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Product Information

FnCas9 Protein
FnCas9 - NLS from *Francisella novicida*, expressed in *Escherichia coli*

Catalog Number **FNCAS9PROT**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

FnCas9 protein is a recombinant Cas9 protein derived from a type II-B *Francisella novicida* Cas9 and expressed in *E. coli*. In contrast to the type II-A SpCas9 from *Streptococcus pyogenes*, FnCas9 cleaves target DNA in a staggered pattern and leaves 5′ overhangs after initiation of a double strand break in DNA. The 5′ overhangs are predominantly 4-nt long, each with a 5′ phosphate group, and proximal to the protospacer adjacent motif (PAM). This unique enzymatic property enables FnCas9 to serve as a programmable "restriction enzyme" for DNA digestion and directional molecular cloning or other genomic applications. The protein is tagged with a SV40 large T antigen nuclear localization signal (NLS) at the C-terminus for nuclear import in cell transfection applications.

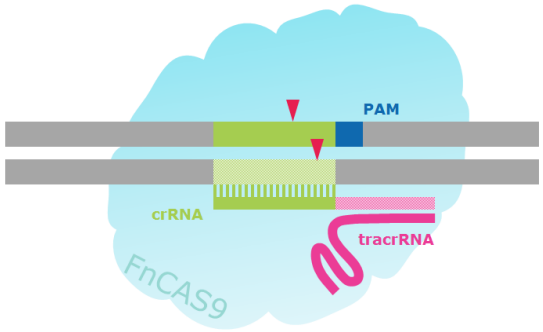
Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) systems are bacterial adaptive immune systems for defense against viruses and plasmids. There are two major classes of CRISPR/Cas systems and several subtypes within each class. In class 2 type II CRISPR/Cas systems, the effector nuclease is a ribonucleoprotein (RNP) consisting of a Cas9 protein, a CRISPR RNA (crRNA), and a trans-activating crRNA (tracrRNA). The nuclease recognizes a target DNA sequence (protospacer) that is immediately followed by a PAM and is complementary to the variable 5′ region of crRNA. The nuclease subsequently uses a RuvC-like nuclease domain to cleave the non-complementary DNA strand (non-target DNA strand) and an HNH nuclease domain to cleave the complementary DNA strand (target DNA strand).

Similar to the type II-A SpCas9, FnCas9 uses a NGG PAM for targeting, but with a preference for an HGG PAM (where H is A, T, or C). However, in contrast to SpCas9, the RuvC-like nuclease domain of FnCas9 cleaves the non-complementary DNA strand 6-7 bp from the PAM, whereas the HNH nuclease domain cleaves the complementary DNA strand 3 bp from the PAM.

This results in the generation of 5′ overhangs after double strand break, with each overhang carrying a 5′ phosphate group.[1] This cleavage feature can be utilized for programmable "restriction digestion" and directional molecular cloning. Furthermore, genome editing in human cells and mouse embryos has been demonstrated for FnCas9.[2]

FnCas9 is orthogonal to SpCas9 and thus requires FnCas9 specific tracrRNA and crRNA. FnCas9 specific synthetic crRNA and tracrRNA can be purchased for preparation of a three-component RNP complex to cleave a target of interest (see Figure 1). Alternatively, the protein can be combined with an *in vitro* transcribed (IVT) FnCas9 specific single guide RNA (sgRNA) to form a two-component RNP complex.

Figure 1.
Target recognition and cleavage by FnCas9 RNP



The FnCas9 ribonucleoprotein consists of FnCas9 protein, a crRNA, and a tracrRNA. The 5′ portion of the crRNA is variable and complementary to the target of interest, while the tracrRNA sequence is constant. The crRNA and the tracrRNA can be joined together to form a single guide RNA (sgRNA) which can be produced by chemical synthesis or *in vitro* transcription. Staggered cleavage results in the generation of 5′ overhangs after double strand breaks in DNA.

Components
FnCas9 protein is provided lyophilized with a Reconstitution Solution, a Dilution Buffer, and a 10× Digestion Buffer.

- One vial of lyophilized recombinant FnCas9 protein, Catalog Number C120030, 50 μg or 250 μg
- Reconstitution Solution, Catalog Number RSOLUTION, 1 mL of 50% glycerol in water
- Dilution Buffer, Catalog Number DBUFFER, 1 mL of 20 mM HEPES, pH 7.5, with 200 mM NaCl
- Digestion Buffer (10×), Catalog Number DIGBUFFER, 1 mL of 200 mM HEPES, pH 7.5, with 1 M KCl and 100 mM $MgCl_2$

Reagents and Equipment Required but Not Provided.
crRNA – Custom synthetic 48 nucleotide FnCas9 crRNA can be ordered via this link. Alternatively, click "Request Quotation for Custom crRNA Synthesis" at the bottom of sigma.com/SygRNA. Enter the 20 bp genomic DNA target sequence (5′ to 3′) plus the adjacent 3′ PAM site. Under special instructions, indicate “FnCas9 crRNA”.

tracrRNA – Catalog Number FNCAS9TRACR

sgRNA – Custom *in vitro* transcribed 129 nucleotide FnCas9 sgRNA can be purchased by sending the 20 bp genomic DNA target sequence (5′ to 3′) plus the adjacent 3′ PAM site via email to CRISPR@sial.com. .

