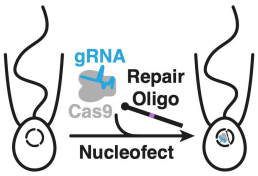
Feb 20, 2020 Version 1

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



In 1 collection

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Protocol status: Working
We use this protocol and it's working

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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* (*Sp*Cas9 RNP) into *S. rosetta*. Upon cleaving the *S. rosetta* genome at locations specified by the guide RNA (gRNA) of the *Sp*Cas9 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 525cm² Rectangular Straight Neck Cell Culture Multi-Flask, 3-layer with Vented Cap **Corning** **Catalog #**353143
- EnGen Cas9 NLS, S. pyogenes - 400 pmol **New England Biolabs** **Catalog #**M0646T
- SF Cell Line 96-well Nucleofector Kit **Lonza** **Catalog #**V4SC-2096
- Salpingoeca rosetta cultured with Echinicola pacifica (SrEpac) **ATCC** **Catalog #**PRA-390
- crRNA **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**Custom Order
- Duplex Buffer **Integrated DNA Technologies, Inc. (IDT)** **Catalog #**11-01-03-01
- Papain from papaya latex **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P3125-100MG

Protocol materials

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Before start

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 [M] 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 *Sp*Cas9 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 [M] 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 [M] 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 [M] 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 *Sp*Cas9 cleavage.

3.1 Dissolve oligonucleotides to a final concentration of [M] 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 *Sp*Cas9/gRNA complex.

4 Assemble *Sp*Cas9 with the gRNA to form the *Sp*Cas9 RNP.

4.1 For one transfection, place 2 µL of [M] 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 [M] 100 micromolar (µM) gRNA ( ) by slowly pipetting up and down with *Sp*Cas9 to gently mix the gRNA together. This solution is called the "*Sp*Cas9 ribonucleoprotein (RNP)."

4.3 Incubate the *Sp*Cas9 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 unti 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* ( )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" ( ) 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 [M] 50000000 cells/ml and split into 100 µL 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 [M] 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 pell in 25 µL of SF Buffer ( ). 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 *Sp*Cas9 RNP (go to step #4.3), which has a total volume of 4 µL .

> Note
>
> For reactions that use two different gRNAs, assemble each *Sp*Cas9 RNP separately then combine each *Sp*Cas9 RNP at this step. After the *Sp*Cas9 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 *Sp*Cas9 RNP and SF Buffer ( ).

10.3 Add 2 µL of "primed cells" (from) to the PCR tube with *Sp*Cas9 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

Control system for performing nucleofection

Lonza

AAF-1002B

https://bioscience.lonza.com/lonza_bs/US/en/Transfection/p/000000000000203684/4
Nucleofector-Core-Unit

Equipment

96-well Shuttle Device

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

Lonza

AAM-1001S

https://bioscience.lonza.com/lonza_bs/US/en/Transfection/p/000000000000191639/9
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 [M] 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 µL 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.