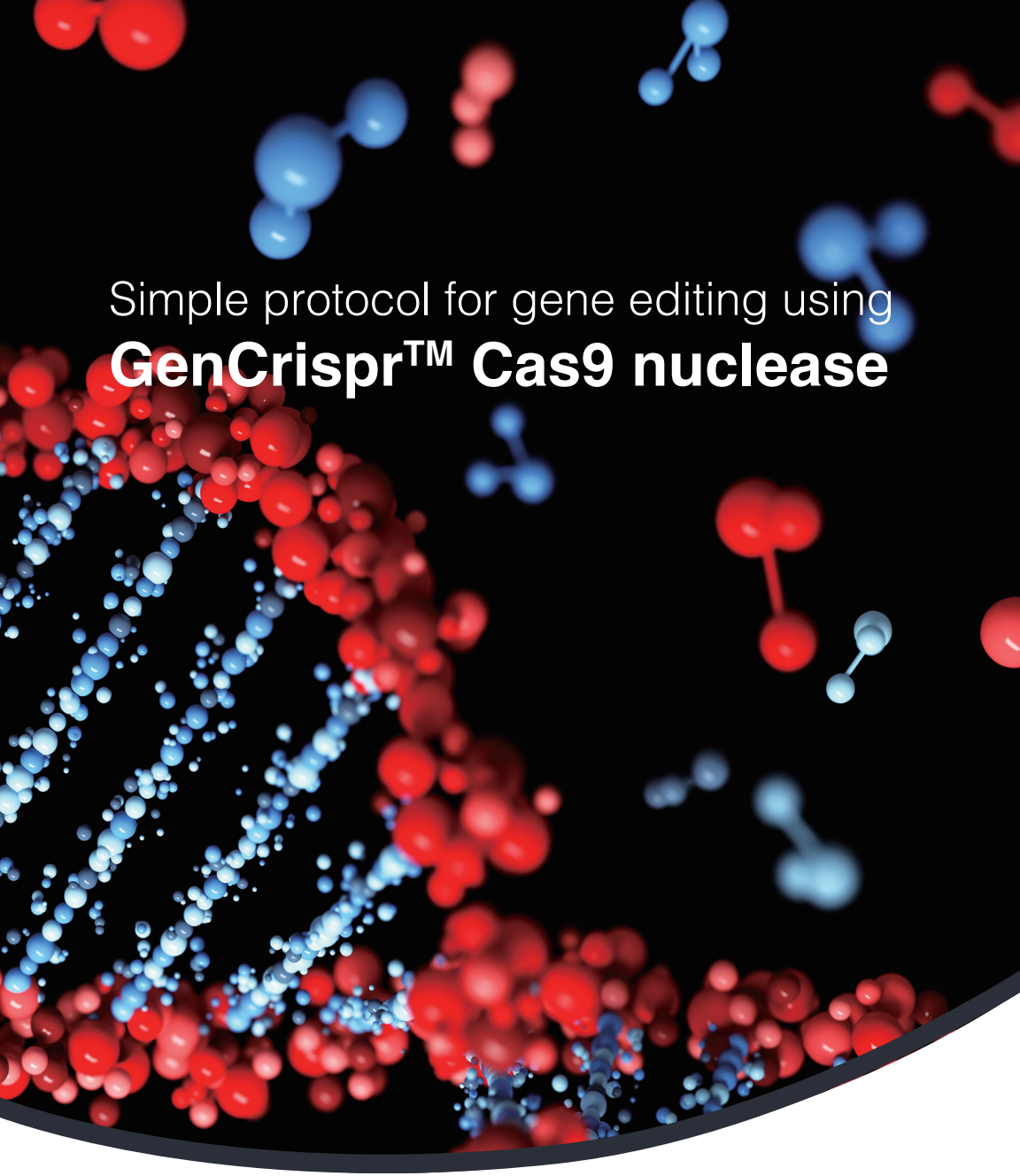


Simple protocol for gene editing using

GenCrispr™ Cas9 nuclease

GenScript

Make Research Easy

Contents

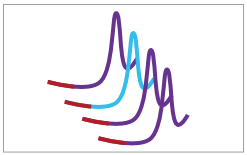
Introduction

Compared with traditional genetic engineering, the CRISPR-Cas9 system is easier to design and simpler to use because targeting a new locus only requires the redesign of a single guide RNA (sgRNA). Moreover, CRISPR-Cas9 allows multiplexing of gene editing, as multiple loci can be targeted simultaneously if multiple sgRNAs are provided. Owing to its easy-to-use and multiplexing capacity, the CRISPR-Cas9 genome editing tool has revolutionized many fields of medical research, including disease modeling, therapeutic explorations, genetic screens, *etc*. and become the most popular and powerful tool for genome engineering.

For successful genomic editing, GenCRISPR™ Cas9 system offers an optimized and efficient experimental program to the customer. The methods described here show great potential as highly efficient gene editing tools for mammalian cells.

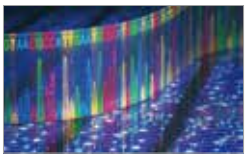
1. Select the target DNA sequence

2. Design sgRNA
http://www.genscript.com/gRNA-design-tool.html



3. Prepare sgRNA
