



CRISPR-Cas9 episome conjugation into *Phaeodactylum tricornutum* V.2

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1 Works for me

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JCVI West Protocols



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ABSTRACT

Bacterial-conjugation methodology used to introduce the CRISPR-Cas9 episome generated using the GG2 assembly protocol.

This method, though, can be adapted for any episomal transfromation from an *E. coli* culture that contains a cargo plasmid (episome) and conjugation plasmid (pta-MOB) to *Phaeodactylum*.

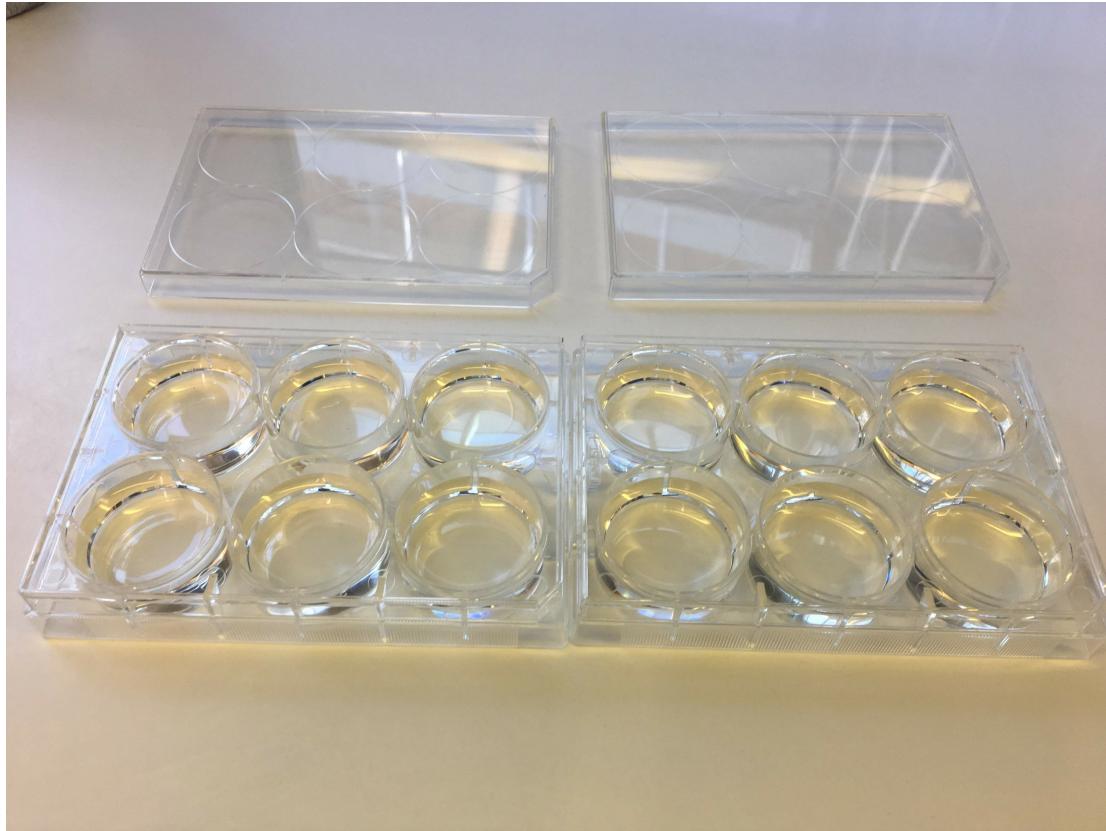
□
CRISPR episome
conjugation in P.t..pdf

GUIDELINES

Take it easy. Conjugation should be easy and conducted without extremely sensitive steps and difficult techniques.

Prepare conjugation transformation plate

1 Prepare conjugation plate



Conjugation transformation 6-well culture plate with ASW (0.8% agarose, 5% LB)

1.1 Autoclave **45 ml** of 1.6% agarose solution (in MQ-H₂O)

Incubate autoclaved bottle/flask in **50 °C** waterbath

1.2 Pipette **50 ml** mL of N-free ASW (Artificial Sea Water) into 250 mL flask

Add **5 ml** LB media

Add **25 µl** NaNO₃ (880uM)

Add **25 µl** NH₄Cl (880uM)

Incubate ASW+LB in **50 °C** waterbath **00:30:00**

(or until agarose has acclimated to 50°C)

1.3 During incubation, place 2 6-well culture plates (flat bottom) in a PCR hood

1.4 Once both solutions have reached **50 °C**, pour the agarose into the ASW flask in the PCR hood to maintain sterility