GenElute™ PCR Clean-Up Kit – Catalog Number NA1020

Gel Loading Buffer – Catalog Number G2526

Precast agarose gels

Tris-Borate EDTA Buffer, 5× concentrate, powdered blend – Catalog Number T3913

Ethidium Bromide Solution, 10 mg/mL in water – Catalog Number E1510

Water, PCR Reagent grade – Catalog Number W1754

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
1. Resuspend the lyophilized FnCas9 protein with the supplied Reconstitution Solution (Catalog Number RSOLUTION).
   a. For 250 μg vials, add 50 μL of Reconstitution Solution to achieve a concentration of ~5 mg/mL (25 pmol/μL).
   b. For 50 μg vials, add 30 μL of Reconstitution Solution to achieve a concentration of ~1.7 mg/mL (8 pmol/μL).

   Note: Precise quantities vary from lot-to-lot, please refer to the certificate of analysis for exact protein content per vial.

2. Gently tap tube to completely dissolve lyophilized powder, incubate for 10 minutes on ice, and spin tube briefly to bring material to bottom of tube.

3. If a lower concentration of FnCas9 protein is required, dilute the FnCas9 protein solution with the supplied Dilution Buffer (Catalog Number DBUFFER) immediately before use. Store diluted protein on ice, up to 6 hours.

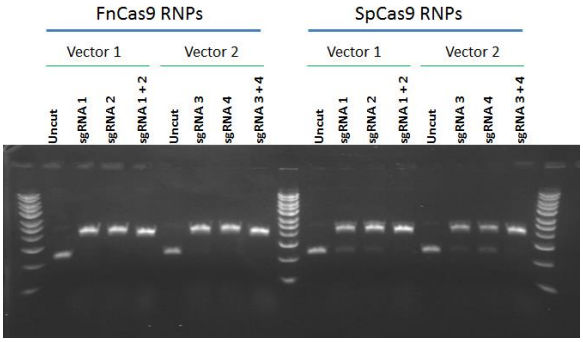
Storage/Stability
All components are shipped on ice. Store all components at –20 °C upon arrival. Once resuspended in the provided Reconstitution Solution, the FnCas9 protein solution should be stored at –20 °C.

Procedures

DNA Digestion with FnCas9

Purified DNA from PCR products, plasmid, or genomic DNA samples may be used as substrates for FnCas9 digestion (see Figure 2).

Figure 2.
Plasmid vector digestion by FnCas9 and SpCas9 RNPs



FnCas9 ribonucleoprotein exhibits more effective digestion of plasmid DNA as compared to SpCas9 ribonucleoprotein.

It is recommended to use 50-100 fmol of DNA (100-200 ng for 2-3 kb DNA) and 10 to 30-fold molar excess of FnCas9 protein in a 20-30 μL reaction to achieve complete digestion. Preparing RNP in a molar ratio from 1:1:1 to 2:2:1 (crRNA:tracrRNA:FnCas9 protein) is suggested. Further optimization of the ratio may be required for certain targets. *In vitro* transcribed sgRNA can be used in the same range of ratios.

1. Dissolve lyophilized crRNA and tracrRNA with TE buffer (10 mM Tris, pH 7.5, with 1 mM EDTA) to reach concentration of 100 μM.

2. Anneal crRNA and tracrRNA by adding 3 μL of crRNA, 3 μL of tracrRNA, and 94 μL of TE buffer to a 1.5 mL tube, heating the tube at 95 °C for 5 minutes on a heat block, and then gradually cooling the tube on bench to room temperature (30-45 minutes). The final crRNA/tracrRNA duplex concentration is 3 μM. Store the solution at -20 °C.

3. Prepare fresh 0.3 μM crRNA/tracrRNA duplex solution by diluting 5 μL of the annealed crRNA/tracrRNA with 45 μL of RNAse-free water. Keep the diluted solution on ice.

4. Prepare FnCas9 RNP digestion mix as follows:

Component	Volume
Nuclease-free water	21 μL
Digestion Buffer (10×)	3 μL
crRNA/tracrRNA (0.3 μM)	4 μL
FnCas9 protein (1 μM)	1 μL
Total Volume	**29 μL**

Note: The volume of water can be adjusted based on the volume of DNA substrate needed.