- *In vitro* gRNA synthesis using GenCRISPR™ sgRNA Synthesis Kit (Cat # L00694)
- Screening active sgRNAs using GenCRISPR™ sgRNA Screening Kit (Cat # L00689)

4. Deliver sgRNA & Cas9 to cells
- Complex Cas9 protein + gRNA
- Lipid-based delivery or electroporation
- Incubate 48-72 h



5. Analyze genome editing efficiency
- Analyze mutation efficiency using GenCRISPR™ Mutation Detection Kit (Cat # L00688)
- DNA Sequencing

Figure 1. The CRISPR-Cas9 gene editing workflow

Protocol

Step 1: Choose the target DNA sequence

Choose the DNA target sequence that will correspond to your actual sgRNA target sequence as shown in figure 2 according to the following guidelines:

a. 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.

b. 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 sgRNA design will predict off-target sequences for a given sgRNA target sequence.

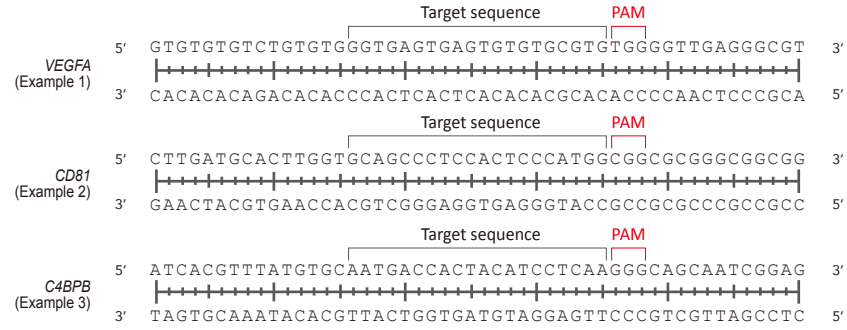


Figure 2. Selection of correct target sequence

Step 2: Design sgRNA

Design sgRNA using online design tool hosted at
http://www.genscript.com/gRNA-design-tool.html

Step 3: Preparation for sgRNA

To make Cas9 protein-driven gene editing, we have to introduce both Cas9 protein and gRNA together into cells. To get specific gRNA, there are two optional and easy approaches.

