Manual

Version: 04

Update: 02/25/2025

GenCRISPR™ Ultra eSpCas9-2NLS-GMP

Cat. No.: Z03624-GMP

Table of Contents

GenScript USA, Inc.

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Product Introduction

GenCRISPR™ Ultra eSpCas9-2NLS-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 both wild-type and mutant Cas9 nucleases in GMP Grade* for selection based on your specific downstream applications. The GMP Grade Cas9 nucleases can be utilized as ancillary materials for Cell, Gene, and Tissue-Based Products and are manufactured under the standards listed below:

- *USP <1043>. Ancillary materials for Cell, Gene and Tissue-engineered products.*
- *ICH Q7 Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients*
- *NMPA: Technical guidelines for pharmaceutical research and evaluation of immune cell therapy products*
- *Guideline and procedure specified in Chinese Pharmacopeia.*

Table 1: GenCRISPR™ Ultra Cas9 nuclease selection

Nucleases	Description
GenCRISPR™ Ultra NLS-Cas9-GMP (Cat. No. Z03623-GMP)	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-GMP (Cat. No. Z03624-GMP)	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-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: 10.0 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±5 °C for up to 12 months from the date of manufacture. Avoid repeated freeze-thaw cycles.

*__GMP Grade__ is a specific term that GenScript uses to describe the Cas9 nucleases manufactured in GMP-complaint facility and in compliance with guidelines of Current Good Manufacturing Practice (cGMP), ISO 9001 and ISO 13485 quality management system standards with stringent process controls and complete documentation records. GenScript

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is capable of providing documents, site audits, 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-GMP	Z03624-GMP-2.5	2.5 mg	10.0 mg/ml	-20±5 °C
	Z03624-GMP-5	5 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

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gRNA and HDR Template Preparation

If GMP Grade sgRNA from GenScript Synthetic sgRNA Service is being used, please keep the RNA oligonucleotides tightly sealed at -20±5 °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 ±5°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 ±5°C for 3 months.

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, 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-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-GMP</td><td>10.0 mg/ml</td><td>1000 ng</td><td>6.25 pmol</td><td>0.1 µl</td></tr>
</table>
Note: the mutant eSpCas9 may have different preference to sgRNA for specific target genes compared to wild type SpCas9, it's recommended to screen a most appropriate sgRNA for eSpCas9 specifically.
</td></tr>
<tr><td>Summary of recommended reaction system</td><td>
1. For Recommended Reaction Conditions Using GenCRISPR™ Ultra NLS-Cas9-GMP (Cat. No. Z03623-GMP) or GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) with Neon™ electroporation system or 4D-Nucleofector® electroporation system, please see Appendix 2 in page 8.
2. For Recommended Reaction Conditions Using GenCRISPR™ Ultra NLS-Cas9-GMP (Cat. No. Z03623-GMP) or GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) with Lonza 4D-Nucleofector® Electroporation System for scale-up transfection, 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 complete 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-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-GMP (Cat. No. Z03624-GMP)</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>
</td></tr>
</table>

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	2. Incubate the mix solution at 37 °C or room temperature for 15-30 min to assemble the RNP complex (~ 25 pmol).
Step 3: Electroporation	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 × DPBS or1 × 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. 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 (Cat. No. L00688) to verify the gene editing efficiency for knock-out assays. 2. Proceed with downstream applications as needed.

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Appendix 1: Quality Control Specifications

GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) is manufactured in compliance with ISO 13485 and GMP quality management system standards and with stringent process controls and complete documentation records. GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) meets the following quality control specifications.

