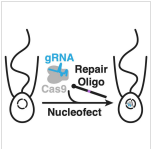

Version 2

Sep 11, 2021

Genome editing in the choanoflagellate *Salpingoeca rosetta* V.2

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dx.doi.org/10.17504/protocols.io.bx58pq9w

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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.

DOI

dx.doi.org/10.17504/protocols.io.bx58pq9w

PROTOCOL CITATION

David Booth 2021. Genome editing in the choanoflagellate Salpingoeca rosetta. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bx58pq9w Version created by David Booth

CREATED

Sep 11, 2021

LAST MODIFIED

Sep 11, 2021

PROTOCOL INTEGER ID

53152

GUIDELINES

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

MATERIALS TEXT

STEP MATERIALS

DNA Oligonucleotide **Integrated DNA Technologies Catalog #Custom Order** Step 3.1

tracrRNA **Integrated DNA Technologies Catalog #1072534** Step 2.2

Falcon 525cm² Rectangular Straight Neck Cell Culture Multi-Flask, 3-layer with Vented Cap **Corning Catalog #353143** Step 1.2

EnGen Cas9 NLS, S. pyogenes - 400 pmol **New England Biolabs Catalog #M0646T** Step 4.1

SF Cell Line 96-well Nucleofector Kit **Lonza Catalog #V4SC-2096** Step 5

Salpingoeca rosetta cultured with Echinicola pacifica (SrEpac) **ATCC Catalog #PRA-390** Step 1.1

crRNA **Integrated DNA Technologies Catalog #Custom Order** Step 2.1

Duplex Buffer **Integrated DNA Technologies Catalog #11-01-03-01** Step 2.1

Papain from papaya latex **Millipore Sigma Catalog #P3125-100MG** Step 6.1

BEFORE STARTING

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** .

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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 **200 Micromolar (µM)** .

crRNA **Integrated DNA Technologies Catalog #Custom Order**

Duplex Buffer **Integrated DNA Technologies Catalog #11-01-03-01**

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

tracrRNA **Integrated DNA Technologies 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 *Sp*Cas9 cleavage.**

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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 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 **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 #2.6**) 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 Reagents

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).

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

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

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 **Millipore**

Sigma Catalog #P3125-100MG

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.

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.

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7.1 Homogenized the **120 mL** culture of *S. rosetta* feeding on *E. pacifica* (**go to step #1.2**) by vigorously 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.

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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.

> 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.

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

LUNA-FL
Dual Fluorescence Cell Counter
Logos Biosystems **L20001**

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

> 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 #6.2**) 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)

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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 (**go to step #5.2**). 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 #5.2**) to the *Sp*Cas9 RNP (**go to step #4.3**), which has a total volume of **4 µl** .

> 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 (**go to step #3.4**) to the PCR tube with *Sp*Cas9 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 *Sp*Cas9 RNP, SF

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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.

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.

4D-Nucleofector Core Unit
Control system for performing nucleofection
Lonza AAF-1002B

96-well Shuttle Device
Add-on for Nucelofector 4d device to perform plate-based nucleofections
Lonza AAM-1001S

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.

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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 **10 mg/ml** of *E. pacifica* to the wells in the 6 well plate.

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 **1 µg/ml** of cycloheximide to the **2 mL** culture of transfected cells after allowing the cells to fully recover.

Cycloheximide is toxic. Handle carefully and properly dispose.

14

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

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