

## Product Information

### Recombinant Cas9, eSpCas9, and Cas9D10A Nickase Proteins For RNP-Based Genome Editing

Cas9 Protein, Catalog Number

**CAS9PROT**

ESPCAS9 Protein, Catalog Number

**ESPCAS9PRO**

CAS9 NICKASE, Catalog Number

**CAS9D10APR**

This product is for R&D use only, not for drug, household, or other uses.

Storage Temperature -20 °C

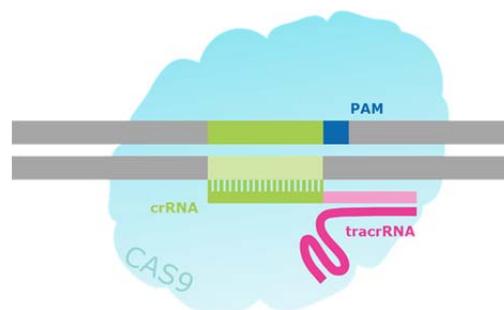
#### Product Description

Multiple different Cas9 proteins are available: wild type NLS-tagged Cas9 from *Streptococcus pyogenes* (SpCas9, Catalog Number CAS9PROT), enhanced specificity SpCas9 (eSpCas9, Catalog Number ESPCAS9PRO), which has been engineered to further increase the specificity of Cas9, and Cas9 nickase (Cas9D10A, Catalog Number CAS9D10APR), which is modified to be used in pairs to enhance specificity.

Each recombinant Cas9 protein is lyophilized from a 0.2 µm filtered solution containing HEPES pH 7.5, Sodium Chloride, Sucrose and Dithiothreitol and is provided with a reconstitution solution to resuspend the protein and a dilution buffer to further dilute the protein before delivery, if desired.

#### Background Information

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system was discovered in bacteria, where it functions as an adaptive immune system against invading viral and plasmid DNA. In this system, short DNA sequences (spacers) from invading viruses are incorporated at CRISPR loci within the bacterial genome and serve as memory of previous infections. Reinfection triggers complementary mature CRISPR RNA (crRNA) to find a matching viral sequence. Together, the crRNA and trans-activating crRNA (tracrRNA) guide CRISPR-associated (Cas) nuclease or nickase to induce breaks in the corresponding foreign DNA sequences<sup>1</sup>.

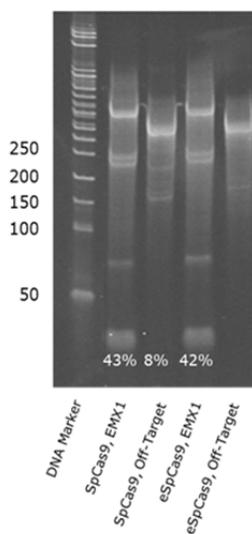


**Figure 1. Three Component CRISPR Cas9 System.** The Cas9 ribonucleoprotein is made up of the Cas9 protein and a guide RNA, which can be divided into a tracrRNA and a crRNA. The crRNA is variable and complementary to the target of interest, while the tracrRNA sequence is static.

The type II prokaryotic CRISPR “immune system” has been engineered to function as an RNA-guided genome-editing tool that is simple, easy, and quick to implement.

Type of Protein	Product Number
Wild Type SpCas9 <sup>1</sup>	CAS9PROT
Enhanced Specificity SpCas9 <sup>2</sup> .	ESPCAS9PRO
Cas9 Nickase <sup>3,4</sup>	CAS9D10APR

The above proteins can be combined with SygRNA<sup>®</sup> synthetic crRNAs and tracrRNAs to form ribonucleoprotein (RNP) complexes that target the specific genomic locus of interest (Figure 1).



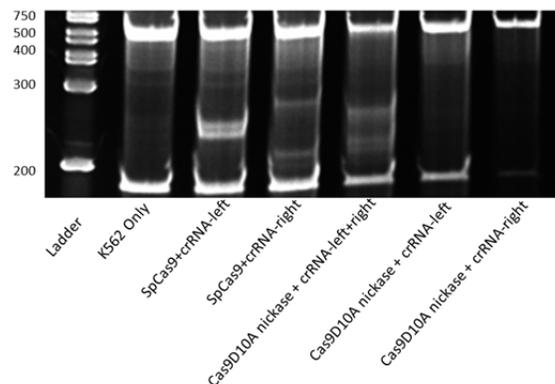
**Figure 2. eSpCas9 Reduces Off-Target Cutting Compared with WT Cas9.** K562 cells were nucleofected with WT SpCas9 or eSpCas9 and synthetic tracrRNA and EMX1-targeted crRNA. A CEL-1 assay showed equal cutting between the Cas9 and eSpCas9, while cutting at a known off-target<sup>7</sup> was reduced when eSpCas9 was used compared to Cas9.

