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Application Note

Recombinant eSpCas9 Protein

For RNP-Based Genome Editing

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Introduction

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 to cleave double-strand 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. Here, we describe two different recombinant Cas9 proteins: Cas9 (wild type SpCas9) and enhanced specificity Cas9 (eSpCas9), which has been shown to reduce off-target cleavage[2]. These proteins can be combined with SygRNA™ synthetic crRNAs and tracrRNAs to form ribonucleoprotein (RNP) complexes that target the specific genomic locus of interest (**Figure 1**).

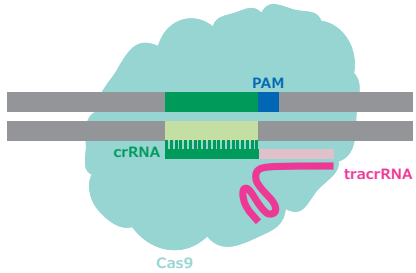

Figure 1. Three Component CRISPR Cas9 System. The Cas9 ribonucleoprotein is made up of the Cas9 protein and a guide RNA, which can be divided into a tracrRNA and a crRNA. The crRNA is variable and complementary to the target of interest, while the tracrRNA sequence is static.

Although the CRISPR system can be delivered to cells via plasmids, direct introduction of Cas9 RNP-sgRNA 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 reduces risk for 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[1,3,4,5]. This RNP technology has broad applications and has been shown to function in both mammalian and plant systems[6]. Furthermore, Cas9 RNP delivery holds great promise for therapeutic applications including the recent successful generation of knock-in primary human T cells[7].

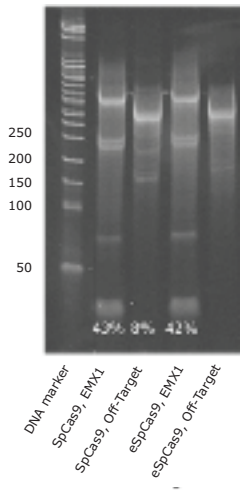

Figure 2. eSpCas9 Reduces Off-Target Cleavage Compared with Cas9. In this experiment, K652 cells were nucleofected with SpCas9 or eSpCas9 and synthetic tracrRNA and EMX1-targeted crRNA. A CEL-1 assay showed equal cleavage efficiency between Cas9 and eSpCas9, while cleavage at a known off-target site[7] was reduced when eSpCas9 was used compared to Cas9.

One of the primary concerns with CRISPR is potential for off-target cleavage; eSpCas9 improves the specificity of the system by reducing these effects. The unwinding of 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 has the potential to increase the requirement for precise match between guide RNA and 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

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made in the chromosome-binding motif of SpCas9[2]. In testing eSpCas9 in combination with select gRNA at Sigma-Aldrich (**Figure 2**), on-target cleavage efficiency was comparable to wild type SpCas9 with undetectable cleavage at select off-target sites.

Procedure Overview

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

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

Methods

1. Resuspend the lyophilized Cas9 protein with the supplied reconstitution solution.
   a. For 250 ug vials, add 50 ul of reconstitution solution to achieve a concentration of approximately 5 mg/ml (30 pmol/µl).
   b. For 50 ug vials, add 30 µl of reconstitution solution to achieve a concentration of approximately 1.7 mg/ml (10 pmol/µl).

Note: Search for additional information about CRISPR-Cas9 Proteins:

SigmaAldrich.com/CRISPRprotein

2. Gently tap tube to completely dissolve of lyophilized powder, incubate for 10 minutes on ice and spin tube 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 immediately before use. Store diluted protein on ice, up to 6 hours.

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. Once resuspended in the provided reconstitution solution, Cas9 and eSpCas9 are recommended to be stored at –20°C.

