Diatom Transformation via Bacterial Conjugation Version 3

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Abstract

Citation: Jeric Harper Diatom Transformation via Bacterial Conjugation. **protocols.io**
dx.doi.org/10.17504/protocols.io.hgpb3vn
Published: 29 Mar 2017

Before start

- Make sure you are using fresh *E. coli* cells streaked for isolation on LB + antibiotics no more than 1 week from -80°C cryostock.

Protocol

Diatom cultivation (liquid)

Step 1.

Grow the diatom culture to mid-log phase (≈ 8.0E^6 cells/ml for *Phaeodactylum tricornutum* grown on L1 medium or 5.0 E^7 cell/ml when grown on BG-11*).* We have investigated transformation efficiency throughout the growth curve and found these to be the optimal densities.

NOTES

Andrew Alverson 29 Mar 2017

- The original protocol plated the culture (250uL of 1.0E^8 cells/ml) on 1/2 strength L1, 1% agar plates for 4 days prior to transformation.

- We have been investigating liquid cultivation because many diatom species do not survive on agar.

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Cultivation Conditions

- Cultivation in 0.2 um-filtered L1 or BG-11 medium prepared using 32 ppt seawater collected from the Gulf of Mexico.

- 80–100 µmol photons m^{-2} s^{-1} provided by Philips Daylight Deluxe 40 watt T12 fluorescent bulbs.
- Semi-continuous operation in 1 L bubble column bioreactors (500–800 ml working volume).
- 0.2 µm filtered air containing 1% CO_2 provides aeration/agitation at 0.2 vvm.

Prepare E. coli starter culture

Step 2.

Grow 1 mL of *E. coli* culture, containing both the mobillity plasmid (Pta-MOB) and carrier plasmid, overnight (16–20 hrs) in LB+antibiotics for each planned transformation. We grow them at 37°C at 270 rpm in a shaking incubator.

DURATION
16:00:00

ANNOTATIONS
Jernej Turnsek 29 Mar 2017

In my experience it has proven beneficial to perform conjugations with multiple donors carrying THE SAME construct. 2/3 worked beautifully, one failed completely.

Outgrow E. coli

Step 3.

On the day of transformation, use the overnight culture to inoculate 50 mL of fresh LB+antibiotic, 1:50 dilution, for each planned transformation.

- Grow to an OD_{600} of 0.8–1.0 (37°C with 270 rpm shaking).
- This takes 3–4 hours.

DURATION
03:00:00

NOTES
Andrew Alverson 29 Mar 2017

The authors of The original protocol reported that the OD_{600} range is flexible. Transformation success has been seen within OD_{600} range of 0.4 to 1.2.

P. tricornutum cell concentration

Step 4.

During the 3–4 hours when the *E. coli* culture is growing, measure the *Phaeodactylum tricornutum* cell concentration with a FlowCam or haemocytometer to calculate the required volume needed to collect $2.5E^8$ cells for each transformation.

DURATION
00:05:00

Concentrate the diatom and E. coli cultures

Step 5.

For each transformation, centrifuge 50 mL of *E. coli* culture and the required *Phaeodactylum tricornutum* volume at 4000 x g for 10 minutes at room temperature.

- Resuspend *E. coli* pellet in 500 µL of SOC medium.
- Resuspend *P. tricornutum* pellet in 500 µL of L1 medium.

Note: The diatom and *E. coli* cultures should be **centrifuged at around the same time** to minimize the amount of time they spend concentrated.

DURATION
00:10:00

NOTES
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The original protocol scraped the agar plates the culture was initiated on using 500uL L1 then adjusted the volume to attain 5.0 E^8 cells/ml. We have found no difference so far in transformation effficiency between liquid- and plate-initiated cultures.

Conjugation

Step 6.

In a 1.5 mL tube, mix 200 µl of *E. coli* cells with 200 µl of *Phaeodactylum tricornutum* cells.

Negative control: In a 1.5 mL tube, mix 200 µl of SOC medium with 200 µl of *Phaeodactylum tricornutum* cells.

Note: Incubate and treat the negative control plates identically to conjugation plates.

NOTES
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- The authors of The original protocol suggested spreading the mixture near, but not touching, the edges of the plate, as this mixture will be scraped and replated in 2 days.

- N concentration seems to be important. We have found greater transformation success using 0.5x BG-11 medium made with seawater instead of 0.5x L1 medium.

- 5% LB is a sufficient concentration to support the E. coli culture without encouraging growth. Increasing the LB concentration results in increased cell death of the diatoms. We have also tried buffering the medium, but that did not increase *P. tricornutum* survivorship.

Conjugation

Step 7.

Spread the mixture (400 µL) on Conjugation Plates. (0.5x BG-11 with 5% LB and 1% agar).

The authors of the original protocol suggested spreading the mixture near, but not touching, the edges of the plate, as this mixture will be scraped and replated in 2 days.

NOTES

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- Nitrogen concentration seems to be important. We have found greater transformation success using 0.5x BG-11 medium made with seawater instead of 0.5x L1 medium.

- 5% LB is a sufficient concentration to support the *E. coli* culture without encouraging growth. Increasing the LB concentration results in increased algal mortality. Buffering the growth medium did not increase *P. tricornutum* survivorship.

Conjugation

Step 8.

Incubate plates for 90 minutes at 30°C in the dark.

DURATION

01:30:00

NOTES

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P. tricornutum can survive transformation temperatures up to 32ºC. Survivorship is severely hampered at 34ºC and above.

