GenScript
Make Research Easy

GenCRISPR™ Ribonucleoprotein Gene Editing Handbook

First Revision

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User must read the protocol in its entirety before starting the GenCRISPR workflow

Introduction

The ability to perform genome editing by manipulating DNA and RNA is one the greatest tools of modern science. In the last two decades, advances in gene editing technologies, specifically Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology, has provided scientists with a reliable, highly precise and efficient methodology for genome editing. Scientists can efficiently inhibit a gene function, alter gene expression, and identify a genomic target for diagnostic purposes in a variety of biological systems.

Scientists can refer to this handbook for best practices and protocols for performing ribonucleoprotein (RNP) complex gene editing using GenScript's GenCRISPR solutions.

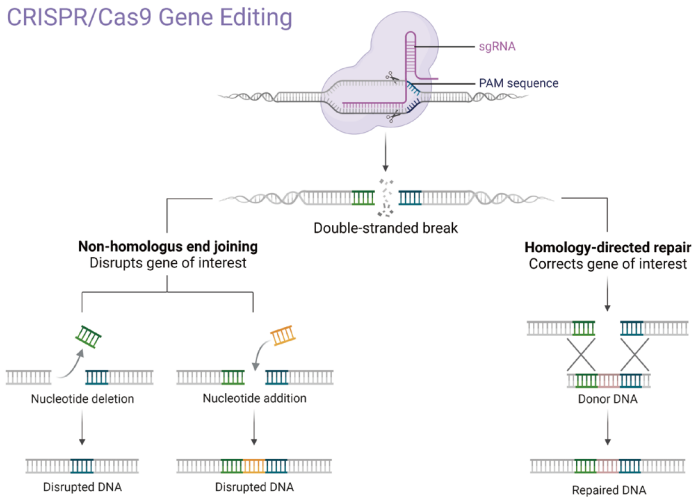

Figure 1. CRISPR Cas9 Gene Editing
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Product Selection

The GenCRISPR™ nuclease product line provides scientists with a wide selection of research use and GMP compliant Cas nucleases for RNP-based RNA and DNA editing. Additionally, GenScript's GenCRISPR™ kits complete the gene editing workflow by providing easy-to-use solutions for identifying functional gRNAs before transfection to evaluate the efficiency of the gene editing experiment. The GenCRISPR antibodies are used for the corresponding Cas nucleases screening and detection.

Direct delivery of CRISPR/CAS system as a ribonucleoprotein (RNP) complex consisting of Cas protein and single guide RNA is a powerful and widespread gene editing method that has been shown to reduce the challenges encountered with other CRISPR gene editing techniques, such as viral and plasmid delivery.

Challenges include off-target effects, reduced cell viability and transcription/translational challenges.

The Ultra Cas9 product line consists of GMP complaint wild type Cas9 and eSpCas9(a mutant form of Cas9). The wild type Cas9 is effective in most gene editing applications, while the eSpCas9 is ideal for applications that are sensitive to off-target effects. In some cases, improvement in editing efficiency can be achieved by optimization of reaction conditions, such as increasing RNP amount.

Figure 2. GenCRISPR Product Offering

GenCRISPR™ Enzyme Selection Guide				
Product	**Target Application**	**PAM orPFS (5' - 3')**	**Features**	**Benefits**
Ultra Cas9 Z03621 Z03623 Z03623-GMP	DNA-Editing	NGG	N-NLS	Enhanced performance GMP compliant recommended for gene knock-out workflows
Ultra eSpCas9 Z03622 Z03624 Z03624-GMP	DNA-Editing with high specificity	NGG	2× NLS	Enhanced specificity GMP compliant recommended for workflows that require low off-target efficiency
Cas9 v1.1 Z03701	DNA-Editing with enhanced knock-in efficiency	NGG	2× Modified NLS	Enhanced Knock-in efficiency
Cas9 v1.2 Z03702	DNA-Editing with enhanced knock-in efficiency	NGG	2× Modified NLS	Enhanced Knock-in efficiency
Cas9 Z03386 - Z03385 Z03388 - Z03468 Z03389 - Z03469	DNA-Editing	NGG	*various* *NLS options*	Affordable *in vitro* gene editing
D10A Cas9 Nickase Z03390	DNA-Editing with high specificity	NGG	N-NLS	Enhanced specificity Single-strand cleavage
EGFP Cas9 Z03393 - Z03467	DNA-Editing	NGG	N-NLS	EGFP fused protein facilitates verification of RNP delivery by Fluorescence-activated cell sorting (FACS)

SaCas9 Z03699	DNA-Editing	NNGRRT	2× NLS	Compact nuclease
Cas12a (Cpf1) Z03502	DNA-Editing and *in vitro* diagnosis	TTTV	C-NLS	Staggered Double-strand cut and collateral ssDNA cut
Cas13a (C2c2) Z03486	RNA-Editing and *in vitro* diagnosis	A, U or C	Cis-cleavage and trans-cleavage	RNA editing and collateral unspecific RNA cutting

