4320 Forest Park Ave
Suite 303
Saint Louis, MO | 63108
+1 (314) 833-9764

CAS9 PROTEIN

Cas9 Protein enables gene editing with the CRISPR-Cas9 system with increased efficiency and fewer off target effects.

INCLUDED

Cas9 Protein
10 µg/µl
50 µg or 250 µg

www.canopybiosciences.com

USER MANUAL CRSP004

CAS9 PROTEIN

PRODUCT DESCRIPTION

Cas9 is provided as a purified protein, which allows for immediate activity without transcription or translation and has been shown to reduce off-target effects. The Cas9 has an N-terminal and a C-terminal nuclear localization signal, which allows for more efficient crossing of the nuclear membrane in order to generate double-stranded breaks in DNA with high efficiency and specificity. Cas9 forms an active ribonucleoprotein complex with the sgRNA. Cas9 protein requires an sgRNA (not included) towards the target sequence for gene editing applications.

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INCLUDED

Store all items at -20°C. Avoid repeated freeze thaws.

- Cas9 62 µM (5 or 25 µl, 62 µM in formulation buffer)

ADDITIONAL ITEMS NEEDED FOR GENE EDITING

- Your cell line of choice (test to confirm it is mycoplasma-free)
- Two crRNAs (45 µl, 200 µM in TE, pH 7.5)
- tracrRNA (45 µl, 200 µM in TE, pH 7.5)
- Forward and Reverse Primers (20 µl, 100 µM in TE, pH 8.0)
- Duplex Buffer (2 tubes, 1 ml each)
- Donor oligo or donor plasmid (knock-in and point mutation applications only)
- Genomic DNA extraction solution
- T7 endonuclease
- Polymerase
- TAE buffer
- Agarose
- DNA gel loading dye
- Ethidium Bromide
- DNA gel ladder

Additional items needed for transfection only, not included:

- Transfection reagents (Invitrogen RNAiMax recommended)
- Opti-MEM (Thermo Fischer Scientific Catalog #31985062 recommended)

Additional items needed for nucleofection only, not included:

- Additional Cas9 (Canopy Biosciences Cat #CRSP004) may be needed for nucleofection kit as nucleofection requires more Cas9 than transfection. Each vial of Cas9 62 µM (5 µl, 62 µM in formulation buffer) is sufficient for 3 nucleofections at the suggested Cas9 concentration. Note that Cas9 concentration optimization may need to be performed for your cell line of interest.
- Nucleofector (Lonza 4D-nucleofector recommended)
- Nucleofection reagents (Lonza kit recommended for your cell line of choice recommended). To determine the appropriate Lonza kit for your cell line of interest visit http://bio.lonza.com/6.html

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EQUIPMENT NEEDED

- Thermocycler
- PCR gel set-up
- FACS (optional, used for single cells plating and for checking transfection efficiency)

SUGGESTED TRANSFECTION AND NUCLEOFECTION PROTOCOLS

Perform transfection or nucleofection to deliver CRISPR-Cas9 reagents into the cells.

Recommended Reactions (recommended to perform in duplicate)

- GFP or transfection reagent alone
- Cas9 alone
- Positive control such as HPRT (optional)
- Donor alone
- Cas9+crRNA1+tracrRNA
- Cas9+crRNA2+tracrRNA
- Cas9+crRNA1+tracrRNA+donor
- Cas9+crRNA2+tracrRNA+donor

TRANSFECTION PROTOCOL

Recommended Parameters for Transfection Protocol

- RNAiMax: 0.2-2.0 µl /96 well range, start with 1.2 µl
- Timing: 24-72 h, start with 48 h
- Cell density: 5,000-250,000 cells/96 well, start with 40,000 cells
- Donor (for knock-in applications): 1-50 nM final concentration, start with 25 nM

1. Prepare crRNA and tracrRNA 1 µM final concentration duplex
   a. 1 µl of 200 µM crRNA
   b. 1 µl of 200 µM tracrRNA
   c. 198 µl of Duplex Buffer
2. Heat duplex in thermocycler for 5 min at 95°C
3. Remove from heat and allow to cool to room temperature on bench top

