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 We use this protocol and it's working

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🌐 Genome editing in the choanoflagellate *Salpingoeca rosetta* V.1

👤 In 1 collection

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ABSTRACT

This protocol details the preparation and execution of CRISPR/Cas9 genome editing in *S. rosetta*. The protocol builds on a [method to transfect macromolecules into *S. rosetta*](#) for delivering a purified Cas9 ribonucleoprotein from *Streptomyces pyogenes* (*SpCas9* RNP) into *S. rosetta*. Upon cleaving the *S. rosetta* genome at locations specified by the guide RNA (gRNA) of the *SpCas9* RNP, *S. rosetta* can use DNA oligonucleotides as templates to repair the double-stranded break. Those repair templates can encode foreign sequences and mutations for editing the *S. rosetta* genome, so long as DNA oligonucleotides have >30 bases of sequence that is homologous to both sides of the Cas9 cleavage site.

GUIDELINES

Perform cell culturing and transfection procedure inside of a biosafety cabinet to maintain sterility.

MATERIALS

STEP MATERIALS

DNA Oligonucleotide Integrated DNA Technologies, Inc. (IDT) Catalog #Custom Order
tracrRNA Integrated DNA Technologies, Inc. (IDT) Catalog #1072534
Falcon 525cm ² Rectangular Straight Neck Cell Culture Multi-Flask, 3-layer with Vented Cap Corning Catalog #353143
EnGen Cas9 NLS, <i>S. pyogenes</i> - 400 pmol New England Biolabs Catalog #M0646T
SF Cell Line 96-well Nucleofector Kit Lonza Catalog #V4SC-2096
<i>Salpingoeca rosetta</i> cultured with <i>Echinicola pacifica</i> (SrEpac) ATCC Catalog #PRA-390
crRNA Integrated DNA Technologies, Inc. (IDT) Catalog #Custom Order
Duplex Buffer Integrated DNA Technologies, Inc. (IDT) Catalog #11-01-0301
Papain from papaya latex Merck MilliporeSigma (Sigma-Aldrich) Catalog #P3125-100MG

BEFORE START INSTRUCTIONS

Please consult the attached file of media recipes for artificial seawater, high nutrient media, and low nutrient media. **MediaRecipes.pdf**

Culture Cells

- 1 **Seed a large culture of *S. rosetta*.**

- 1.1 Two days prior to transfection, inoculate 120 mL of high nutrient media with a culture of *S. rosetta* feeding on *E. pacifica* to a final concentration of *S. rosetta* of 8000 cells/mL .

Salpingoeca rosetta cultured with *Echinicola pacifica* (SrEpac) **ATCC Catalog #PRA-390**

- 1.2 Grow the culture for 48:00:00 in a 3-layer flask at 22 °C with 60% humidity .

Falcon 525cm² Rectangular Straight Neck Cell Culture Multi-Flask, 3-layer with Vented Cap **Corning Catalog #353143**

Prepare Gene Editing Cargo

- 2 Prepare a guide RNA (gRNA) that binds to *SpCas9* and targets DNA by annealing CRISPR RNA (crRNA) with the trans-activating CRISPR RNA (tracrRNA) .

- 2.1 Resuspend crRNA in duplex buffer (30 mM HEPES-KOH, pH 7.5; 100 mM potassium acetate) to a final concentration of 200 micromolar (μM) .

crRNA **Integrated DNA Technologies, Inc. (IDT) Catalog #Custom Order**

Duplex Buffer **Integrated DNA Technologies, Inc. (IDT) Catalog #11-01-03-01**

- 2.2 Resuspend tracrRNA in duplex buffer to a final concentration of 200 micromolar (μM) .

tracrRNA **Integrated DNA Technologies, Inc. (IDT) Catalog #1072534**

- 2.3 Mix equal volumes of crRNA (go to step #2.1) and tracrRNA (go to step #2.2) to have a final concentration of 100 micromolar (μM) gRNA, which is the annealed complex of crRNA and tracrRNA.

- 2.4 Incubate the gRNA solution at 95 °C in an aluminum block for 00:05:00 .

2.5 Place the aluminum block was placed at Room temperature to slowly cool the gRNA to 25 °C .

2.6 Store the gRNA at -20 °C .

3 Prepare DNA oligonucleotides that serve as repair templates after *SpCas9* cleavage.

3.1 Dissolve oligonucleotides to a final concentration of 250 micromolar (μM) in 10 mM HEPES-KOH, pH 7.5.

DNA Oligonucleotide **Integrated DNA Technologies, Inc. (IDT) Catalog #Custom Order**

3.2 Incubate the dissolved oligonucleotides at 55 °C for 01:00:00 .

3.3 Store oligonucleotides at -20 °C .

3.4 Before starting nucleofections, ensure that the oligonucleotides are fully dissolved by incubating them at 55 °C for 01:00:00 , which concurs with the assembly of the *SpCas9*/gRNA complex.

4 Assemble *SpCas9* with the gRNA to form the *SpCas9* RNP.

- 4.1 For one transfection, place 2 μL of 20 micromolar (μM) *SpCas9* in the bottom of a 0.2 ml PCR tube.