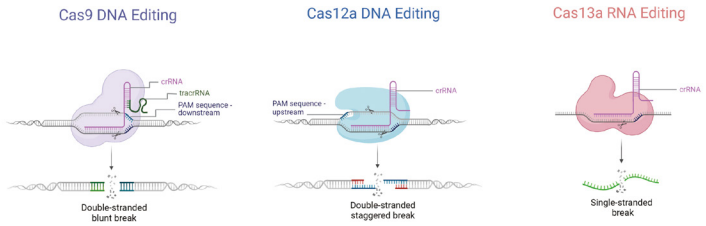


Figure 3. Cas9, Cas12a and Cas13a Gene Editing
Created with BioRender.com

GenCRISPR Nuclease Family			
Feature	**Cas9**	**Cas12a**	**Cas13a**
Targeted Nucleic Acid	ds DNA	ds or ss DNA	ss RNA
Size of the protein	~160 kDa	~150 kDa	~140 kDa
Guide	sgRNA (tracrRNA + crRNA)	crRNA	crRNA
Type of cut	Double-strand, blunt cut	Double-strand, staggered cut	ss RNA
Cleavage	Cis-cleavage	Cis-cleavage Trans-cleavage	Cis-cleavage Trans-cleavage
Targeting restriction	GC-rich sequences	AT-rich sequences	
PAM/PFS (5'-3')	NGG downstream	TTTV upstream	A, U or C
GenScript variants	eSp, eGFP, Nickase, WT	WT	WT
Species	Various	*Acidaminococcus sp. (strain BV3L6)*	*Leptotrichia wade*
Compliance	Research Use Basic GMP GMP	RUO	RUO

Cas9 Protocol Overview

GenCRISPR™ Kit Offerings		
Product	**Application**	**Benefits**
GenCrispr gRNA Synthesis Kit 20 reactions - L00694-20	*In vitro* transcription of single-gRNA (gRNA)	Easy to use, cost effective
GenCrispr gRNA Screening Kit 30 reactions - L00689-30	Designed to screen gRNA/Cas9 efficiency before transfecting cells	Used for ensuring the optimal conditions and gRNA design suitable for the experiment
GenCrispr Mutation Detection Kit 25 reactions - L00688-25 T7 Endonuclease I Z03396	Simple protocol for detecting targeting efficiency in genome editing experiments	T7 Endonuclease-based detection of site specific genome editing events
FnCpf1 Antibody (9H6) and (14H3), mAb, Mouse A01957 - A01958	Nuclease detection	Evaluation of transfection efficiency by Immunofluoresce or Western Blotting
SpCas9 Antibody (4A1) and (14B6), mAb, Mouse A01935 - A01936		
SaCas9 Antibody (11C12) and (26H10), mAb, Mouse A01951 - A01952		
Cas9 Antibody, pAb, Rabbit A01885		

A Solution for Every Step of the Gene Editing Workflow

Ribonucleoprotein (RNP) complex delivery

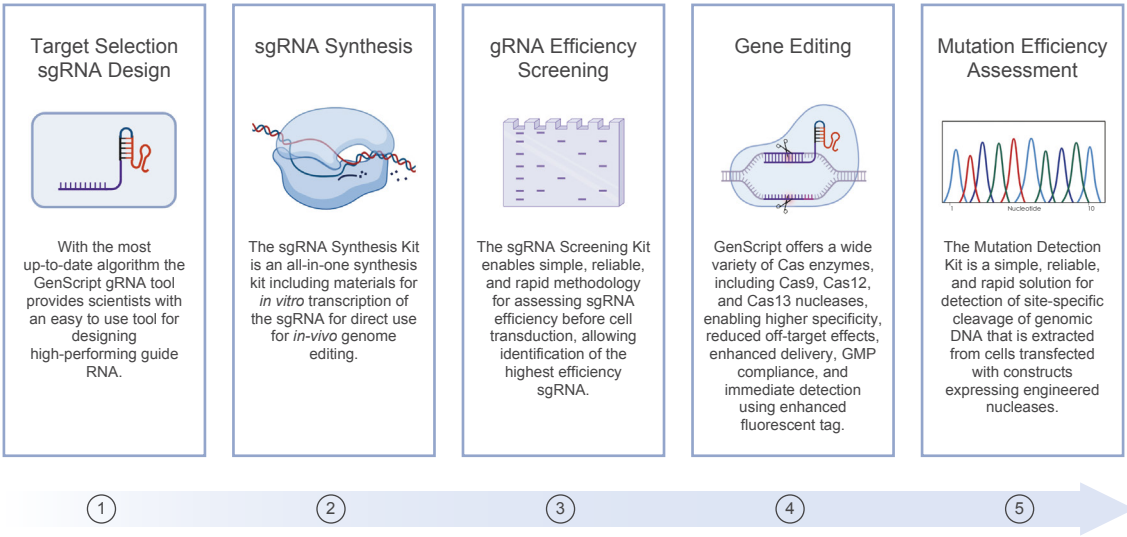


Figure 4. GenCRISPR Comprehensive Solutions
Created with BioRender.com

Step 1. Design of experiment – target selection. Scientists are encouraged to use the GenCRISPR gRNA design tool

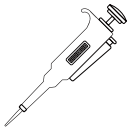
Step 2. gRNA Synthesis – the gRNA can be synthesized using GenScript gRNA synthesis services or using the GenCRISPR gRNA synthesis kit for Cas9 applications.