4. Pipette 1 µl of 62 µM Cas9 into 61 µl of Duplex Buffer and mix to obtain 1 µM Cas9 solution
5. Assemble 25 µl of Ribonucleoprotein (RNP) Complex by mixing
   a. 1.5 µl of µM complexed crRNA:tracrRNA
   b. 1.5 µl of µM cas9
   c. 22 µl of opti-MEM
6. Incubate 5 min at room temperature (unused RNP complexes can be stored up to one month at 4°C
7. Reverse Transfect RNP Complex in 96 well plate (this is per well)
   a. 25 µl of RNP
   b. If using Knockin Kit add donor
   c. 1.2 µl RNAiMax
   d. 24 µl opti-MEM
   e. total 50 µl, incubate 20 min
      i. during incubation prepare cells
      ii. dilute to 400,000 cells/ml with complete media with no antibiotics
   f. add 50 µl of incubated RNP complex to each well
   g. add 100 µl diluted cells to each well (40,000 cells/well final RNP concentration 10 nM)
   h. Incubate for 24-72 hours (recommended to start with 48 hours)

NUCLEOFECTION PROTOCOL

Recommended Parameters for Nucleofection Protocol

- Timing: 24-72 h, start with 48 h
- Cell density: 200,000-500,000 cells/96 well, start with 350,000 cells
- Donor (for knock-in applications): 0.2-5.0 µM final concentration, start with 1 µM

1. Lonza recommends using low cell passage number and cells that have been in culture for at least 2-3 days.
2. Add entire Nucleofector Supplement supplied by Lonza to Nucleofector Solution prior to use.
3. Selector Nucleofector Program recommended for your cells of interest (supplied with Nucleofector kit)
4. Form the crRNA:tracrRNA duplex by mixing (amount for 8, 96 wells)
   a. 5 µl of 200 µM crRNA
   b. 5 µl of 200 µM tracrRNA
5. Heat at 95°C for 5 min
6. Remove from heat and allow to cool to room temperature on the bench
7. Form the RNP complex by mixing (amount needed for 1 condition/96 well plate)

   a. 2.2 µl PBS
   b. 1.2 µl crRNA:tracrRNA duplex
   c. 1.6 µl of 62 µM Cas9
8. Incubate at room temperature for 15 min
9. Add 175 µl of culture media to each 96 well, warm to 37°C
10. Warm an additional 75 µl/well of culture media to 37°C in a conical tube
11. Harvest cells and transfer total cells needed for experiment in a 15 ml conical tube.
12. Wash cell pellet with PBS
13. Resuspend cell pellet in 20 µl of Nucleofector Solution (supplement has already been added) per well
14. Pipette 20 µl of cell suspension into each well of an empty V-bottom 96 well plate or individual tubes
15. Add 5 µl of crRNA:tracrRNA:Cas9 RNP to each well
16. Add donor (if using knock-in kit)
17. Pipette up and down to mix and transfer 25 µl of cell/RNP complex mixture to Nucleocuvette.
18. Gently tap the Nucleocuvette to remove air bubbles
19. Put Nucleocuvette in Shuttle device
20. Perform Nucleofection
21. Add 75 µl of pre-warmed media and gently pipette up and down
22. Transfer 25 µl from the Nucleocuvette into the tissue culture plate with pre-warmed module
23. Incubate 24-72 hours (recommended to start with 48 hours)

EXTRACTION OF GENOMIC DNA

1. Wash cells with 100 µl PBS
2. Add genomic DNA extraction lysis buffer such as 50 µl epicentre quick extract DNA solution
3. Transfer cell lysate to PCR tubes
4. Vortex
5. Incubate for 65°C 15 min
6. Incubate for 95°C 5 min

