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Product Information

Recombinant Cas9-GFP and eSpCas9-GFP Fusion Proteins for Fluorophore-Assisted RNP-Based Genome Editing

Cas9-GFP Protein
Product Number **CAS9GFPPRO**
eSpCas9-GFP Protein
Product Number **ECAS9GFPPR**

Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Fluorescent Cas9 and eSpCas9 fusion proteins were developed for visualization of Cas9. In both products, an Enhanced Green Fluorescent Protein (EGFP) is fused, via a proprietary linker to the N-terminus of either wild type *Streptococcus pyogenes* Cas9[1] (Cas9-GFP Protein) or enhanced specificity Cas9[2] (eSpCas9-GFP Protein). Both proteins contain three varied nuclear localization sequences positioned for optimal activity.

The molecular mass of Cas9-GFP Protein is 194 kDa, whereas eSpCas9-GFP Protein is 192 kDa. The EGFP has an excitation peak at 488 nm, with an emission peak at 509 nm.[3]

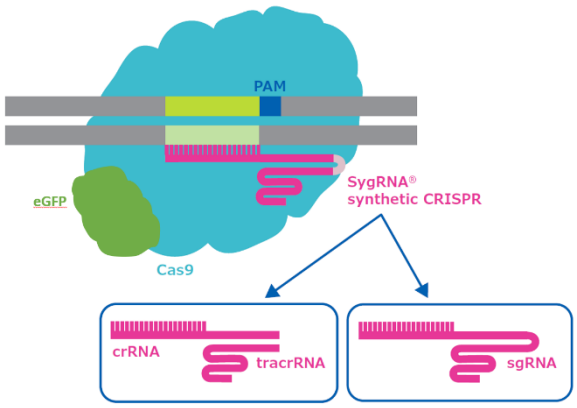
Background Information
The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) system was discovered as a microbial adaptive immune system against invading viral and plasmid DNA. In this system, short DNA sequences (spacers) from invading viruses are incorporated at CRISPR loci within 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 to induce double-stranded breaks in the corresponding foreign DNA sequences.[1]

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 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.[4,5,6,7] This RNP technology has broad applications and has been shown to work in both mammalian and plant systems.[8] Furthermore, Cas9 RNP delivery holds great promise for therapeutic applications including the recent successful generation of knock-in primary human T cells.[9]

Sigma-Aldrich offers a variety of lyophilized recombinant Cas9 proteins. All of these proteins can be combined 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 (see Figure 1).

Figure 1.
CRISPR Cas9 RNP

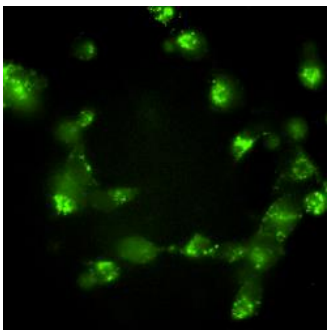


The Cas9 ribonucleoprotein is made up of the Cas9 protein and a guide RNA, which can be delivered as a single guide RNA or as a tracrRNA and a crRNA. The crRNA portion is variable and complementary to the target of interest, while the tracrRNA sequence is static.

The Cas9-GFP Protein and eSpCas9-GFP Protein are additions to our Cas9 protein portfolio with new utilities. The green fluorescence can be used for visual confirmation of RNP complex delivery, more importantly, it can be used to enrich Cas9 RNP transfected cells through cell sorting. This is especially useful for increasing genome editing efficiency in difficult-to-transfect cell lines.

Both Cas9-GFP and eSpCas9-GFP proteins yield strong green fluorescence that allows viewing cellular localization of the Cas9 RNP after transfection (see Figure 2).

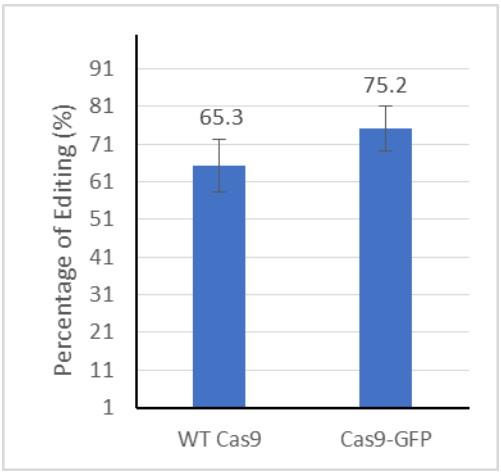
Figure 2.
Microscopy Image of Cas9-GFP Transfected HEK293 Cells



HEK293 cells were transfected with Cas9-GFP RNP complex. Cell image was taken 24 hours post transfection with 40× magnification.