Step 3. gRNA Efficiency Screening – after the gRNA has been synthesized, the Scientist is highly encouraged to test the gRNA efficiency using the screening kit.

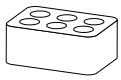
Step 4. After the gRNA has been screened for efficiency, the gRNA is complexed with the GenCRISPR nuclease and delivered into the host where gene editing will take place.

Step 5. After gene editing has been conducted, the Scientist is encouraged to assess the mutation efficiency using next generation sequencing or the GenScript Mutation Detection Kit.

Equipment

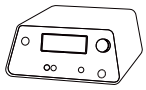


Calibrated hand-held pipette set

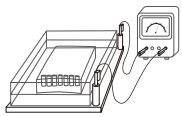


Heating block

PCR instrument



Electroporation system



PAGE equipment or agarose gel electrophoresis equipment

Fluorescence-Activated Cell Sorting (FACS)

Resources

GenCRISPR gRNA Design Tool
https://www.genscript.com/gencrispr-grna-design-tool.html

Reagents service – CRISPR Synthetic gRNA Services
gene@genscript.com

GenScript features educational webinars on a wide range of topics, such as Gene Editing https://www.genscript.com/webinars

Materials

Step of the Process	Product	Recommended Product and Supplier
General reagents	Nuclease-free water	Supplied from a third party vendor
	Nuclease-free labware (pipettes, tubes, plates, etc.)	Supplied from a third party vendor

gRNA and DNA Synthesis	gRNA containing the targeting sequence in the genomic region of interest	Option #1: GenCRISPR gRNA Synthesis Kit GenScript Catalog Number: L00694 Option #2: CRISPR Synthetic gRNA Services gene@genscript.com
	Substrate DNA containing the target sequence for *in vitro* digestion of DNA	Single-strand DNA and Double-strand DNA Synthesis Services gene@genscript.com
	HDR donor template containing the target sequence for knock-in application	GenScript PCR Reagents https://www.genscript.com/pcr.html
gRNA Efficiency Screening	gRNA screening	GenCRISPR gRNA Screening Kit GenScript Catalog Number: L00689
Gene Editing	GenCRISPR Cas Nucleases	Example: GenCRISPR™ Ultra NLS-Cas9-GMP GenScript Catalog Number: Z03623-GMP
	Proteinase K for *in vitro* digestion of DNA	Supplied from a third party vendor
	Reagents for RNP transfection	Electroporation or lipofection reagents
Genome Mutation Efficiency Screening	Mutation Detection	GenCRISPR Mutation Detection Kit GenScript Catalog Number: L00688 GenCRISPR T7 Endonuclease I GenScript Catalog Number: Z03396 NGS or Sanger Sequencing (seq@genscript.com)

Design of Experiment

Design the target sequence

GenCRISPR Cas9 gRNA Design Tool
https://www.genscript.com/gencrispr-grna-design-tool.html

A commonly used tool is the CCTop: An Intuitive, Flexible and Reliable CRISPR/-Cas9 Target Prediction Tool
https://cctop.cos.uni-heidelberg.de:8043/

Best practices for gRNA design

• PAM must not be in the gRNA.
• The length of gRNAs is about 19-22 nucleotides.
• The length and design of the various gRNAs should be dependent on the Cas nucleases type. Please refer to the table listing the gRNA sequences by Cas Type.
• If the genome editing is tested by gel electrophoresis, it is highly recommended that the gRNA is designed with distinguishable restriction enzyme cutting sites that can be detected later on using gel electrophoresis.
• The GC content of 40%-60% is preferable for gRNA.
• For gene knock out, the binding site of gRNA should be as close to the coding region downstream of ATG as possible. To induce frameshift mutation, the first or second exon is preferred.
• Whole genome off-target effect analysis is suggested. At least 5 bases can be allowed for the base mismatch, and whether the off-target is located in the gene encoding regions needs to be confirmed. In addition to the off-target analysis, we recommend detecting base insertion or deletion in off-targets by sequencing.

gRNA sequences by Cas Type	
Cas Type	**gRNA Sequence**
SpCas9	5'NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAG CAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT GGCACCGAGTCGGTGCTTTT3'CACCGAGTCGGTGCTTTT3'
SaCas9	5'NNNNNNNNNNNNNNNNNNNNGTTTTAGTACTCTGGAAA-CAGAATCTACTAAAACAAGG-CAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGATTT3'
AsCas12a	5'UAAUUUCUACUCUUGUAGAUNNNNNNNNNNNNNNNNNNN NNN3'
LwaCas13a	5'GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACNNN-NNNNNNNNNNNNNNNNNNNNNNNNN3'

Choose the DNA target sequence that will correspond to your actual gRNA target sequence as shown in figure 5 according to the following guidelines:

1. The DNA target sequence you choose must end with the proto-spacer adjacent motif (PAM) sequence, NGG, on its 3' end. Only DNA sequences that are 20 nucleotides upstream of a PAM sequence can be used for CRISPR/Cas9.
2. Any target sequence can be used if the sequence is followed by the PAM sequence, NGG. However, to minimize off-target cleavage events, the entire target sequence (including the PAM) should have at least three base mismatches with any other non-targeted genomic sequence. Off-target events should be especially low if the mismatches are in, or adjacent to, the PAM. The majority of

online tools for gRNA design will predict off-target sequences for a given target sequence.

