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# Protocols for Recombinant Cas9 protein (Streptococcus pyogenes)

## 1. Reconstitution protocol for Cas9 protein (lyophilized).

- We recommend dissolving lyophilized Cas9 protein in 50 ul of nuclease-free water (1 mg/ml stock solution) by pipetting when you receive the package.
- Allow the solution to sit at least 10 minutes at room temperature (20-23℃) to completely dissolve in solution. Vortex and centrifuge immediately before use.
- The final buffer condition is 20 mM Hepes (pH 7.5), 150 mM KCl, 1% sucrose.
- Store the appropriate aliquot of proteins at or below – 70℃. Do not store in a frost-free freezer.

## 2. Digestion of target sequence in vitro using CRISPR sgRNAs.

**1)** Set up the reaction mixture as below.

| Components | Amount | |
|---|---|---|
| Cas9 protein | 500 ng | (100 ng~1,000 ng) |
| CRISPR sgRNA | 250 ng | (100 ng~700 ng) |
| Targeting substrate | 100~150 ng | PCR product |
| | 80 ng | (Plasmid) |
| 10X NEB buffer. 3 | 1 ul | |
| 10X BSA | 1 ul | |
| D.W | To 10 ul | |

**2)** Incubate the reaction mixture at 37℃ for 1 hr.

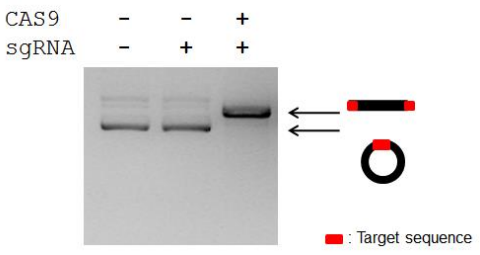
**3)** Add 4 ug of RNase and incubate for 15 min at 37℃

**4)** Add 1 ul of STOP solution to the reaction mixture and incubate for 15 min at 37℃ .

[STOP solution: 30% glycerol, 1.2% SDS, 250 mM EDTA (pH 8.0)]

**5)** Analyze on 2% agarose gel.

**<Example of efficient cleavage of circular plasmid using CRISPR in vitro>**




## 3. General guidelines for the establishment of KO animal by direct injection of CRISPR RNP into one-cell embryo.

**1)** Make a mixture of Cas9 protein and guide RNA Recommended parameters.

| Mouse | Concentration (/ul) |
|---|---|
| Cas9 protein | 20 ng (10~100 ng) |
| CRISPR sgRNA | 40 ng (10~80 ng) |

**2)** Incubate for 15 min at 37℃.

**3)** Inject diluted solution into one-cell embryos.

## 4. General guidelines for the application of CRISPR RNP to cultured cells by Electroporation. (Neon electroporation device, Thermo Fisher)

1) Day 0, seed the cells into a flask/dish with fresh medium such that the cells are 60~80% confluent in the day of the electroporation.
2) Day 1, prepare culture medium containing serum without antibiotics.
3) Take cells and determine the cell number
4) Centrifuge cells at 300 x g for 5 minutes at room temperature.
5) Aspirate the supernatant and wash cells with PBS (without $Mg^{2+}$ and $Ca^{2+}$) and centrifuge cells at 300 x g for 5 minutes at room temperature.
6) Aspirate the supernatant and re-suspend the cell pellet in Resuspension Buffer R at a final density of 2.4 x $10^5$ cells/9.6 µl. Gently pipette the cells to obtain homogenous single cell suspension.
7) Prepare 24-well plates by filling the wells with 0.5 ml of culture medium containing serum without antibiotic and pre-incubate plates in an incubator.
8) Prepare Cas9 and sgRNA expressing plasmids or Cas9 protein and sgRNA in Resuspension Buffer R into a sterile, 1.5 ml microcentrifuge tube at the suggested concentration below.

| | Cas9 protein | CRISPR sgRNA | Total Volume |
|---|---|---|---|
| **RNP** | 4 ug | 1 ug | 2.4 ul |

**< We routinely use the 4:1 ratio in the amount of Cas9 protein and CRISPR sgRNA. The optimal amount of CRISPR RNP mixture should be determined empirically. Usually, an electroporation of 0.4~10 ug of Cas9 (0.1~2.5 ug of CRISPR sgRNA) support a highly efficient genome editing in various cell types.>**

9) Add cells to the tube containing CRISPR-Cas9 and gently mix.
10) Set the desired pulse conditions on the device.
(Optimal electroporation conditions vary depending on cell types.)
Fill the Neon pipette with cell/CRISPR-Cas9 mixture. Insert the Neon pipette into Neon tube and deliver the electric pulse.
11) Remove the Neon pipette from Neon tube and immediately

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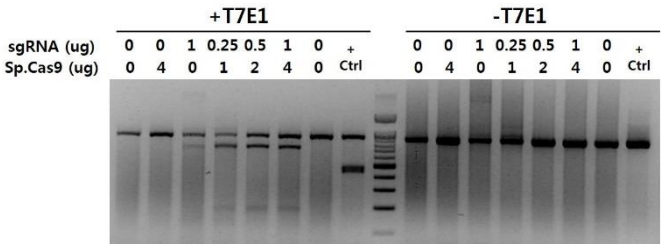
transfer the samples into the prepared culture plate containing pre-warmed medium.

