

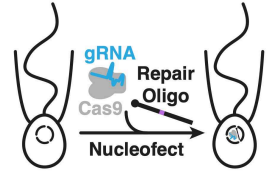
Sep 12, 2021

Version 2

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

DOI

<https://dx.doi.org/10.17504/protocols.io.bx58pq9w>



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DOI: <https://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**



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Protocol status: Working

We use this protocol and it's working

Created: September 12, 2021

Last Modified: September 12, 2021

Protocol Integer ID: 53152

Keywords: genome editing in the choanoflagellate salpingoeca, cas9 genome editing, genome editing, execution of crispr, crispr, choanoflagellate salpingoeca rosetta, purified cas9 ribonucleoprotein, guide rna, dna oligonucleotide, dna oligonucleotides as template, choanoflagellate salpingoeca, cas9 ribonucleoprotein from streptomyces pyogene, cas9 cleavage site, spcas9 rnp, rna, dna, mutation, macromolecule

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 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.



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 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). 
- 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 buffer is warmed to room temperature prior to use.

Wash Cells

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



- 7.1 Homogenized the  120 mL culture of *S. rosetta* feeding on *E. pacifica* ( go to step #1) 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.
- 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 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 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$ 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 *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 ([⇒ go to step #3.4](#)) 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

Control system for performing nucleofection

Lonza

AAF-1002B

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

Equipment

96-well Shuttle Device

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

Lonza

AAM-1001S

https://bioscience.lonza.com/lonza_bs/US/en/Transfection/p/000000000000191639/96-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 [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 $\mu\text{g/ml}$ 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.

