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CRISPR-Cas9 genome editing in *Corallochytrium limacisporum*

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We use this protocol and it's working

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Abstract

This protocol walks you through how to achieve genome editing in *Corallochytrium limacisporum*, a unicellular marine eukaryote which is closely related to animals. Here we describe how to achieve resistance to rapamycin or carboxin. In brief, the cells are Nucleofected, to introduce a sgRNA-Cas9 RNP complex. Next, the cells are selected on agar plates and isolated for genotyping.

Materials

- Marine Broth (BD Difco #2216)
- Marine Agar (Scharlau #01-291)
- T-25 flasks (Corning #430639)
- 90 mm cell culture dishes (Falcon #353002)
- 12-well plate (Corning #3513)
- 96-well plate (Nunc #167008)
- Cell scraper (Corning #3010)
- 5 mm glass beads (Sigma-Aldrich #18406)
- P3 Primary Cell 4D-Nucleofector X Kit (Lonza #V4XP-3032)
- Amaxa ® 4D-Nucleofector ®
- EnGen Spy Cas9 NLS (New England Biolabs, #M0646M)
- PBS (Sigma #P5368-10PAK)
- Rapamycin Sigma-Aldrich #37094
- Carboxin Sigma-Aldrich #45371
- Ca-Mg Free Sea Water: NaCl 460 mM, $NaHCO_3$ 2.15 mM, KCl 10.7 mM, Na_2SO_4 7 mM, Tris-HCl pH 8.0 10 mM

Before start

- *C. limacisporum* is prone to contamination as its growth medium is very rich, always proceed under sterile conditions (laminar flow cabinet) to avoid contamination.
- Unless otherwise stated, *C. limacisporum* is grown in Marine Broth, its usual growth medium.
- All reagents and their references can be found in the Materials section.

Seed cells for transfection

20m

1 Prepare a T-25 flask of *C. limacisporum* so that it is growing exponentially on the day of transfection. Usually, 100-200 µL from a confluent culture in a 25 cm^2 flask with 5 mL of Marine Broth works well.

20m

2 Incubate the cells at 23 °C Overnight in an incubator without agitation.

Transfection

40m

3 Before starting, prepare the following reagents and keep them on ice:

- 1X Filter-sterilized PBS
- Lonza nucleofector strip (part of P3 Primary Cell 4D-Nucleofector X Kit)
- Lonza P3 Buffer (part of P3 Primary Cell 4D-Nucleofector X Kit)
- Repair DNA template (if needed)

Note

Ensure that the supplement has been added to the Lonza P3 buffer.
Supplemented Lonza P3 Buffer expires 3 months after preparation. To avoid wasting P3 buffer, aliquot the components to prepare 1/3 of the volume:

- 225 µL P3 solution
- 50 µL supplement

4 Prepare RNP complex (sgRNA+Cas9) mix. One mix should be prepared spearately for each sgRNA to be used. When adding the final component, gently swirl it with the pipette tip and incubate at RT for at least 30 min.

30m

RNP mix:

- 10 µL Lonza P3 Buffer
- 2.5 µL sgRNA at [M] 60 micromolar (µM)
- 2 µL EnGen Spy Cas9 NLS

Proceed with the next steps during the 00:30:00 incubation.

Note

For co-transfection experiments, prepare the two mixes independently:
Mix 1:
- 5 µL of P3 buffer
- 2 µL EnGen Spy Cas9 NLS
- 2.5 µL sgRNA1

Mix 2:
- 5 µL of P3 buffer
- 2 µL EnGen Spy Cas9 NLS
- 2.5 µL sgRNA2

5 Scrape and homogenize the cells, then determine the concentration by counting a 1:100 dilution of the homogenized cell suspension using a Neubauer chamber.

6 Pellet 1.5 × 10^6 cells per reaction at 2000 x g, 00:05:00 5m

Note

Cells for multiple transfections can be pelleted together in the same tube.

Note

During centrifugation: Prepare a 12-well plate with 1 mL of Marine Broth for every transfection

7 Remove supernatant from pelleted cells and wash the pellet with 100 µL **ice-cold** sterile PBS.

8 Spin the cells again 2000 x g, 00:05:00 5m

Note

During centrifugation: Prepare the Lonza nucleofector.
Turn on Lonza nucleofector, select the vessel (strip) and select the wells that are going to be used.
- Solution: **Primary cell P3**
- Pulse Code: **EN138**

9 Remove supernatant from the washed cells and resuspend in 6.5 µL ice-cold P3 buffer for every transfection that will be done.