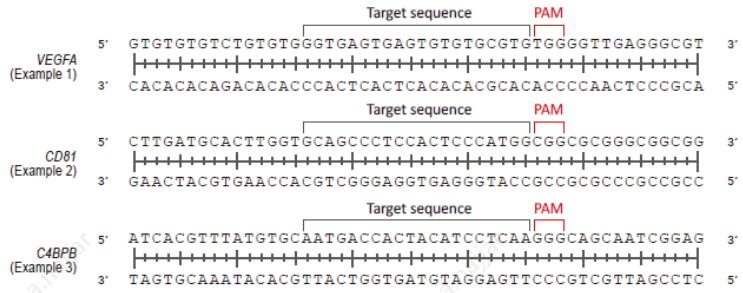


Figure 5. Example of how the DNA target sequence can be selected

gRNA Synthesis and Efficiency Screening

Synthesize gRNA

We recommend the GenCRISPR gRNA Synthesis Kit (L00694) for *in vitro* transcription of gRNA, or CRISPR Synthetic Guide RNA Services gene@genscript.com.

sgRNA Synthesis – *In Vitro* Transcription of sgRNA

Prepare the gRNAs by *in vitro* transcription (IVT) using the GenCRISPR™ gRNA Synthesis Kit (Cat # L00694).

1. The gRNA DNA template can be assembled using PCR reaction. Order the primers containing target gRNA DNA sequence and PCR amplify the gRNA DNA template under the guide of gRNA synthesis kit manual. (Please find the details in the product manual. For example, HPRT target gRNA sequence: GCATTTCTCAGTCCTAAACA Order primers:
   HPRT Target F1: TAATACGACTCACTATAG + GCATTTCTCAGTCCTA
   HPRT Target R1: TTCTAGCTCTAAAAC + TGTTTAGGACTGAGAAAT
2. Purify the PCR product.
3. Generate the gRNA by *in vitro* transcription. Perform the IVT reaction following the gRNA synthesis kit manual.
4. Purify the gRNA.

RNA Oligo Preparation for Lyophilized gRNA

Note: Scientists can skip this section if the RNA Oligo is in liquid form.

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, 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, 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 (100 µM) can be stored at -20 °C for 12 months.

4. To prepare working solutions:

   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 (25 µM) can be stored at -20 °C for 3 months.
   If working with gRNA system, for 25 µM working concentration gRNA solution.

gRNA Oligo (100 µM)	10 µl
Nuclease-free water	30 µl

gRNA Efficiency Screening

Screen the high efficiency gRNAs by using GenCRISPR gRNA Screening Kit (Cat# L00689).

1. Prepare the experimental DNA substrate by amplifying target fragments from your samples.
2. Set up a cleavage reaction containing your experimental gRNA sample and your experimental target DNA substrate, in parallel with a positive control reaction.

Reagents	Experimental cleavage reaction	Positive Control cleavage reaction
Experimental gRNA	100-500 ng	-
Positive Control gRNA	-	1 µl (100 ng)
GenCRISPR Cas9 Nuclease	~0.25 µl (50 ng)	~0.25 µl (50 ng)
10X Reaction Buffer	2 µl	2 µl
Nuclease-free water	0-17 µl	Up to 18 µl
Incubate the above mixture for 10 min at 37 °C		
Experimental DNA substrate	~160 ng	-
Positive control substrate	-	2 µl
Total volume per reaction	20 µl	20 µl

3. Mix gently and Incubate at 37 °C for 1-2 hours.
4. Analyze 10 µl reactions on a 1% agarose gel alongside a negative control.

Example: The *in vitro* cleavage of different gRNAs.
Only gRNA9 was observed to induce cleavage.

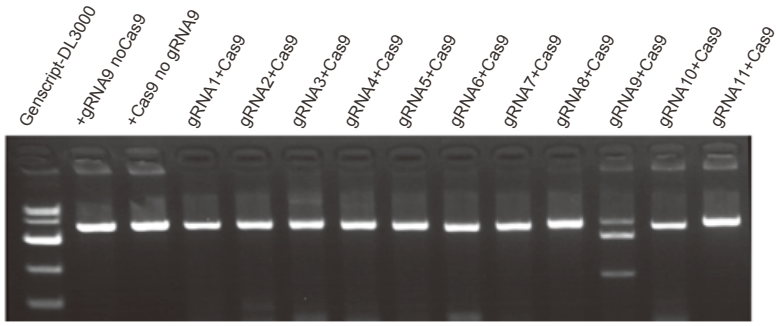


Figure 6. gRNA efficiency screening using GenCRISPR gRNA screening kit. Eleven unique synthesized gRNAs were incubated with Cas9 nuclease for 1 h at 37 °C, then detected on DNA agarose gel.

Genome Editing Using RNP Complex

Transfection of the RNP complex

Note: The following protocols are based on the Ultra Cas9 and Ultra eSpCas9 products: Z03621, Z03622, Z03623, Z03624, Z03623-GMP, Z03624-GMP.

