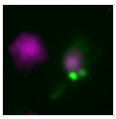

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Electroporation of the protist *Cafeteria roenbergensis* cells using the Neon Transfection System

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1 *Works for me* dx.doi.org/10.17504/protocols.io.qvhdw36

Protist Research to Optimize Tools in Genetics (PROT-G)

Sarah Duponchel

Cell preparation

1 Before the experiment (it can be the day before without affecting too much the cell viability)

Density-based removal of bacteria from a culture of the marine heterotrophic flagellate *Cafeteria roenbergensis*
by Sarah Duponchel

PREVIEW RUN

1.1 Determine the cell density of a *Cafeteria roenbergensis* culture: stain 10 µL of *Cafeteria* culture with 1 µL of Lugol's acid iodine solution and count them on a haemocytometer (Neubauer Chamber)

1.2 Dilute the *Cafeteria* culture to 5 x 10^5 cells/mL in f/2 medium + 0.03% yeast extract and let them grow O/N at 20-25°C

1.3 Centrifuge the *Cafeteria* cells for 5 min at 4,500 g, 20°C

If it is possible use 50 mL Falcon-types to reduce cell loss

1.4 Resuspend the cell pellet in 900 µL of 1X Gradient Buffer (0.5M NaCl, 1x PBS).

Pipette several times to break up cellular aggregates

1.5 In SW40 Ultra-Clear centrifuge tubes, load not more than 5 ml of the cells suspension in 1X Gradient Buffer.

1.6 Under-layer the cell suspensions approx. 4 ml of 10% solution of OptiPrep using a syringe and a flat needle.

The OptiPrep stock should be diluted in 1X Gradient Buffer

1.7 Load approx. 4 ml of 20% solution of Optiprep underneath the 10% solution using a syringe and a flat needle.

The OptiPrep stock should be diluted in 1X Gradient Buffer

1.8 Centrifuge the tubes using an ultra-centrifuge at 20000 rpm, 20°C for 20 min **with slow braking**.

1.9 Recover Cafeteria cells from the gradient, at the interphase between the 10% and 20% Optiprep layers. You can collect them by pipetting from the top or use a syringe and needle.

1.10 Checked the samples under the light microscope for the presence of bacteria and to determine flagellate density.

It is easier to dilute the collected samples in at least 10 ml of fresh f/2 media, before observing the cells.

Depending on the bacterial populations, separation can not be as effective. You can use a gradient of OptiPrep (from 30 to 10%) to determine better conditions.

1.11 It is recommended to remove the OptiPrep from the flagellates by centrifuging them at 4,500 g, 5 min, room temperature.

1.12 *C. roenbergensis* can be left in f/2 medium (no added yeast extract) overnight without affecting flagellate viability

Account for about 30% of cell loss from the initial stock (from step 4)

2 Centrifuge the *Cafeteria* cells at 4,500 g, 5 min, room temperature

3 Wash twice the cell pellet with 1X PBS
=> use small volume of PBS to concentrate the cells

4 Count the cells: stain 10 µL of Cafeteria culture with 1 µL of Lugol's acid iodine solution and load them in a haemocytometer (Neubauer Chamber)

Maximum volume of the desirable number of cells needed for the electroporation = **1.5 µl**

Electroporation buffer preparation

5 Prepare the different reagents ($CaCl_2$ and 2X HEBS) according to the followed protocol from Pia Elustondo

Calcium phosphate transfection of Oxyrrhis marina with an Alexa488-labelled DNA fragment
by Pia Elustondo

PREVIEW RUN

Aliquot and autoclave the 2X HEBS buffer to avoid contamination

5.1 -O marina cultures in F/2 medium supplemented with heat inactivated bacteria. The cultures were maintained with the heat inactivated bacteria supplanted every week with an extra addition an hour prior to the transfection. (It has not been tested yet if this addition one hour before the transfection is critical or not).

-ULYISIS Nucleic acid labeling kit (Molecular Probes, Cat # U21650). This kit is used to prepare the DNA and label it with a fluorophore. In our case, we used the ULYSIS Alexa 488 labeling kit.

