Development of GMP-Manufactured Cas9 Proteins Including Wild Type and High-Fidelity Versions for Therapeutic Applications

Bringing therapy from the bench to the clinic is more seamless than ever before with the availability of GMP-Manufactured Cas9 Proteins for Therapeutic Applications

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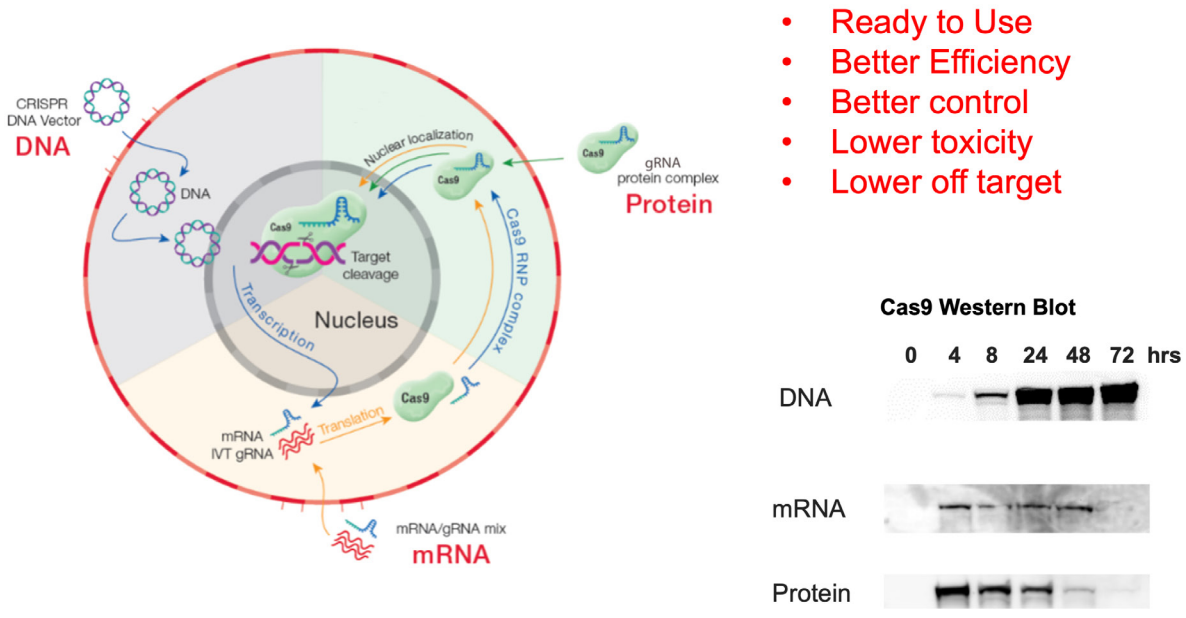
OVERVIEW

There's a growing interest in cell and gene therapies, both in the news and in scientific literature. This includes current therapies, as well as the promise of new treatments thanks to several ongoing trials. The question at the top of the scientific community's mind is: how do we take these types of enzymes from the early research and development stages through clinical trials and, finally, to commercialization? The key is utilizing higher grades of manufacturing technology and ingredients at the outset so that the same enzymes can be proven safe at the lab bench, before transitioning to approved large scale clinical use.

UNMATCHED MANUFACTURING: CREATING CTS TRUECUT CAS

Cas9 is often the enzyme of choice for gene therapy researchers because there are many ways to deliver it. The ribonucleoprotein (RNP) complex of the Cas9 protein and bound sgRNA outperforms DNA and mRNA delivery since it requires fewer steps and yields better efficiency, better control, lower toxicity, and fewer off-target effects. As you can see in the Western blot in **FIGURE 1**, DNA delivery of a Cas9 plasmid results in a high level of Cas9 over the course of 72 hours, and Cas9 mRNA delivery builds up quickly to a moderate level before disappearing as it's degraded in the cells. With direct Cas9 protein delivery, however, the initial high dose is immediately observed and is rapidly degraded throughout the course of the experiment. For this reason, Cas9 can rapidly do its job, allow for editing within the first 24 to 48 hours, and then clear from the system. This ability to precisely control the dose of Cas9 protein results in high editing efficiency with a significantly lower off-target risk than with DNA delivery.

FIGURE 1: Why use RNP format?