5. Mix gently and spin briefly (5 seconds). Incubate at room temperature for 10 minutes.

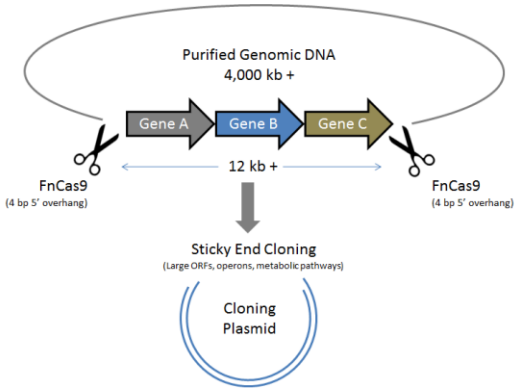
6. Add 1 μL of DNA substrate and mix gently and spin briefly (5 seconds). Incubate at 37 °C for 1 hour. Longer incubation time may be needed for genomic DNA samples.

7. Add 5× gel loading dye and heat the sample at 60 °C for 10 minutes. Run the sample on an agarose gel. Alternatively, purify the digestion with the GenElute PCR Clean-Up Kit before use in downstream applications.

Molecular Cloning with FnCas9

The staggered cleavage feature of FnCas9 can be utilized for directional ligation and molecular cloning (see Figure 3). To ensure compatible cohesive ends are generated on both the vector and the insert DNA substrate, it is recommended that both the vector and the insert DNA substrate are digested with the same set of guide RNAs. The selected target sites can be cloned into the vector using oligonucleotides.

Figure 3.
Schematic of molecular cloning with FnCas9 RNPs



1. Synthesize a dsDNA oligonucleotide containing the selected target sites (protospacer plus PAM for each site) in the same orientation as is in the insert DNA substrate. Separate the two target sites by 20 bp.

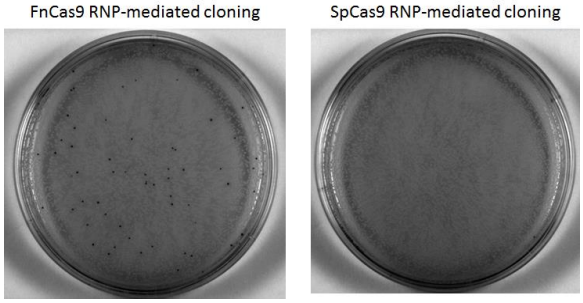
2. Clone the dsDNA oligo into an appropriate vector.

3. Digest the vector and insert DNA substrate with FnCas9 as described in the above **DNA Digestion with FnCas9** section. For genomic DNA digestion, higher amounts of crRNA/tracrRNA and FnCas9 protein (up to 5-fold increase) may be used.

4. Gel purify the digested vector and the insert fragment. For genomic DNA substrate, purify the digested DNA sample using the GenElute PCR Clean-Up Kit or using phenol/chloroform extraction followed by ethanol precipitation.

5. Perform ligation and transformation (see Figure 4) using a standard molecular cloning practice.

Figure 4.
FnCas9 and SpCas9 genomic DNA cloning



FnCas9 ribonucleoprotein promotes more effective cloning of a 12 kb lacZ locus as compared to SpCas9. Cloning performed in bacterial strain BL21-DE3.

Cell Transfection with FnCas9

In general, the steps required for successful introduction of FnCas9 RNP into cultured and primary cells are as follows:

- Prepare crRNA and tracrRNA or *in vitro* transcribed (IVT) sgRNA reagents
- Prepare cells
- Assemble FnCas9 RNP
- Transfect cells with FnCas9 RNP

RNP Preparation Recommendations

- Assemble guide RNA:FnCas9 Protein RNP complexes on ice, immediately before use.

- In all instances, combine equal molar amounts of crRNA:tracrRNA. Alternately, use IVT sgRNA.

- Preparing RNP in a molar ratio from 1:1:1 to 5:5:1 (crRNA:tracrRNA:FnCas9 protein) is suggested. Further optimization may be required for certain targets.

Preparation and Microinjection of FnCas9 RNP into One-Cell Embryo

Microinjection protocols vary greatly depending on embryo type and researcher preferences. Microinjection of FnCas9 RNP has been demonstrated in *Mus musculus* (mouse).[2]

References

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Troubleshooting Guide

Suspected Issue	Solution
FnCas9 protein has denatured after long term storage in Dilution Buffer.	Dilution of the FnCas9 protein in the provided Dilution Buffer is only recommended for immediate use. For long term storage, keep the protein lyophilized or resuspended in the provided Reconstitution Solution and stored at –20 °C.
crRNAs and tracrRNAs need to be annealed before complexing with the FnCas9 protein.	Anneal the crRNA and tracrRNA by mixing them in the desired ratio, incubating the mixture for 5 minutes at 95 °C followed by 20 minutes on ice.
crRNAs and tracrRNAs are degraded.	For certain cell lines, modification of crRNA and tracrRNA may be necessary to increase the stability. Modifications are available through Sigma-Aldrich.
Transfection fails.	Optimize transfection protocol for each cell line used. Refer to the manufacturer's protocol for further assistance.
Guide RNA is low quality or degraded.	For optimal performance, only quality-verified guide RNA should be used.

US Patents pending

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