO Cas9 (7.5 pmol) were each mixed with Invitrogen™ TrueGuide™ Synthetic sgRNA (7.5 pmol) to form two RNP complexes. Each RNP complex was used to transfect 500,000 T cells using the Neon Transfection System (10 µL kit). Cells were harvested after 72 hours of culture. **(A)** Cell viability was measured and analyzed by flow cytometry. **(B)** Summary of NGS-based analysis of editing efficiency as measured by targeted amplicon-seq validation (TAV) using an Ion Torrent™ NGS system for all target loci. All reactions were performed in triplicate.

Performance of CTS Cas9 at 10x process scale-up

To assess the performance of CTS Cas9 at a larger electroporation scale that is more representative of an autologous T cell development process, we used the larger-scale Neon Transfection System (100 µL kit).

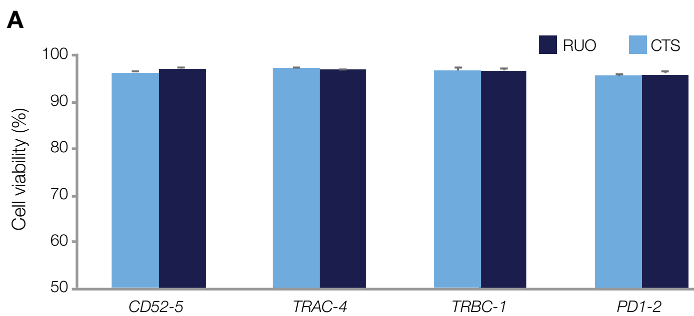
Similar to the smaller-scale electroporation results in Figure 2, the cell viability from both Cas9 proteins remains high (over 90%) at the larger scale (Figure 5A). The results suggested that a 10x scale-up in the electroporation process did not negatively affect the performance of the CTS Cas9. The performance of the CTS Cas9 protein was comparable to that of RUO Cas9 at both electroporation scales.

HDR-based knock-in efficiency of CTS Cas9

Given the importance of precise genome editing for cell and gene therapy applications, we also evaluated the efficiency of homology-directed repair (HDR)-based knock-in (KI) of the CTS Cas9 in primary T cells. Four CAR T cell–related genes—*TRAC, TRBC, PD1*, and *CD52*—were selected, and one gRNA was used per gene. A single-stranded oligodeoxynucleotide (ssODN) was used as the donor DNA to evaluate the percentage of HDR. The electroporation was performed with the Gibco™ CTS™ Xenon™ Genome Editing Buffer, a buffer optimized for improved performance for HDR knock-in–based applications.

CTS Cas9 was comparable to the RUO Cas9 in total editing efficiency as well as percentage of indels and HDR, across all targets (Figure 5B). The total editing efficiency for both CTS Cas9 and RUO Cas9 was 60–90% and the HDR percentage was 40–70%. These results suggest that the CTS Cas9 offers high knock-in performance and results comparable to the RUO Cas9.

A



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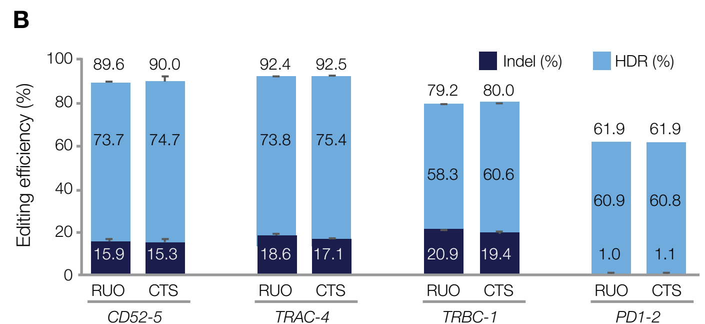

Figure 5. CTS Cas9 achieved high HDR-based knock-in efficiency at 10x electroporation scale. CTS Cas9 and RUO Cas9 (12.5 µg or 75 pmol) was mixed with TrueGuide Synthetic sgRNA (75 pmol) and ssODN donor (150 pmol) to form RNP-ssODN. Each RNP was transfected into 5,000,000 T cells using the larger-scale Neon Transfection System (100 µL kit). Cells were harvested after 72 hours of culture. **(A)** Cell viability was measured and analyzed by flow cytometry. **(B)** Summary of editing efficiency calculated as percentage of donor integration through HDR and indel as measured by targeted amplicon-seq validation (TAV) using an Ion Torrent NGS system. All reactions were performed in triplicate.