Gibco CTS TrueCut Cas9 Protein is a GMP version of the classic TrueCut Cas9. Like the TrueCut Cas9, CTS Cas9 demonstrates strong delivery into the nucleus of all the cells tested, including primary immune cells (>90% editing in T cells). It is manufactured in compliance with GMP principles and meets 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. The process, from start to finish, is enclosed and controlled in clean rooms and tested for sterility, endotoxin, mycoplasmas, and residual host nucleic acids and proteins.

Why switch to CTS? The CTS process can help scientists confidently transition cell therapy from the lab bench to the clinic. They are 21 CFR Part 820 compliant for medical devices and manufactured at FDA-registered sites with ISO 13485 certified quality management systems. The CTS process for Cas9, from fermentation through purification and filing, is designed to have a high yield. In fact, Thermo Fisher can generate around 10 to 20 grams for each lot size, all in an enclosed process that significantly reduces the risk of environmental contamination. All animal origin components have been removed from the process and extensive release testing is performed (**FIGURE 2**). Thermo Fisher provides traceability documentation, including Drug Master Files and Certificates of Origin. So far, the CTS process has been used in several FDA-approved CAR-T therapies—including the first FDA-approved therapeutic cancer vaccine—and over 100 clinical trials.

EVALUATING CTS TRUECUT CAS9 PROTEIN

To ensure that the enzyme could perform as needed in a clinical setting, Thermo Fisher designed several different assays.

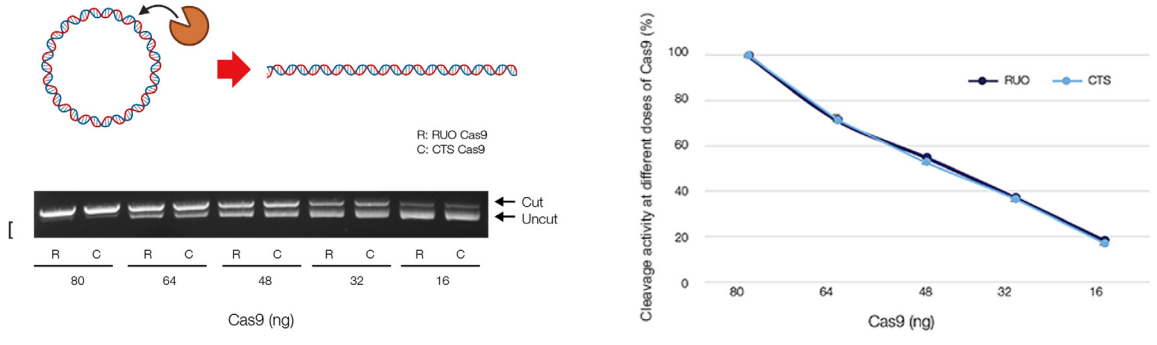
First, an in vitro cleavage assay confirmed performance. Second, they assessed the protein editing activity in primary T cells using the Invitrogen™ Neon™ Transfection System (10 µL sample size). Finally, for clinical use, they validated the second assay's findings at a much larger scale (several hundred thousand cells) using the Neon system as well as the Xenon Transfection system, which can accommodate up to 18 million cells (in this case, activated T cells).

As shown, there is no performance difference between the research-use-only (RUO) version of Cas9 and CTS TrueCut Cas9 (**FIGURE 3**). They are, in fact, the same construct; the difference is in CTS Cas9's purification process. Similarly, there is negligible difference between them in the effects of freeze/thaw, their performance across multiple doses, and how they perform across varying protein doses. In fact, CTS Cas9 has been shown to have no change in activity up to one month at both room temperature and 4°C, potentially allowing for the use of formulated RNPs over multiple doses or multiple processes. CTS and Cas9 also have comparable viabilities and editing efficiencies across multiple gene targets (**FIGURE 4**), which is helpful for researchers who want to begin with the research-grade Cas9 and then switch to CTS Cas9 at a later stage of work. Thermo Fisher compared CTS Cas9 with samples from other suppliers and found that, generally

FIGURE 2: Importance of testing and specifications.

