

## Technical Bulletin

## Cas9 Plus Protein for RNP-Based Genome Editing

from *Streptococcus pyogenes*, recombinant, expressed in *E. coli*, 3X NLS

**Catalog Number CAS9PL**

### Product Description

Cas9 Plus recombinant protein was developed for high specificity and efficient genome editing using RNP complex delivery. The protein was engineered with proprietary mutations that greatly improve editing specificity while maintaining high on-target activity. The protein contains three varied nuclear localization sequences positioned for optimal activity. The molecular mass of Cas9 Plus is 162 kDa and the theoretical pI is 9.1.

### Background Information

The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system was discovered as a microbial adaptive immune system against invading viral and plasmid DNA. In this system, short DNA sequences (protospacers) 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 induce double-stranded breaks in the corresponding foreign DNA sequences.<sup>1</sup>

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.

Although the CRISPR system can be delivered to cells via plasmids, direct introduction of the Cas9:gRNA RNP complex 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>2-5</sup> This RNP technology has broad applications and has been shown to work in both mammalian and plant systems.<sup>6</sup> Furthermore, Cas9 RNP delivery holds great promise for therapeutic applications including successful generation of knock-in primary human T cells.<sup>7</sup>

Sigma-Aldrich offers a variety of lyophilized recombinant Cas9 proteins. All of these proteins can be combined either with SygRNA® synthetic single guide RNA (sgRNA) or with synthetic crRNAs and tracrRNAs to form ribonucleoprotein (RNP) complexes that target the specific genomic locus of interest.

## Components

- CAS9 Plus Protein 1 vial  
Catalog Number C9PLPR  
One vial contains lyophilized Cas9 Plus recombinant protein, 50 µg or 250 µg.
- Transfection Buffer for Cas9 Plus protein 1 vial  
Catalog Number PLXBUF  
One vial contains 25 µL or 125 µL
- Reconstitution Solution for Cas9 proteins 1 mL  
Catalog Number RSOLUTION
- Dilution Buffer for Cas9 proteins, 1 mL  
Catalog Number DBUFFER

## Reagents and Equipment Required but Not Provided

- SygRNA<sup>®</sup> synthetic single guide RNA (sgRNA) or synthetic crRNA and tracrRNA
- Electroporation System for Mammalian Cells  
Note: We recommend the Amaxa Nucleofector<sup>®</sup> 2b device (Lonza, Catalog Number AAB-1001) with Nucleofector Kit (Lonza, different kits suitable for different cell lines)
- 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)
- Custom DNA primers
- Mutation Detection
  - T7 Endonuclease Detection Assay (Catalog Number T7E1001)
  - NGS based analysis
  - Sanger based sequence analysis
- 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)
- Appropriate cell culture media and cultureware

## Precautions and Disclaimer

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.

## Storage/Stability

The lyophilized protein is shipped on wet ice. Once resuspended in the provided Reconstitution Solution, the proteins are recommended to be stored at -20 °C or at -80 °C for long term storage.

## Preparation Instructions

### Notes:

- Minimum deliverable protein quantities are listed as package sizes. Precise quantities vary by lot number; please refer to the Certificate of Analysis for exact protein amounts per vial.
- It is CRITICAL to resuspend the lyophilized proteins in the recommended volume of Reconstitution Solution to maintain the correct formulation for optimized stability. Do NOT use more than the recommended volume. Increasing the volume will reduce salt concentration that is essential for Cas9 protein stability. If lower protein concentrations are desired, the reconstituted proteins can be further diluted with the provided Dilution Buffer (Catalog Number DBUFFER).
- 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, which does not contain glycerol.

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 ~5 mg/mL.
  - b. For 50 µg vials, add 30 µL of Reconstitution Solution to achieve a concentration of ~1.7 mg/mL.
  - c. Refer to Table 1 for the weight to picomole conversion of the Cas9 Plus protein.

**Table 1.**

Weight to picomole conversion of the Cas9 Plus protein

Weight (µg)	Picomole
1.7	10.5
5	30.8

2. Gently flick the tube to dissolve lyophilized powder and place the tube on ice for at least 30 minutes with flicking 2-3 times during incubation to ensure a homogeneous solution. Spin the tube to bring material to bottom of tube.
3. After reconstitution, protein should be stored at -20 °C for frequent uses. Alternatively, protein can be stored at -80 °C for long term storage. If the lyophilized protein is reconstituted in the Dilution Buffer (see Note above), it cannot be stored at -20 °C and freeze-thawed in the absence of glycerol.
4. 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 for up to 6 hours. Discard the diluted protein after use. Diluted protein cannot be stored at -20 °C.

