Manual

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GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP

Cat. No.: Z03624

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Product Introduction

GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP is utilized for CRISPR gene editing applications. The Cas9 nuclease forms a stable ribonucleoprotein (RNP) complex with the guide RNA (gRNA) component. With the help of two nuclear localization signals (NLS) expressed with the Cas9 nuclease, the RNP complex enters the nucleus and cleaves target gene. When compared with a plasmid-based delivery system, the RNP delivery system has been observed to increase the on-target gene editing efficiency and decrease off-target effects.

GenScript provides two types of Cas9 nucleases in basic GMP* for selection based on your specific downstream applications.

Table 1: GenCRISPR™ Ultra Cas9 nuclease selection

Nucleases	Description
GenCRISPR™ Ultra NLS-Cas9-basic GMP (Z03623)	Recombinant Cas9 nuclease (wild type) of *Streptococcus pyogenes*; ideal choice for most CRISPR gene editing applications where high editing efficiency is preferred.
GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624)	Recombinant enhanced specificity Cas9 nuclease (mutant) of *Streptococcus pyogenes*; ideal for CRISPR gene editing applications which require low off-target effects and robust on-target cleavage.

Product Name: GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP
Source: Recombinant mutant Cas9 with nuclear localization signal (NLS) at both N-terminal and C-terminal expressed in *E.coli*
Species: *S. pyogenes*
Tag: Tag-free
Theoretical Molecular Weight: ~160 kDa
Concentration: 10 mg/ml
Active Temperature: optimal at 37 °C.
Application: sgRNA-dependent double-stranded DNA cleavage.
Storage Buffer: 25 mM Tris-HCl, 300 mM NaCl, 0.1 mM EDTA, 50% Glycerol, pH 8.0.
Storage & Stability: Store at -20 °C for up to 12 months from the date of manufacture. Avoid repeated freeze-thaw cycles. ***Do not store below -20 °C!***

***basic GMP** is a branding term that GenScript uses to describe reagents manufactured in compliance with ISO 9001 and ISO 13485 quality management system standards and with more stringent process controls and relatively complete documentation records. GenScript is capable of providing documents, site audit and other support to help with the applications of your projects in specific regions.

Contents

Contents	Cat. No.	Amount	Concentration	Storage
GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP	Z03624-100	100 μg	10 mg/ml	-20 °C
	Z03624-500	500 μg		
	Z03624-1	1 mg		

Reagents Required But Not Provided

1. Guide RNA
   - GenCRISPR sgRNA Synthesis Kit (Cat. No. L00694) or
   - CRISPR Synthetic sgRNA Services (see **genscript.com**)
2. Homology-Directed Repair (HDR) Knock-in Templates
   - Single-stranded DNA Synthesis Services (see **genscript.com**) or
   - Double-strand DNA Synthesis Services (see **genscript.com**)
3. Reagents and instrument for electroporation
4. GenCRISPR Mutation Detection Kit (Cat. No. L00688)
5. GenCRISPR T7 Endonuclease I (Cat. No. Z03396)
6. 1 × TE buffer, pH 8.0 and nuclease-free water
7. Proteinase K (for *in vitro* cleavage)
8. Substrate DNA containing the target sequence for *in vitro* cleavage of DNA

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gRNA and HDR Template Preparation

If SafeEdit sgRNA or EasyEdit sgRNA from GenScript Synthetic sgRNA Service is being used, please keep the RNA oligonucleotides tightly sealed at -20 °C for long-term storage and avoid repeated freeze-thaw cycles. We recommend working in a sterile environment, and using Nuclease-free pipette tips and tubes.

1. Centrifuge tube(s) at 12,000 rpm for 2 min at 4 °C before opening to ensure RNA oligos are at the bottom of the tube(s).
2. Resuspend oligos in nuclease-free TE buffer to reach the appropriate stocking solution concentration. For example, for making 100 µM stocking solution:

Normalized Oligo Quantity Delivered (nmol)	Nuclease-free TE buffer (µl)
2	20
4	40
10	100
50	500

3. Vortex for 15 seconds and centrifuge for 1 minute at 12,000 rpm, make sure the gRNA is completely dissolved.

   Note: Avoid repeated freeze-thaw cycles after dissolving. If necessary, divide the stocking solution into small aliquots, and centrifuge for 30 seconds at 4,000 rpm to ensure all solutions are at the bottom of the tubes. Stocking solutions with high concentration (e.g. 100 µM) can be stored at -20 °C for 12 months.
4. Prepare working solutions by adding appropriate amount of nuclease-free water to the stocking solution.