	Parameter	Specification
Product-related impurities	Purity by RP-HPLC	≥95.0%
	Purity by SDS-PAGE	≥95.0%
	Aggregates	≤5.0%
	Identity by HPLC-DAD	Conforms
	Identity by SDS-PAGE	Conforms
Process-related impurities	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
	Mycoplasma	Negative
Characterization	Concentration	10 mg/mL
	pH	7.0–7.8
	Activity (*in vitro*)	≥90% cleavage of a DNA reference
	Color and clarity	Color: Not more intense than Y6; clarity: <30 NTU

FIGURE 3: CTS Cas9 maintains in vitro cleavage activity across doses.



The cleavage reactions containing uncut and cut plasmids were resolved on an agarose gel, and cleavage activity was assessed.

FIGURE 4: Comparable editing efficiency and viability in T cells.

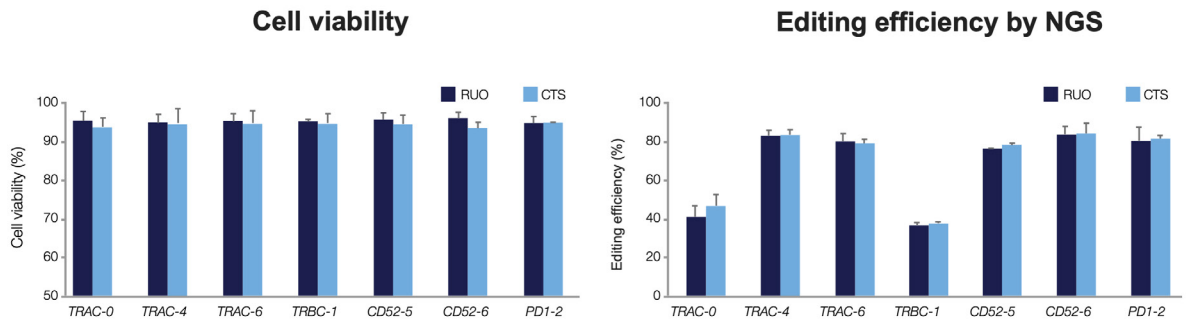
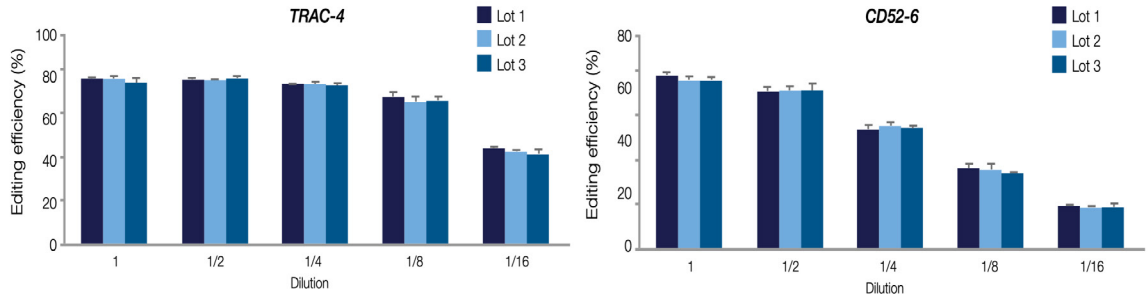