EnGen Cas9 NLS, *S. pyogenes* - 400 pmol **New England Biolabs Catalog #M0646T**

- 4.2 Add 2 μL of 100 micromolar (μM) gRNA (go to step #2) by slowly pipetting up and down with *SpCas9* to gently mix the gRNA together. This solution is called the "*SpCas9* ribonucleoprotein (RNP)."

- 4.3 Incubate the *SpCas9* RNP at Room temperature for 01:00:00 (roughly the time to complete the preparation of *S. rosetta* for priming, see below).

Prepare transfection rReagents

5 Prepare SF Buffer (Lonza) for transfections.

SF Cell Line 96-well Nucleofector Kit **Lonza Catalog #V4SC-2096**

- 5.1 Add all of buffer B (smaller volume that may also be called supplement 1) to buffer A (larger volume).

- 5.2 Store on ice until ready for use. The combined buffer can also be stored at 4°C for up to 3 months.

Note

The combined buffer can be stored at 4°C for up to 3 months.

Note

Because the Lonza kits can be so expensive, we recommend aliquoting large volumes of the SF components (900 µl aliquots for buffer A and 200 µl aliquots for buffer B) to prevent SF buffer from spoiling after buffers A and B have been combined.

6 Prepare the priming buffer.

6.1

Dilute papain to a final concentration of 100 µM in dilution buffer (50 mM HEPES-KOH pH 7.5, 200 mM sodium chloride, 20% [v/v] glycerol, and 10 mM cysteine) from a stock solution of 1 mM papain (Millipore Sigma, St. Louis, MO; Cat. No. P3125-100MG), and incubate at room temperature just before priming cells for transfection.

Papain from papaya latex **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P3125-100MG**

Note

The dilution buffer [50 mM HEPES-KOH pH 7.5, 200 mM sodium chloride, 20% (v/v) glycerol and 10 mM cysteine] should be sterile filtered through a 0.22 µm filter.

The dilution buffer may also be prepared ahead of time and stored in a -80°C freezer just before its use.

6.2

Make a solution of the remaining components of the priming buffer (40 mM HEPES-KOH, pH 7.5; 34 mM lithium citrate; 50 mM L-cysteine; 15% [wt/vol] PEG 8000). DO NOT combine the papain and priming buffer until just before adding the priming buffer to cells.

Note

The priming buffer without papain should be sterile filtered through a 0.22 µm filter.

The priming buffer without papain can also be made ahead of time and stored at -80°C until it is used. Be sure that the priming before is warmed to room temperature prior to use.

Wash Cells

7 Prepare *S. rosetta* for transfection by washing away feeder bacteria.

7.1 The 120 mL culture of *S. rosetta* feeding on *E. pacifica* (go to step #1) was homogenized by vigorous shaking and then split into 40 mL aliquots in 50 ml conical tubes.

7.2 Vigorously shake the aliquots and centrifuge the cells for 00:05:00 at 2000 x g and 22 °C in a swinging bucket rotor.

7.3 Use a serological pipette to gently remove from the cell pellet all but 2 ml of the supernatant, which remains cloudy with *E. pacifica* bacteria. With a fine tip transfer pipette, gently remove the remaining liquid near the pellet.

7.4 The three cell pellets were resuspended in a total volume of 50 mL artificial seawater, combined into one conical tube, and vigorously shaken to homogenize the cells.

7.5 For a second time, the resuspended cells were centrifuged for for 00:05:00 at 2000 x g and 22 °C in a swinging bucket rotor.

7.6 The supernatant was removed as before (go to step #7.3).

7.7 The pellet was resuspended in 50 mL of artificial seawater, and the cells were homogenized by vigorous shaking.

7.8 The cells were centrifuged for a third time for 00:05:00 at 2200 x g and 22 °C .

7.9 Remove the supernatant as before (go to step #7.3).

7.10 Resuspend the cell pellet in 400 µL of artificial seawater. This resuspension is called the "washed cells."

8 Prepare 100 µL aliquots of [M] 50000000 cells/ml .

8.1 Dilute 2 µL of "washed cells" (go to step #7.10) into 196 µL of artificial seawater.

8.2 Fix the diluted cells with 2 µL of 37.5% formaldehyde and homogenize by vortexing.

8.3 Pipet the fixed cells into a fixed chamber slide and determine the cell concentration.

Note

Remember that concentration of diluted and fixed cells is a 100-fold dilution from the "washed cells." Be sure to factor that dilution into your concentration.

Note

Cells can be counted on a hemacytometer (Neubauer with brightlines) or with an automated cell counter. We recommend a Luna-FL automated cell counter.

Equipment

LUNA-FL	NAME
Dual Fluorescence Cell Counter	TYPE
Logos Biosystems	BRAND
L20001	SKU
https://logosbio.com/automated-cell-counters/fluorescence/luna-fl	LINK

- 8.4 After determining the cell concentration, dilute the "washed cells" to final concentration of **50000000 cells/ml** and split into **100 μ L** aliquots.

