NLS-Cas9-NLS

Cat# PR-137211

Background	Cas9 (CRISPR associated protein 9) is an RNA-guided DNA endonuclease enzyme associated with the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) adaptive immunity system in Streptococcus pyogenes, among other bacteria. S. Pyogenes utilizes Cas9 to remember and later probe and cleave foreign DNA, such as invading bacteriophage DNA or plasmid DNA. In case that the DNA substrate is complementary to the guide RNA, Cas9 cleaves the invading DNA. In addition to its original role in bacterial immunity, the Cas9 protein has been heavily employed as a genome engineering tool to induce site-directed double strand breaks in DNA. These disruptions may lead to gene inactivation or the presentation of heterologous genes via non-homologous end joining and homologous recombination respectively in many laboratory model organisms. Cas9 NLS, S. pyogenes contains Simian virus 40(SV40) T agtigen nuclear logcalization sequence(NLS) on the N- and C- termini of the protein.	
Concentration	50 µg/ 50 µl (313 pmol)	
Source	*E. coli*	
Buffer	10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 300 mM NaCl, and 50% (v/v) Glycerol	
Appearance	Sterile filtered colorless solution.	
Formulation	Recombinant Cas9 (D10A & H840A) protein expressed in E. coli supplied in a buffer of 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM DTT, 300 mM NaCl, and 50% (v/v) Glycerol.	
Storage	-70°C. Avoid freeze thaw cycles.	
10X Reaction Buffer	Concentration	10X (1 ml)
	Buffer	20 mM HEPES, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, pH 6.5
	Storage	-20 ^{o}C
Reaction Conditions	Use 1X Cas9 Reaction Buffer and incubate at 37 ^{o}C.	

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Functional Assay	Incubation of linearlized DNA (3 nM) and sgRNA (30 nM) with 100 nM NLS-Cas9-NLS nuclease in 1X reaction buffer at 37°C for 1 hour resulted in >90% digestion of DNA substrate (agarose gel electrophoresis).

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Protocol

The protocol listed below is for reference only. The user may optimize the protocol according to their own experiments.

In vitro Cas9 mediated digestion of DNA

1. Add the following components to a sterile, nuclease-free tube on ice:

Components	Volume	Final Concentration
sgRNA (300 nM)	2 µl	~30 nM
Cas9 Nuclease Protein (1000 nM)	0.60 µl	~30 nM
10X Cas9 Nuclease Reaction Buffer	2 µl	1X
Nuclease-free H2O	12.4 µl	-
Pre-Incubate for 15 minutes at room temperature		
Substrate DNA (30 nM)	3 µl	3 nM
Total Volume	20 µl	-

2. Collect all components by a brief centrifugation. Incubate the reaction at 37 °C for 1 hour.

3. Analyze fragments by running an agarose gel electrophoresis.

RNP Complex Formation

Gently mix the reaction and incubate at room temperature for 10 minutes. Then place on ice for following transfection by electroporation or liposome.

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Components	Volume	Final Concentration
sgRNA (1000 nM)	1.2 µl	~120 nM
Cas9 Nuclease Protein (1000 nM)	1.2 µl	~120 nM
Opti-MEM	12.6 µl	-
Total	15 µl	

Trypsinize and Prepare HEK293T Cells

1. Seed the cells so that they will be around 70-90% confluent on the day of transfection.
2. During the RNP/liposome incubation, trypsinize the cells, washing once to remove any traces of trypsin. Resuspend the cells in 10 ml of media and count.
3. Calculate the dilution and volume needed to get the cells to 3.2 x 10^5 cells per ml. You will need 125 µl of cells per well.

Transfect Cells with Liposome Complexes

1. From each tube of RNP/liposome complex, aliquot 25 µl into 3 wells of a 96-well plate.
2. Add 125 µl of cells (3.2 x 10^5 cells/ml) to each well containing RNP/liposome complex and pipette up and down gently a few times.
3. Incubate the cells in a humidified 37°C, 5% CO_2 incubator for 48-72 hours.

Harvest DNA and Amplify Target Region

1. Gently aspirate the media from the cells and wash twice with 100 µl 1X PBS.
2. Add 75 µl of DNA Extraction Solution and shake/vortex for 5 minutes. Transfer the solution to a PCR plate or tubes and place in a thermocycler, running the following program:

   65°C for 15 min
   95°C for 15 min
   Hold at 4°C

3. Dilute the DNA 1:10 in nuclease-free water.
4. Follow the protocol detailed in the T7E1 assay kit

Quality Control:

Exonuclease, endonuclease and non-specific nuclease activities not detected.

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