FIGURE 5: Consistent lot-to-lot performance in primary T cells



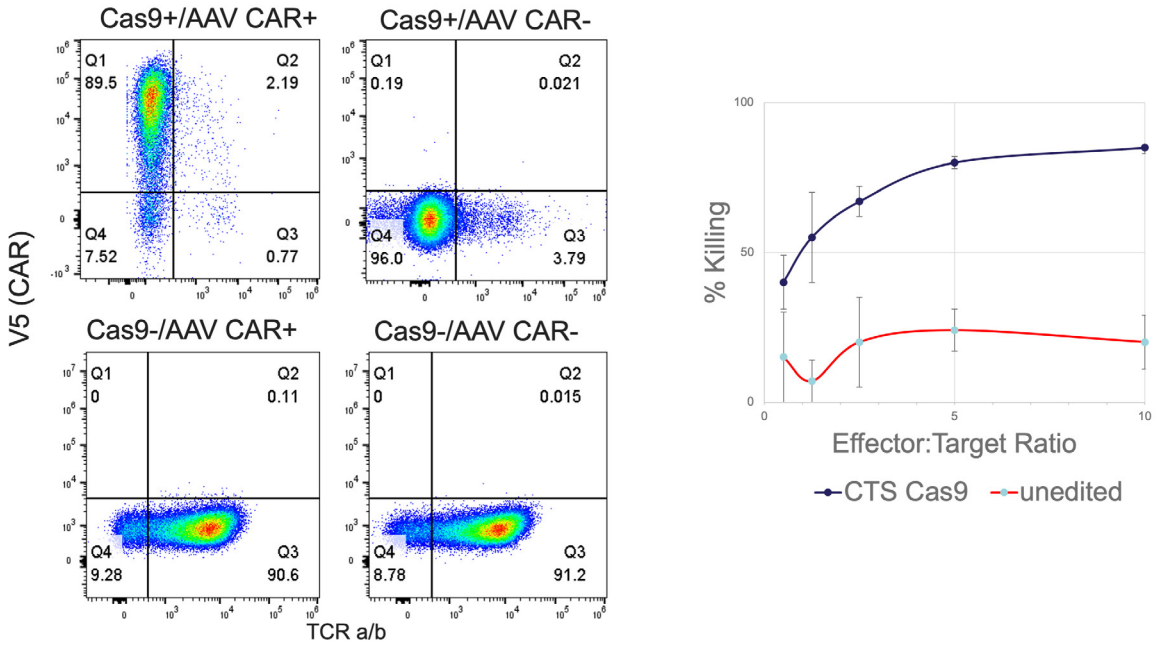
CTS Cas9 from three manufacturing lots was diluted and mixed with sgRNAs targeting TRAC-4 and CD52-6. The amplicons were barcoded and sequenced using an Ion Torrent NGS system.

speaking, they perform similarly. Lot-to-lot performance was also confirmed by comparing the functional activity of three full-scale lots manufactured under GMP conditions (**FIGURE 5**).

Next, Thermo Fisher wanted to confirm the compatibility of Cas9 with HDR-based methods. There was no change in viability with a single-strand small donor. In this case, editing efficiency for the first two targets was high. When tested in a CAR-T system, Cas9 RNP targeted to the TRAC locus was delivered along with the anti-CD19 CAR AAV donor via two steps: delivering the RNP by electroporation and shortly thereafter delivering the CAR donor by AAV transduction at high MOI. This resulted in very efficient insertion of the CAR donor (88.5%), due to the high Cas9 RNP cutting efficiency (~96%). The anti-CD19 CAR-T cells displayed strong killing activity compared to unedited, normal T cells in the standard CD19 expressing NALM6 cytotoxicity assay (**FIGURE 6**).

Thermo Fisher wanted to take this testing into a larger-scale system. So far, these experiments have been run in the Neon electroporator, which accommodates a 10 to 100 microliter scale. Utilizing the CTS Rotea, counterflow centrifugation concentrates the starting isolated primary T cells, exchanging any buffers for the desired editing buffer. There are several benefits to the Rotea system. It utilizes intuitive, user-

FIGURE 6: CTS Cas9 RNP with a AAV Donor Performance – T cell knock-in of a AAV CAR donor containing a V5 epitope and cytotoxicity killing assay of CD19 expressing NALM6 cells with the modified CAR T cells.



programmable software, it has low-output volumes (as little as 5 mL of cell concentrate), it has a fluid bed which allows for >95% cell recovery and no loss in viability, and it can be used beginning in the research stage all the way through commercial manufacturing.

Next, Thermo Fisher used CTS DynaBeads to activate the T cells for three days. They utilized a large-scale electroporator called the Xenon System to add the editing cocktail. The CTS Xenon is a single-use system that uses replaceable consumables for minimizing contamination and is equipped with compliance software. After that, the edited T cells can be expanded out. After 6-12 days, they can be characterized by microscopy and flow as well as the killing assay.