Both Cas9-GFP and eSpCas9-GFP Proteins were designed so the fused EGFP domain does not reduce activity of the Cas9 nuclease, while the three added NLS increase nuclear localization of the RNP and thereby increase genome editing activity (see Figure 3).

Figure 3.
Comparison of Gene Editing Activities of Cas9 and Cas9-GFP



RNP complexes were transfected into U2OS cells targeting a VEGFA3 target. Genome editing activity was assessed by next generation sequencing.
WT Cas9: Product Number CAS9PROT
Cas9-GFP: Product Number CAS9GFPPRO

Components

Cas9-GFP Protein 1 vial
(Product Number CAS9GFPPRO)
One vial contains lyophilized Cas9-GFP recombinant protein (Product Number C120040), 50 µg or 250 µg.

or

eSpCas9-GFP Protein 1 vial
(Product Number ECAS9GFPPR)
One vial contains lyophilized eSpCas9-GFP recombinant protein (Product Number E120030), 50 µg or 250 µg.

Each protein kit includes the following components:

Reconstitution Solution for Cas9 proteins 1 mL
(Product Number RSOLUTION)
1×, 50% glycerol in water

Dilution Buffer for Cas9 proteins, 1 mL
(Product Number DBUFFER)
1×, 20 mM Na-HEPES, pH 7.5, with 200 mM NaCl

Reagents and Equipment Required but Not Provided

- SygRNA synthetic single guide RNA (sgRNA) or SygRNA synthetic crRNAs and tracrRNA (Product Number VC40003)
- Electroporation System for Mammalian Cells
  Note: We recommend the Amaxa Nucleofector® 2b device (Lonza, Catalog Number AAB-1001) with Nucleofector Kit (Lonza, different kits suitable for different cell lines)
- GenElute™ Mammalian Genomic DNA Miniprep Kit (Product Number G1N70)
- JumpStart™ Taq ReadyMix™ (Product Number P2893
- Water, PCR Reagent (Product Number W1754)
- Custom DNA primers
- Mutation Detection
  - NGS based analysis
  - Mismatch detection kit
  - Sanger based sequence analysis
- Gel Loading Buffer (Product Number G2526)
- Tris-Borate EDTA Buffer, 5× concentrate, powdered blend (Product Number T3913)
- Ethidium Bromide Solution, 10 mg/mL in water (Product 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.

Preparation Instructions

1. Resuspend the lyophilized protein with the supplied Reconstitution Solution (Product Number RSOLUTION).
   a. For 250 µg vials, add 50 µL of Reconstitution Solution to achieve a concentration of approximately 5 mg/mL.
   b. For 50 µg vials, add 30 µL of Reconstitution Solution to achieve a concentration of approximately 1.7 mg/mL.
   c. Please refer to Table 1 for the weight to pmole conversion of the Cas9-GFP proteins.

Table 1.
Weight to pmole Conversion of the Cas9-GFP Proteins

Weight (µg)	pmole
1.7	8.8
5	26.0

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 (Product 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.

2. Gently tap tube to completely dissolve lyophilized powder, incubate for 10 minutes on ice, and spin tube to bring material to bottom of tube.
3. After reconstitution, proteins should be stored at –20 °C, protected from light. If the lyophilized protein is reconstituted in the Dilution Buffer (see Note above), it cannot be stored at –20 °C and freeze-and-thawed in the absence of glycerol.
4. If a lower concentration of Cas9 protein is required, dilute the Cas9 protein with supplied Dilution Buffer (Product Number DBUFFER) immediately before use. Store diluted protein on ice up to 6 hours and protect the tube from light by wrapping the tube with aluminum foil. Discard the diluted protein after use. Diluted protein cannot be stored at –20 °C.

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, protected from light.

Procedure

Researchers should use their preferred method to introduce Cas9 RNP into the 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-GFP Protein and ESPCAS9-GFP Protein may be used in the following protocols. The term "Cas9 Protein" is subsequently used to represent either of the proteins.

