GeneHero™ Cas9 Nuclease

Catalog No.	**GE001**	**Cas9-NLS**	**25 µg**
	GE002	**Cas9-NLS**	**100 µg**
	GE003	**Cas9-NLS-His**	**25 µg**
	GE004	**Cas9-NLS-His**	**100 µg**

User Manual

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I. Description

GeneHero™ Cas9 nuclease is the recombinant *Streptococcus pyogenes* Cas9 (wt) protein, purified from *E. coli*. Cas9 protein forms a ribonucleoprotein (RNP) complex with guide RNA *in vitro* that catalyzes site-specific double strand breaks that when repaired by nonhomologous end joining creates small insertions and deletions. Incorporation of nuclear localization signals (NLS) at the N-terminal enhances the rate of genome editing by facilitating its delivery to the nucleus. Cas9 RNPs can cleave genomic targets with similar or higher efficiency as compared to Cas9/sgRNA plasmids. RNPs can be delivered to the cell as functional complexes and do not need transcription and translation; it is cleared rapidly from the cell that may increase CRISPR specificity and reduce off-target mutations.

II. Contents and Storage

Contents and storage recommendations for the GeneHero™ Cas9 Nuclease are provided in the following table.

Catalog no.	Content	Conc.	Amount	Storage
GE001	Cas9 protein with NLS sequence	1 µg/µl	25 µl (25 µg)	-20 ºC
GE002	Cas9 protein with NLS sequence		100 µl (100 µg)	
GE003	Cas9 protein with NLS sequence and C-terminal 6X His-tag		25 µl (25 µg)	
GE004	Cas9 protein with NLS sequence and C-terminal 6X His-tag		100 µl (100 µg)	

Storage buffer: 10 mM Tris-HCl (pH 7.4), 0. 3 M NaCl, 1 mM DTT, 0.1 mM EDTA, 50 % glycerol.
Store the GeneHero™ Cas9 Nuclease at -20 ºC until required for use.
Maintain RNase-free conditions by using RNase-free reagents, tubes, and barrier pipette tips while setting up your experiments.

III. Reaction Condition

Reaction buffer (1X): 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 100 µg/ml BSA.

Dilution buffer is the same as storage buffer.

IV. Protocol: Cas9 protein cleavage of target DNA fragments *in vitro*

Overview

The protocol describes the *in vitro* digestion of DNA fragments using the Cas9/gRNA RNP complex. This method can be used to validate the activity of sgRNA before application in *in vivo* studies.

Materials required but not provided

Synthetic sgRNA or *in vitro* transcribed (IVT) sgRNA
DNA substrate containing the target sequence (plasmids, PCR products, or synthetic DNA oligo duplex)
Cas9 nuclease dilution buffer: 10 mM Tris-HCl (pH 7.4), 0.3 M NaCl, 1 mM DTT, 0.1 mM EDTA, 50 % glycerol.
10X Reaction Buffer: 500 mM Tris-HCl (pH 7.9), 1 M NaCl, 100 mM $MgCl_2$, 1mg/ml BSA.
Nuclease-free water

Tips

- Ensure the DNA substrate contains the 20 nt sgRNA targeting sequence, followed by the Cas9 PAM site (NGG);
- Prepare 50 nM DNA substrate with the target sequence by diluting the stock with nuclease-free water on ice.

  ***Note**: for synthetic DNA oligo duplex (≤100 bp), prepare 20-50 μM DNA substrate; for PCR fragments (100-2000 bp), prepare 20-500 nM DNA substrate; for linearized plasmid (≥ 2000 bp), prepare 10-20 nM DNA substrate.*

  The final concentration of DNA substrate in the reaction can range from 2 nM to 5 μM depending on its format (linearized plasmid, PCR fragment) and length;
- The molar ratio of Cas9: sgRNA: DNA substrate should be at least 10: 10: 1 to obtain optimal cleavage efficiency;

Procedure

- Prepare 300 nM sgRNA by diluting the stock with nuclease-free water on ice.
- Prepare 150 ng/μl Cas9 nuclease (~900 nM) using dilution buffer on ice.
- Assemble the reaction at room temperature in the following order:

Component	Volume	Final Concentration
Nuclease-free water	20 μl	
10X Reaction Buffer	3 μl	
300 nM sgRNA	3 μl	30 nM

900 nM Cas9 Nuclease	1 µl	30 nM
Reaction Volume	**27 µl**	
Pipette to mix and incubate for 10 min at 25 ºC		
30 nM DNA substrate	3 µl	3 nM
Total reaction volume	**30 µl**	

- Mix thoroughly and incubate at 37 ºC for 15 min.
- Add 1 µl of Proteinase K to the reaction, mix thoroughly
- Incubate at room temperature for 10 min.
- Analyze the digestion by agarose gel electrophoresis or Fragment Analyzer.

V. Protocol: Cas9 protein for genome modification in mammalian cell lines

Overview

This protocol describes the genome modification of HEK293 cell line by lipid-based transfection of the Cas9/sgRNA RNP complex.
Validation of the mutations can be conducted with the T7E1 assay. Adjustment on cell confluence, reagent concentration and culture incubation time may be required if other mammalian cell lines are used.

Materials required but not provided

Synthetic sgRNA or *in vitro* transcribed (IVT) sgRNA
CRISPR-Fectin™ Transfection Reagent (GeneCopoeia, Cat# EF015)
IndelCheck™ CRISPR insertion or deletion detection system (GeneCopoeia, Cat# IC001, IC002)
Opti-MEM™ I Reduced Serum Medium (Life Technologies, Cat# 31985-088)
HEK293 cell line
Nuclease-free water
6-, 24- or 96-well plate

Tips

- For successful lipofection, low-passage cell lines should be used.
- The optimal cell density for transfection varies for different cell lines based on cell size and growth characteristics. In general, a cell confluence of 30-70% on the day of transfection is recommended for lipid-based transfection.
- The molar ratio of Cas9: sgRNA should be at least 1: 1 to obtain optimal cleavage efficiency. It is recommended to optimize the dosage of RNP for transfection based on the cell lines.

Procedure

Day 0. Seed cells

- If the cells are from a recent liquid nitrogen stock, passage the cells at least 2 times before transfection.
- The day before transfection, trypsinize and count the cells.
- Adjust the cell density and media volume according to the table below. Do not include antibiotics.

	6-well	24-well	96-well
Cell density per well	250,000-450,000 cells	40,000-90,000 cells	8,000-18,000 cells

Volume of media per well	2 ml	0.5 ml	100 µl

Day 1.

Grow cells in culture plate

– The number of cells plated in each well should be 50% confluent on the day of transfection.

Cas9-sgRNA RNP preparation

– For each well, mix sgRNA, GeneHero™ Cas9 Nuclease and Opti-MEM™ I Reduced Serum Medium according to the table below. Mix well using pipette, reduce bubbles during pipetting.

	6-well	24-well	96-well
sgRNA	1200 ng (37.5 pmol)	240 ng (7.5 pmol)	50 ng (1.5 pmol)
GeneHero™ Cas9 Nuclease	6250 ng (37.5 pmol)	1250 ng (7.5 pmol)	250 ng (1.5 pmol)
Opti-MEM™ I Medium	125 µl	25 µl	5 µl

– Incubate at room temperature for 5 min to assemble the RNP complexes.

Reverse transfect the RNP complex

– Dilute CRISPR-Fectin™ transfection reagent in Opti-MEM™ I Medium in an RNase-free microcentrifuge tube according to the table below. Mix well.

	6-well	24-well	96-well
CRISPR-Fectin™ reagent	7.5 µl	1.5 µl	0.3 µl
Opti-MEM™ I Medium	125 µl	25 µl	5 µl

– Incubate the CRISPR-Fectin™ transfection reagent in Opti-MEM™ I Medium at room temperature for 1 minute.
– Add the CRISPR-Fectin™ transfection reagent to the Cas9-sgRNA RNP mixture. Mix well by pipetting.
– Incubate the mixture of RNP and transfection reagent at room temperature for 15 to 20 min, do not exceed 30 min.
– Add the mixture to the cells at 30-70% confluence according to the table and mix gently by rocking the plate back and forth.

