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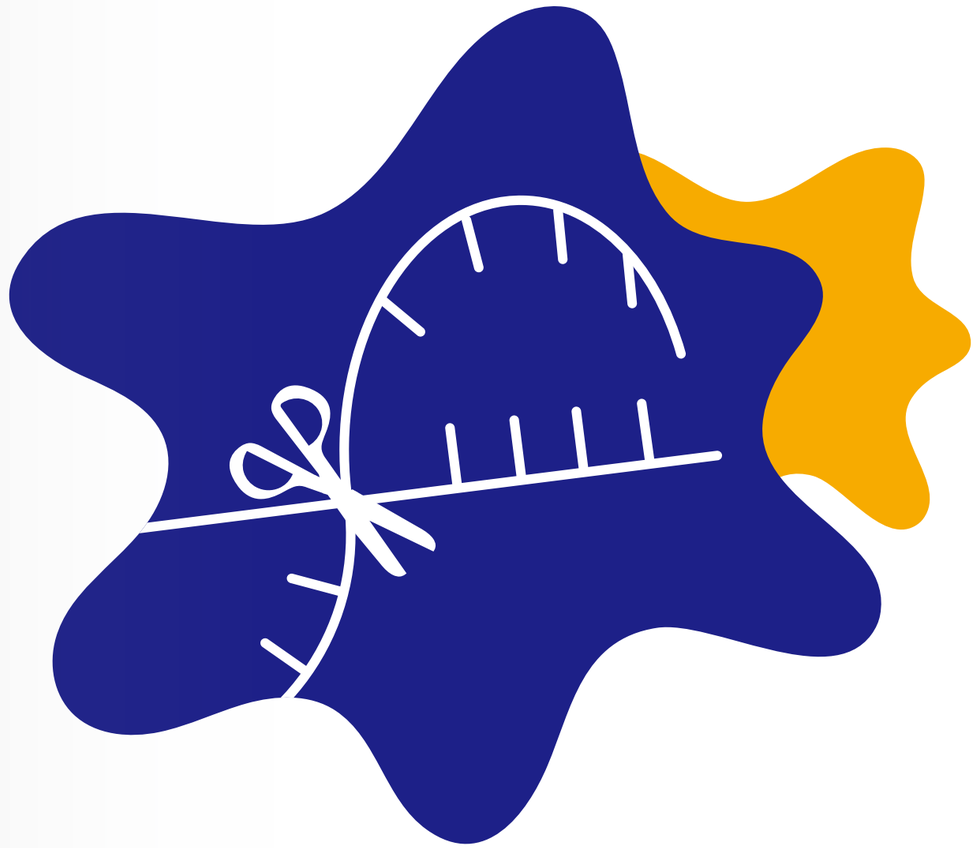

CRISPR

Ribonucleoprotein (RNP) User Manual

A guide on how to use GenScript's RNP products for targeted genome editing

Overview

Using CRISPR ribonucleoprotein (RNP) system for genome editing has many advantages over the traditional CRISPR plasmid method, including improved transfection efficiency in hard-to-transfect cells and reduced off-target effects.

To meet your specific research needs, GenScript offers purified SpCas9-2NLS nuclease proteins for optimized nuclear compartmentalization, as well as two forms of guide RNAs :

- synthetic crRNA and tracrRNA ready for duplex,
- synthetic full length single-guide RNA (sgRNA).

This user manual describes how to use GenScript's RNP products for targeted genome editing. For additional information, please visit: ***https://www.genscript.com/crispr-cas9-protein-crRNA.html***

GenScript CRISPR RNP Products

	Reagents	Catalog Number
sgRNA	CRISPR/Cas9 sgRNA	Cat# SC1968
crRNA & tracrRNA	CRISPR/Cas9 crRNA CRISPR/Cas9 tracrRNA Nuclease-Free Annealing Buffer, 5X	Cat# SC1838 Cat# SC1933 Cat# SC1957-B
Cas9 Protein	GenCRISPR NLS-Cas9-NLS Nuclease (1 μg/μL) Cas9 Nuclease Reaction Buffer, 10X	Cat# SC1841
HPRT Control	HPRT Primer Mix (Human) HPRT Positive Control crRNA (Human)	Cat# SC1940-HM-P Cat# SC1839-HP
Other Products	Lipofection or Electroporation Materials	

Step 1: Prepare RNA Oligos

Note: Keep the RNA oligonucleotides tightly sealed at -20°C prior to use and avoid repeated freeze-thaw cycles. We recommend working in a sterile environment, using RNase-free pipette tips and tubes.