Conjugation

Step 9.

Move plates to light incubator (18°C and 100 µmol photons m^{-2} s^{-1}) for 2 days.

DURATION

48:00:00

NOTES

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- According to Diner et. al, the conjugation occurs during the 2-day incubation. Transformation efficiency increased as incubation time increased.

Selection

Step 10.

1. Collect cells by adding 1 mL of L1 medium. Use a cell scraper to concentrate cells and medium to one side of the plate. We often add 500 µL L1 to collect the majority of the cells from the plate We then repeat with an additional 500 µl L1 to collect the remaining cells.
2. Transfer resuspended cells into a 1.5 ml microcentrifuge tube with a P1000 pipette and filter tips.

NOTES

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It is important to use filter tips as smalll peices of agar that are accidentaly scraped up can cause the pipette to cavitate resulting in contamination.

We often add 500 µL L1 to collect the majority of the cells from the plate into the 1.5 mL tube, then repeat with an additional 500 µl L1 to collect the remainder.

Selection

Step 11.

Spread 200 µl of the cell suspension on a Selection Plate.

NOTES

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- Dilution of the selection volume (using L1 as makeup volume) can help facilitate colony enumeration if 200µl results in too many colonies.

- To maximize the number of colonies the entire volume of resuspended cells can be plated to multiple plates.

- Collect 1 µL of the cell suspention and dilute 1:1000 for FlowCam cell count.

Selection

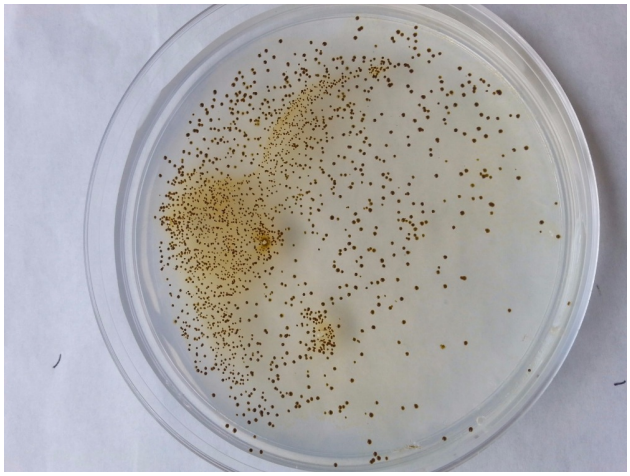
Step 12.

Incubate at 18°C and 100 µmol photons m^{-2} s^{-1} until colonies appear.

Colony identification

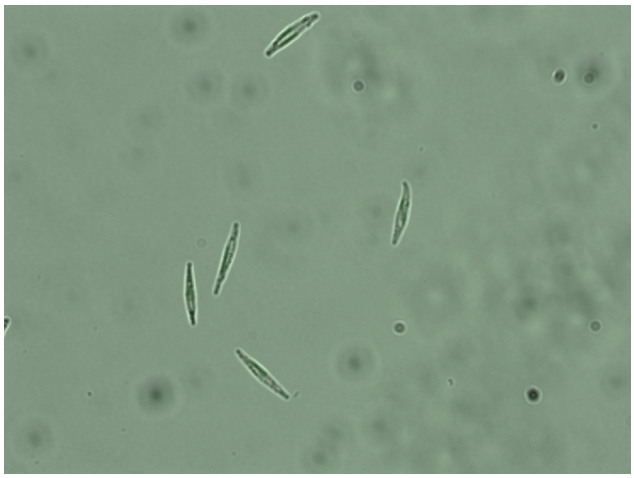
Step 13.

After a minimum of 8-12 days, untransformed *Phaeodactylum tricornutum* cells die off, and colonies of transformed cells begin to appear. In some cases, this can take 3–4 weeks.

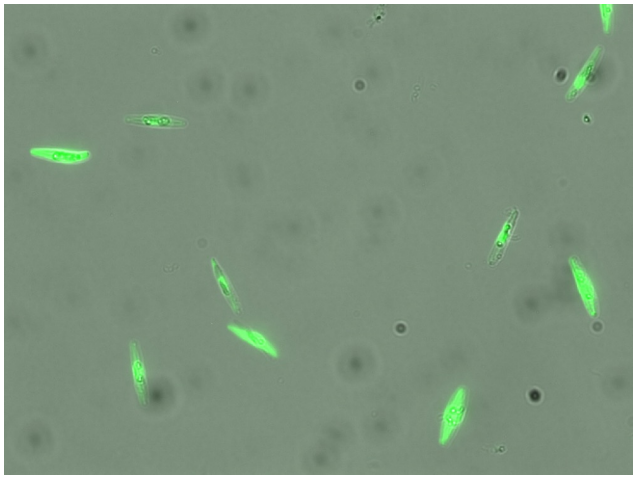


Alternatively, selection can be done in liquid BG-11 selection medium using eGFP as a reporter and sorted using FACS.

un-transformed Pt



eGFP expression



NOTES

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- For plate selection, use the ImageJ protocol for colony enumeration.

- Calculate transformation efficiency using the following equation:

Efficiency = (number of colonies on plate) ÷ (selection volume cell density [cell/mL] **x** volume [mL] put on selection plate)

Note: Be sure to include a dilution factor in the calculation if it was used in plating or counting.

Step 14.

This protocol was modified based on the original procedure and correspondence with the authors.