12) Incubate the plate at 37°C in a humidified $_{CO2}$ incubator in a cell culture incubator for 1~2 days and assay samples to determine the genome editing efficiency by T7E1 assay (see below) or targeted deep sequencing.




**<T7E1 assay after CRISPR-Cas9 RNP delivery into Jurkat cells.>**
CRISPR sgRNA and Cas9 proteins were delivered into Jurkat cells at each amounts as indicated above using Neon electroporation device (1400 V, 20 ms, 2 pulses). gDNA was prepared 1 day after electro-poration.

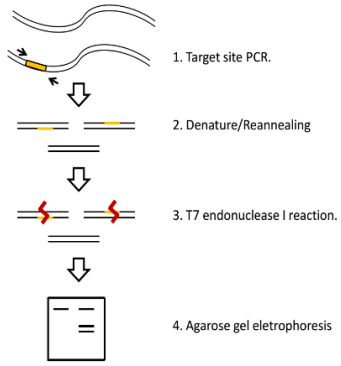
## 4. General guidelines for the application of CRISPR RNP to cultured cells by Electroporation. (Lipofection, Thermo Fisher)

The amounts of the reagents given in the protocol below are for one well of a 24-well plate. For other reaction formats, scale the amounts of reagents up or down accordingly.

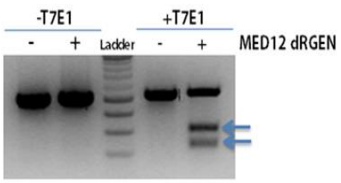
1) Add Cas9 RNP complex (0.5 µg of Cas9 Nuclease and 250 ng of CRISPR sgRNA) to 50 µl Opti-MEM I Reduce Serum Medium.
2) In a separate tube, dilute the transfection reagent by adding 4 µl of the Lipofectamine 2000 transfection reagent to 50 µl of Opti-MEM I Reduce Serum Medium. Mix gently.
3) Incubate for 5 min at RT
4) Add the diluted transfection reagent to the tube containing Cas9 protein/gRNA RNP complexes and mix gently.
5) Incubate at room temperature for 20 minutes to allow the formation of Cas9/gRNA-lipid complexes.
6) Add the Cas9/gRNA-lipid complexes to the 1x10$^5$ NIH3T3 cells to be transfected. Swirl the plates gently to allow the mixing of the transfection mixture with the medium.
7) Incubate the plate at 37°C in a humidified $CO_2$ incubator in a cell culture incubator for 2~3 days and assay samples to determine the genome editing efficiency by T7E1 assay (see below) or targeted deep sequencing.

# Protocols for T7E1 assay

T7 endonuclease I is a surveyor nuclease. T7 endonuclease recognizes mismatched DNA, heteroduplex DNA. And T7 endonuclease I cleaves at the first, second or third phosphodiester bond that is 5´ to the mismatch.




*** Example**




| | |
|---|---|
| PCR product | 507 bp |
| Expected cut product | 287 bp<br>220 bp |

## I. Genomic DNA Extraction

## II. PCR Amplification

1. PCR amplify the genomic DNA purified from transfected CRISPR RNP.
2. Check PCR on 0.8% agarose gel.

| | |
|---|---|
| DNA polymerase Taq | 0.2 ul |
| 10X buffer | 2 ul |
| dNTP (10mM) | 0.4 ul |
| Primer-F (10pmol) | 0.5 ul |
| Primer-R (10pmol) | 0.5 ul |
| gDNA | 50~100 ng |
| DW | Up to 20 ul |

| | | | |
|---|---|---|---|
| Initial Denaturation | 95℃ | 4:00 | |
| Denaturation | 95℃ | 0:30 | 35~40 Cycles |
| Annealing | X℃ | 1:00 | |
| Extension | 72℃ | 1:00 | |
| Final Extension | 72℃ | 10:00 | |
| Hold | 4℃ | ∞ | |

## III. T7E1 Assay

1. Denature and annealing PCR reaction.
2. Take 15 µl PCR reaction and add 0.2~0.8 µl T7E1 enzyme to each reaction. Incubate at 37℃ 15 minutes.
3. Run the digestions on 2% agarose gel.
4. Visualize with UV illuminator.

| Denature and annealing condition | |
|---|---|
| 95℃ | 4:00 |
| 95℃ to 85℃ | -2℃/s |
| 85℃ to 25℃ | -0.1℃/s |

| PCR reaction | 15 ul |
|---|---|
| T7E1 enzyme (ToolGen) | 0.2~0.8 ul |
| 10X T7E1 buffer | 2 ul |
| DW | Up to 20 ul |