Consistency of CTS Cas9 performance across multiple material lots

To further evaluate the reproducibility of the manufacturing processes, three lots of CTS Cas9 were produced over the course of several months at full production scale. For each lot of CTS Cas9, a dilution series was made, the cleavage activity for each dose was measured, and the editing efficiency at *TRAC-4* and *CD52-6* in primary T cells was assessed. No significant lot-to-lot variation was detected at the target loci in T cells (Figure 6).

Conclusions

The application of CRISPR-Cas9 for the development of cell and gene therapy products holds great promise. In this work, we highlighted the extensive quality specifications and the consistent lot-to-lot performance of the CTS TrueCut Cas9 Protein. We also demonstrated performance of the CTS TrueCut Cas9 Protein comparable to that of our flagship, research-grade TrueCut Cas9 Protein v2, at different electroporation scales in primary T cells. This confirmed that the scale-up of manufacturing processes to GMP standards did not negatively affect the performance of the CTS Cas9. We also showed that a high level of HDR was achieved when using the Cas9 proteins with the optimized CTS Xenon Genome Editing Buffer. With the launch of the CTS TrueCut Cas9 Protein, you can now accelerate your therapeutics more confidently, knowing that Thermo Fisher can supply a high-quality product at the scale you need for your cell and gene therapy development.

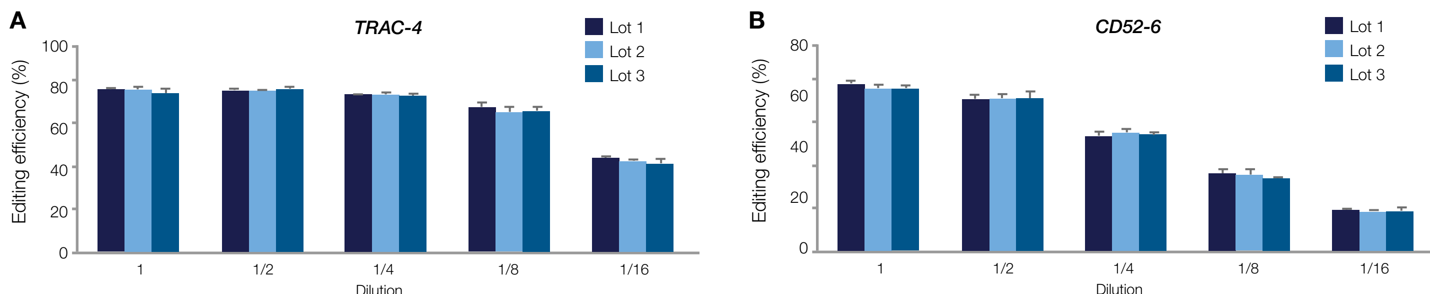

Figure 6. Consistent performance across multiple lots of CTS Cas9 in T cells. CTS Cas9 proteins from three manufacturing lots were serially diluted. Different amounts of CTS Cas9 (7.5 pmol for undiluted) were mixed with 7.5 pmol of **(A)** TRAC-4 or **(B)** CD52-6 TrueGuide Synthetic sgRNA and transfected into T cells using the Neon Transfection System (10 µL kit). The cells were lysed and amplified using a pair of primers flanking the cleavage sites, 72 hours after transfection. The amplicons were barcoded and sequenced using an Ion Torrent NGS system and analyzed using an editing efficiency analysis tool developed in-house. Reactions were performed in triplicate.

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