1. *In vitro* Digestion
2. Knock Out
3. Knock In

1. Operation procedures for *in vitro* digestion of DNA

1.1 Reagents preparation

a. Prepare the specific gRNA - We recommend the GenCRISPR gRNA Synthesis Kit (L00694) for the gRNA *in vitro* transcription of gRNA or CRISPR Synthetic gRNA Services (gene@genscript.com).

b. Prepare the specific substrate DNA - We recommend GenScript DNA Synthesis Service (gene@genscript.com) for this application, or PCR amplification of the substrate DNA (Note: the PCR product needs to be purified prior to further assay).

c. Prepare the gRNA working solution at an appropriate concentration (e.g. 50 ng/μl) by diluting the stock solution with nuclease-free water on ice.

d. Prepare the substrate DNA work solution at an appropriate concentration (e.g., 80 ng/μl) by diluting the stock solution with nuclease-free water on ice.

e. Prepare the 10 × Reaction Buffer (if needed, please contact us for free stock buffer).

f. A reaction volume of 20 μl is used, but can be adjusted according to the user's specific applications.

1.2 Assay procedure

Assemble the reaction in a nuclease-free microcentrifuge tube or a PCR strip tube at room temperature on a clean bench in the following order:

Components	Volume SpCa9 (Z03623)	Volume eSpCa9 (Z03624)
10× Reaction Buffer	2 μl	2 μl
sgRNA	100 ng 2 μl (50 ng/μl)	200 ng 2 μl (100 ng/μl)
50 ng GenCRISPR™ Ultra Cas9 Nuclease	2 μl (25 ng/μl)	2 μl (25 ng/μl)
Nuclease-free water	12 μl	12 μl
Mix thoroughly, incubate for 10 min to prepare RNP complex at 37 ºC;		
160 ng Substrate DNA	2 μl (80 ng/μl)	2 μl (80 ng/μl)
Mix thoroughly, incubate at 37 ºC;	Incubate for 1 hour	Incubate for 2 hours
Add 1 μl Proteinase K (20 μg/μl), incubate for 20 min at 55 ºC;		
After incubation, determine the digestion efficiency by agarose gel electrophoresis.		

1.3 Example of *in vitro* DNA digestion

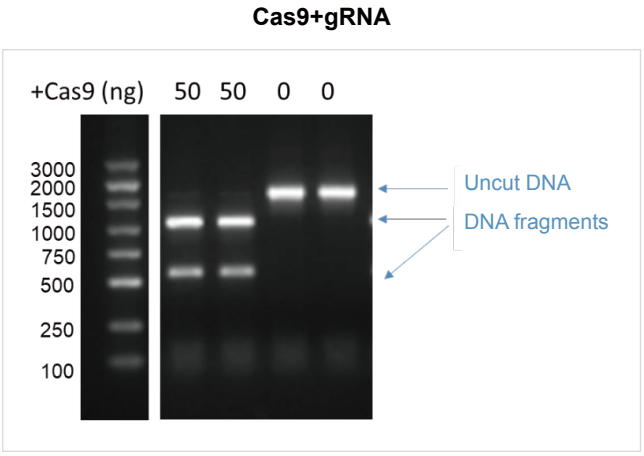


Figure 7. A 20 µl reaction in 1 × Cas9 Nuclease Reaction Buffer containing 160 ng linearized plasmid, 100 ng gRNA, and 50 ng GenCRISPR™ Ultra NLS-Cas9 (Z03623) for 1 hour at 37 °C results in a digestion efficiency of over 90%, as determined by agarose gel electrophoresis.

2. Operation procedure for gene knock-out

2.1 Reagent preparation

a. Prepare the specific gRNA - We strongly recommend GenScript's gRNA Synthesis Service (gene@genscript.com) for this application.

b. Prepare the gRNA working solution at an appropriate concentration (e.g., 125 pmol/µl) by diluting the stock solution with nuclease-free water on ice.

c. Transfer your cells to a new complete media according to experimental groups 1-2 days before transfection. Adherent cells should be around 80% confluent on the day of transfection.

d. Prepare the transfection reagents for Cas9 transfection. Electroporation is typically used in our laboratory to get higher transfection efficiency for various cell types.

Note: the user may use other methods such as lipofection for specific assays referring to suppliers' protocol.

2.2 Recommended electroporation system for SpCas9 and eSpCas9

a. Recommended reaction system using different electroporation instrument

Neon electroporation system for cell lines and T cells		
	SpCas9 (Z03623)	**eSpCas9 (Z03624)**
Cell numbers	2.0 × 10^5	2.0 × 10^5
Cas9: gRNA (molar ratio)	1:1-1:3	1:1-1:3

RNP amount	2.5-15 pmol	5-15 pmol
Electroporation volume	10 µl	10 µl

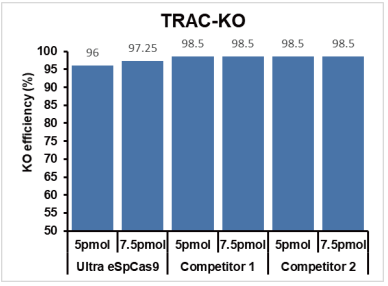
Lonza 4D electroporation system for cell lines and T cells		
	SpCas9 (Z03623)	**eSpCas9 (Z03624)**
Cell numbers	0.4-1.0 × 10^6	0.4-1.0 × 10^6
Cas9: gRNA (molar ratio)	1:1-1:5	1:1-1:5
RNP amount	18.75-80 pmol	25-80 pmol
Electroporation volume	20 µl	20 µl

