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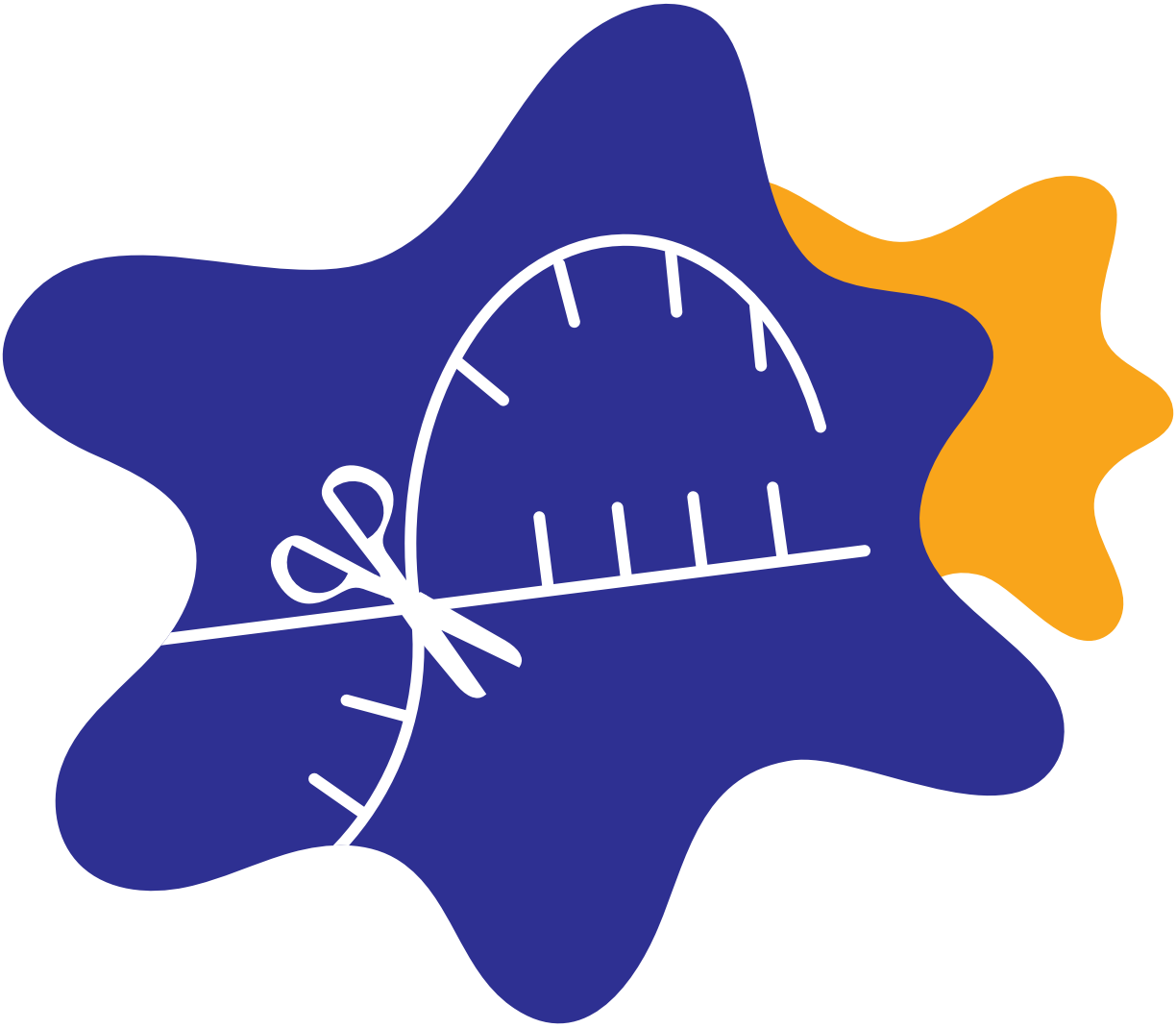