Although the CRISPR system can be delivered to cells via plasmids, direct introduction of Cas9 RNP strengthens and expands the applications of CRISPR genome modification technology by eliminating the possibility of plasmid DNA integration into the host genome. This method also results in fewer off-target effects due to the rapid degradation of the RNP after delivery; in many cases Cas9 RNP results in efficient genome modification with higher specificity when compared to cells transfected with Cas9 plasmid<sup>4,5,6,7</sup>. This RNP technology has broad applications and has been shown to work in both mammalian and plant systems<sup>8</sup>. Furthermore, Cas9 RNP delivery holds great promise for therapeutic applications including the recent successful generation of knock-in primary human T cells<sup>9</sup>.

One of the primary concerns of the CRISPR system is off-target cutting of sequences similar to the target; eSpCas9 improves the system by reducing detectable off-target cutting. The unwinding of the target DNA by SpCas9 is driven by the sum of two forces: the positive charge of the chromosome-binding motif of the protein, and the RNA:DNA interaction between the guide RNA and the target DNA. Therefore, weakening the binding efficiency of SpCas9 will increase the criteria for a more precise match between the guide RNA and the target DNA for unwinding of the target to occur.

To create eSpCas9, wild type SpCas9 was engineered to possess a relaxed binding efficiency, resulting in higher on-target fidelity without the loss of cleavage efficiency. To engineer this protein, alanine point mutations were made in the chromosome-binding motif of SpCas9<sup>2</sup>. When tested in combination with select gRNA (Figure 2), our eSpCas9 demonstrated on-target cleavage efficiency comparable to wild type SpCas9 with undetectable cleavage at select off-target sites.

Another solution to off-target cutting is to use a paired Cas9 nickase system. Wild-type Cas9 possesses two protein domains, RuvC and HNH, each responsible for cutting a strand of DNA. In this system the RuvC domain has been modified with a D10A (Aspartic Acid to Alanine) mutation to make the Cas9 protein function as a nickase rather than as a nuclease. Two single-strand nicks at proximal locations form a double-stranded break that minimizes off-target cutting through doubling of DNA recognition requirements<sup>3,4</sup>. Predicted off-target sites for these guides may only incur single strand nicks, which are then repaired by the cell to a level of off-target mutagenesis indistinguishable from background<sup>3,4</sup>. Our Cas9 nickase proteins when complexed with a pair of guides demonstrated on-target cleavage efficiency comparable to wild type SpCas9, but no cleavage when only one of the guides was used (Figure 3).



**Figure 3. Cas9 Nickase Cuts Target Area Only when Paired.** K562 cells were nucleofected with WT SpCas9 or Cas9D10A and synthetic tracrRNA and EMX1-targeted crRNA. A CEL-1 assay using nickase protein showed detectable cutting with paired gRNAs only.

### Preparation Instructions

1. Resuspend the lyophilized 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 approximately 5 mg/ml (30 pmol/µL).
  - b. For 50 µg vials, add 30 µL of Reconstitution solution to achieve a concentration of approximately 1.7 mg/ml (10 pmol/µL).

### Notes:

1. Minimum deliverable protein quantities are listed above. Precise quantities vary by lot number; please refer to the certificate of analysis for exact protein per vial amounts.
2. The reconstitution solution provided is 50% glycerol. In case this is not suitable for your specific application, it is recommended to use the provided dilution buffer. Do not reconstitute in water.
2. Gently tap tube to completely dissolve lyophilized powder, incubate for 10 minutes on ice, and spin to bring material to bottom of tube.
3. If a lower concentration of Cas9 protein is required, dilute the Cas9 protein with supplied Dilution buffer (Catalog Number DBUFFER) immediately before use. Store diluted protein on ice, up to 6 hours.

### Procedure Overview

In general, the steps required for successful introduction of Cas9 RNP into cultured and primary cells are as follows:

1. Prepare SygRNA crRNA and tracrRNA reagents
2. Prepare cells
3. Assemble Cas9 RNP
4. Transfect cells with Cas9 RNP
5. Harvest genomic DNA and assay mutations

### Precautions and Disclaimers

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

### Storage and Stability

This lyophilized protein is shipped at ambient temperature and should be stored at -20°C upon arrival. Once resuspended in the provided reconstitution solution, the proteins are recommended to be stored at -20°C.