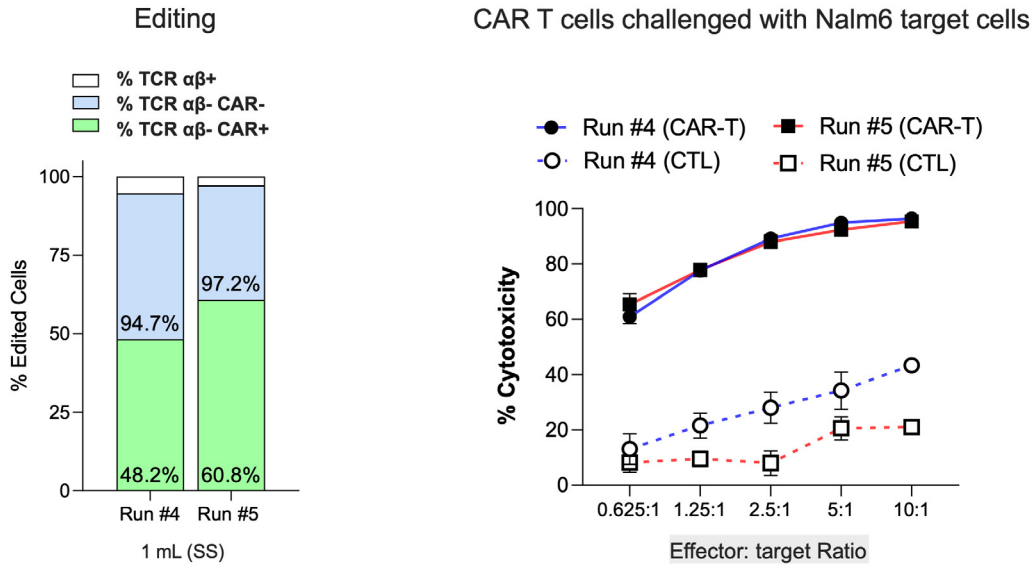
The tests examined a fixed amount of TrueCut Cas9 and a guide RNA. After complexing those together into an RNP, Thermo Fisher took a non-viral approach in which they delivered the CAR donor directly as double-strand DNA (as opposed to an AAV). Examining two separate runs with two separate T cell donors yielded 94% to 97% overall TRAC editing efficiency with 48% and 60% CAR knock-in efficiency. This test also yielded efficient killing of the targeted cells compared to control (**FIGURE 7**).

OFF-TARGET AND HIGH-FIDELITY CAS9

While the risks of off-target editing are not as dangerous when working with a cell model, they become much more concerning when working with therapeutics that will be used by real patients. As a result, the goal is always to eliminate as much risk as possible by minimizing and eliminating off-target cutting.

It's important to understand where off targets or unwanted edits come from. The main source is the Cas9 RNP itself where the nuclease can cut at genomic sequences similar to that of the guide RNA. Unwanted chromosomal rearrangements can occur

FIGURE 7: CTS Cas9 RNP with a dsDNA CAR Donor Performance – T cell knock-in of a dsDNA CAR donor and cytotoxicity killing assay of CD19 expressing NALM6 cells with the modified CAR T cells.



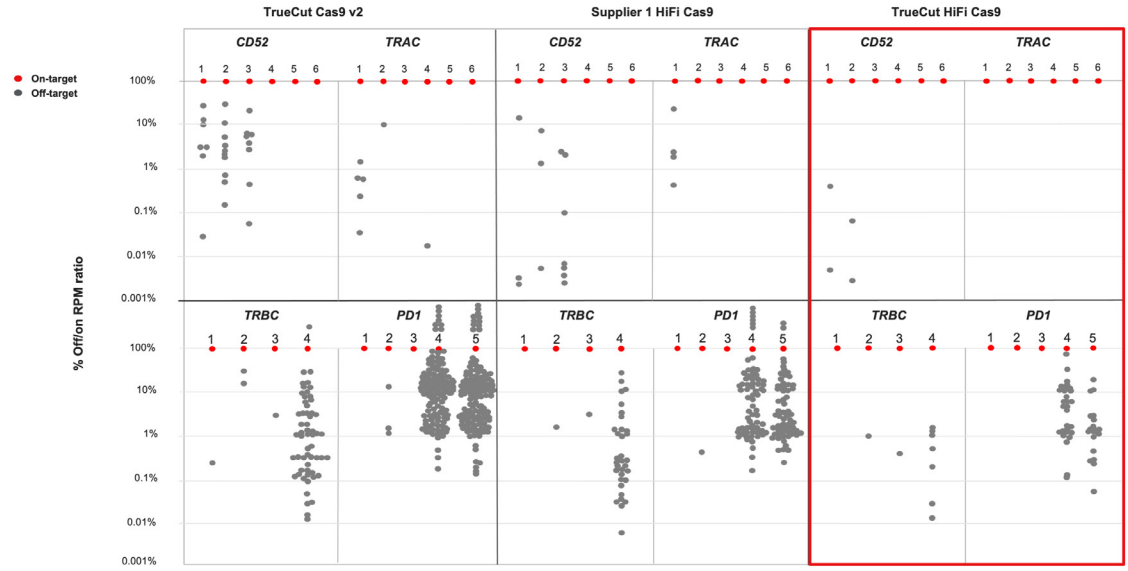
whenever there are multiple double-strand breaks, potentially between on and off-target cuts or in a multiplex reaction where multiple gRNA are used. Unwanted edits can also come from double-strand breaks at the targeted or off-target location leading to large deletions of hundreds to thousands of bases which can result in the loss of nearby genes or even the loss of a chromosomal arm. When dealing with donor DNA, it's also possible for the donor DNA to integrate randomly into the genome.