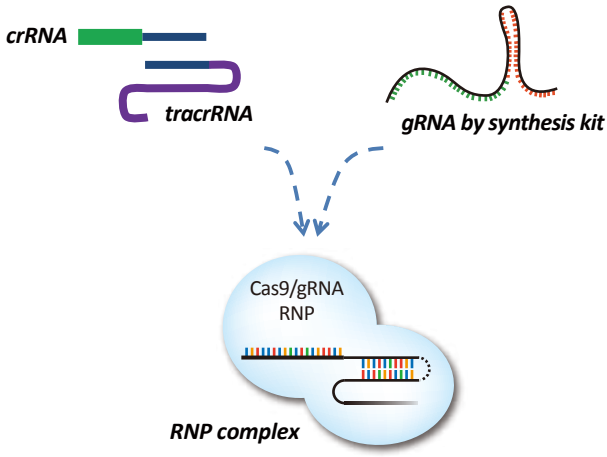


Figure 3. Approaches to make Cas9 and gRNA complex (RNP complex)

3.1 *In vitro* transcription of sgRNAs

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

a. PCR assemble the gRNA DNA template

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 product manual.)

For example, HPRT target gRNA sequence: GCATTTCTCAGTCCTAAACA

Order primers: HPRT Target F1: TAATACGACTCACTATAG + GCATTTCTCAGTCCTA

HPRT Target R1: TTCTAGCTCTAAAAC + TGTTTAGGACTGAGAAAT

b. Purify the PCR products.

c. Generate the gRNA by *in vitro* transcription
Perform the IVT reaction under the guide of gRNA synthesis kit manual.

d. Purify the gRNA

3.2 Chemically synthesis of sgRNAs

a. Order the RNA oligo at GenScript directly. The length optimized crRNA and tracRNA will be chemically synthesized according to the specific target site provided.

b. Re-suspend each RNA oligo with DEPC water or annealing buffer to final concentrations of 100 µM, or order pre-annealed TracrRNA:CrRNA duplex.

c. Form the TracrRNA:CrRNA duplex.

Components	Volume
100 µM TracrRNA	3 µL
100 µM CrRNA	3 µL
GenCrispr 5X annealing buffer	2 µL
DEPC water	2 µL
Total volume	10 µL

d. Heat the reaction at 95 °C for 10 min.

e. Remove from heat and allow to cool room temperature (25 °C) on the bench top.

Step 4: sgRNA screening

Screen the high efficient sgRNA by using GenCRISPR™ gRNA screening kit (Cat# L00689)

a. Prepare experimental DNA substrate by amplifying target fragments from your samples.

b. Set up a cleavage reaction containing your experimental sgRNA sample and your experimental target DNA substrate, in parallel with a positive control reaction.

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

c. Mix gently and Incubate at 37 °C for 2 hours.

d. Analyze 10 µL reactions on a 1% agarose gel alongside a negative control.

Example: The *in vitro* cleavage of different sgRNA.

Only sgRNA9 was observed to induce cleavage.

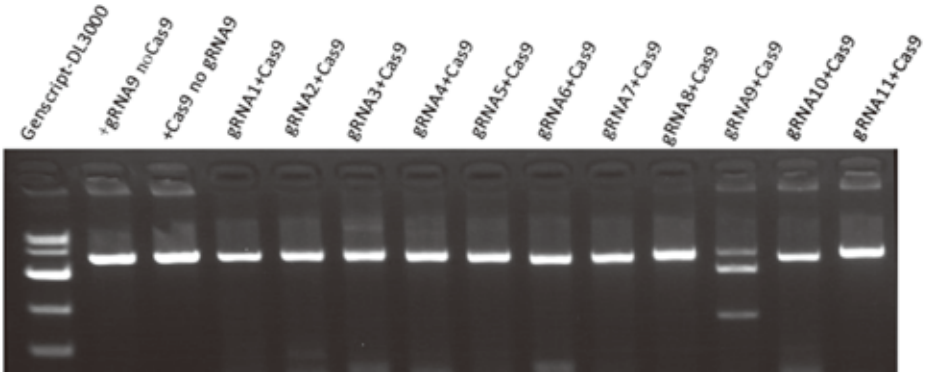


Figure 4. gRNA efficiency screening using GenCRISPR™ sgRNA screening kit. 12 unique synthesized gRNAs were incubated with Cas9 nuclease for 1 h at 37 °C, then detected on DNA agarose gel.