Continually mix in flask until mixed thoroughly

1.5 Pipette **8 mL** of ASW (0.8% agarose, 5% LB) mix into each well

Avoid bubbles on plate by pipetting up 10 mL while only dispensing 8 mL

1.6 Solidify and dry plates in PCR hood **00:30:00**

During, prepare *P. tricornutum* cells

Prepare *Phaeodactylum tricornutum* cells

2 Obtain an axenic culture of *Phaeodactylum tricornutum* (CCAP1055/1) growing in exponential phase

2.1 Determine culture health by Fv/Fm measurement using Dual-PAM fluorometer

(Exponentially growing culture typically has a Fv/Fm value of 0.6 to 0.7)

2.2 Measure cell concentration

(Ideally by flow cytometry side scatter (SSC) vs. FL3 (longpass filter, 570nm))

2.3 Collect enough culture volume for 6e8 cells (total)

Calculation:

100 µl *P. tricornutum* at concentration of 5e8 cells/mL per well

12 wells X **100 µl** = **1.2 mL** (5e8 cells/mL) --> 6e8 cells

Ex:

P.t. concentration = 1.1e6 cells/mL

6e8 (cells) / 1.1e6 (cells/mL) = **545.5 mL**

*** I round up to **600 mL** ***

2.4 Pellet cells

3000 xg

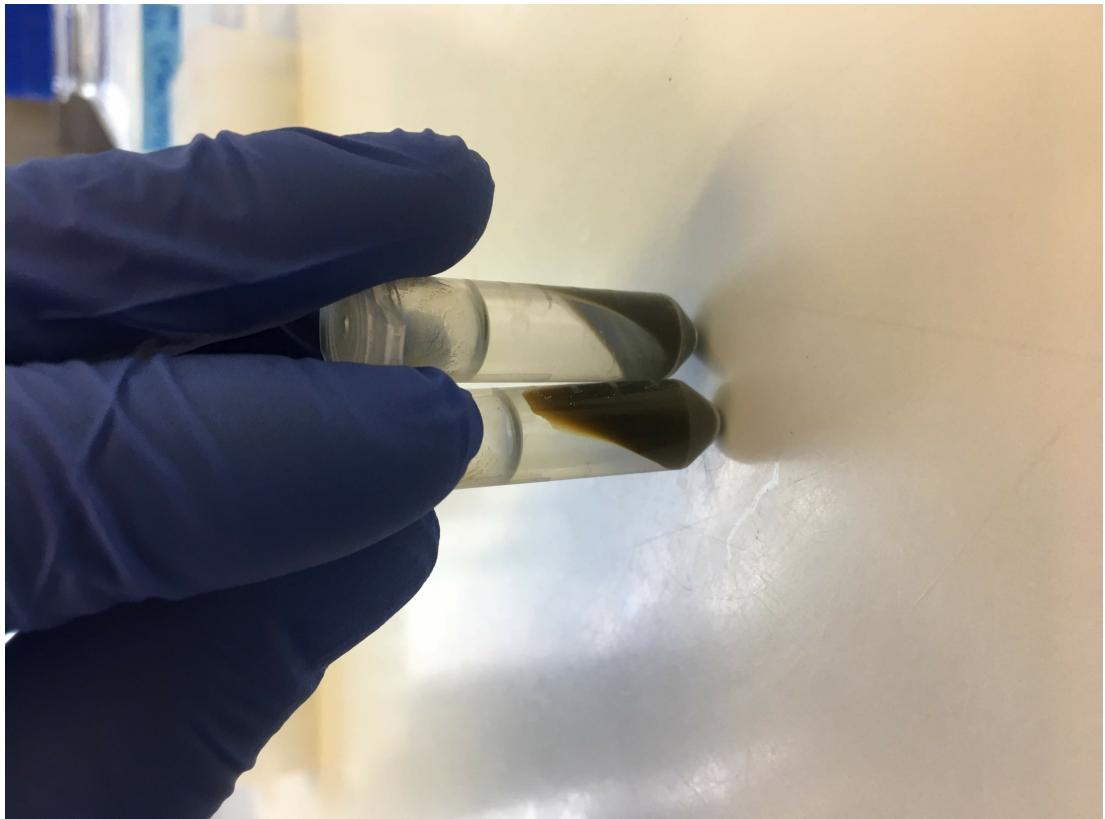
00:15:00

2.5 Remove supernatant with graduated pipette-man so to not disturb pellet

Add cells to 2 2mL tubes

2.6 Pellet cells again

2000 xg



00:05:00

After second pelleting

2.7 Remove supernatant with pipette

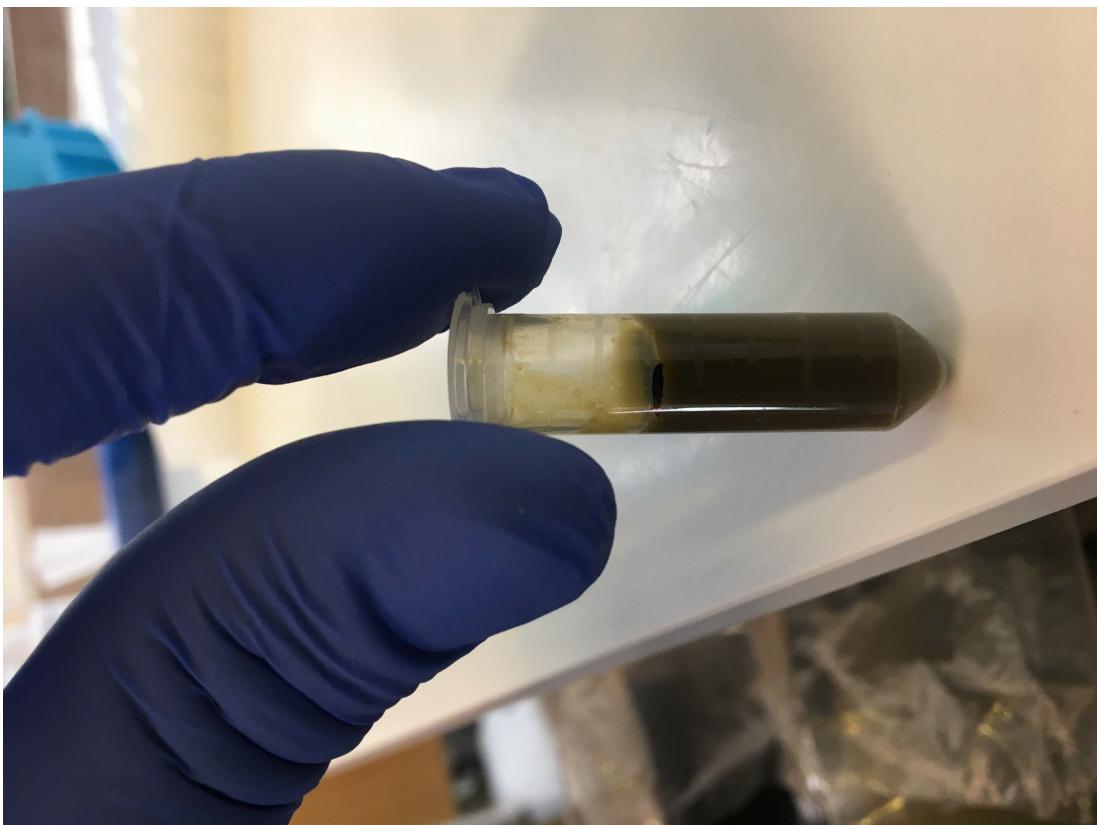
2.8 Add 100-200 uL NO₃-ASW to one 2 mL tube and mix cells with pipette