Assay	Specifications
Appearance	Clear, colorless, liquid
Identity	Consistent with the theoretical sequence by LC-MS/MS
Purity	≥ 95.0% as analyzed by SDS-PAGE
	≥ 95.0% as analyzed by SEC-HPLC
Concentration	10.0 mg/ml ± 1.0 mg/ml as analyzed by A280
Bioactivity	≥ 85.0% as analyzed by in *vitro assay*
Residual DNase	≤ 10 ng/mg by Ambion DNaseAlert
Residual RNase	≤ 1 ng/mg by Ambion RNaseAlert
Endotoxin Level	< 10 EU/mg as analyzed by gel clotting method
Host Cell Protein	≤ 10 ng/mg by ELISA
Host Cell DNA	≤ 1 ng/mg by qPCR
Mycoplasma	Non-detectable by Culture method
Sterility	No growth by membrane filtration method

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Appendix 2: Recommended Reaction Conditions Using Different Electroporation Instruments

The following reaction conditions for cell transfection using GenCRISPR™ Ultra NLS-Cas9-GMP (Cat. No. Z03623-GMP) or GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) with Neon™ electroporation system or 4D-Nucleofector® electroporation system are recommended as a starting point. Further optimization may be needed for best performance.

1. Transfection using Neon™ or 4D-Nucleofector® electroporation system in small scale assays

Neon™ electroporation system for cell lines and primary T cells		
Cas9 nuclease	GenCRISPR™ Ultra NLS-Cas9-GMP (Cat. No. Z03623-GMP)	GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP)
Cell numbers	2.0×10^5-5.0×10^5	
Cas9: sgRNA (molar ratio)	1:1-1:3	
RNP amount	2.5-15 pmol	5-15 pmol
HDR donor	0.5-1 μg	
Electroporation volume	10 μl	
4D-Nucleofector® electroporation system for cell lines and primary T cells		
Cas9 nuclease	GenCRISPR™ Ultra NLS-Cas9-GMP (Cat. No. Z03623-GMP)	GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP)
Cell numbers	0.4×10^6-1.0×10^6	
Cas9: sgRNA (molar ratio)	1:1-1:5	
RNP amount	18.75-80 pmol	25-80 pmol
HDR donor	1-3 μg	
Electroporation volume	20 μl	

2. Transfection using 4D-Nucleofector® electroporation system in large scale (100μl) assays in primary T cells

4D-Nucleofector® electroporation system for primary T cells		
Cas9 nuclease	GenCRISPR™ Ultra NLS-Cas9-GMP (Cat. No. Z03623-GMP)	GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP)
Cell numbers	5.0×10^6	
Cas9: sgRNA (molar ratio)	1:2	
RNP amount	125 pmol	
Electroporation volume	100 μl	

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Appendix 3: Case Studies

1. Consistent high knockout efficiency in primary T cells using different grade Cas9 nucleases

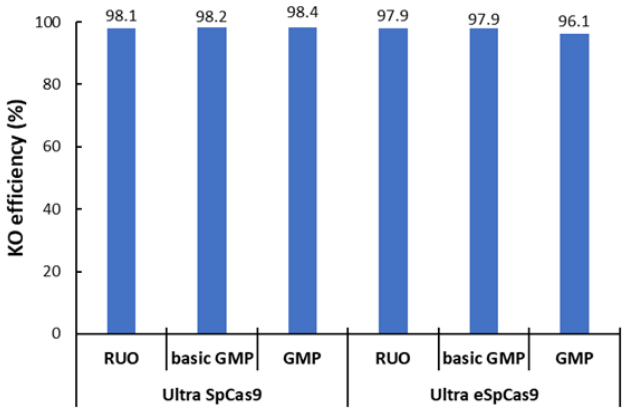

Figure 1: *TRAC* knock-out in primary T cells using GMP, basic GMP and RUO Grade GenCRISPR™ Ultra Cas9 or GenCRISPR™ Ultra eSpCas9 with Lonza 4D-Nucleofector® electroporation system. The cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-Research (Cat. No. Z03621), GenCRISPR™ Ultra NLS-SpCas9-basic GMP (Cat. No. Z03623), GenCRISPR™ Ultra NLS-SpCas9-GMP (Cat. No. Z03623-GMP) or GenCRISPR™ Ultra eSpCas9-2NLS-Research (Cat. No. Z03622), GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Cat. No. Z03624), GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) and sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) for human *TRAC* gene knock-out by Lonza 4D-Nucleofector® electroporation. After transfection and cell culture, the *TRAC* knock-out efficiency was measured by FACS. This data indicated that GMP, basic GMP and RUO Grade GenCRISPR™ Ultra Cas9 or GenCRISPR™ Ultra eSpCas9 enable consistent high gene editing efficiency in primary T cells.