-Transfection reagents
2.5 M CaCl2
2XHEBS (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM D-glucose, 42 mM HEPES (free acid), pH 7.10
HBS0/2: 144 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES pH 6.7 (alternatively other protocols use PBS pH 7.4). It was also possible to see the transfection with no washes.

5.2 Use only fragments that are 100 bp to 1000 bp
Long DNA can be degraged with DNAse into fragments 100 to 1000 bp. The ULYSIS kit includes DNAse and a protocol to produce fragments in that range. Alternative PCR products can be used. The labelling kit suggests not to use longer fragments to avoid precipitation of the labelled fragments.
For labeling 1 ug of DNA, cleaned PCR products or DNA fragments should be precipitated in 3M sodium acetate (pH5.2) and two volumes of absolute ethanol, freeze at -70 °C for 30 min and then for 15 min at 12,000 g. Wash the pellet with 70 % ethanol and allow it to air dry. Resuspend the pellet in 20 ul of the labeling buffer from the ULYSIS kit. Alternatively I have eluted the PCR products from the mini column in 30 ul of the labelling buffer form the ULYISIS kit.

5.3 The following two steps were performed exactly as indicated in the instruction manual of the kit. It was up scaled to label 5 ug of DNA

- Denature the DNA at 95 °C for 5 min and then snap cool on ice. Centrifuge the tube briefly to redeposit the sample to the bottom of the tube
- Add 1 ul of Alexa fluo Ulysis reagent per ug of DNA
- Incubate at 80 °C for 15 min. Stop the reaction by placing the tube on ice. Centrifuge briefly

5.4
- Purify the product from the excess of free ULS labelling reagent using a gel filtration-based spin column. The instructions recommend Bio-Rad Micro Bio-Spin ®P-30 or Princeton Separations Centri-Sep. I used Zeba spin desalting columns (Zeba, Thermo Scientific, cat # 89882)

5.5 -Prepare DNA/$CaCl_2$ mix for transfection of 1 ml of dense culture:

DNA 1-8 ug
2.5 M $CaCl_2$ 5 ul
Nuclease free H_2O to 50 ul final volume

If there is a desire to increase the amounts of DNA to 3-24 ug , the $CaCl_2$ should be increased to 15 ul and the final volume to 150 ml.

-Mix the DNA/$CaCl_2$ with equal volumes of HeBS. **IMPORTANT:** add 1/10th of the volumes of the DNA/$CaCl_2$ mix at a time mixing well by vortexing 3 s after each addition. This gradual mixing avoids local over concentrations of $CaCl_2$, which creates an unevenly sized precipitate. Supposedly, only very fine precipitate is efficiently taken up by the cells.

-Incubate 20 min at RT

-Add 800-1000 ul of O marina culture

-Incubate at RT. Image at different time points. I saw the fluorescence after about 4-5 and collected images 24 h later.

-The efficiency seems to be 30-40 % but a lot of them show fluorescence in the nucleus, suggesting that this could be potentially an appropriate method to generate stable transfected O marina.

-There is a wash step in which the cells are supposed to be rinsed with HBS0/2 three times and then resuspended in their medium but I noticed a lot of cell damage with centrifugation so I skipped the washes and did a dilution in F/2 medium in half the first day.

This protocol was adapted from Matz J, Gilyan A, Kolar A, McCarvill T, Krueger SR. Rapid structural alterations of the active zone lead to sustained changes in neurotransmitter release. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(19):8836-8841. doi:10.1073/pnas.0906087107.