   Note: If starting with frozen stocking solution, leave the solution at 4 °C for 30 min to thaw. Then vortex for 15 seconds and centrifuge for 30 seconds at 4,000 rpm to ensure all solutions are at the bottom of the tubes. Working solutions with lower concentration (e.g. 25 µM) can be stored at -20 °C for 3 months.

RNA Oligo Preparation for gRNA generated by *in vitro* transcription (Optional)

If using *in vitro* transcribed gRNA with GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP in CRISPR gene editing, GenCRISPR sgRNA Synthesis Kit (Cat. No. L00694) is also recommended for the gRNA preparation for obtaining gRNA quickly and simply. Please find the GenCRISPR sgRNA Synthesis Kit manual for detailed guidelines of *in vitro* transcribed gRNA generation at **genscript.com**.

Homology-Directed Repair (HDR) Knock-in Templates Preparation (Optional)

If HDR DNA templates are being used to perform gene knock-in with CRISPR gene editing system, the Homology-Directed Repair (HDR) Knock-in Templates Synthesis Service from GenScript is highly recommended. Please find the detailed user manual for HDR knock-in templates preparation at **genscript.com**.

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Transfect Primary T cells with GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP using Lonza 4D-Nucleofector® Electroporation System

<table>
<tr><th colspan="2">Before starting</th></tr>
<tr><td>Prepare reagents and cells</td><td>
1. Prepare required amount of primary T cells for gene editing

Isolate primary T cells from peripheral blood mononuclear cells (PBMC) and activate the cells using appropriate reagents and methods.
Culture the cells in an incubator (37 °C, 5% CO_2) for 3 days before electroporation to obtain optimal gene editing efficiency.

2. Conversion formula for eSpCas9 nuclease:
<table>
<tr><th>Cat. No.</th><th>Concentration</th><th>Mass</th><th>Molar weight</th><th>Volume</th></tr>
<tr><td>Z03624</td><td>10 mg/ml</td><td>1000 ng</td><td>6.25 pmol</td><td>0.1 µl</td></tr>
</table>
</td></tr>
<tr><td>Summary of recommended reaction system</td><td>Note: For Recommended Reaction Conditions Using GenCRISPR™ Ultra NLS-Cas9-basic GMP (Z03623) or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) with Neon™ electroporation system or 4D-Nucleofector® electroporation system, please see Appendix 2 in page 8.</td></tr>
<tr><th colspan="2">Cell Transfection</th></tr>
<tr><td>Step 1: Prepare cell culture medium</td><td>
1. On the day of transfection, add primary T cell-specific growth medium to a 48-well plate and pre-warm it at 37 °C. Transfer 240 µl complete growth medium for primary T cell culture into each well of the 48-well-plate, incubate at 37 °C again to pre-warm.
2. Prepare the electroporation transfection reagents for transfection. Set the electroporation program prior to transfection.
</td></tr>
<tr><td>Step 2: Prepare the RNP complex</td><td>
1. Mix GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP and sgRNA at an appropriate molar ratio (e.g. 1:2) with the electroporation buffer in a nuclease-free centrifuge tube as shown below: Note: The molar ratio of Cas9:sgRNA should be optimized mainly based on the target genes, cell types and transfection methods.
<table>
<tr><th>Reagent</th><th>Amount</th></tr>
<tr><td>GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624)</td><td>0.4 µl, 25 pmol (~4000 ng)</td></tr>
<tr><td>sgRNA</td><td>0.4 µl, 50 pmol (~1515 ng)</td></tr>
<tr><td>Electroporation buffer</td><td>3.2 µl</td></tr>
<tr><td>Total reaction volume</td><td>4 µl</td></tr>
</table>
2. Incubate the mix solution at 37 °C or room temperature for 15-30 min to assemble the RNP complex (~ 25 pmol).
</td></tr>
<tr><td>Step 3: Electroporation</td><td>
1. Centrifuge the cells for 10 minutes at 300 g at room temperature, completely remove the cell culture supernatant.
2. Wash the cells with 1 × PBS, centrifuge the cells for 10 min at 300 g at room temperature, and completely remove the supernatant.
3. Aspirate 16 µl electroporation buffer to suspend approximately 1.0 × 10^6 cells, mix thoroughly.
</td></tr>
</table>