GenCRISPR

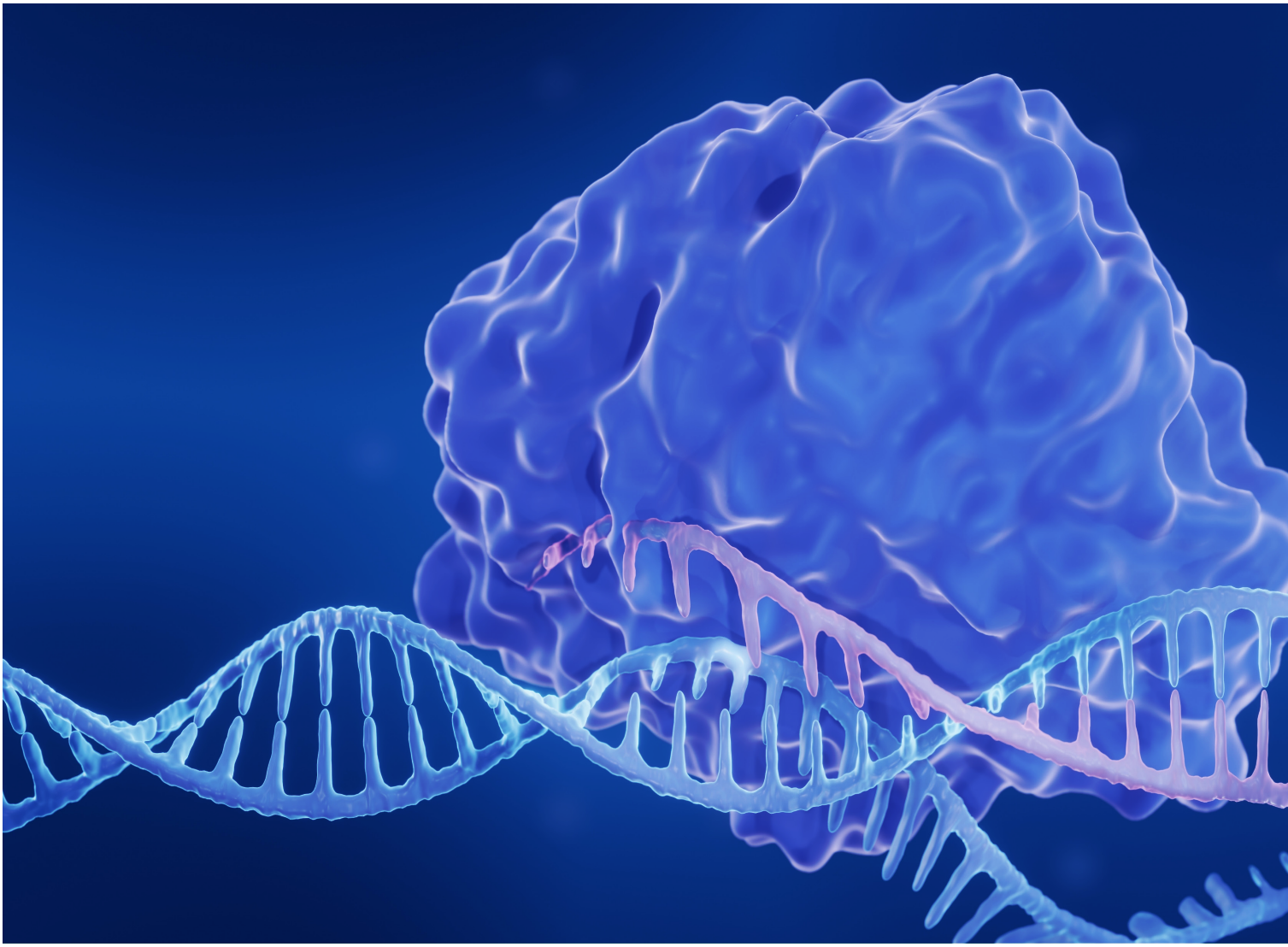
Ribonucleoprotein (RNP) User Manual

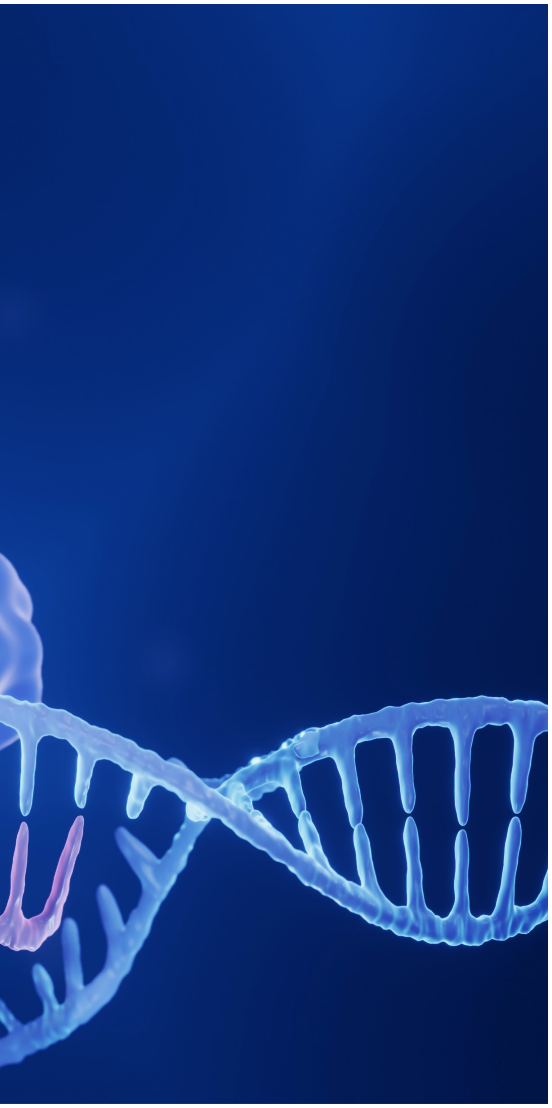
A guide on how to use GenScript's RNP products for targeted genome editing

The GenCRISPR RNP System for Non-viral Gene Editing

The CRISPR ribonucleoprotein (RNP) complex consists of a nuclease protein and guide RNA, which can be co-delivered to the cell for transient, non-viral genome editing.

Genome editing with an RNP system offers many advantages over traditional plasmid methods, including more rapid editing and reduced off-target effects.





User Manual Table of Contents

Overview

The GenCRISPR™ RNP system offers ready-to-use purified SpCas9 nuclease proteins for optimized nuclear compartmentalization, as well as synthetic single guide RNA (sgRNA) modified for enhanced stability and performance.

GenScript CRISPR RNP Products

	Reagents	Catalog Number
sgRNA	EasyEdit sgRNA SafeEdit sgRNA	Cat# SC1969 Cat# SC1968
Cas9 Protein	Ultra NLS-Cas9 Nuclease (10mg/ml) Ultra eSpCas9-2NLS Nuclease (10mg/ml)	Cat# Z03621 Cat# Z03622
Controls and Primers	EasyEdit Human HPRT Positive Control sgRNA SafeEdit Human HPRT Positive Control sgRNA Human HPRT Primer Mix Custom Primer for CRISPR sgRNA	Cat# SC1969-EC Cat# SC1968-SC Cat# SC1940-HM-P Cat# SC1968-P
Other Required Products	Nuclease-Free Water and TE buffer Lipofection or Electroporation Materials	

1. Kim S, Kim D, et al. (2014) Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res 24(6):1012–1019.
2. Hoshijima K, Jurynec MJ, et al. (2019) Highly efficient CRISPR-Cas9-based methods for generating deletion mutations and F0 embryos that lack gene function in zebrafish. Dev Cell 51:1–13.
3. Lee B, Lee K, et al. (2018) Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours. Nat Biomed Engin 2:497–507.

Step 1: Prepare RNA Oligos

Note: 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 RNase-free pipette tips and tubes.

1. Centrifuge tube(s) at 12000rpm for 2 minutes 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 100μM 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 12000rpm, make sure sgRNA 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 4000rpm to ensure all solutions are at the bottom of the tubes. Stocking solutions with high concentration (100μM) 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 minutes to thaw. Then vortex for 15 seconds and centrifuge for 30 seconds at 4000rpm to ensure all solutions are at the bottom of the tubes. Working solutions with lower concentration (25μM) can be stored at -20 °C for 3 months.