Mix all cells together in 1 tube



All cells mixed in one tube. Line drawn on tube indicates 1.2 mL position

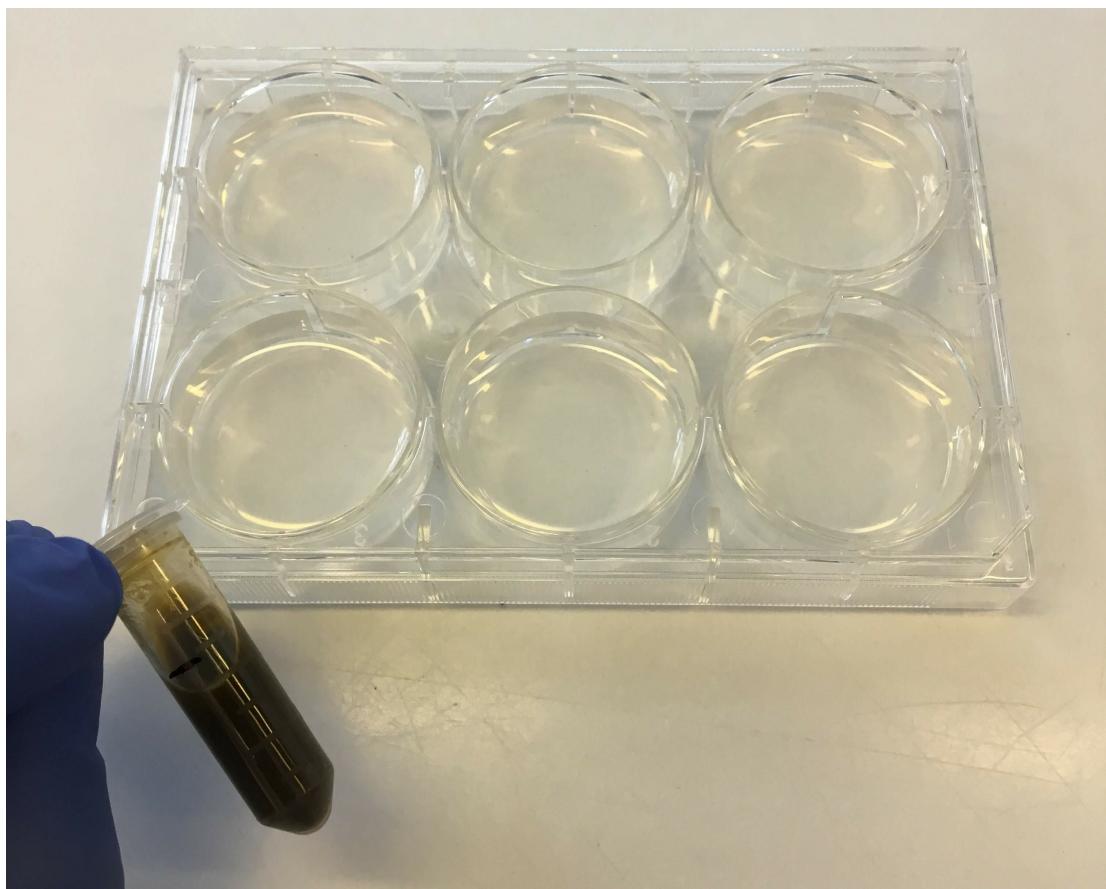
2.9 Add appropriate volume of NO₃-ASW to cells to bring final volume to **1.2 ml**



Mixed cells in final volume of 1.2 mL

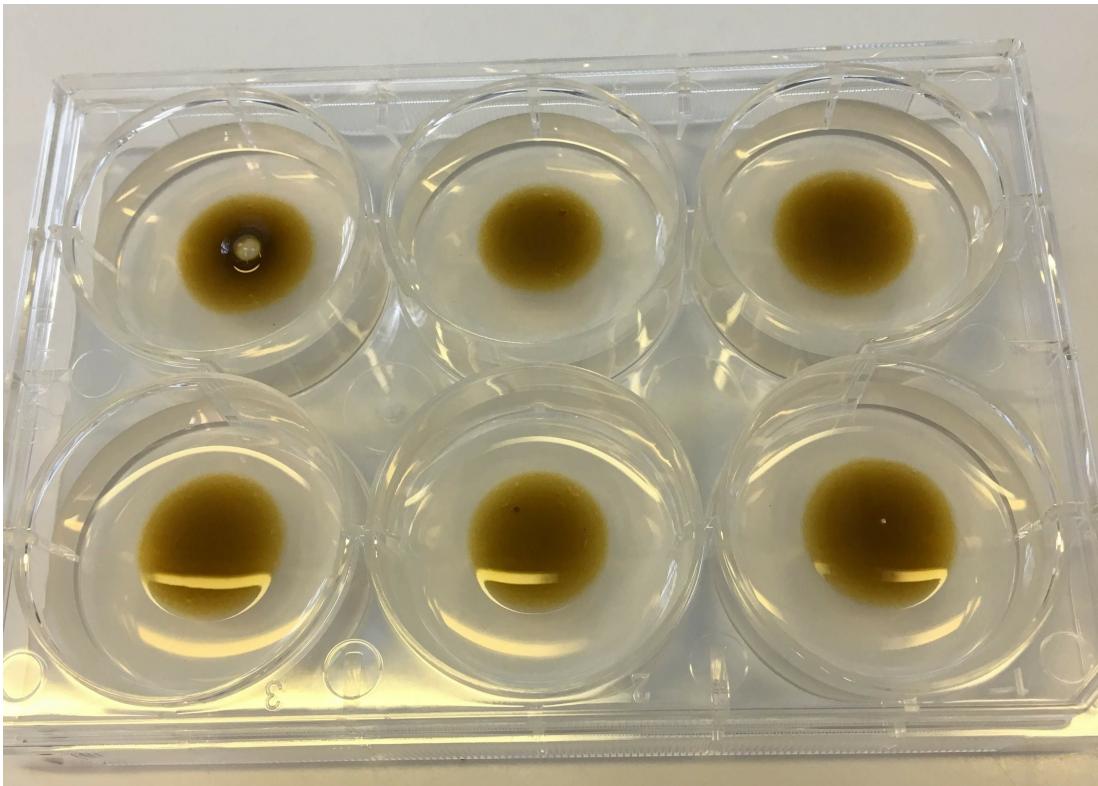
Plate *P. tricornutum* cells onto conjugation plates