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	4. Aspirate 16 µl of the cell suspension (from Step 3.3) and add to the RNP complex in electroporation buffer (from Step 2). If performing gene knock-in experiment, aspirate 16 µl cell suspension (from step 3.3) and 1-3 µg of HDR donor template to the RNP complex in electroporation (from step 2), mix thoroughly. 5. Use primary T cells-specific electroporation reagents from Lonza to proceed with the electroporation according to Lonza's P3 Primary Cell 4D-Nucleofector™ X Kit (Cat. No. SV4XP-3032) protocol. If electroporation reagents from other vendors are applied, please refer to the specific protocols. **Note:** 1) Avoid creating bubbles when pipetting and mixing reagents; 2) After mixing the RNP complex with the cells, immediately proceed with the transfection. 6. After the electroporation, transfer the cell mixture immediately to the pre-warmed complete media (from Step 1.1). Culture the cells in an incubator (37 °C, 5% CO_2) for 3-6 days.
Gene Editing Efficiency Analysis	
Step 4: Analyze gene editing efficiency and perform downstream applications	1. Methods for gene editing efficiency analysis at different levels: • Genome level- NGS and Sanger sequencing are generally used to determine gene editing efficiency precisely. GenScript is one of the most reliable vendors providing sequencing service, please see **genscript.com** or contact seq@genscript.com for more information. • Translational level- fluorescence-activated cell sorting (FACS) is generally used to determine the gene editing efficiency at functional level. ***Note:*** *Specific antibodies against target protein are required if performing FACS assay.* • For genome-wide off-target efficiency, GenScript is providing off-target monitoring service based on iGUIDE assay. Please see **genscript.com** or contact seq@genscript.com for more information. • Alternatively, use the GenCRISPR Mutation Detection Kit (L00688) to verify the gene editing efficiency for knock-out assays. 2. Proceed with downstream applications as needed.

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Evaluation of *In Vitro* Cleavage Efficiency of GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP

<table>
<tr><th colspan="2">Before Starting</th></tr>
<tr><td>Reagents preparation</td><td>1. Prepare the specific substrate DNA - We recommend GenScript DNA Synthesis Service (see genscript.com) for this application, or PCR amplification of the substrate DNA (Note: the PCR product needs to be purified prior to use).
2. Prepare the substrate DNA working solution at an appropriate concentration (e.g. 80 ng/µl) by diluting the stock solution with nuclease-free water on ice.
3. Prepare the 10 × Reaction Buffer (if needed, please contact us for free stock buffer).
4. A reaction volume of 20 µl is used, but can be adjusted according to the user's specific applications.</td></tr>
<tr><th colspan="2">In Vitro Cleavage</th></tr>
<tr><td>Cleavage procedures</td><td>Assemble the reaction in a nuclease-free centrifuge tube or a PCR strip tube at room temperature on a clean bench in the following order:
Table 2: Reaction process for in vitro cleavage of DNA
<table>
<tr><th>Components</th><th>Volume</th></tr>
<tr><td>10 × Reaction Buffer</td><td>2 µl</td></tr>
<tr><td>200 ng sgRNA</td><td>2 µl (100 ng/µl)</td></tr>
<tr><td>50 ng GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624)</td><td>2 µl (25 ng/µl)</td></tr>
<tr><td>Nuclease-free water</td><td>12 µl</td></tr>
<tr><td colspan="2">Mix thoroughly, incubate for 10 min to prepare RNP complex at 37 °C;</td></tr>
<tr><td>160 ng Substrate DNA</td><td>2 µl (80 ng/µl)</td></tr>
<tr><td colspan="2">Mix thoroughly, incubate for 2 hours at 37 °C;</td></tr>
<tr><td colspan="2">Add 1 µl Proteinase K (20 µg/µl), incubate for 20 min at 55 °C;</td></tr>
<tr><td colspan="2">After incubation, determine the in vitro cleavage efficiency by agarose gel electrophoresis.</td></tr>
</table></td></tr>
</table>

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Appendix 1: Quality Control Specifications

GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) is manufactured in compliance with ISO 9001 and ISO 13485 quality management system standards and with more stringent process controls and relatively complete document records. GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) meets the following quality control specifications.