Step 5: Deliver Cas9 protein & sgRNA to cells

With the increase of Cas9 protein used in the gene editing field, multiple reagents and methods have been developed to facilitate the introduction of RNP complex (Cas9 protein and gRNA complex). Here, two approaches (reagents and electroporation) are described.

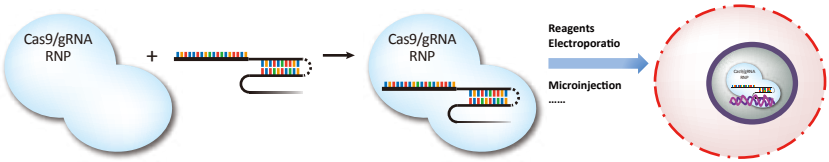


Figure 5. Multiple approaches to introduce RNP into cells.

5.1 Lipid-based delivery of Cas9 protein & sgRNA RNP complex

a. Plate cells
   a. Trypsinize and count cells.
   b. Plate cells to achieve 70-80% confluence the next day. For example, plate 4-5×10^5 HEK293T cells in a 6-well plate.
   c. Incubate cells at 37 °C in 5% CO_2 overnight.

b. Prepare RNP complexes with lipid transfection agent
   a. Prepare your choice of lipid transfection reagent (LTR) for use according to the manufacturer's recommendations.
   b. Add appropriate amount of guide RNA and Cas9 protein with the molar ratio of 1:1 or 2:1. For example, 50 nM of guide RNA and 25 nM purified Cas9 protein (final concentration).
   c. Pipet gently to mix completely. Incubate at room temperature for 10 minutes.
   d. Add recommended amount of LTR to the diluted RNP mixture.
   e. Pipet gently to mix completely. Incubate at room temperature for 15-30 minutes.

c. Distribute the complexes to cells in complete growth medium
   a. Add the LTR:RNP complexes to different areas of the wells.
   b. Gently rock the culture vessel back-and-forth and from side-to-side to distribute the LTR:RNP complexes.
   c. Incubate for 48-72 hours and remove culture medium and rinse cells with 1 mL PBS, lyse with 20-250 µL lysis buffer, and perform genomic cleavage detection assay.

5.2. Delivery of RNP complex via electroporation into cells

a. Prepare 6-well plates by transferring 2 mL of pre-warmed culture medium to each well required for the number of samples and replicates in the experiment. Pre-incubate/equilibrate by placing at 37 °C in 5% CO_2 while preparing samples.

b. Combine 150 pmol of Cas9 protein with 300 pmol of crRNA:tracrRNA working solution for a final concentration of 1.5 µM and 3 µM, respectively.

c. Incubate at room temperature for 10 minutes

d. Trypsinize and Collect 1×10^6 cells for each sample, aspirate medium from the cell pellet and re-suspend in 100 µL of appropriate electroporation buffer, Transfer re-suspended cells to the tube containing Cas9 RNP complex. Gently mix components and transfer the entire volume to an electroporation cuvette.

e. Electroporate sample according to the suggested program.

f. Transfer to one well of a 6-well plate. Gently aspirate cells from the bottom of the cuvette and transfer to the well.

g. Incubate cells at 37 °C in 5% CO_2 for a total of 48 to 72 hours after electroporation before proceeding with gene knockout analysis.

Step 6: Analyze genome editing efficiency

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

6.1 Harvest cells:

a. Spin down cells transfected with CRISPR at 12000 rpm for 1 minute 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.

6.2 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.

Content	Samples	Control
5X PCR Reaction Buffer	5 µL	5 µL
10 uM dNTP mixture	0.5 µL	0.5 µL
10 uM 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 minutes
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.

6.3 Heteroduplex formation:

Assemble the reaction as follows:

Reagent	Amount
PCR reaction	10 µL
10x Reaction buffer	2 µL
Nuclease-free water	Up to 19 µL

Denature and then anneal the products in a thermocycler using the following program:

Cycle Step	TEMP	RAMP RATE	TIME
Initial Denaturation	95 °C		5 min
Annealing	95-85 °C	-2 °C/second	
	85-25 °C	-0.1 °C/second	
Hold	4 °C		

Alternatively, if a thermocycler is not available with these ramp speeds, the samples can be heated to 95 °C for 10 minutes and then allowed to cool down to room temperature gradually.