Kit Components

Each kit contains the following components:

- One vial of lyophilized Cas9 recombinant protein
- One vial of 1X reconstitution solution (1 ml, 50% glycerol in water)
- One vial of 1X dilution buffer (1 ml, 20 mM Na-Hepes pH 7.5, 200 mM NaCl)

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

- SygRNA™ synthetic crRNAs and TRACRRNA05N
- Nucleofector® Kit V (Lonza, Cat# VCA-1003)
- GenElute Mammalian Genomic DNA Miniprep Kit. (**Cat. No. G1N70**)
- JumpStart™ Taq ReadyMix™ (**Cat. No. P2893**)
- Water, PCR Reagent (**Cat. No. W1754**)
- Mutation Detection Method of choice
- Gel Loading Buffer (**Cat. No. G2526**)
- Tris-Borate EDTA Buffer (**Cat. No. T3913**)
- 10 mg/µl Ethidium Bromide Solution (**Cat. No. E1510**)

Protocols

CRISPR is amenable to a variety of transfection methods; therefore it is recommended to optimize methods to suit 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, we have suggested protocols below.

General Recommendations

- Assemble RNA:Cas9 Protein complexes (RNP) on ice, immediately before use.
- The gRNA can by 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.

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 µl each of 20 µM SygRNA™ crRNA and tracrRNA stock solutions to sterile tube on ice.
   c. Optional: anneal the crRNA and tracrRNA by incubating the mixture for 5 minutes at 95°, then placing the mixture on ice for 20 minutes.
   d. Add 1 to 2 µl of Cas9 protein (30 to 60 pmol) 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 µl of serum-free or reduced serum medium to Cas9 RNP.
   g. Add 5–6.25 µl of TransIT-CRISPR® reagent to Cas9 RNP.
      i. This volume was optimized in adherent U2-OS cells; the volume of TransIT-CRISPR® reagent should be optimized for each cell type.
   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 dropwise throughout well.
   b. Gently rock culture vessel back-and-forth and from side-to-side to distribute TransIT-CRISPR®:SygRNA™ RNP complexes.
   c. Incubate cells 24–72 hours before harvest for assay. It is not necessary to replace the medium.

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

3. Prepare Nucleofector® Solution and Cells
   a. Prepare Nucleofector® Kit 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 µl of solution per well.
   e. Place 0.5 ml of complete medium to each well of a 12-well plate.
4. Prepare SygRNA™ RNP
   a. Dilute SygRNA™ crRNA and tracrRNA to a 10 µM working solution using a 10 mM Tris-containing buffer of pH between 7 and 8.
   b. Pipet 0.6 to 7.5 µl (6 to 75 pmol) of each RNA to a sterile microfuge tube on ice.
   c. Optional: anneal the crRNA and tracrRNA by incubating the mixture for 5 minutes at 95°, 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 µl (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 µl.
5. Nucleofect SygRNA™ RNP
   a. Pipet 100 µl of resuspended cells in Nucleofector® solution 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.
6. Distribute nucleofected cells to each well
   a. Immediately add 400 µl 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)[9]
2. *Mus musculus* (mouse)[10,11]
3. *Rattus norvegicus* (rat)[11]
4. *Danio rerio* (zebrafish)[12]

Note: For more information about CRISPR gene editing and assay development, please visit:

SigmaAldrich.com/CRISPRlinks

SigmaAldrich.com/AssayDevelopment

Conclusion

We present a new development in Cas9 technology for addressing the problem of CRISPR off-target effects. In addition, we provide detailed protocols for use of Cas9 RNP which has broad applications in both animal and plant models.

Troubleshooting

If cleavage is not observed, the following considerations may aid the researcher in troubleshooting:

Solution	Suspected Issue
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°.	The Cas9 protein has denatured after long term storage in dilution buffer.
The Cas9 protein has been shown to withstand several rounds of freezing and thawing without sacrificing cutting activity, but aliquoting the protein into smaller quantities upon resuspension will allow this potential issue to be avoided.	The Cas9 protein has been thawed and refrozen too many times.
While an annealing step is generally not needed, it has shown to increase cutting in rare cases[13]. To anneal the crRNA and tracrRNA, mix them in the desired ratio and incubate the mixture for 5 minutes at 95°, then place the mixture on ice for 20 minutes.	The crRNAs and tracrRNAs need to be annealed before complexing with the Cas9 protein.
Under normal cell culture conditions, synthetic RNA modifications are not needed; however, for certain cell lines, this may be necessary. Modifications are available through MilliporeSigma.	The crRNAs and tracrRNAs are degraded.
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.	The transfection or nucleofection is not working or is too toxic.
For optimal performance, only quality-verified IVT RNA should be used.	IVT RNA is low quality or degraded.

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