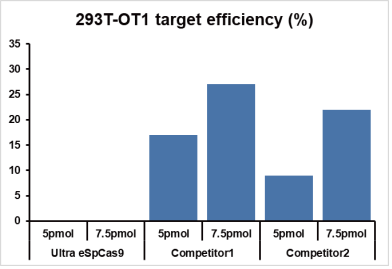
b. Case Study

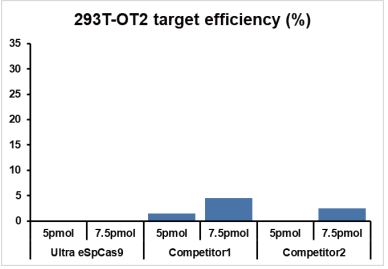
Case 1 Reduced off-target using eSpCas9

*Conduct gene knock-out at TRAC site in 293T cell following the above protocol using neon electroporation system.ol.

Human 293T cells were cultured for the test. The cells were transfected with GenCRISPR™ Ultra eSpCas9-2NLS-basic GMP (Z03624) + gRNA (synthesized from GenScript gene@genscript.com) for human TRAC gene knock-out by electroporation. After transfection and cell culture, the gene editing and off-target efficiency were measured by Sanger sequencing. GenScript Ultra eSpCas9 shows a comparative editing efficiency to competing products, but a greatly lower off-target efficiency.



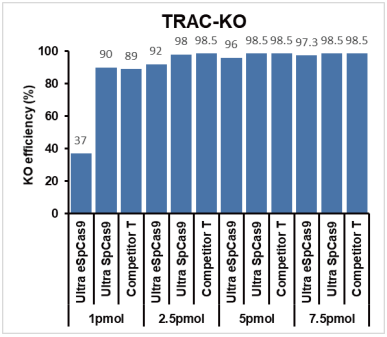




Case 2 Comparison between SpCas9 and eSpCas9 in gene editing performance

*Conduct gene knock-out at TRAC site in 293T cell following the above protocol using neon electroporation system.

Human 293T cells were cultured for the test. The cells were transfected with RNP of different Cas9 nucleases and same gRNA 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 eSpCas9 demonstrates a lower gene editing efficiency with a small amount of RNP than the wild type SpCas9 and competing product. The higher concentration of RNP amount showed an increase in the editing efficiency.



2.3 Assay procedures

a. Protocol recommended for gene knock-out in T cells using Lonza 4D system.

<table>
<tr><th>Steps</th><th>Operations</th></tr>
<tr><td>Step 1:
Prepare cell culture media</td><td>a. On the day of transfection, add cell type-specific growth media to the plate and pre-warm it at 37 °C. Transfer 240 µl complete media for T cell culture into each well of a 48-well-plate, incubate at 37 °C to pre-warm.
b. Set the electroporation program prior to transfection.</td></tr>
<tr><td>Step 2:
Prepare the RNP complex</td><td>a. Mix the GenCRISPR™ Ultra Cas9 Nuclease and gRNA at a 1: 2 molar ratio with the electroporation buffer in a nuclease-free microcentriuge tube or a PCR strip tube as shown below:
<table>
<tr><th>Reagent</th><th>Amount</th></tr>
<tr><td>GenCRISPR™
Ultra Cas9 and eSpCas9 Nuclease</td><td>0.4 µl, 25 pmol (~4000 ng)</td></tr>
<tr><td>gRNA</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>
b. Incubate the mix solution at 37 °C or room temperature for 15 min (up to 30 min) to assemble the RNP complex (~ 25 pmol).</td></tr>
<tr><td>Step 3:
Electroporate using cell type-specific transfection reagents</td><td>a. Centrifuge the cells for 10 min at 300 g at room temperature, completely remove the cell culture supernatant.
b. Wash the cells with 1 × PBS, centrifuge the cells for 10 min at 300 g at room temperature, and thoroughly remove the supernatant.
c. Aspirate 16 µl electroporation buffer to suspend 1.0 × 10^6 cells, mix thoroughly.</td></tr>
</table>

	d. Aspirate 16 µl of the cell suspension (from step 3c) to the RNP complex (from step 2), mix thoroughly. e. Use the cell type-specific electroporation reagents (from other suppliers) to proceed with the transfection referring to the suppliers' protocol. Note: 1) Avoid creating bubbles when pipetting and mixing reagents; 2) After mixing the RNP complex with the cells, immediately proceed with the transfection. f. After the electroporation, transfer the cell mixture immediately to the pre-warmed complete media (from Step 1a). Culture the cells in an incubator (37ºC, 5% CO_2) for 3-6 days.
Step 4: Gene editing analysis and perform downstream applications	a. It is recommended to perform FACS, NGS, or Sanger Sequencing (seq@genscript.com) to determine the knock in efficiency. Alternatively, use the GenCRISPR Mutation Detection Kit (L00688) to verify the gene editing efficiency. b. Proceed with the downstream application as needed.