6.4 Heteroduplex digestion:

Reagent	Amount
Annealed PCR product	19 µL
T7 Endonuclease I	1 µL

Mix well and briefly spin. Incubate each reaction at 37 °C for 15 minutes. Detect it directly by agarose gel electrophoresis or add 1 µL protease K and incubate for at 37 °C for 5 minutes to stop the reaction.

6.5 Detection

Add loading buffer to the reaction mixture directly, and detect the cleavage efficiency by agarose gel electrophoresis. (The theoretical size of positive control PCR products is 589 bp, which will be partially cleaved into 341 bp and 248 bp fragments)

Example:

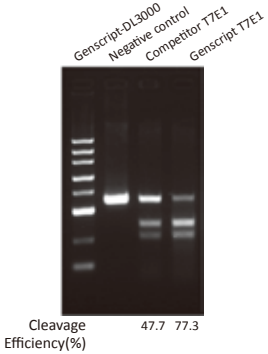


Figure 6. The cleavage efficiency of T7E1 from different companies. The annealed target PCR products were added 1 ul T7E1 and incubated at 37 °C for 15 min.

Ordering information

Cat	Name	Applications
Z03386-10	GenCrispr Cas9 Nuclease	For *in vitro* gRNA cleavage
Z03386-50	GenCrispr Cas9 Nuclease	
Z03385-100	GenCrispr Cas9-C-NLS Nuclease	For *in vivo* gene editing; NLS ensure nuclear localization
Z03385-50	GenCrispr Cas9-C-NLS Nuclease	
Z03388-100	GenCrispr Cas9-N-NLS Nuclease	
Z03388-50	GenCrispr Cas9-N-NLS Nuclease	
Z03389-100	GenCrispr NLS-Cas9-NLS Nuclease	
Z03389-50	GenCrispr NLS-Cas9-NLS Nuclease	
Z03390-10	GenCrispr NLS-Cas9-D10A Nickase	Lower off-target effect
Z03390-100	GenCrispr NLS-Cas9-D10A Nickase	
Z03390-50	GenCrispr NLS-Cas9-D10A Nickase	
Z03393-100	GenCrispr NLS-Cas9-EGFP Nuclease	For sorting and enrichment by FACS
Z03393-50	GenCrispr NLS-Cas9-EGFP Nuclease	
L00688-25	GenCrispr Mutation Detection Kit	For detecting cleavage after DNA editing
L00688-100	GenCrispr Mutation Detection Kit	
L00689-30	GenCrispr sgRNA Screening Kit	For detecting sgRNA cleavage efficiency
L00689-100	GenCrispr sgRNA Screening Kit	
L00690-25	High-Efficiency gRNA-Cas9-GFP Plasmid (linear) Assembly Kit	For assembly of sgRNA into a linearized Cas9-EGFP fusion protein expressing vector
L00690-10	High-Efficiency gRNA-Cas9-GFP Plasmid (linear) Assembly Kit	
L00691-25	High-Efficiency gRNA-Cas9-Puro Plasmid (linear) Assembly Kit	For assembly of sgRNA into a linearized Cas9-puromycin fusion protein expressing vector
L00691-10	High-Efficiency gRNA-Cas9-Puro Plasmid (linear) Assembly Kit	
L00692-25	High-Efficiency gRNA-Cas9-GFP Plasmid Assembly Kit	For assembly of sgRNA into a Cas9-EGFP fusion protein expressing vector
L00692-10	High-Efficiency gRNA-Cas9-GFP Plasmid Assembly Kit	

Cat	Name	Applications
L00693-25	High-Efficiency gRNA-Cas9-Puro Plasmid Assembly Kit	For assembly of sgRNA into a Cas9-puromycin fusion protein expressing vector
L00693-10	High-Efficiency gRNA-Cas9-Puro Plasmid Assembly Kit	

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