Protocol I: Transfection of Salpingoeca rosetta with Lonza 4d nucleofector Version 2

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Abstract

The protocol describes the transformation of the choanoflagellate *Salpingoeca rosetta* using a Nucleofector 4d Device and SF kit from Lonza. Plasmids that use *S. rosetta* promoters and regulatory elements to drive the expression of luciferase and fluorescent protein reporters are being deposited at Addgene.

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Guidelines

1. To maintain sterility, perform all steps in a biosafety cabinet unless otherwise noted.
2. For protocols on starting and maintaining choanoflagellate cultures and preparing media, see King N, Young SL, Abedin M, Carr M, and Leadbeater BSC (2009) *Emerging Model Organisms, Vol. 1, CSHL Press* and Levin TC and King N (2013) *Current Biology* 23:2176-80.
3. For seawater recipe, see Cembella AD (1995) *Manual on Harmful Marine Microalgae*, Chapter Three: Culture Methods. UNESCO (http://unesdoc.unesco.org/images/0013/001317/131711e.pdf)

Before start

Place the following items in the biosafety cabinet to make the procedure proceed rapidly.

- 4 x 50 ml conical tubes
- 1.5 ml centrifuge tubes
- Artificial Seawater (ASW) filtered through a 0.22 µm filter
- 500 ml bottle to collect waste
- Sterile fine-tip transfer pipettes
- Serological pipettes
- Aliquot of thawed pretreatment buffer from Protocol E
- Pipettes and tips for transferring volumes of 1-1000 µl
- Fine-tips (gel-loading tips) for transferring 100-200 µl volumes
- Multichannel pipette for transferring 100 µl volumes
- Reservoir for multichannel pipette if doing multiple samples
- 96-well nucleofection plate (Lonza V4SC-2096)
- Papain stock solution (Step 3)
- 12 well plate(s) (Corning 353225) containing 1 ml of 1% ASWC (See Step 1 for guidelines on culture media)

Before you start the procedure, place the following items in an ice bucket next to the biosafety cabinet

- Recovery buffer from Protocol F
- BSA from Protocol D
- Reporter DNA mixture (Step 2)
- SF Buffer (Lonza V4SC-2096)

Materials

Lonza Nucleofector 4d AAF-1002X by Lonza

SF Cell Line 4D-Nucleofector® X Kit S (32 RCT) V4XC-2032 by Lonza

Salpingoeca rosetta cultured with Echinicola pacifica (SrEpac) PRA-390 by ATCC

Falcon® 875cm² Rectangular Straight Neck Cell Culture Multi-Flask, 5-layer with Vented Cap 353144 by Corning

Protocol

Seed large culture of S. rosetta (2 days prior to transfection)

Step 1.

1. Maintain a small (6 ml in 25 cm^2 vented flask) or medium (18 ml in 75 cm^2 vented flask) culture *Salpingoeca rosetta* feeding on *Echinicola pacifica* (SrEpac) grown in 5% Artificial Seawater Complete Media (ASWC).
2. Count the concentration of cells using a haemocytometer.
3. Use the starter culture to innoculate 200 ml of 5% ASW in a Falcon 5-layer flask to a final cell concentration of $5x10^3$ cells/ml.
4. Grow culture for 36-48 hours at 22°C and 60% humidity.

Prepare mixture of reporter DNA for nucleofection

Step 2.

1. For one nucleofection reaction, prepare one tube with 2 µl of 20 µg/µl of pUC19 carrier plasmid (Protocol A),1 µl of 250 mM adenosine triphosphate-NaOH, pH 7.5 (Protocol B), 1 µl of 100 mg/ml heparin (Protocol C), 1 µl of 2.5-10 µg/µl reporter plasmid (Protocol G).
2. To save time, prepare a 400 µl master mix of addtives for nucleofection by mixing 200 µl of 20 µg/µl of pUC19 carrier plasmid, 100 µl of 250 mM adenosine triphosphate-NaOH, pH 7.5, 100 µl of 100 mg/ml Heparin. Add 4 µl of this master mix to 1 µl of reporter plasmid.

NOTES

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This step can be performed outside of the hood. Take care to use sterile filtered solutions of ATP and Heparin.

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The amount of reporter plasmid should be adjusted by performing a dilution series to determine the best concentration for your particular assay. For nanoluc reporter assays, we use 2.5 µg of reporter plasmid. For fluorescence microscopy, we use 10 µg of total reporter plasmid

Prepare a sterile stock solution of 100 µM papain

Step 3.

1. Dilute papain to a final concentration of 100 µM in dilution buffer [50 mM HEPES-KOH pH 7.5, 200 mM NaCl, 20% (v/v) glycerol, and 10 mM cysteine] from a stock solution of 1 mM papain (Sigma Aldrich).