Assay	Specifications
Appearance	Clear, colorless, liquid
Purity	≥ 95% as analyzed by SDS-PAGE
	≥ 95% as analyzed by SEC-HPLC
Concentration by A280	10 mg/ml ± 1 mg/ml
Bioactivity (*in vitro*)	≥ 85%
Residual DNase	≤ 10 ng/mg
Residual RNase	≤ 1 ng/mg
Endotoxin Level	≤ 10 EU/mg
Residual HCP	≤ 10 ng/mg
Residual HCD	≤ 1 ng/mg
Mycoplasma	< LOD
Bioburden	< 1 CFU/ml

Appendix 2: Recommended Reaction Conditions Using Different Electroporation Instruments

The following reaction conditions for cell transfection using GenCRISPR™ Ultra NLS-Cas9-basic GMP (Z03623) or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) with Neon™ electroporation system or 4D-Nucleofector® electroporation system are recommended as a starting point. Further optimization may be needed for best performance.

Neon™ electroporation system for cell lines and primary T cells		
Cas9 nuclease	GenCRISPR™ Ultra NLS-Cas9-basic GMP (Z03623)	GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624)
Cell numbers	2.0×10^5	
Cas9: sgRNA (molar ratio)	1:1-1:3	
RNP amount	2.5-15 pmol	5-15 pmol
HDR donor	1-3 μg	
Electroporation volume	10 μl	
4D-Nucleofector® electroporation system for cell lines and primary T cells		
Cas9 nuclease	GenCRISPR™ Ultra NLS-Cas9-basic GMP (Z03623)	GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624)
Cell numbers	$0.4\text{-}1.0 \times 10^6$	
Cas9: sgRNA (molar ratio)	1:1-1:5	
RNP amount	18.75-80 pmol	25-80 pmol
HDR donor	1-3 μg	

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Electroporation volume	20 µl

Appendix 3: Case Studies

1. ***TRAC* and *PD-1* knock-out in primary T cells using GenCRISPR™ Ultra NLS-SpCas9-basic GMP or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP with Lonza 4D-Nucleofector® electroporation system**

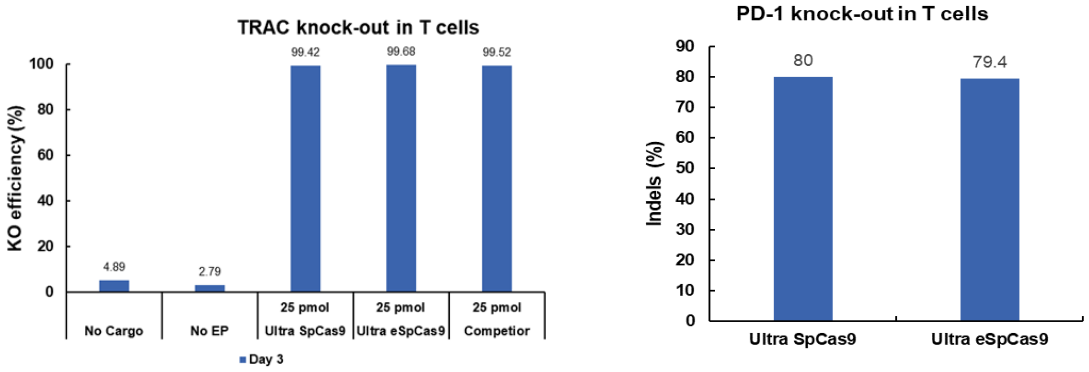

Figure 1: *TRAC* and *PD-1* knock-out efficiency in human primary T cells. The cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Z03623) or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) or wild type Cas9 and sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) for human *TRAC* and *PD-1* gene knock-out by electroporation. After transfection and cell culture, the *TRAC* knock-out efficiency was measured by FACS while *PD-1* knock-out efficiency was measured by Sanger sequencing. Both GenScript Ultra SpCas9 and eSpCas9 show high editing efficiency.