Procedure Overview and Recommendations

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

1. 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.
2. Preparation of guide RNA reagents
   - The guide RNA can be synthetic or *in vitro* transcribed (IVT). Sigma-Aldrich provides custom SygRNA synthetic single guide RNA (sgRNA) or synthetic crRNAs and tracrRNAs.
   - 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
   - Assemble guide RNA:Cas9 Protein complexes (RNP) on ice, immediately before use.
   - It is recommended to prepare RNP in a molar ratio between 1:1 to 5:1 (guide RNA:Cas9 protein). Further optimization may be required.
   - The Cas9-GFP RNP complex can be easily visualized as a fluorescent green color in the test tube.
4. Cell transfection with Cas9 RNP
   - Transfect the Cas9 RNP into the cells with the chosen transfection reagent.
5. Harvest transfected cells and mutation detection
   - Allow the cells to grow 48–72 hours post transfection before harvesting.
   - There are many methods to detect indels produced by CRISPR systems. The most commonly used methods include NGS based sequence analysis, mismatch detection assay, and Sanger based sequence analysis.

RNP Preparation and Nucleofection® (6 Well Plate Format)

1. Prepare Nucleofector solution and cells
   a. Prepare Nucleofector Kit reagents according to manufacturer's instructions.
   b. Obtain enough cells for approximately 2.5×10^5 cells per well in a 6 well plate.
   c. Concentrate the cells by centrifugation and remove the medium by aspiration, washing the cells twice with Hanks' balanced salt solution.
   d. Add 2 mL of complete medium to each well of a 6 well plate, prewarm at 37 °C until use.
   e. Resuspend the cells in enough Nucleofector Solution (with supplement added) to allow the distribution of 100 µL of solution per well.
   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 suggested to resuspend the cells in the Nucleofection Solution AFTER the Cas9 RNP complex is prepared.
2. Prepare SygRNA RNP complex
   a. If using SygRNA crRNA and tracrRNA, dilute SygRNA crRNA and tracrRNA to 20–100 µM (20–100 pmole/µL) working solutions using a10 mM Tris buffer, pH 7.4.
   b. If using SygRNA single guide RNA, dilute the single guide RNA to 20–100 µM (20–100 pmole/µL) working solution using 10 mM Tris buffer, pH 7.4.
   c. Pipette desired amount of each RNA to a sterile microfuge tube on ice. The RNA amount depends on the amount of Cas9 protein used and the desired RNA:Cas9 ratio.
   d. 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.
   e. Dilute Cas9 protein to 1 mg/mL using the supplied Dilution Buffer and store on ice.
   f. Pipette 1–5 µL (5.2–26 pmole) of Cas9 protein to the prepared guide RNA, mix gently, and incubate at room temperature for 5 minutes. The final volume of RNP complex should be less than 20 µL.

3. Nucleofect Cas9 RNP
   a. Pipette 100 µL of resuspended cells in Nucleofector Solution from Step 1e to the tube containing Cas9 RNP, and pipette gently to mix completely.
   b. Transfer cell/RNP complex suspension to a compatible 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 500 µL of prewarmed medium from the 6 well plate to the cuvette, 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 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:

- *Caenorhabditis elegans* (nematode)[10]
- *Mus musculus* (mouse)[11,12]
- *Rattus norvegicus* (rat)[12]
- *Danio rerio* (zebrafish)[13]

Related Sigma-Aldrich Cas9 Protein Products

Wild type *Streptococcus pyogenes* Cas9
(Product Number CAS9PROT)

Enhanced specificity Cas9 (eSpCas9)
(Product Number ESPCAS9PRO)

Cas9D10A nickase (Cas9D10A)
(Product Number CAS9D10APR)

Catalytically inactive dCas9
(dCas9-3XFLAG™-biotin protein,
Product Number DCAS9PROT)

Cas9 from *Francisella novicida* (FnCas9)
(Product Number FNCAS9PROT).

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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 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 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.
Green fluorescence in Cas9-GFP has been diminished.	Protect the proteins from light by long term storage in a dark box at –20 °C and wrapping the tube with aluminum foil during experiment under normal light.
The crRNAs and tracrRNAs need to be annealed before complexing with the Cas9 protein.	While an annealing step is generally not needed, it has been shown to increase cutting in rare cases.[14] 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.

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