T7 ASSAY TO VERIFY NUCLEASE ACTIVITY

1. Set up PCR for T7 Assay
   a. Have a water/no template control
   b. Have a positive control (control template and primers typically come with kit)
   c. 2.5 µl genomic DNA (after it has been diluted 1:5 in water)

   d. 1.25 µl 10 µM Primer for 0.5 µM final
   e. 1.25 µl 10 µM Primer for 0.5 µM final
   f. 12.5 µl 2X Q5 HiFi Master Mix
   g. Bring up to 25 µl in water
   h. mix and spin
2. PCR machine (will need to optimize based on primers)
   a. 98°C, 30 sec, 1 cycle
   b. 35 cycles
      i. 98°C, 20 sec
      ii. 67°C (temperature will change based on primers), 15 sec
      iii. 72°C, 1 min
   c. 72°C, 2 min
   d. hold 4°C
3. Run a portion on a gel to confirm PCR was successful
4. Form Heteroduplexes for T7E1 digestion (18 µl total)
   a. 10 µl PCR reaction
   b. 2 µl T7E1 Reaction buffer
   c. 6 µl nuclease free water (to bring up to 18 µl)
   d. Heat 95°C for 10 min
   e. 95-85°C, ramp rate -2°C /sec
   f. 85-25°C, ramp rate -0.3°C /sec
5. Heteroduplex Digestion (20 µl total)
   a. 18 µl annealed PCR product
   b. 2 µl T7E I
   c. incubate 37°C for 60 min
   d. use or store at -20 °C
6. Gel Analysis or Fragment Analyzer
   a. run samples on 2% agarose gel or fragment analyzer
   % modification = 100*(1-(1-fraction cleaved)1/2)
   % cleavage=100*(cut products/cut products+uncut)

Note: Negative control wells should only have full length fragments

NGS

1. If cutting is difficult to see by gel or fragment analyzer, NGS is recommended.
2. If Knockin kit is being used NGS is recommend to confirm HDR has occurred in the cell pool before single cell expansion is performed.

SINGLE CELL EXPANSION

1. If co-transfection of GFP was performed, single cell sort and plate 8, 96 well

plates per condition.
2. If FACS machine is available single cell sort and plate 8, 96 well plates per condition.
3. If FACS machine is unavailable single cells plate 8, 96 well plates per condition by calculation half a cell per 96 well.

SEQUENCING ANALYSIS

Once single cells clones have become confluent split each 96 well plate into two duplicate 96 well plates. Keep one plate for growing up and passaging the cells. Harvest genomic DNA from second plate and send off for sequencing to determine if the insertion/deletion was successful. Grow up positive clones.

TROUBLESHOOTING GUIDE

Problem: It has been several weeks and my single cell clones are not growing up
Answer: If cells do not start to divide after 3 weeks adjust growth conditions.
Potential changes to growth media:

- Increase FBS concentration in growth media
- Add NaPyruvate to growth media
- Add Rock Inhibitor Y27632 to the growth media
- Add conditioned media to the growth media

Problem: I don't see a parental band in the PCR
Answer: Optimize the PCR conditions. First, try lowering the annealing temperature and increasing the elongation time. Confirm that the annealing temperature is correct based on primer Tm.

Problem: My transfection is not working
Answer: Optimize transfection.

- Try using fluorescent tracrRNA (requires fluorescent microscope or FACS machine, requires fluorescent tracrRNA).
- Try transfection GFP plasmid.
- Adjust cell number, amount of transfection reagent, concentrations of RNP complex, timing.
- Try HPRT control kit.

Problem: I am performing a knockin and am seeing cutting by the crRNAs, but no HDR.
Answer: The transfection/nucleofection is working because the crRNAs are cutting. Try adjusting the amount of donor. If necessary, try experiment in control cells such as K562 (for nucleofection) or U2OS (for transfection) that have high rates of HDR.

Problem: I need to determine if my knockin sequence has been inserted into my gene of interest.
Answer: Sanger sequencing or Next Generation Sequencing is used to further confirm insertions and deletions. Don't have NGS access? Email info@canopybiosciences.com to find out about the Canopy Biosciences NGS offering.

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