### Components

Cas9 Protein (Catalog Number CAS9PROT) contains:

- One vial of lyophilized Cas9 recombinant protein, Catalog Number C120010, 50 µg or 250 µg

eSpCas9 Protein (Catalog Number ESPCAS9PRO) contains:

- One vial of lyophilized eSpCas9 recombinant protein, Catalog number E120020, 50 µg or 250 µg

Cas9 Nickase Protein (Catalog Number CAS9D10APR) contains:

- One vial of lyophilized Cas9D10A nickase recombinant protein, Catalog number C120020, 50 µg or 250 µg

Each protein is provided with the following components:

- Reconstitution solution for Cas9 proteins, 1×, 50% glycerol in water, Catalog Number RSOLUTION, 1 mL
- Dilution buffer for Cas9 proteins, 1×, 20 mM Na-Hepes pH 7.5, 200 mM NaCl, Catalog Number DBUFFER, 1 mL

The following equipment and reagents are recommended but **not** provided in this kit:

- SygRNA synthetic crRNAs (Catalog Number VC4003)
- tracrRNA (Catalog Number TRACRRNA05N)
- TransIT-CRISPR<sup>®</sup> Transfection Reagent (Catalog Number T1701)
- Electroporation System for Mammalian Cells
  - We recommend the Amaxa Nucleofector<sup>®</sup> 2b device (Lonza, Catalog Number AAB-1001) with Nucleofector Kit V (Lonza, Cat# VCA-1003)
- GenElute<sup>™</sup> Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70)
- JumpStart<sup>™</sup> Taq ReadyMix<sup>™</sup> (Catalog Number P2893)

- Water, PCR Reagent (Catalog Number W1754)
- Mutation Detection (either kit may be used)
  - Surveyor<sup>®</sup> Mutation Detection Kit (IDT, Catalog Number 706025)
  - EnGen<sup>®</sup> Mutation Detection Kit (NEB, Catalog Number E3321)
- Gel Loading Buffer (Catalog Number G2526)
- Tris-Borate EDTA Buffer, 5× concentrate, powdered blend (Catalog Number T3913)
- Ethidium Bromide Solution, 10 mg/ml in water (Catalog Number E1510)

### Protocols

We recommend using your preferred method to introduce nucleic acids into your cells of interest. Sigma-Aldrich provides a variety of transfection reagents, cell culture media and plates, and custom DNA primers for detection of CRISPR-mediated genome editing. For your reference, suggested protocols are below.

**NOTE:** Cas9 Protein, eSpCas9 Protein, or Cas9 Nickase may be used in the following protocols. The term "Cas9" is subsequently used to represent one of the proteins.

### General Recommendations

- Assemble RNA:Cas9 Protein complexes (RNP) on ice, immediately before use.
- The gRNA can be synthetic or *in vitro* transcribed (IVT); however, the protocols included here have been optimized for SygRNA synthetic crRNAs and tracrRNAs.
- In all instances, combine equal molar amounts of crRNA:tracrRNA.
- We suggest preparing RNP in a molar ratio between 1:1:1 to 5:5:1 (crRNA:tracrRNA: Cas9 protein). Further optimization may be required.
- For Cas9 nickase, 2 different RNA:Cas9 Protein complexes will be assembled separately, then combined after formation before use.

### Preparation of Cells

Approximately 18–24 hours before use, plate cells in complete growth medium. For most cell types, cultures should be 50–80% confluent at the time of transfection.

### Preparation of SygRNA RNP and transfection with TransIT-CRISPR (6-Well Plate Format)

1. Prepare TransIT-CRISPR:SygRNA RNP immediately before transfection
  - a. Warm TransIT-CRISPR Transfection Reagent to room temperature and vortex gently.
  - b. Pipet 1.5 to 15  $\mu$ L each of 20  $\mu$ M SygRNA crRNA and tracrRNA stock solutions to sterile tube on ice.
  - c. **Optional step:** Anneal the crRNA and tracrRNA by incubating the mixture for 5 minutes at 95 °C, then placing the mixture on ice for 20 minutes.
  - d. Add 30 to 60 pmol of Cas9 protein to tube containing synthetic crRNA and tracrRNA. Gently pipet up-and-down to mix.
  - e. Incubate on ice 30 minutes for complex formation.
  - f. Add 250  $\mu$ L of serum-free or reduced serum medium to Cas9 RNP.
  - g. Add 5–6.25  $\mu$ L of TransIT-CRISPR reagent to Cas9 RNP.
 