1. Centrifuge tubes at 12000 rpm for 2 minutes at 4°C before opening to ensure RNA oligos are at the bottom of the tubes.
2. Resuspend oligos in nuclease-free water to reach the appropriate final concentration, for example, 100 μM.

Normalized Oligo Quantity Delivered (nmol)	Nuclease-Free Water (μl)
2	20
5	50
10	100
20	200

3. Vortex for 15 seconds and centrifuge for 1 minute at 12000 rpm
4. Prepare the following reagents:

➤➤ **If working with crRNA and tracrRNA system:**
To anneal components of 25 μM final duplex concentration:

Nuclease-Free Water	12 μl
Annealing Buffer (5X)	8 μl
crRNA Oligo (100 μM)	10 μl
tracrRNA Oligo (100 μM)	10 μl
Total Volume for Annealing	40 μl

➤➤ **If working with sgRNA system:**
To anneal components of 25 μM final sgRNA concentration:

Nuclease-Free Water	22 μl
Annealing Buffer (5X)	8 μl
sgRNA Oligo (100 μM)	10 μl
Total Volume for Annealing	40 μl

5. Heat at 95°C for 5 minutes.
6. Remove from heat and put in 60°C water, and let it to cool to room temperature.
7. If necessary, divide the annealed sample into smaller aliquots. Store at –20°C.

Step 2: Prepare RNP Complex

Note: Keep the Cas9 vial sealed until use and avoid repeated freeze-thaw cycles, as either may reduce the activity of Cas9 protein.

TIP Prior to use, you can dilute the Cas9 protein solution using a diluent buffer (10 mM Tris, 300 mM NaCl, 0.1 mM EDTA,1 mM DTT, 50% Glycerol to pH7.4 at 25°C) for easier quantification of the protein.

For different cell lines, different transfection reagents or methods should be used for best genome editing results. Typically, Lipofectamine™ CRISPRMAX or electroporation is recommended for transfection in easy-to-transfection cell lines. For hard-to-transfect cell lines, electroporation (e.g. Celetrix, Nucleafector, Neon) can be a good choice. Please check what is the most appropriate transfection reagent or method for your specific cell line, and follow the recommended protocol for it, if other than Lipofectamine™ CRISPRMAX™.

➤➤ The following protocol is recommended for **Lipofectamine™ CRISPRMAX™.**

1. Prepare the following reagents based on your needs:

	For Each well / 6-Well Plates		For Each well / 24-Well Plates		For Each well / 96-Well Plates	
	Solution A	Solution B	Solution A	Solution B	Solution A	Solution B
Opti-MEM	100 μl	100 μl	25 μl	25 μl	5 μl	5 μl
Cas9 Nuclease （1mg/ml）	2.5μl (15 pmol)	/	0.5 μl (3 pmol)	/	0.1 μl (0.6 pmol)	/
RNA Oligos Annealed from Step 1	1.25 μl (30 pmol)	/	0.25 μl (6 pmol)	/	0.05 μl (1.2 pmol)	/
Lipofectamine™ CRISPRMAX™	/	7.5 μl	/	1.5 μl	/	0.3 μl

2. Gently add solution A to solution B
3. Incubate the tube at room temperature for 10 minutes.

Test RNP Efficacy in Cells

RNP testing is best performed in easy-to-handle cell lines, such as HEK293 cells, to confirm gene editing efficiency. For such cells, Lipofectamine™ CRISPRMAX™ are recommended for transfection. The following protocol is recommended for Lipofectamine™ CRISPRMAX™.

CRISPRMAX™ Procedure

1. Seed well-dissociated cells one day (16–24 hours) prior transfection in D10 medium without antibiotics.

TIP 30–70% confluency at the time of transfection is recommended. If confluency is too high, this can negatively impact transfection efficiency.

2. Prepare RNP complex and transfect cells following Lipofectamine™ CRISPRMAX™ protocol.
3. Harvest the cells approximately 48 hours after transfection. Extract the genomic DNA for further analysis.
4. PCR amplify the fragment containing the target (for best results, design the primers to target >200 bp away from the target) and test the genome editing efficiency by T7E1 or by Sanger Sequencing.

How to Use the HPRT Positive Control?

It is recommended to use GenScript's human HPRT positive control and a non-coding negative control gRNA to optimize transfection conditions and find the condition that gives the highest gene editing efficiency and cell viability.

