

Oct 02, 2019 Version 2

Modified Bacterial Conjugation Protocol For Pseudo-nitzschia multiseres V.2

Version 1 is forked from [Modified Bacterial Conjugation Protocol For Pseudo-nitzschia multiseres](#)

Nature Methods

DOI

dx.doi.org/10.17504/protocols.io.7vhhn36

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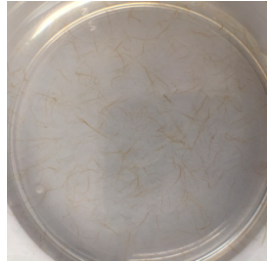
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DOI: dx.doi.org/10.17504/protocols.io.7vhhn36

Protocol Citation: G Jason Smith, April L Woods 2019. Modified Bacterial Conjugation Protocol For Pseudo-nitzschia multiseres. [protocols.io https://dx.doi.org/10.17504/protocols.io.7vhhn36](https://dx.doi.org/10.17504/protocols.io.7vhhn36)

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Protocol status: Working

We use this protocol in our group and it is working, but it can always be improved

Created: October 02, 2019

Last Modified: October 02, 2019

Protocol Integer ID: 28297

Keywords: Pseudo-nitzschia multiseres, actin gene, EGFP

Abstract

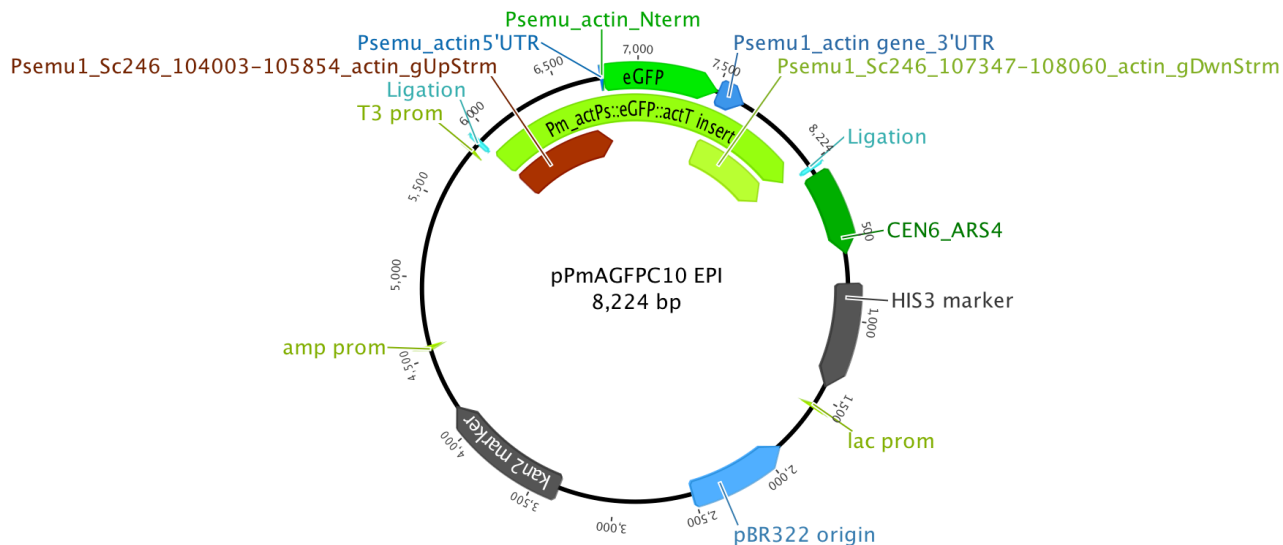
This is a modified version of 'Conjugation of *Thalassiosira pseudonana*', published by J. Turnsek [dx.doi.org/10.17504/protocols.io.f55bq86](https://doi.org/10.17504/protocols.io.f55bq86).

The protocol was modified to enhance viability in the diatom *Pseudo-nitzschia multiseriis*.

This version was used to transform *Pseudo-nitzschia multiseriis* isolate 15091C3 with an episomal plasmid derived from pPtPUC3 to express a egfp gene under control of the *P. multiseriis* actin promoter and termination domains.

PLASMID INFO

pPmAGFPC10 EPI : A *P. multiseriis* derived expression cassette under control of *P. multiseriis* ca 1000 bp of actin promoter and termination domains. *Pm actPs::eGFP::actTs*



EGFP expression vector pPmAGFPC10. Upstream and downstream genomic regions bracketing the *P. multiseriis* actin CDS targeted for constitutive expression of EGFP. Expression cassette inserted into modified pPtPUC3 episomal plasmid backbone. Plasmid sequence is available in attached file below.