Note

Don't leave the cells in P3 buffer for longer than necessary.

10 Prepare mix for nucleofection:
14.5 µL RNP mix go to step #4
6.5 µL cells resuspended in P3 go to step #9
2 µL repair DNA template (if needed)

Note

For co-transfection with two guides:
- 9.5 µL RNP mix 1
- 9.5 µL RNP mix 2
- 6.5 µL cells resuspended in P3
- 2 µL repair template (if needed)

Note

When doing HDR, different length repair templates can be used at 100 uM. No repair template is necessary for NHEJ.

11 Add transfection mixes to the Lonza strip, place the strip in the machine and apply **EN138** pulse

12 Quickly remove strip from Lonza and add 80 µL Marine Broth to allow the cells to recover

13 Transfer transfected cells to 12-well plate with 1 mL Marine Broth

14 Grow cells at 23 °C Overnight for selection on rapamycin, or 48:00:00 for selection on carboxin

Plating onto Marine Agar

15 Melt 20 mL of Marine Agar (Scharlau #01-291) for every plate that you are going to prepare.

Note

If you are using rapamycin always use fresh plates and wait until it is quite cool to the touch but not solidifying yet, as rapamycin is very sensitive to heat. Carboxin plates can be prepared in advance and stored at 4^{o}C for up to a week.

16 Add selection drug to the agar and add 20 mL agar per dish. Allow them to solidify until it is dry.

Note

Rapamycin selection concentration: 125 ng/mL
Carboxin selection concentration: 30 µg/mL

17 Scrape the transfected wells and add 100-200 µL of the culture onto the agar.

Note

If pelleting a larger volume, spin the cells down first and resuspend the pellet in 100 µL of Marine Broth first.

18 Add 3-4 glass beads (Sigma-Aldrich #18406) and shake the plate until the agar is completely dry. Gently remove the glass beads by pouring them from the plate. Seal the plate with parafilm.

19 Incubate the plates at 23 °C for 10-14 days.

Note

Monitor the plates to ensure contaminants don't appear, these will usually appear faster than *C. limacisporum* colonies. *C. limacisporum* will usually start to be detectable after around 5-7 days and appear as very small, transparent colonies. Eventually, they will become round and pink.

Colony picking and streaking

20 Prepare Marine Agar plates containing the selection drug as above go to step #15 and label them with the approximate number of colonies that have appeared, or however many you want to isolate and screen.

21 Gently pick the colonies by touching them with a sterile pipette tip and gently streaking onto a fresh agar plate labelled with clone identifers. Seal the plate with parafilm.

22 Incubate the plates at 23 °C allowing the streaks to grow for 2-3 days until the streak is visible.

Transfer clones to liquid medium

23 Once the streak is visible, pick a part of the streak using a sterile tip and place it in 100 µL Marine Broth with selecting drug in a 96-well plate.

24 Allow the cells to grow by incubating the plates at 23 °C for 2-3 days before genotyping.

Genotyping

25 Pipette up and down to resuspend cells in the 96-well plate.

26 Transfer 50 µL of the culture to a tube. Fresh Marine Broth can be added to the well to maintain the culture if needed, otherwise they can also be retrieved from the streaked agar plates.

Note

50 µL of culture in a 0.2 mL tube or PCR strip is enough to genotype with, but larger volumes can be used by scaling up the amount of Ca-Mg free sea water used to wash the cells in step 28.

27 Pellet the cells at 2500 x g, 00:03:00. If using PCR strips, use a Minispin with strip adaptor for 3 minutes.

Note

From this step forward, sterile environment is no longer required.

28 Remove the supernatant and wash the cells with 100 µL Ca-Mg free sea water. Repeat the wash once.

Note

Ca-Mg free sea water:
NaCl 460 mM, NaHCO3 2.15 mM, KCl 10.7 mM, Na2SO4 7 mM, Tris-HCl pH 8.0 10 mM

29 After the second wash, heat the cell suspension for 00:10:00 at 95 °C in a Thermoblock or PCR machine.

30 Spin down cell remains at 2500 x g, 00:05:00 and use 1-2 µL of the supernatant as template in genotyping PCR.

Note

Lysed cells can be stored at 4ºC before genotyping for up to a week.