Note: This experiment used the following:

Cell Type:	Primary T cells
Transfection Method:	Electroporation
Target Gene:	*TRAC*
Cell Amount:	1.0 × 10^6 cells
Cas9:sgRNA ratio:	1:2
RNP Amount:	25 pmol

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2. Consistent high editing efficiency in primary T cells in different electroporation scale

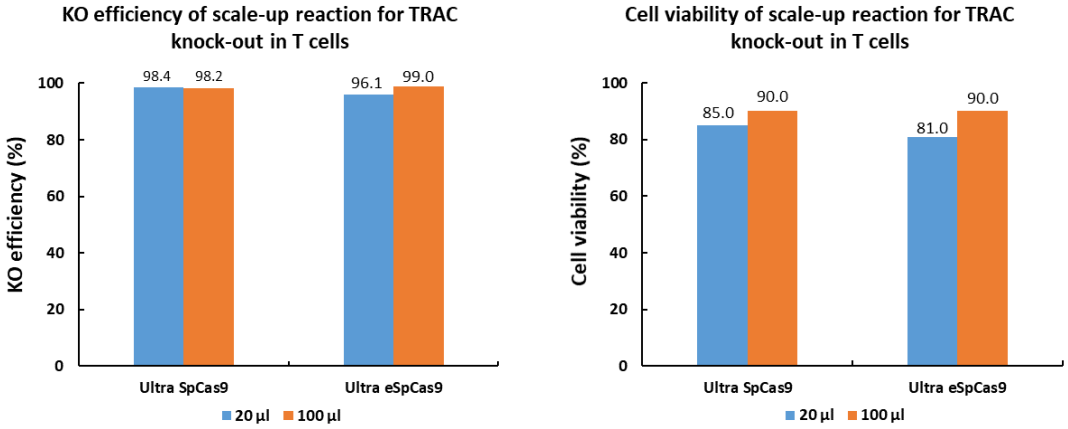

Figure 2: *TRAC* knock-out in primary T cells using GMP Grade GenCRISPR™ Ultra Cas9 or GenCRISPR™ Ultra eSpCas9 with Lonza 4D-Nucleofector® electroporation system in different electroporation scale. Human primary T cells were cultured for the test. The cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-GMP (Cat. No. Z03623-GMP) or GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP), and sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) for human *TRAC* gene knock-out by electroporation in a small scale of 20 µl reaction volume and a scale-up of 100 µl reaction volume, respectively. After transfection and cell culture, the *TRAC* knock-out efficiency was measured by FACS. The data indicated that GMP Grade GenCRISPR™ Ultra Cas9 and GenCRISPR™ Ultra eSpCas9 maintain consistent high editing efficiency in different electroporation scale.

Note: This experiment used the following:

Electroporation Scale	20 µl	100 µl
Cas9 Nuclease Amount	4 µg / 25 pmol	20 µg / 125 pmol
Cell Amount	1.0 × 10^6 cells	5.0 × 10^6 cells
Cas9: sgRNA (molar ratio)	1:2	1:2
RNP Amount	25 pmol	125 pmol

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3. **High knock-in efficiency in primary T cells using GMP Grade GenCRISPR™ Ultra Cas9 and Ultra eSpCas9 Nuclease**