b. Case Study

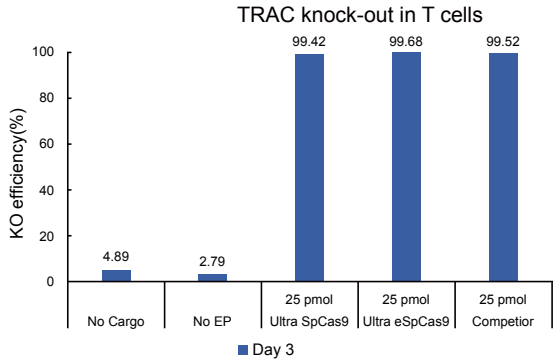
*** Conduct gene knock-out at TRAC site in T cells using Lonza 4D system**

Human T cells were cultured for the test. The cells were transfected with GenCRISPR™ Ultra SpCas9 (Z03623) or eSpCas9 (Z03624)+ sgRNA (synthesized from GenScript gene@genscript.com) for human TRAC gene knock-out by electroporation. After transfection and cell culture, the gene editing efficiency was measured by FACS. Both GenScript Ultra SpCas9 and eSpCas9 show high editing efficiency.

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

RNP amount: ~ 25 pmol

Cell number: 1.0 × 10^6 cells



3. Operation procedures for gene knock-in

3.1 Reagent preparation

a. Prepare the specific gRNA - We strongly recommend GenScript's gRNA Synthesis Service (gene@genscript.com) for this application.

b. Prepare the gRNA working solution at an appropriate concentration (e.g.125 pmol/µl) by diluting the stock solution with nuclease-free water on ice.

c. Prepare the specific HDR donor template - We strongly recommend GenScript's DNA Synthesis Service (gene@genscript.com) for this application.
d. Transfer your cells to a new complete media according to experimental groups 1-2 days before transfection. Adherent cells should be around 80% confluent on the day of transfection.
e. Prepare the transfection reagents for Cas9 transfection. Electroporation is typically used in our laboratory for higher transfection efficiency for various cell types.

Note: the user may use other methods such as lipofection for specific assays referring to suppliers' protocol.

3.2 Assay procedures

a. Protocol recommended for gene knock-in in T cells using Lonza 4D system

<table>
<tr><th>Steps</th><th>Operations</th></tr>
<tr><td>Step 1:
Prepare cell culture media</td><td>a. On the day of transfection, add cell type-specific growth media to the plate and pre-warm it at 37 °C. Transfer 240 µl complete media for T cell culture into each well of a 48-well-plate, incubate at 37 °C to pre-warm.
b. Set the electroporation program prior to transfection.</td></tr>
<tr><td>Step 2:
Prepare the RNP complex</td><td>a. Mix the GenCRISPR™ Ultra Cas9 Nuclease and gRNA at a 1: 2 molar ratio with the electroporation buffer in a nuclease-free microcentrifuge tube or a PCR strip tube as shown below:
<table>
<tr><th>Reagent</th><th>Amount</th></tr>
<tr><td>GenCRISPR™
Ultra Cas9 and eSpCas9 Nuclease</td><td>0.4 µl, 25 pmol (~4000 ng)</td></tr>
<tr><td>gRNA</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>
b. Incubate the mix solution at 37 ℃ or room temperature for 15-30 min to assemble the RNP complex (~ 25 pmol).</td></tr>
<tr><td>Step 3:
Electroporate using cell type-specific transfection reagents</td><td>a. Centrifuge the cells for 10 min at 300 g at room temperature, completely remove the cell culture supernatant.
b. Wash the cells with 1 × PBS, centrifuge the cells for 10 min at 300 g at room temperature, and thoroughly remove the supernatant.
c. Aspirate 16 µl electroporation buffer to suspend 1.0 × 10^6 cells, mix thoroughly.
d. Aspirate 16 µl of the cell suspension (from step 3c) to the RNP complex (from step 2), mix thoroughly.
e. Use the cell type-specific electroporation reagents (from other suppliers) to proceed with the transfection referring to the suppliers' protocol.
Note:
1) Avoid creating bubbles when pipetting and mixing reagents;
2) After mixing the RNP complex with the cells, immediately proceed with the transfection.
f. After the electroporation, transfer the cell mixture immediately to the pre-warmed complete media (from Step 1a). Culture the cells in an incubator (37℃, 5% CO_2) for 3-6 days.</td></tr>
</table>

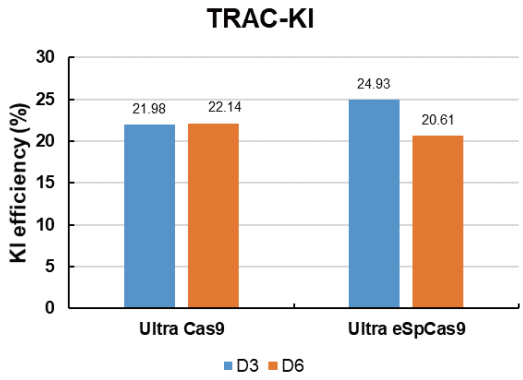
Step 4: Gene editing analysis and perform downstream applications	a. It is recommended to perform FACS, NGS, or Sanger Sequencing (seq@genscript.com) to determine the knock in efficiency. Alternatively, use the GenCRISPR Mutation Detection Kit (L00688) to verify the gene editing efficitency. b. Proceed with the downstream application as needed.

b. Case Study

*Knock-in at TRAC site in T cells

Human T cells were cultured for the test. The cells were transfected with GenCRISPR™ Ultra Cas9-Cas9 (Z03623) and GenCRISPR™ Ultra Cas9-eSp Cas9 (Z03624) with the specific gRNA (synthesized from GenScript gene@genscript.com) + dsDNA template (synthesized from GenScript gene@genscript.com) respectively for knock-in test at the TRAC site by electroporation. After transfection and cell culture, the editing efficiency was measured by FACS.

