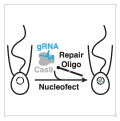

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Genome editing in the choanoflagellate *Salpingoeca rosetta*

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1 Works for me dx.doi.org/10.17504/protocols.io.89fhz3n

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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.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
crRNA	Custom Order	Integrated DNA Technologies
Duplex Buffer	11-01-03-01	Integrated DNA Technologies
tracrRNA	1072534	Integrated DNA Technologies
DNA Oligonucleotide	Custom Order	Integrated DNA Technologies
EnGen Cas9 NLS, S. pyogenes - 400 pmol	M0646T	New England Biolabs
Salpingoeca rosetta cultured with Echinicola pacifica (SrEpac)	PRA-390	ATCC
Falcon 525cm² Rectangular Straight Neck Cell Culture Multi-Flask, 3-layer with Vented Cap	353143	Corning
SF Cell Line 96-well Nucleofector Kit	V4SC-2096	Lonza
Papain from papaya latex	P3125-100MG	Millipore Sigma

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)
by 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
by 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
by Integrated DNA Technologies
Catalog #: Custom Order

Duplex Buffer
by Integrated DNA Technologies
Catalog #: 11-01-03-01

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

tracrRNA
by 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.

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

DNA Oligonucleotide
by 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 **[M]20 Micromolar (µM) SpCas9** in the bottom of a 0.2 ml PCR tube.

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

4.2

Add **2 µl** of **[M]100 Micromolar (µM) gRNA** (**go to step #2**) 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
by 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.

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
by 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.**

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.

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**) 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 pell 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 *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 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 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.

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 mg/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.