1. Seed well-dissociated cells one day (16-24 hours) prior to transfection in media lacking antibiotics.
2. Determine different transfection conditions that need to be tested, including cell density at the time of transfection, different gRNA to Cas9 ratio, transfection reagent quantity, incubation time period, etc.
3. Prepare RNP complex and transfect cells following Lipofectamine™ CRISPRMAX™ protocol. Harvest cells approximately 48 hours to 72 hours after transfection and extract genomic DNA for analysis.
4. PCR amplify genomic fragments using GenScript Human HPRT Primers and verify genome editing efficiency via sequencing or T7E1 digestion assay.

Electroporation Procedure for Suspension Cell Line (THP-1, U937)

The following procedure is for electroporation of suspension cells with an electroporator, Celetrix. It is highly suggested to optimize the electroporation condition for each cell line by electroporating a positive control (e.g. HPRT). The RNP can also be electroporated by other electroporators following manufacturer's manuals, e.g. Nucleafector (Amaxa) or Neon (Life Technology).

1. Collect $3*10^6$ cells and spin cells at 800 rpm for 5 minutes. Decant supernatant and wash cells with PBS. Spin cells again and decant PBS.
2. Resuspend cells with 65 µl electroporation buffer.
3. Mix 16 µl Cas9 nuclease (1 mg/ml) and 4 µl annealed crRNA:tracRNA or sgRNA in a 1.5 ml EP tube. Add electroporation buffer in the tube to a final volume 65 µl and mix gently. Incubate at room temperature for 10 minutes.
4. Mix the solution of step 3 and the resuspended cells of step 2 gently. Incubate for 10 minutes.
5. Transfer the mixture of step 4 to the electroporation tube.
7. Electroporate following Celetrix operation procedure using an appropriate voltage (700V suggested to THP-1, 660V suggested to U937).
7. Harvest the cells approximately 48-72 hours after transfection. Extract the genomic DNA for further analysis.
8. PCR amplify the fragment containing the target (for best results, design the primers to target >200 bp away from the target) and test the genome editing efficiency by T7E1 or by Sanger Sequencing.

Note: The electroporation can be used for iPS or ES cells following manufacturer's manuals.

Test RNP Efficacy in Embryos

RNP testing can also be performed in embryos, such as mouse or zebrafish, to confirm gene editing efficiency. The following zebrafish protocol can be used as an example:

1. Prepare 10 µl RNP complex in a nuclease-free tube:

10X Cas9 Nuclease Reaction Buffer	1 µl
RNA Oligos Annealed from Step 1	20-40 pmol (0.83-1.66 µl)
Cas9 Nuclease （1mg/ml）	12-24 µg (2-4 µl)
Nuclease-Free Water	Up to 10 µl

2. Incubate the tube at 37°C for 10 minutes to allow RNP complexing.
3. Microinject ~1 nl RNP Mix into embryos at the 1-cell stage.
4. When embryos reach 24 hpf, collect at least 5 of the injected embryos and extract their genomic DNA.
5. PCR amplify the fragment containing the target (for best results, design the primers to target >200 bp away from the target) and test the genome editing efficiency by T7E1 or by Sanger Sequencing.

Test RNP *in vitro* Cutting Efficiency

Depending on the target site, it may be necessary to perform *in vitro* testing of the CRISPR/Cas9 system prior to introducing RNP into cells.

1. Prepare the PCR amplicon as the substrate of CRISPR/Cas9 RNP digestion. When designing the amplicon, add at least 200 bp on either side of the guide RNA target.

TIP A longer amplicon will give a clearer band when the sample is run on a gel to verify successful cutting. We typically use amplicons around 1kb.

2. Prepare 16 µl RNP complex in a nuclease-free tube:

RNA Oligos Annealed from Step 1	3.6 pmol (0.15 µl or 1.5 ul after 10X dilution)
Cas9 Nuclease（1mg/ml）	1.5 pmol (0.25 µl or 2.5 ul after 10X dilution)
Cas9 Nuclease Reaction Buffer (10X)	2 µl
Nuclease-Free Water	13.6 µl
Total Volume	16 µl

3. Incubate the tube at 37°C for 10 minutes to allow RNP complexes to assemble.
4. Add 450 ng of PCR amplicon into 16µl RNP Mix. Bring the final volume to 20 µl with nuclease-free water and mix gently.
5. Incubate the reaction for at least 30 minutes at 37°C.
6. Assess the reaction by gel electrophoresis. If the reaction works correctly, two distinct bands will appear on the gel.

To learn more about

GenScript's CRISPR services and resources, please visit

www.genscript.com/CRISPR.html

- CRISPR sgRNA Database
- CRISPR sgRNA Design Tool
- CRISPR Webinars
- CRISPR Handbook
- CRISPR Plasmid User Guide
- CRISPR Case Studies
- CRISPR FAQs
- CRISPR News & Blogs