Note: This experiment used the following:

Cas9: sgRNA (molar ratio) =1:2

RNP amount: ~ 25 pmol

Cell number: 1.0×10^6 cells

2. ***NKG2A* knock-out in NK92 cells using GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP with 4D-Nucleofector® electroporation system**

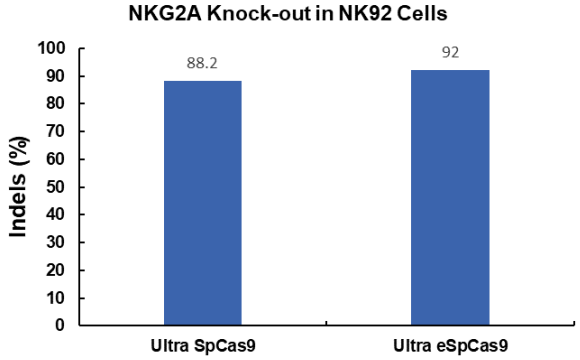

Figure 2: *NKG2A* knock-out efficiency in NK92 cells. The cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Z03623) or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) and sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) for human *NKG2A* gene knock-out by

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electroporation. After transfection and cell culture, the *NKG2A* knock-out efficiency was analyzed by Sanger sequencing. Both GenScript GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Z03623) or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) show high gene editing efficiency.

Note: This experiment used the following:

SpCas9/eSpCas9: sgRNA (molar ratio) = 1:1.2

RNP amount: 80 pmol

Cell number: 4.0×10^5 cells

3. *TRAC* knock-out in 293T cells using GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP with Neon™ electroporation system

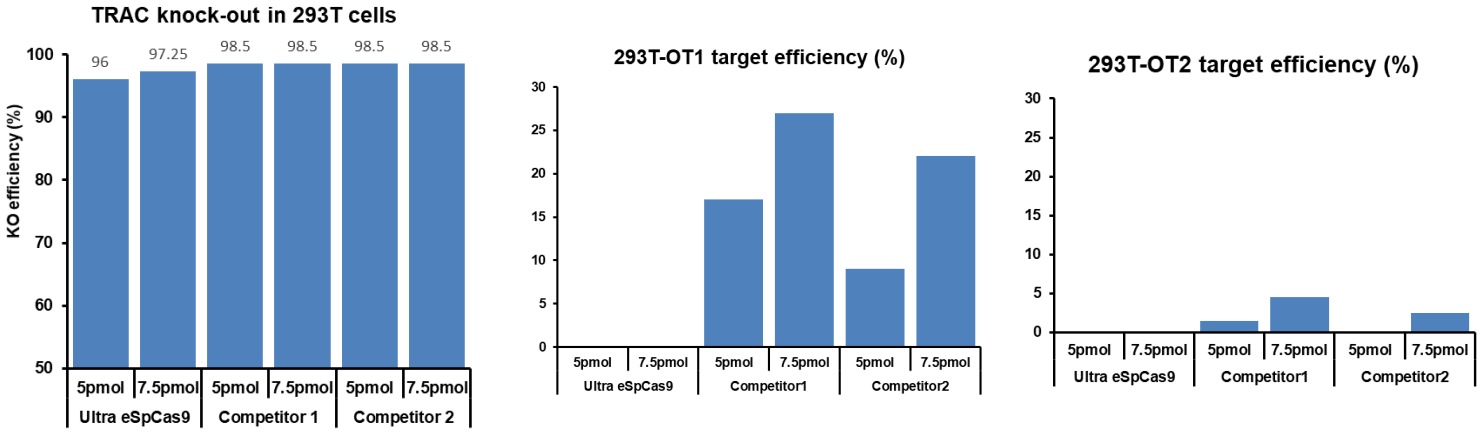

Figure 3: *TRAC* knock-out and off-target effect analysis in 293 T cells. The cells were transfected with GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) or wild type Cas9 proteins from competitors and sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) for human *TRAC* gene knock-out by electroporation. After transfection and cell culture, the *TRAC* knock-out efficiency and off-target effect were analyzed by Sanger sequencing. GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) shows a comparative gene editing efficiency to competing products (wild type SpCas9), but a greatly lower off-target effect.

