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

CRISPR COMPLETE GENE EDITING KITS

CRISPR Complete Gene Editing kits contain everything you need to genetically engineer your cell line, in one simple box.

INCLUDED

crRNA
tracrRNA
Cas9 Protein
Forward and Reverse Primers
Duplex Buffer
Plasmid or Oligo Donor (for knock-in and point mutation projects only)

www.canopybiosciences.com

USER MANUAL CRISPR-001

CRISPR COMPLETE GENE EDITING KITS

PRODUCT DESCRIPTION

The Canopy CRISPR Complete Kit contains everything you need to genetically engineer your cell line. 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 tracrRNA:crisprRNA complex. The tracrRNA is a universal RNA oligo that duplexes with the crisprRNA (crRNA). It contains chemical modifications to protect from degradation by cellular RNAses. Two custom designed crRNAs are provided in the kit to target your gene-of-interest with high specificity, creating a double strand break. The crRNAs contain chemical modifications to protect from degradation by cellular RNAses. Knock-in kits will contain a single stranded DNA donor oligo (45-200 bases) for small insertions or a donor plasmid (200 bp-4,000 bp) for large insertions. Donors are endogenously inserted into your gene-of-interest by homology-directed repair. Cas9, tracrRNA, crRNA, and donor can be delivered into cells by lipid-based transfection or by nucleofection. Custom designed primers are included to confirm the insertion or deletion upon completion of CRISPR cell line generation.

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ITEMS INCLUDED IN THE KIT

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

- Cas9 62 µM (5 µl, 62 µM in formulation buffer)
- Two crRNAs (10 nmol each)
- tracrRNA (50 µl, 200 µM in TE, pH 7.5)
- Forward and Reverse Primers (25 nmole each)
- Duplex Buffer (2 tubes, 1 ml each)
- Donor oligo or donor plasmid (knock-in and point mutation kits only)

ADDITIONAL ITEMS NEEDED

- Your cell line of choice (test to confirm it is mycoplasma-free)
- Genomic DNA extraction solution
- T7 endonuclease
- Polymerase
- TAE buffer
- Agarose
- DNA gel loading dye
- Ethidium Bromide
- DNA gel ladder

Additional items need for transfection only, not included in kit:

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

Additional items needed for nucleofection only, not included in kit:

- Additional Cas9 (Canopy Biosciences Cat #CRISPR-002) 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)

TRANSFECTION AND NUCLEOFECTION PROTOCOL

Perform transfection or nucleofection to deliver CRIPSR Complete 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

Recommended Parameters

- CRISPR-Cas9 RNP complex: 1-50 nM range, start with 25 nM
- 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

TRANSFECTION PROTOCOL

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

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5. Assemble 25 µl of Ribonucleoprotein (RNP) Complex by mixing
   d. 1.5 µl of µM complexed crRNA:tracrRNA
   e. 1.5 µl of µM cas9
   f. 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

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, 96 well)
   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 minutes
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. Optimal cell number per well will depend on your cell line. 3.5x105/well is a good starting point.

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12. Wash cell pellet with PBS
13. Resuspend cell pellet in 20 µl of Nucleofector Solution (supplement has already been added) per well
14. Pipet 20 µl of cell suspension into each well of a V-bottom 96 well plate or tube
15. Add 5 µl of crRNA:tracrRNA:Cas9 RNP to each well.
16. Pipet up and down to mix and transfer 25 µl of cell/RNP complex mixture to Nucleocuvette.
17. Gently tap the Nucleocuvette to remove air bubbles
18. Put Nucleocuvette in Shuttle device
19. Perform Nucleofection
20. Add 75 µl of pre-warmed media and gently pipette up and down
21. Transfer 25 µl from the Nucleocuvette into the tissue culture plate with pre-warmed module.
22. 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 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

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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 (see step 6)
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)
6. 18 µl annealed PCR product
7. 2 µl T7E I
8. incubate 37°C for 60 min
9. use or store at -20 °C
10. 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 using a knockin kit 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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