Both GenScript Ultra Cas9 and eSpCas9 show high gene editing efficiency.



Analyze Genome Editing Efficiency

Analyze genome editing efficiency - Mutation detection - T7E1

You can analyze the efficiency of your gene editing experiment by using GenCRISPR™ mutation detection kit (Cat# L00688) or T7E1 (Cat# Z03396).

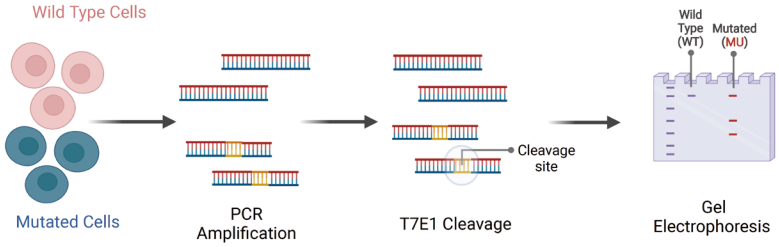


Figure 8. Overview of analysis of the efficiency of gene editing experiment

1. Harvest cells:

a. Spin down cells transfected with CRISPR at 12000 rpm for 1 min at 4 °C and carefully remove supernatant.
b. Use a genomic DNA extraction kit to extract genomic DNA from harvested cells,or lyse the cell pellets directly with cell lysis buffer (e.g. QuickExtract DNA Solution from Epicenter) for the following GenCRISPR cleavage detection assay.

2. PCR amplification:

Set up a 25 µl PCR reaction using ~100 ng of genomic DNA as a template. Add the following components to PCR tubes.

Contents	Samples	Control
5X PCR Reaction Buffer	5 µl	5 µl
10 µM dNTP mixture	0.5 µl	0.5 µl
10 µM Primer F/R Mix	1 µl	-
Control primer mix	-	1 µl
Template (~100 ng)	~100 ng	1 µl
High-Fidelity DNA polymerase	0.25 µl	0.25 µl
Nuclease-free water	Up to 25 µl	Up to 25 µl

Run a PCR reaction according to the following program:

Step	Temp	Time
Initial Denaturation	98 °C	30 seconds
25-35 Cycles	98 °C	5-10 seconds
	50-72 °C	10-30 seconds
	72 °C	30-40 seconds/kb
Final Extension	72 °C	2 min
Hold	4-10 °C	

Note: Thermocycling conditions for positive control is as follows: 98 °C/10 s, 60 °C/15 s, 72 °C/15 s, for 35 cycles.

Supplements: If non-specific bands are present, PCR reactions should be purified by gel extraction kit prior to the further fragment analysis.

Troubleshooting and Technical Assistance

Frequently Asked Questions

Does Cas9 protein have the accompanying dilution buffer?

No, the Cas9 protein does not have the dilution buffer. For diluting the Cas9 protein for long-term storage, please refer to the product manual for dilution buffer preparation.

What is the primary use for the accompanying Cas9 10× reaction buffer?

The 10x reaction buffer is used for cutting linearized plasmids *in vitro*. The buffer can used for diluting Cas9; however, the use of 10× reaction buffer to dilute Cas9 protein for long-term storage is not recommended. Please refer to the product manual for dilution buffer preparation.

What is the recommended buffer for RNP incubation prior to transfection?

The Opti-MEM reagent or the electroporation buffer are the recommended incubation buffers.

What is the recommended ratio of Cas9 to gRNA when performing *in vivo* transfection?

The molar ratio of Cas9 and gRNA is generally between 1:1-1:5, and needs to be experimentally optimized as it can vary depending on the type of cells and the electroporator used. Some reference values are provided as examples:

1. For chemical transfection in 24-well plate: 9-10×10^4 cells/well, usually 36 pmol gRNA+ 36 pmol Cas9 protein;
2. For 24-well plate electroporation (Lonza 4D electroporator): 10^6 cells/well, usually 25 pmol Cas9+50 pmol gRNA

What is the molecular weight of Cas proteins? How do I convert the mass to moles?

Product	Molecular Weight (kDa)	Quantity (μg)	Molar Weight (pmol)
Cas9 (without GFP)	160 kDa	4 μg	25 pmol
Cas9 with GFP tag	190 kDa	4 μg	21 pmol
Cas12a	150 kDa	4 μg	26.7 pmol
Cas13a	140 kDa	4 μg	28.6 pmol

What is the conversion formula for molecular weight and mol weight?

1Da = 1 g/mol

100 kDa = 100000 g/mol

For additional information please contact product@genscript.com

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