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Innovating Epigenetic Solutions

PROTOCOL

Transfection of CRISPR/Cas9 Nuclease NLS ribonucleoprotein (RNP) into adherent mammalian cells using Lipofectamine® RNAiMAX

INTRODUCTION

The **CRISPR/Cas genome editing system** consists of a single guide RNA (sgRNA) and the Cas9 endonuclease. To create gene disruptions, a single guide RNA is generated to direct the Cas9 nuclease to a specific genomic location. Cas9-induced double strand breaks are repaired via the Non Homologous End Joining (NHEJ) DNA repair pathway. The repair is error prone, and thus insertions and deletions (INDELs) may be introduced that can disrupt gene function. Recombinant Cas9 Nuclease NLS Protein can be combined with sgRNA to form an RNP complex to be delivered to cells for rapid and highly efficient genome editing.

There are several ways in which to introduce Cas9-guide RNA RNP complexes into cells: transfection using lipid mediated transfection reagents, electroporation or micro-injection. Here we present a method for the forward transfection of Cas9 Nuclease NLS RNP's into adherent mammalian cells using conventional lipofection reagents.

Transient or stable transfection of large construct(s) for Cas9 and single guide RNA are not needed. In addition, this methods avoids other common pitfalls associated with transfection of CRISPR/Cas9 plasmids:

- **Off-target mutations rarely occur**
  Prolonged expression of Cas9 increases off-target editing but RNP delivery is naturally transient
- **Transfection/injection is easy**
  Cas9-NLS bypasses transcription and translation by delivering the protein directly
- **Assay is DNA-free**
  No risk of DNA integration that can happen using plasmids plus no cloning required

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Europe Diagenode sa / LIEGE SCIENCE PARK // Rue du Bois Saint-Jean, 3 // 4102 Seraing (Ougrée) // Belgium // Phone: (+32) 4 364 20 50 // E-mail: info@diagenode.com
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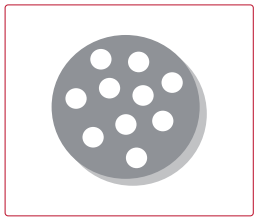
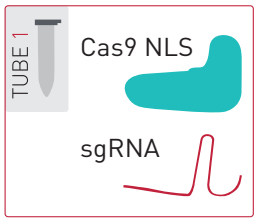
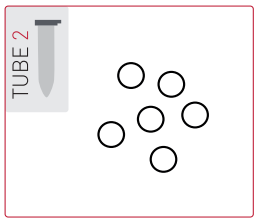
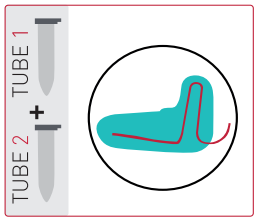
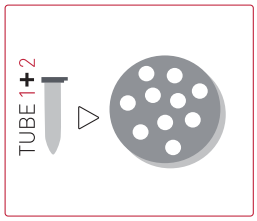
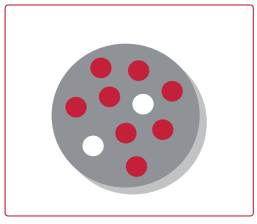
METHOD OVERVIEW AND TIME TABLE

			Time needed	Day
STEP 1		Seed cells	30 minutes	1
STEP 2		Prepare RNP complex	5 minutes	2
		Dilute transfection reagent		
STEP 3		Prepare RNP and transfection complex	20 minutes	2
STEP 4		Add complexes to cells	5 minutes	2
STEP 5		Analyze transfected cells		4-5

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REQUIRED MATERIALS

- Cas9 Nuclease protein NLS 75 µg (3 µg/µl), *S. pyogenes* (Diagenode, Cat. No. C29010001)
- 10x Reaction Buffer (10 x H Buffer, 1.0 ml: 500 mM Tris-HCl Ph7.9, 1 M NaCl, 100 mM MgCl2, 10 mM DTT) - provided with the Cas9 Nuclease protein NLS.

Required materials not provided:

- sgRNA containing the targeting sequence in the region of interest (user supplied)

  Note: sgRNAs must contain the target sequences (20 nucleotides) adjacent to the Protospacer Adjacent Motif (PAM, NGG) in the target DNA.
- Gloves to wear at all steps
- RNase/DNase-free sterile 1.5 ml tubes
- HEK293 cells (or other cell line) at 30-70% confluency
- RNP Transfection reagent (e.g., Lipofectamine® RNAiMAX Transfection Reagent; ThermoFisher)
- Sterile 1X PBS without Ca^{2+} and Mg^{2+}
- DMEM with Glutamax (or appropriate growth medium) with 10 % FBS
- Serum free cell culture medium (e.g., Opti-MEM™ Reduced Serum Medium; ThermoFisher)
- Cell culture plates

REMARKS BEFORE STARTING

- We strongly recommend wearing gloves and using nuclease-free tubes and reagents to avoid RNase contamination.
- Cell density at the time of transfection is critical, as high confluency can negatively impact efficiency. It is recommended to optimize your conditions for each cell type.
- The following protocol is a "forward transfection method" that uses a final concentration of 10 nM RNP per transfection in a 24-well culture plate. For optimal transfection, we recommend testing RNP concentrations ranging from 10 to 30 nM final in well. Cas9 Nuclease NLS (Diagenode, Cat. No. C29010001) concentration is 3 µg/µl equal to 18 µM. Please go to page 6 (Annex) to scale up your transfection experiment according to the type of culture vessel you are using.
- In this protocol, we recommend using Lipofectamine® RNAiMAX. Other RNP transfection reagents may work as well, however, this might need to be optimized.