## Procedure

Researchers should use their preferred method to introduce Cas9 RNP into the cells of interest.

Sigma-Aldrich provides a variety of transfection reagents, synthetic sgRNA, cell culture media and plates, and custom DNA primers for detection of CRISPR-mediated genome editing. For your reference, suggested protocols are described below.

### Procedure Overview and Recommendations

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

1. Preparation of cells. Plate cells in complete growth medium approximately 18–48 hours before use, depending on cell types. For most cell types, cultures should be about 50–80% confluent at the time of transfection.
2. Preparation of guide RNA reagents.
  - a. The guide RNA can be synthetic or *in vitro* transcribed (IVT). Sigma-Aldrich provides custom SygRNA® synthetic single guide RNA (sgRNA) or synthetic crRNA and tracrRNA.
  - b. When using synthetic crRNA and tracrRNA, the two RNA molecules should be used in a molar ratio of 1:1. Annealing of the crRNA and tracrRNA is optional.
3. Assembly of Cas9 RNP complex.
  - a. Assemble guide RNA and Cas9 protein complex (RNP complex) on ice and incubate at room temperature for 10-15 minutes. If RNP samples are stored on ice after complexing, warm up the samples to room temperature for 5-10 minutes prior to transfection.

- b. It is recommended to prepare RNP in a molar ratio between 1:1 to 5:1 (guide RNA: Cas9 protein). Further optimization of guide RNA: Cas9 protein ratio may be required.
- 4. Cell transfection with Cas9 RNP complex. Transfect the Cas9 RNP into the cells with the chosen transfection reagent.
- 5. Harvest transfected cells and perform mutation detection.
  - a. Allow the cells to grow 48–72 hours post-transfection before harvesting.
  - b. There are many methods to detect indels produced by CRISPR systems. The most used methods include mismatch detection assay using T7E1 Endonuclease Detection Assay (Catalog Number T7E1001), NGS based sequence analysis, and Sanger based sequence analysis.
- f. Add the desired amount of Cas9 Plus protein (30-50 picomole) to the tube and mix gently by flicking. Spin the tube briefly and incubate at room temperature for 10-15 minutes. If RNP samples are stored on ice after complexing, warm up the samples to room temperature for 5-10 minutes prior to transfection. The final volume of RNP complex should not exceed 15  $\mu$ L. If desired, dilute Cas9 Plus protein to a desired concentration using the supplied Dilution Buffer before RNP assembly. Store diluted Cas9 Plus protein on ice for up to 6 hours. Do not freeze diluted Cas9 Plus protein.

RNP Preparation and Nucleofection (6-Well Plate Format)

1. Prepare SygRNA and Cas9 Plus protein RNP complex:
  - a. If using SygRNA single guide RNA, reconstitute the single guide RNA to 100  $\mu$ M (100 picomole/ $\mu$ L) in 10 mM Tris buffer, pH 7.4.
  - b. If using SygRNA<sup>®</sup> crRNA and tracrRNA, reconstitute SygRNA crRNA and tracrRNA each to 100  $\mu$ M (100 picomole/ $\mu$ L) in 10 mM Tris buffer, pH 7.4.
  - 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. Pipette 5  $\mu$ L of the supplied Dilution Buffer to a sterile microcentrifuge tube on ice. The volume of Dilution Buffer may be adjusted according to the desired final RNP volume.
  - e. Add the desired amount of guide RNA to the tube. The RNA amount depends on the amount of Cas9 protein used and the desired guide RNA: Cas9 protein ratio.
2. Prepare Nucleofactor Solution and cells:
  - a. Prepare Nucleofactor Kit reagents according to manufacturer's instructions. Bring nucleofection solution to room temperature before experiment.
  - b. Add 2 mL of complete medium to each well of a 6-well plate and prewarm the plate at 37 °C and 5% CO<sub>2</sub> until use.
  - c. Obtain enough cells for  $\sim 2.5 \times 10^5$  cells per Nucleofection.
  - d. Collect the cells by centrifugation and remove the medium by aspiration.
  - e. Wash the cells twice with Hanks' Balanced Salt Solution.
  - f. Transfer an aliquot of Nucleofactor Solution (with Supplement added) needed for the experiment to a sterile tube. Add 1  $\mu$ L of Transfection Buffer (Catalog Number PLXBUF) per 100  $\mu$ L and mix thoroughly.
  - g. Resuspend the cells with the prepared Nucleofactor Solution at  $\sim 2.5 \times 10^5$  cells per 100  $\mu$ L of solution.