Note: This experiment used the following:

eSpCas9: sgRNA (molar ratio) =1:3

RNP amount: 5-7.5 pmol

Cell number: 2.0×10^5 cells

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4. **Comparison of *TRAC* knock-out in 293 T cells between using GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Z03623) and using GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) with Neon™ electroporation system**

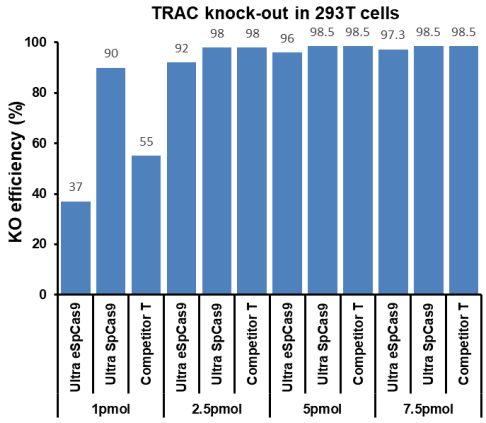

Figure 4. *TRAC* knock-out efficiency in 293 T cells. The cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Z03623) or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) or wild type Cas9 from competitor and same sgRNA for human *TRAC* gene knock-out by electroporation. After transfection and cell culture, the gene editing efficiency were measured by Sanger sequencing**.** Data shows that GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) demonstrate a lower gene editing efficiency with a small amount of RNP than wild type SpCas9 from both GenScript and competitor, while increasing a little RNP amount exhibits a comparable editing efficiency with wild type SpCas9.

Note: This experiment used the following:

Cas9: sgRNA (molar ratio) =1:3

RNP amount: 1-7.5 pmol

Cell number: 2.0×10^5 cells

5. **Gene knock-in at *TRAC* site in primary T cells using GenCRISPR™ Ultra NLS-SpCas9-basic GMP or GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP with 4D-Nucleofector® electroporation system**

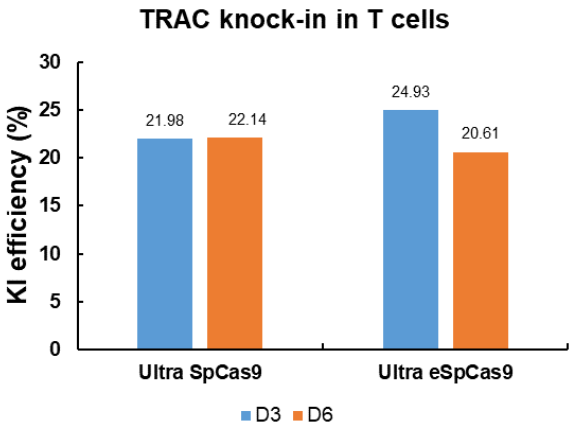

Figure 5: Gene knock-in efficiency at *TRAC* site in primary T cells. The cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Z03623) or GenCRISPR™ Ultra eSpCas9-2NLS-basic

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GMP (Z03624), sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) and dsDNA HDR template (synthesized by GenScript Double-strand DNA Synthesis Services) for gene knock-in at *TRAC* site in primary T cells by electroporation. After transfection and cell culture, the gene knock-in efficiency was analyzed by FACS. Both GenScript GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Z03623) and GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) show high gene knock-in efficiency.

Note: This experiment used the following:

SpCas9/eSpCas9: sgRNA (molar ratio) =1:2

RNP amount: ~ 25 pmol

Cell number: 1.0×10^6 cells

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References

1. Jinek, Martin, et al. "A programmable dual-RNA–guided DNA endonuclease in adaptive bacterial immunity." *science* 337.6096 (2012): 816-821.
2. Chen, Sean, et al. "Highly efficient mouse genome editing by CRISPR ribonucleoprotein electroporation of zygotes." *Journal of Biological Chemistry* 291.28 (2016): 14457-14467.
3. Larson, Matthew H., et al. "CRISPR interference (CRISPRi) for sequence-specific control of gene expression." *Nature protocols* 8.11 (2013): 2180-2196.
4. Ran, F. Ann, et al. "Genome engineering using the CRISPR-Cas9 system." *Nature protocols* 8.11 (2013): 2281-2308.
5. Kim, Sojung, et al. "Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins." *Genome research* 24.6 (2014): 1012-1019.

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