If working with sgRNA system, for 25μM working concentration sgRNA solution

sgRNA Oligo (100μM)	10μl
Nuclease-Free Water	30μl

If working with crRNA and tracrRNA system, for 25μM working concentration duplex solution

crRNA Oligo (100μM)	10μl
tracrRNA Oligo (100μM)	10μl
Annealing Buffer (5X)	8μ
Nuclease-Free Water	12μl

Heat the mixture at 95°C for 5 minutes.
Remove from heat and put in 60°C water, and let it to cool to room temperature.

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 (10mM Tris, 300mM NaCl, 0.1mM EDTA,1mM DTT, 50% Glycerol to pH7.4 at 25°C) for easier quantification of the protein.

Maximum nuclease activity is typically achieved at a molar ratio of 1–2 molecules of sgRNA per molecule of Cas9 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 can be a good choice. Please check what is the most appropriate transfection reagent or method for your specific cell line.

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

1. Prepare the following reagents based on your needs:

		For each well /6-well plate	For each well /24-well plate	For each well /96-well plate
Solution A * Incubate at room temperature for 10 minutes after mixing	Opti-MEM	125µl	25µl	5µl
	Cas9 Nuclease (10mg/ml)	0.8µl (50pmol)	0.16µl (10pmol)	0.03µl (2pmol)
	RNA Oligos from Step 1 (25µM)	4µl (100pmol)	0.8µl (20pmol)	0.16µl (4pmol)
Solution B	Opti-MEM	125µl	25µl	5µl
	Lipofectamine™ CRISPRMAX™	7.5µl	1.5µl	0.3µl

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

See the **Comprehensive CRISPR Knock-in Guide**
for additional RNP delivery protocols
https://www.genscript.com/crispr-knock-in-comprehensive-guide.html

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 200bp 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 from Page 2, Step 1 (25µM)	0.24µl or 6pmol (200ng)
Cas9 Nuclease (10mg/ml)	0.08µl or 5pmol (800ng)
Cas9 Nuclease Reaction Buffer (10X)	2µl
Nuclease-Free Water	13.68µl
Total Volume	16µl

3. Incubate the tube at 37°C for 10 minutes to allow RNP complexes to assemble.
4. Add 160ng of PCR amplicon into 16µl RNP Mix. Bring the final volume to 20µl 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.

Test RNP Efficacy in Cells

For initial RNP testing, it is recommended to start with an easy-to-handle cell line such as HEK293 cells. The following Lipofectamine™ CRISPRMAX™ protocol is recommended for RNP transfection in such cells.

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 >200bp away from the target) and test the genome editing efficiency by T7E1 or by Sanger Sequencing.

Using 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, such as Celetrix. It is highly suggested to optimize the electroporation condition for each cell line using a positive control (e.g. HPRT). The RNP can also be electroporated by other electroporators following manufacturer's manuals.

1. Collect $3*10^6$ cells and spin cells at 800rpm for 5 minutes. Decant supernatant and wash cells with PBS. Spin cells again and decant PBS.
2. Resuspend cells with 65μl electroporation buffer.
3. Mix 1.6μl Cas9 nuclease (10mg/ml) and 200pmol sgRNA in a 1.5ml EP tube. Add electroporation buffer in the tube to a final volume 65μl 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.
6. 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 >200bp 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 10µl RNP complex in a nuclease-free tube:

10X Cas9 Nuclease Reaction Buffer	1µl
RNA Oligos Annealed from Page 2, Step 1 (25µM)	20-40pmol (0.83-1.66µl)
Cas9 Nuclease (10mg/ml)	12-24µg (0.2-0.4µl)
Nuclease-Free Water	Up to 10µl

2. Incubate the tube at 37°C for 10 minutes to allow RNP complexing.
3. Microinject ~1nl RNP Mix into embryos at the 1-cell stage.
4. When embryos reach 24hpf, 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 >200bp away from the target) and test the genome editing efficiency by T7E1 or by Sanger Sequencing.

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