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PROTOCOL

Day 1: Step 1: Cell plating prior to transfection

- Trypsinize cells as per standard tissue culture procedure. Count the cells using a hematocytometer to determine the appropriate volume of cells in complete media without antibiotics to obtain 0.8-2.4 x 10^5 cells per ml.
- Add 500 µl of diluted cell (0.4-1.2 x 10^5) to each well of a 24-well culture plate. Gently rock the plate back and forth and from side to side to distribute the cells evenly.

  Note: Cells should be ~30-70 % confluent at the time of transfection. High confluency can negatively impact transfection efficiency.
- Incubate the cells overnight in a humidified 37°C, 5% CO_2 incubator.

Day 2, Step 2: RNP complex formation

- Dilute Cas9 Nuclease NLS protein to 1 µg/µl (~6 µM) in 1X Reaction buffer (Buffer H) and store on ice. *Note: It is not recommended to store diluted Cas9 Nuclease NLS at -20°C for later use.*
- Dilute the sgRNA to 250 ng/µl (~6 µM) by diluting the stock with nuclease-free water
- Form the RNP complexes in **tube 1** as indicated in Table 1. Volumes are for a single well. Scale the volumes proportionally for additional wells.

Table 1. RNP complex formation

Components	24-well
Cas9 Nuclease NLS 1 µg/µl	1 µl
sgRNA 300 ng/µl	1 µl
Opti-MEM™	25 µl

- Gently mix the reaction by pipetting up and down and incubate at room temperature for 5 minutes.

  Note: This incubation step is important to allow RNP complex to form.

- Dilute the Lipofectamine RNAiMax in Opti-MEM™ in **tube 2** as indicated in Table 2. Incubate at room temperature for 5 minutes.

Table 2. Lipofectamine® RNAiMAX dilution

Components	24-well
Lipofectamine RNAiMAX	1.5 µl
Opti-MEM™	25 µl

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Step3. Prepare RNP and transfection reagent complex

- Add solution from **tube 1** (RNP complex) to diluted Lipofectamine® RNAiMax in **tube 2** and mix well by pipetting up and down. Incubate at room temperature for 20 minutes to allow transfection complexes to form.

  Note: Do not incubate for more than 30 minutes.

Step 4.Transfect Cells with RNP and liposome complexes

- Add 50 µl of transfection complexes to the cells. Gently rock the plate back and forth and from side to side to distribute the complexes evenly.
- Incubate the cells in a humidified 37°C, 5% CO_2 incubator for 48-72 hours. It is not necessary to remove complexes or change the medium after transfection.

Day 4-5: Step 5: Validate your genome editing

- Analyze transfected cells and determine the efficiency of your genome editing experiment.

It is important to carefully monitor the genome editing process to validate that the RNP complex has been successfully incorporated into cells and that the target gene has been modified. This monitoring can be broken down into different categories:

1 . Detect on target mutations.

The Mismatch Cleavage Assay provides a simple and reliable method for the cleavage efficiency of genome editing tools at a given locus. In this assay, a sample of the edited cell population is used as a direct PCR template for amplification with primers specific to the targeted region. The PCR product is then denatured and reannealed to produce heteroduplex mismatches where double-strand breaks have occurred, resulting in insertion/deletion (indel) introduction. These mismatches are recognized and cleaved by the detection enzyme, and the cleavage is easily detectable and quantifiable by gel analysis.

To detect on target mutations, follow the protocol detailed in the "CRISPR/Cas9 editing: mutation detection".

2. Monitor the delivery of CRISPR components in your cells.

The presence of Cas9 in cells can be effectively measured by western blot or immunofluorescence using specific anti-Cas9 antibodies. Due to the transient nature of RNP transfection, we recommend to verify Cas9 efficient delivery 4-24 hours after transfection. Diagenode offers the broad range of antibodies raised against the N- or C-terminus of the Cas9 nuclease. These highly specific polyclonal and monoclonal antibodies are validated in Western blot and immunofluorescence.

CRISPR/Cas9 antibodies

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3. Analyze changes in protein expression.

The CRISPR-Cas9 system is routinely used for knockout, knock-in, or modulation of gene expression. Western blotting is a simple and efficient method to track changes in the level of expression of your targeted protein.

CRISPR/Cas9 antibodies

ANNEX

Table 3. Scaling up or down RNP transfections

Single well only								
Culture vessel	Starting cell number	Volume of growth medium	*Tube 1*			*Tube 2*		Transfection complexes
			Volume Optimem	Cas9 Nuclease NLS (µg)	sgRNA (µg)	Volume Optimem	Lipofectamine	
96-well	0.7-2 x10^4	100 µl	5 µl	0.2	0.05	5 µl	0.3 µl	10 µl
48-well	0.21-0.6 x10^5	250 µl	12.5 µl	0.5	0.125	12.5 µl	0.75 µl	25 µl
24-well	0.42-1.2 x10^5	500 µl	25 µl	1	0.25	25 µl	1.5 µl	50 µl
12-well	0.84-2.4 x10^5	1 ml	50 µl	2	0.5	50 µl	3 µl	100 µl
6-well	2.1-6 x10^5	2 ml	125 µl	5	1.25	125 µl	7.5 µl	250 µl
60 mm	0.46-1.3 x10^6	5 ml	250 µl	10	2.5	250 µl	16.5 µl	500 µl
10 mm	1.2-3.5 x10^6	10 ml	500 µl	20	5	500 µl	43 µl	1 ml

Volumes in each column are for a single well. Scale the volumes proportionally for additional wells. Reaction mix volumes are for one well and account for pipetting.

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