**NOTE:** The volume of TransIT-CRISPR reagent should be optimized for each cell type. This protocol volume was optimized using adherent U2-OS cells
  - h. Pipet up-and-down gently to mix.
  - i. Incubate at room temperature for 15-30 minutes to allow transfection complex formation.
2. Distribute transfection complexes to cells in complete growth medium
  - a. For each sample, distribute TransIT-CRISPR:SygRNA RNP complex dropwise throughout well.
  - b. Gently rock culture vessel back-and-forth and from side-to-side to distribute TransIT-CRISPR:SygRNA RNP.
  - c. Incubate cells 24–72 hours before harvest for assay. It is not necessary to replace the medium.

**Note:**

Additional information can be found at the TransIT-CRISPR technical information page.

[sigma.com/transitcrispr](http://sigma.com/transitcrispr)

**Preparation and Nucleofection® of SygRNA RNP (12-Well Plate Format)**

1. Prepare Nucleofector solution and cells
  - a. Prepare Nucleofector Kit V reagents according to manufacturer's instructions.
  - b. Obtain enough cells for approximately 250K cells per well in a 12-well plate (final volume per well will be 1 ml).
  - c. Concentrate the cells by centrifugation and remove the medium by aspiration.
  - d. Resuspend the cells in enough Nucleofector Solution (with supplement added) to allow the distribution of 100  $\mu$ L of solution per well.
  - e. Add 0.5 ml of complete medium to each well of a 12-well plate.
2. Prepare SygRNA RNP complex
  - a. Dilute SygRNA crRNA and tracrRNA to a 10  $\mu$ M working solution using a 10 mM Tris-containing buffer of pH between 7 and 8.
  - b. Pipet 0.6 to 7.5  $\mu$ L (6 to 75 pmol) of each RNA to a sterile microfuge tube on ice.
  - c. **Optional step:** Anneal the crRNA and tracrRNA by incubating the mixture for 5 minutes at 95 °C, then placing the mixture on ice for 20 minutes.
  - d. Dilute Cas9 protein to 1 mg/ml using the supplied Dilution buffer and store on ice.
  - e. Pipet 1 to 5  $\mu$ L (6 to 30 pmol) of Cas9 protein to the synthetic crRNA and synthetic tracrRNA, mix gently, and incubate at room temperature for 5 minutes. The final volume of SygRNA crRNA and tracrRNA plus Cas9 protein should be less than 20  $\mu$ L.

3. Nucleofect SygRNA RNP
  - a. Pipet 100  $\mu$ L of resuspended cells in Nucleofector Solution from Step 1e to the tube containing RNA and Cas9 protein and pipet gently to mix completely.
  - b. Transfer cell/RNP complex suspension to a certified cuvette.
  - c. Select the appropriate Nucleofector Program and process cells according to manufacturer's directions.
4. Distribute nucleofected cells to each well
  - a. Immediately add 400  $\mu$ L of complete medium to the cuvette and gently transfer the sample into the appropriate well of the prepared 12-well plate. Use the pipettes supplied with the Nucleofector kit and avoid repeated aspiration of the sample.
  - b. Allow cells to grow for 24–72 hours before harvesting for assay. It is not necessary to replace the medium

**Preparation and Microinjection of SygRNA RNP into One-Cell Embryo**

Microinjection protocols vary greatly depending on embryo type and researcher preferences. Microinjection of Cas9 RNPs has been demonstrated in the following organisms:

1. *Caenorhabditis elegans* (nematode)<sup>10</sup>
2. *Mus musculus* (mouse)<sup>11,12</sup>
3. *Rattus norvegicus* (rat)<sup>12</sup>
4. *Danio rerio* (zebrafish)<sup>13</sup>

**Mutation Detection**

There are many methods to detect indels produced by CRISPR systems. Two commonly used kits are the Surveyor Mutation Detection Kit (IDT, Catalog Number 706025) and the EnGen Mutation Detection Kit (NEB, Catalog Number E3321).

## Troubleshooting

If no cutting is observed and there is reason to suspect an experimental flaw is at fault, the following considerations may aid the researcher in troubleshooting the experiment.

Suspected Issue	Solution
The Cas9 protein has denatured after long term storage in dilution buffer.	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 at -20 °C.
The crRNAs and tracrRNAs need to be annealed before complexing with the Cas9 protein.	While an annealing step is generally not needed, it has shown to increase cutting in rare cases <sup>14</sup> . To anneal the crRNA and tracrRNA, mix them in the desired ratio and incubate the mixture for 5 minutes at 95 °C, then place the mixture on ice for 20 minutes.
The crRNAs and tracrRNAs are degraded.	Under normal cell culture conditions, synthetic RNA modifications are not needed; however, for certain cell lines, this may be necessary. Modifications are available through Sigma-Aldrich.
The transfection or nucleofection is not working or is too toxic.	For any transfection reagent or nucleofection, the protocol should be optimized 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.

## References

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