	6-well	24-well	96-well

RNP/CRISPR-Fectin	250 µl	50 µl	10 µl

- Incubate the cells at 37°C in a CO_2 incubator for 2-3 days.

Day 3-4. Harvest cells

- Remove culture supernatant and rinse cells with 50-500 µl of PBS.
- Validate CRISPR modification using the IndelCheck™ CRISPR insertion or deletion detection system
    - Option 1. Extract genomic DNA from cells
        - Harvest cells (no fewer than ~10^6 cells per well).
        - Extract genomic DNA using your method of choice or following the provided protocol of extraction kit manufacturer. Make sure the concentration of genomic DNA solution is above 25 ng/µl.
        - Thaw 2 × SuperHero PCR Mix on ice. For PCR from extracted genomic DNA, prepare a Master Mix with target PCR primers flanking the insert as follows:

Reagent	**Amount**
Genomic DNA	50-200 ng
Primers (5 µM each)	1.25 µl
2X SuperHero PCR Mix	12.5 µl
ddH_2O	to 25 µl
Final	25 µl

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    - Option 2. Cell lysate preparation
        - Collect cells from cell culture dish, 6- well plate or 96-well plate. Centrifuge at 3,000 rpm at 4 ºC for 5 min and carefully remove the supernatant.
        - Add 300 µl 1×PBS. Pipette gently to suspend cells. Centrifuge at 3,000 rpm at 4 ºC for 5 min and remove the supernatant.
        - Add 300 µl 1×PBS and resuspend cells. Sample the suspension to calculate the cell number if necessary. Centrifuge at 3,000 rpm at 4 ºC for 5 min and remove the supernatant as completely as possible. Proceed to lyse or store the pellet at -80 ºC.
        - Add 25 µl Lysis Buffer and lyse cells at 65°C for 15 min, then 95 ºC for 10 min. Quickly put it on ice afterward.
        - Frozen centrifuge at 12,000 rpm for 1 min.
        - Proceeded to PCR reaction with Target PCR kit. The cell lysate can be stored at 4 ºC for no more than one week or -20 ºC for several months until use.
        - Prepare a master mix with target PCR primers as follows:

Reagent	**Volume**
Cell lysate	1 µl

Primers (5 µM each)	1.25 µl
2X SuperHero PCR Mix	12.5 µl
ddH_2O	to 25 µl
Final	25 µl

- Proceed with PCR using the following program

Temp	Time	Cycles
95 ºC	5 min	1
95 ºC	30 s	35
58 ºC	30 s	
72 ºC	1 min	
72 ºC	5 imn	1

- Purification or gel extraction of correct-sized band from non-specific PCR background.
- PCR produces fragments containing both wild-type and mutant target sequences. The DNA double strands are denatured at 95 ºC, and then gradually annealed with primers at room temperature, resulting in forming mismatched heterozygous DNA (such as wild-type/insertion deletion mutant mismatch, or mutant 1/ mutation 2 mismatch).
- Mix PCR products with T7E1 buffer as following:

Reagent	Amount
DNA	200-500 ng
10X T7E1 buffer	2 µl
ddH_2O	To 19 µl
Total	19 µl

- Mix and centrifuge for a few seconds, heat at 95 ºC for 5 min.
- Re-anneal by allowing the denatured PCR products to cool down to RT.
- Add 1 µL of 2 U/µl T7 Endonuclease I, and incubate at 37 ºC for 20-60 min.
- Run gel analysis. Pick the clones that show the highest cleavage efficiency to use in your downstream experiments.

VI. Limited Use License and Warranty

Limited Use License
Following terms and conditions apply to use of all GeneHero™ Cas9 Nulcease (the product). If the terms and conditions are not acceptable, the product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the product. The Product shall be used by the purchaser for internal research purposes only. The product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The product must not be resold, repackaged or modified for resale, or used to manufacture commercial products without prior written consent from GeneCopoeia. This product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the product constitutes acceptance of the above terms.

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GeneCopoeia warrants that the product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the product fails to meet these specifications, GeneCopoeia will replace the product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the product. GeneCopoeia's liability is expressly limited to replacement of product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the product for a particular purpose.

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