NOTES

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• Prepare a solution of of 50 mM HEPES-KOH pH 7.5, 200 mM NaCl, 20% (v/v) glycerol and 10 mM cysteine.
• Sterile filtered through a 0.22 µm filter.
• Prepare a ten fold dilution of dilute papain to a final concentration of 100 µM from a stock solution of 1 mM papain (Sigma Aldrich P3125-100mg). Pipette 20 µl of 1 mM papain stock a180 µl of 50 mM HEPES-KOH pH 7.5, 200 mM NaCl, and 20% (v/v) glycerol.
• NOTE: Reducing reagents such as cysteine should be prepared fresh or extra care should be taken to maintain their reductive potential

Wash bacteria away from S. rosetta

Step 4.

1. Split *S. rosetta* culture between four 50 ml conical tubes. Vigorously shake tubes in hand for 30 sec.
2. Harvest cells by spinning at 2000 x g for 5 min at room temperature in a swinging bucket rotor. Pipette off supernatant with a 50 ml serological pipette, leaving a little bit of liquid on top of the pellet. Then use a fine tip transfer pipette to remove the rest of the liquid on the opposite side of the pellet. Resuspend each pellet in 25 ml of ASW and combine into two 50 ml conical tubes. Vigorously shake in hand for 30 sec.
3. Spin cells at 2200 x g for 5 min at room temperature in a swinging bucket rotor. Pipette off supernatant with 50 ml serological pipette, leaving a little bit of liquid on top of the pellet. Then use a fine tip transfer pipette to remove the rest of the liquid on the opposite side of the pellet.
4. Resuspend each pellet in 50 ml of ASW. Vigorously shake in hand for 30 sec.
5. Spin cells at 2400 x g for 5 min at room temperature in a swinging bucket rotor. Pipette off supernatant with 50 ml serological pipette, leaving a little bit of liquid on top of the pellet. Then use a fine tip transfer pipette to remove the rest of the liquid on the opposite side of the pellet.
6. Resupsend each cell pellet in 50 µl of ASW and combine them into one tube.

Determine cell concentration in preparation for pretreatment

Step 5.

1. Add 2 µl of concentrated and washed *S. rosetta* cells to 196 µl of ASW.
2. Fix cells with 2 µl of 37.5% (w/v) formaldehyde.
3. Count cells on haemocytomer and calculate cell concentrations. Make sure to account for the 100x dilution factor.

4. Dilute cells to a final concentration of $5x10^7$ cells/ml with ASW.
5. Aliquot 100 µl of cells to 1.5 ml centrifuge tubes. Each aliquot contains $5x10^6$ cells.

Pretreat S. rosetta cells.

Step 6.

1. Centrifuge 100 µl aliquots of $5x10^6$ cells at 2750 x g and room temperature in a table-top centrifuge with a fixed-angle rotor for 5 min. If your centrifuge has the option, turn off the brake or set to a soft setting. This prevents cells from experiencing high centrifugal forces.
2. During the centrifugation, add 10 µl of 100 µM papain prepared in Step 3 to 1 ml of pretreatment buffer (Protocol E). Mix well by pipetting up and down.
3. After centrifugation, remove supernatant with a fine pipette tip.
4. Resuspend the cells pellets in 100 µl of in pretreatment buffer with papain.
5. Incubate cells at room temperature for 30 min.
6. Quench reaction by adding 1 µl of 50 mg/ml BSA (Protocol D). Mix well by pipetting up and down.
7. Centrifuge cells at 1250 x g and room temperature in a table-top centrifuge with a fixed-angle rotor for 5 min. Again, use the soft brake setting or no brake at all.
8. Remove the supernatant and resuspend the cell pellet in 25 µl of SF Buffer (Lonza)

Transformation of S. rosetta cells with Lonza 4d Nucleofector

Step 7.

1. Add 16 µl of SF Buffer to DNA mixture prepared in step 2. Total volume should be 21 µl
2. Add 2 µl of pretreated cells the DNA mixture diluted in SF Buffer. Mix well by pipetting up and down.
3. Pipette 23 µl of the complete nucleofection mixture to one well of a 96-well nucleofection plate.
4. Prepare recovery buffer (Protocol F) to add immediately after nucleofection. For nucleofecting multiple samples, pour buffer into a liquid reservoir
5. Transfect cells by applying pulse CM-156 with a Lonza nucleofector.

Recovery of S. rosetta cells from nucleofection

Step 8.

1. Immediately after nucleofection, add 100 µl of ice-cold recovery buffer (Protocol F)
2. Incubate the cells in recovery buffer for 5 min at room temperature. Tap gently on the sides of the plate to mix the nucleofected cells with buffer.
3. Gently pipette cells up and down and then transfer cells to 1 ml of 5% ASWC media.
4. One hour after transferring cells to media, add 20 µl of a frozen 10 mg pellet of *E. pacifica* resuspended in 1 ml of ASW.
5. Incubate for 48 h at 22°C and 60% humidity. After this time, cells will be ready for fluorescence microscopy or reporter assays.