- Note:** Limit the exposure of cells to the Nucleofector Solution to less than 30 minutes to ensure the best cell fitness. Plan the experimental steps accordingly. It is recommended to resuspend the cells in the Nucleofection Solution AFTER Cas9 RNP complex has been prepared.
3. Transfect cells with Cas9 RNP complex.
    - a. Pipette 100  $\mu$ L of resuspended cells from Step 2g above to the tube containing Cas9 RNP complex and pipette up and down gently to mix completely. Avoid introducing air bubbles.
    - b. Transfer the mixture to a Nucleofection cuvette.
    - c. Perform electroporation immediately using the appropriate Nucleofector program according to manufacturer's recommendation.
  4. Transfer nucleofected cells to medium.
    - a. Immediately add a pipette-full ( $\sim$  500  $\mu$ L) of prewarmed medium from a well of the 6-well plate to the cuvette and gently transfer the sample back to the same well. Use the pipettes supplied with the Nucleofector kit and avoid repeated aspiration of the sample.
    - b. Allow cells to grow for 48–72 hours at 37 °C and 5% CO<sub>2</sub> before harvesting for assay. It is not necessary to replace the medium.

#### Preparation and Microinjection of Cas9/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:

- *Caenorhabditis elegans* (nematode)<sup>8</sup>
- *Mus musculus* (mouse)<sup>9,10</sup>
- *Rattus norvegicus* (rat)<sup>10</sup>
- *Danio rerio* (zebrafish)<sup>11</sup>

## Related Cas9 Protein Products

- PURedit™ CAS9 Protein for RNP-Based Genome Editing, from *Streptococcus pyogenes*, recombinant, expressed in *E. coli*, 3X NLS (Catalog Number PECAS9)
- Wild type *Streptococcus pyogenes* Cas9 Protein (Catalog Number CAS9PROT)
- Cas9-GFP Protein from *Streptococcus pyogenes*, fused with enhanced GFP, recombinant, expressed in *E. coli*, 3X NLS (Catalog Number CAS9GFPPRO)

## References

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8. Cho, S.W., et al., Heritable gene knockout in *Caenorhabditis elegans* by direct injection of Cas9- sgRNA ribonucleoproteins. *Genetics*, **195**, 1177–80 (2013).
9. Cebrian-Serrano, A., et al., Maternal supply of Cas9 to zygotes facilitates the efficient generation of site-specific mutant mouse models. *PLoS ONE*, **12** (2017).
10. Aida, T., et al., Cloning-free CRISPR/Cas system facilitates functional cassette knock-in mice. *Genome Biol.*, **16**, 87 (2015).
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12. Mekler, V. et al., Kinetics of the CRISPR-Cas9 effector complex assembly and the role of 3'-terminal segment of guide RNA. *Nucleic Acids Res.*, **44**, 2837–2845 (2016).

## Troubleshooting Guide

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 Plus protein has denatured after long term storage in Dilution Buffer.	The provided Dilution Buffer is recommended only for immediate use. For long term storage, keep the protein lyophilized or resuspended in the provided Reconstitution Solution at –20 °C.
The Cas9 Plus protein has been thawed and refrozen too many times.	The protein is sensitive to several rounds of temperature cycling. Aliquoting and/or stable low-temperature storage methods will allow for this potential issue to be avoided.
The crRNAs and tracrRNAs need to be annealed before complexing with the Cas9 Plus protein.	While an annealing step is generally not needed, it has been shown to increase cutting in rare cases. <sup>12</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.
IVT RNA is low quality or degraded.	For optimal performance, only quality verified IVT RNA should be used. For mammalian cell transfection, remove the 5’ triphosphate group with a phosphatase to avoid cell toxicity.

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