6 Mix the $CaCl_2$ (final concentration 125 mM) with the material to electroporate
- for DNA electroporation complete to the final volume with water
- for protein electroporation complete to the final volume with 1X PBS or the buffer the most suitable for the protein

For one electroporation, final volume of the $CaCl_2$ mixture
10-µl tips: 3.5 µl
100-µl tips: 48.5 µl

7 Add the $CaCl_2$ mixture onto the 2X HEBS

For one electroporation, 2X HEBS volume
10-µl tips: 5 µl
100-µl tips: 50 µl

Option 1: volumes are big enough to vortex constantly the HEBS while the $CaCl_2$ mixture is added drop by drop

Option2: 1/10th of the $CaCl_2$ mixture is added at a time with a 3-secondes vortexing-step between each addition

8 Add the 1.5 µl volume of the appropriate number of *C. roenbergensis* cells to the $CaCl_2$/HEBS mixture

Electroporation

9 Electroporate the cells at 1600V, for 20 ms, 1 pulse using either the 10-µl or 100-µl tips

Cell recovery

10 Place the electroporated cells into 10 time more f/2 media than the electroporated volume for 30 min

11 Cells can either be fixed or maintained in culture by adding 5 time fresh f/2 media complement with 0.02% yeast extract and 1/500th of *E.coli* culture

Fluorescence microscopy with the marine heterotrophic flagellate Cafeteria roenbergensis
by Monica Berjon-Otero
PREVIEW RUN

11.1 Follow the protocol: "*Lysozyme-based removal of bacteria from cultures of the marine heterotrophic flagellate Cafeteria roenbergensis*"

If the culture is to be treated with other reagents, for example L-Azidohomoalanine, dilute the cells without bacteria to the required density and apply the treatment.

11.2 Aliquot 5 x 10^6 *Cafeteria* cells per 1.5 ml microfuge tube for each microscopy sample

11.3 Centrifuge for 5 min at 5,000 g, 20ºC

11.4 Resuspend each pellet in 500 µL of 4% paraformaldehyde (PFA) in 1X PBS

11.5 Incubate for 20 min at 20ºC

11.6 Centrifuge for 5 min at 7,000 g, 20ºC

You can keep the sample overnight at 4 ºC by resuspending the pellet with 250 µL 1X PBS
On the following day, centrifuge for 5 min at 5,000 g, 20ºC and continue with the next step

11.7 Resuspend the pellets in 500 µL of 0.5% Triton in 1X PBS

11.8 Incubate for 15 min at 20ºC

11.9 Clean the filter-support grids of a vacuum manifold system with pure water (e.g. ELGA, milliQ)

11.10 Add a drop of ELGA water on each grid of the vacuum system that you want to use and place a 0.45 µm Millipore support filter on top of it

11.11 Apply vaccum to remove the drop of water

11.12 Add another drop of ELGA water on top of the Millipore filter

11.13 Place a 0.2 µm, 25 mm Anodisc Whatman filter on top of the ELGA water drop

11.14 Apply vacuum with a pressure between 5-10 Hg to remove the water

11.15 If neccesary, block or wash the filter with the required solution.
Remove the solution by applying vacuum

11.16 Place the *Cafeteria* sample from step 8 on top of the Whatman filter

11.17 Apply vacuum with a pressure between 5-10 Hg to remove the permeabilization buffer and to immobilize the cells on the filter

Perform all the neccesary blocking and washing steps following the same instructions

11.18 Place the filter with the *Cafeteria* sample on a piece of Parafilm. Add the staining solution of interest on top of the filter and incubate following the manufacturer's instructions

11.19 Place the filter on the vacuum system again

11.20 Apply vacuum with a pressure between 5-10 Hg to remove the remaining staining buffer

11.21 If required, wash the filter again with the buffer of interest

11.22 If desired, stain DNA by incubating with an appropriate reagent (DAPI, SYBR, Hoechst)

11.23 Apply vacuum with a pressure between 5-10 Hg to remove the remaining liquid

11.24 Add 10 µL of 70% glycerol on a glass slide and place the filter on top of the glycerol

The filter must be placed with the Cafeteria cells in the top!

11.25 Add 10 µL of 70% glycerol on the coverslip and place it on top of the filter

11.26 Apply pressure on the sample during 20 min at room temperature

11.27 Seal the coverslip with nail polish

Samples can be kept at 4 °C or analyzed with the microscope immediatly