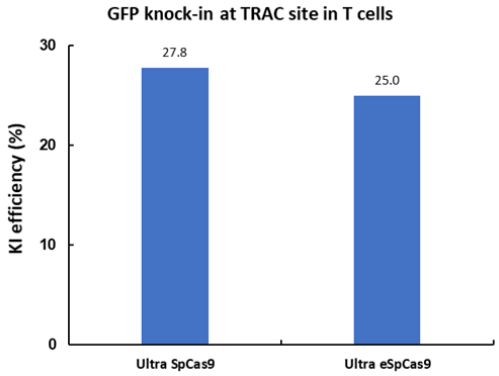

Figure 3: GFP gene knock-in at *TRAC* site in primary T cells using GMP Grade GenCRISPR™ Ultra Cas9 or GenCRISPR™ Ultra eSpCas9 with Lonza 4D-Nucleofector® electroporation system. The primary T cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-GMP (Cat. No. Z03623-GMP) or GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP), and sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) as well as dsDNA HDR template (synthesized by GenScript Double-strand DNA Synthesis Services) for *GFP* 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-GMP (Z03623-GMP) and GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Z03624-GMP) maintain high gene knock-in efficiency in primary T cells.

Note: This experiment used the following:

Cell Type:	Primary T cells
Transfection Method:	Electroporation
Target Gene:	*TRAC*
Gene Knocked-in:	*GFP*
Cell Amount:	1.0 × 10^6 cells
Cas9:sgRNA ratio:	1:2
RNP Amount:	25 pmol

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4. **High knockout efficiency in cell lines using GMP Grade GenCRISPR™ Ultra Cas9 and Ultra eSpCas9 Nuclease**

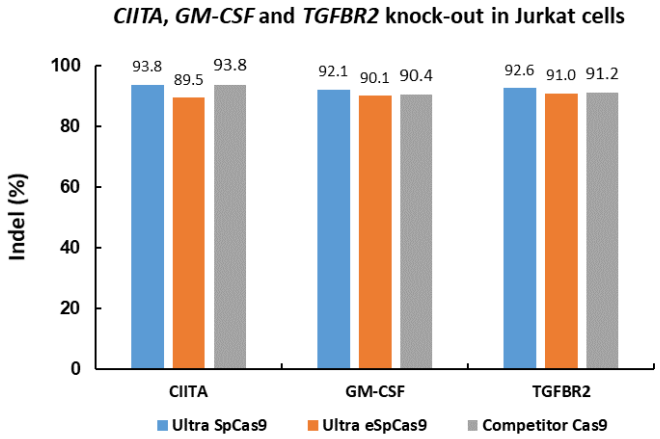

Figure 4: *CIITA*, *GM-CSF* and *TGFBR2* knockout in Jurkat cells using GMP Grade GenCRISPR™ Ultra SpCas9 or GenCRISPR™ Ultra eSpCas9 Nuclease with Neon™ electroporation system. Jurkat cells were cultured for the test. The cells were transfected with GenCRISPR™ Ultra NLS-SpCas9-GMP (Cat. No. Z03623-GMP) or GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) or Cas9 proteins from other vendors, and sgRNA (synthesized by GenScript CRISPR Synthetic sgRNA Services) for *CIITA, GM-CSF* and *TGFBR2* gene knockout by electroporation. After transfection and cell culture, the gene editing efficiency was analyzed by Sanger sequencing. Both GenScript GenCRISPR™ Ultra NLS-SpCas9-GMP (Cat. No. Z03623-GMP) and GenCRISPR™ Ultra eSpCas9-2NLS-GMP (Cat. No. Z03624-GMP) maintain high gene knockout efficiency in Jurkat cells.

Note: This experiment used the following:

Cell Type:	Jurkat cells
Transfection Method:	Electroporation
Target Gene:	*CIITA, GM-CSF, TGFBR2*
Cell Amount:	0.5 × 10^6 cells
Cas9:sgRNA ratio:	1:3
RNP Amount:	7.5 pmol

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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.
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3. Larson, Matthew H., et al. "CRISPR interference (CRISPRi) for sequence-specific control of gene expression." *Nature protocols* 8.11 (2013): 2180-2196.
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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.

For laboratory research or clinical research and manufacturing of cell-based products. Direct human use, including taking orally and injection are forbidden.