Indeed, minimizing off targets is critical. Typically, off targets occur when the guide RNA matches something similar. The best way to avoid this is to use guide RNAs that are unique and don't have any other existing similar sequences elsewhere in the genome.

Finding the location of the off target is also important. While those located in intergenic regions are lower risk, those in regulatory regions or an exon—especially an oncogene—can have serious consequences. One way to find the location of the off target is to use silico prediction tools to see if the target sequence exists elsewhere in the genome.

Thermo Fisher has a tool called TrueDesign, which allows the scientist to see where they want to make cuts, chooses guide RNAs, and provides a score to indicate how unique the target is as well as potential off targets and their genomic coordinates. For potential therapeutic guide RNA targets, we also recommend using a direct discovery method named TEG-Seq which is an improved variation of Guide-Seq. It works by inserting a small double-strand DNA tag at any Cas9 derived double-strand DNA breaks in the edited cell population. This tag can be used as a primer to sequence the regions next to the break. Performing an amplification in both directions allows the scientist to see the left and right borders. Adding a barcode helps them keep track of how many unique events occurred. The amplified library is sequenced by NGS to identify potential off-target locations. These targets are then confirmed by targeted amplicon NGS sequencing in the absence of the tag to see if the off-target locations contain indels and are legitimate. Deep sequencing can determine the frequency and thus the risk of each off target. Ideally for a therapeutic target, the off-target incidence should be below

FIGURE 8: Performance: fewer off-target T cells with TrueCut HiFi Cas9.



0.01%. In **FIGURE 8**, we show the results of a TEG-Seq analysis across 21 targets in 4 genes using wild type TrueCut Cas9 and commercially available HiFi versions of Cas9. As seen with PD-1 guides 4 and 5, even with HiFi Cas9 there is still very high off-target risk. However, with many of the other guide RNAs, TrueCut HiFi Cas9 significantly reduced or eliminated the detectable risk of any off-target cuts.

MINIMIZING OFF-TARGET RISK

There are three ways to reduce off-target risk:

1. Fast Turnover of the Nuclease

Using Cas9 protein allows scientists to control the dose and clears out within 48 to 72 hours as opposed to a viral or plasmid system, which persists for a long time. This reduces the risk of off targets that can develop over prolonged exposure to the nuclease.

2. Design gRNA with Unique Sequences

Choosing a sequence with a high score yields low incidence of off targets, measuring down to 0.01%, which is the standard benchmark for therapeutics.

3. Improve Cutting Fidelity of the Cas9 Protein

Unfortunately, many of the high-fidelity enzymes in the literature tend to have the poor cutting activity. We developed TrueCut high-fidelity Cas9 enzyme to have good cutting activity (average of 80% on target cutting of the wild type TrueCut Cas9) and further reduced off-target risk versus other commercially available enzymes (**FIGURE 8**).

CONCLUSION

Thermo Fisher offers a range of products for today's cell and gene researchers. The CTS TrueCut Cas9 offers improved quality via robust manufacturing process controls and extensive release testing. It's capable of producing large lot sizes to minimize risk of performance variability and assist in supply continuity. It's a high-performing solution with minimal lot-to-lot variation, which is critical for therapeutic programs. The TrueCut HiFi Cas9 is ideal for minimizing off-target risk in experiments where there are limited available guides and a significant off-target risk. Thermo Fisher also offers off-target discovery services (TEG-Seq) for any cell target, and experts are available to create a custom solution.