Note

One aliquot provides enough cells for 12 nucleofections.

Prime Cells

- 9 Prime cells for nucleofection by degrading the glycocalyx that surrounds *S. rosetta*.

9.1 Spin the 100 µL aliquots of washed cells (go to step #8.4) at 800 x g and 22 °C for 00:05:00 .

9.2 Gently remove the supernatant from the cell pellet with a gel-loading pipette tip.

9.3 Combine the priming buffer components (go to step #6) to make a final priming buffer (40 mM HEPES-KOH, pH 7.5; 34 mM lithium citrate; 50 mM l-cysteine; 15% [wt/vol] PEG 8000; and 1 µM papain)

9.4 Resuspend each cell pellet in 100 µL of priming buffer.

9.5 Incubate cells for 00:35:00 at Room temperature .

9.6 Add 10 µL of 50 mg/ml Bovine Serum Albumin to each aliquot of primed cells for quenching proteolysis from the priming buffer.

9.7 Centrifuge cells at 1250 x g and 22 °C for 00:05:00 .

9.8 Gently remove the supernatant from the cell pellet with a gel-loading pipette tip.

9.9 Resuspended each cell pellet in 25 μL of SF Buffer (go to step #5). This suspension of cells is called the "primed cells."

9.10 Store the "primed cells" on ice while preparing nucleofection reactions.

Transfect Cells

10 Deliver gene editing cargo via nucleofection.

10.1 Add 16 μL of ice-cold SF Buffer (go to step #5) to the *SpCas9* RNP (go to step #4.3), which has a total volume of 4 μL .

Note

For reactions that use two different gRNAs, assemble each *SpCas9* RNP separately then combine each *SpCas9* RNP at this step. After the *SpCas9* RNPs have been combined, add 16 μL of ice-cold SF Buffer

10.2 Add 2 μL of the repair oligonucleotide template to the PCR tube with *SpCas9* RNP and SF Buffer (go to step #10.1).

10.3 Add 2 μL of "primed cells" (from go to step #9.10) to the PCR tube with *SpCas9* RNP, SF Buffer, and the repair template (go to step #10.2). This solution, which is called the "nucleofection mix," should have a total volume of 24 μL .

10.4 Transfer the entire nucleofection mix into one well of a 96-well nucleofection plate.

Note

At this point, prepare for the recovery step, by transferring the recovery buffer into a convenient vessel and setting the pipette to 100 µL .

10.5 Pulse the nucleofection plate with the CM156 pulse.

Equipment

4D-Nucleofector Core Unit

NAME

Control system for performing nucleofection

TYPE

Lonza

BRAND

AAF-1002B

SKU

[https://bioscience.lonza.com/lonza_bs/US/en/Transfection/p/000000000000203684](https://bioscience.lonza.com/lonza_bs/US/en/Transfection/p/000000000000203684/4D-Nucleofector-Core-Unit) ^{LINK}
/4D-Nucleofector-Core-Unit

Equipment

96-well Shuttle Device

NAME

Add-on for Nucelofector 4d device to perform plate-based nucleofections

TYPE

Lonza

BRAND

AAM-1001S

SKU

[https://bioscience.lonza.com/lonza_bs/US/en/Transfection/p/000000000000191639](https://bioscience.lonza.com/lonza_bs/US/en/Transfection/p/000000000000191639/96-well-Shuttle-Device) ^{LINK}
/96-well-Shuttle-Device

Rest and Recover Cells

- 11 Allow membranes to reseal by resting cells in recovery buffer before growing cells again in media.**
- 11.1** Immediately after transfection, add 100 μL of ice-cold recovery buffer (10 mM HEPES-KOH, pH 7.5; 0.9 M sorbitol; 8% [wt/vol] PEG 8000) to each nucleofection transfection and gently mixed by firmly tapping the side of the plate.
- 11.2** Allow cells to rest in recovery buffer for 00:05:00 .
- 11.3** Gently mix the well in the nucleofection plate by pipetting up and down before transferring the entire volume in nucleofection well (the nucleofection mix plus the recovery buffer) into to 2 mL of low nutrient media in one well of a 6 well plate.
- 11.4** Incubate at 22 °C and 60% humidity for 00:30:00 .

12 Add *E. pacifica* food and grow transfected cells.

- 12.1** Add 10 μL of [IM] 10 mg/ml of *E. pacifica* to the wells in the 6 well plate.

Note

Prepare the *E. pacifica* solution by resuspending a frozen, 10 mg pellet of *E. pacifica* in 1 ml of artificial seawater.

- 12.2** Incubate the 6 well plate at 22 °C and 60% humidity for 24:00:00 before using in downstream experiments.

(Optional) Select for Cycloheximide Resistance

- 13** Add 10 μ L of [M] 1 mg/ml of cycloheximide to the 2 mL culture of transfected cells after allowing the cells to fully recover.

*

Safety information

Cycloheximide is toxic. Handle carefully and properly dispose.

- 14** Incubate the cells in cycloheximide for 96:00:00 prior to genotyping and clonal isolation.