 [pPmAGFPC10 EPI.gb](#)

Growth and preparation of E. coli pTA-MOB host and episomal plasmid donor

- 1 Innoculate G418+Kan plates with the appropriate host strain and grow overnight (use within 7 days). Pick isolated colonies and inoculate into 10 mL LB medium. Grow overnight at 37°C, 200 rpm.

Preparation of E. coli donor (con

- 2 Measure OD₆₀₀ and start a 150 mL LB subculture (recommended starting OD₆₀₀ either 0.05 or 0.1).

Preparation of E. coli donor (cont)

- 3 Grow at 37°C, 200 rpm until OD₆₀₀ reaches 0.3-0.4
- 4 Centrifuge at 4,000 rpm, 10°C, for 10 min.
Decant supernatant and resuspend bacterial cell pellet in 100 µL SOC*

Growth and preparation of Pseudo-nitzschia cells

- 5 *P. multiseri* stock cultures are maintained in filtered (0.2µ) autoclaved seawater (FASW) + Guillard's Marine Enrichment f/2 medium.
Subcultures are maintained in active mid-log phase growth, by serial batch culture in FASW+f/2
Typical cell numbers at log phase harvest are in the 10-30 ×10³ cells/mL range.
Centrifugal concentration of 100mL of culture yields around 2×10⁶ cells for conjugation.

Note

- I've never tested if cell density before spinning cells down matters with respect to final conjugation outcome same as the Alverson Lab did for *P. tricornutum*. They observe ~8 x 10⁶ cells/mL to be a sweet spot. In my hands spinning cells down at ~4-8 x 10⁶ cells/mL seemed to work fine. According to my information harvesting *T. pseudonana* at ~0.8 x 10⁵ cell/mL works best for biolistic experiments.
- I counted cells with a Sedgewich-Rafter cell.

Harvest Pseudo-nitzschia cells



- 6 Spin down cells at 4000 rpm, 10°C, for 20 min in 50mL falcon tubes. Remove supernatant with vacuum pipette.
Add additional 50mL culture and repeat centrifugation. Pellet will contain cells from a total of 100mL culture.

Re-suspend cell pellet in 1 mL of seawater enrichment media. Allow suspension to come to room temperature.

Conjugation

- 7 Mix 450 µL *Pseudo-nitzschia* cells and 50 µL *E. coli* cells in a 1.5 mL tube.

Mix fully by gentle pipeting.

Incubate mixture at 30 °C for 5 min.

Note


This is the major modification for *Pseudo-nitzschia* conjugation. A final concentration below 10% SOC greatly enhances *Pseudo-nitzschia* viability.

- 8 Gently spread mixture on pre-warmed 1% agar plates containing 5% (v/v) LB and 50% (v/v) filter sterilized seawater - L1 media.

Note

Plates were incubated at 37° for an hour before plating cells.

- 9 Incubate in dark at 30°C for 90 minutes.

 01:30:00

- 10 Move plates to standard *Pseudo-nitzschia* growth conditions - in my case 15°C and constant light - for 4 hours.

- 11 Add 1 mL reduced Nitrogen L1 medium*.

Gently scrape agar surface with a cell scraper or L spreader to resuspend bacterial and diatom cells.

Expect to recover ~500 µL co-culture suspension after scraping.

**Note**

At this point, we use L1 media enrichment and adjust the amount of Nitrogen so that Silica is not a limiting nutrient. Nitrogen:Silica ratio is 1:3.

Prepare for Solid Phase Growth in 1% (w/v) LGTA

- 12 LGTA has been prepared following <https://www.protocols.io/view/low-gel-temperature-agarose-lgta-media-gsibwce>
LGTA has been maintained at 20 °C. LGTA can also be microwaved to reliquify from solid storage, be sure to cool to 20 °C.
- Aliquot 4 mL, molten LGTA (20 °C) into sterile culture tubes (e.g. Falcon 2059).
 - Add as much cell suspension as you are able to scrape off the conjugation plate (ca. 500uL) **into** LGTA.
 - Gently vortex to mix.

Note

The LGTA contains the same adjusted 1:3 NO₃:Si ratio.

PLATING CULTURES:

- 13 Transfer 1 mL to well of Costar 12-well culture plate avoiding introducing bubbles.
Repeat for 4 wells.
For selection experiments may reduce volume to 500uL per well and include selection-free matched control wells.
(LGTA will solidify during subsequent incubation at 15 °C.)

Screening

- 14 Observe plates under fluorescence microscopy for viability and growth (chlorophyll fluorescence).
Screen for expression of egfp under fluorescence microscopy.



As cells grow, some may emerge from the agarose and into the liquid overlay. These are available for isolate pics.

Gel plugs may be removed and resuspended into liquid growth.

Isolate cultures may be screened for presence of plasmid by PCR.

DNA extraction of isolate cultures may be used to transform *E. coli* and selected for Kanamycin resistance.