3



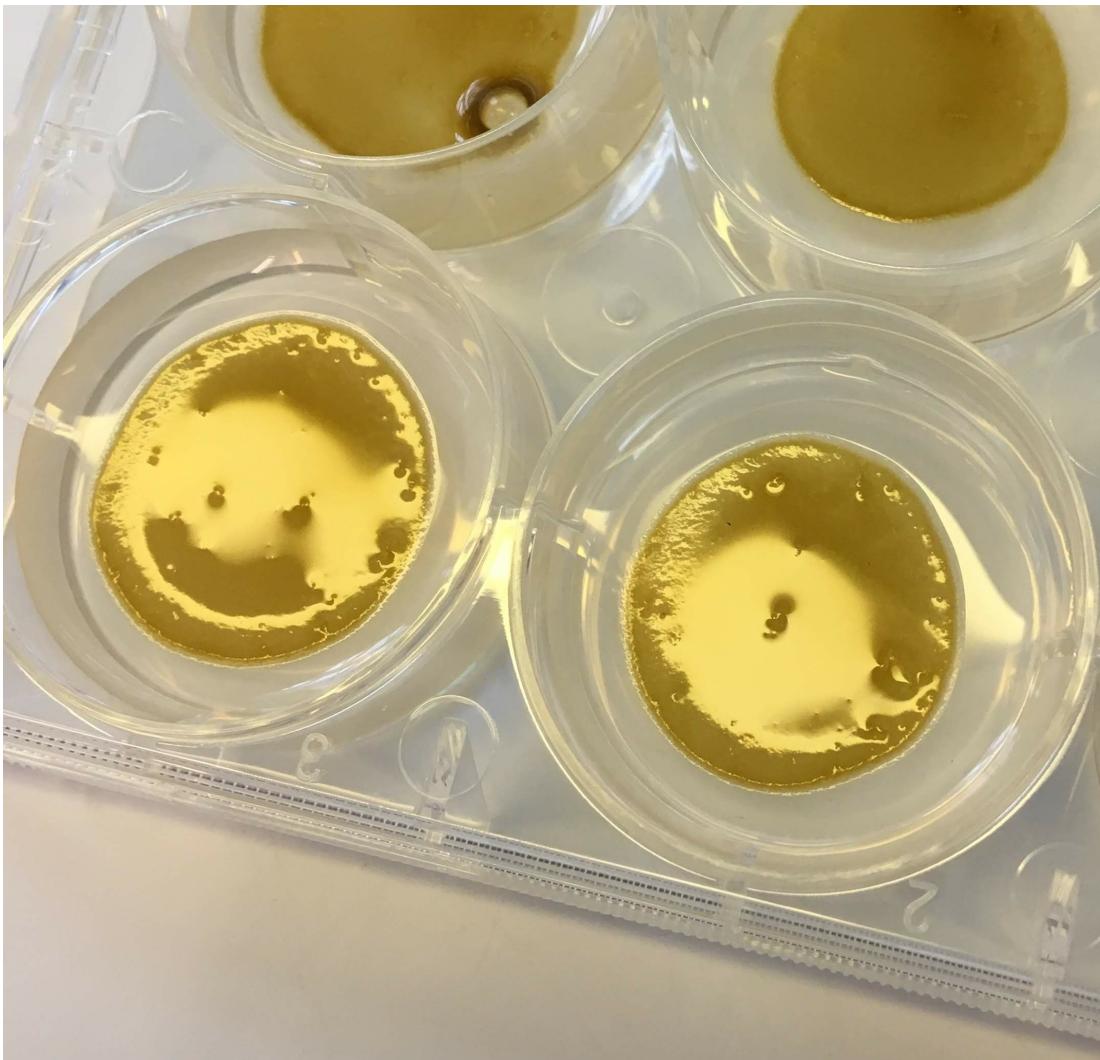
Prepare to plat cells onto drying 6-well ASW (0.8% agarose, 5% LB)

3.1 Pipette  *P. tricornutum* cells on each well, directly in the middle of the well

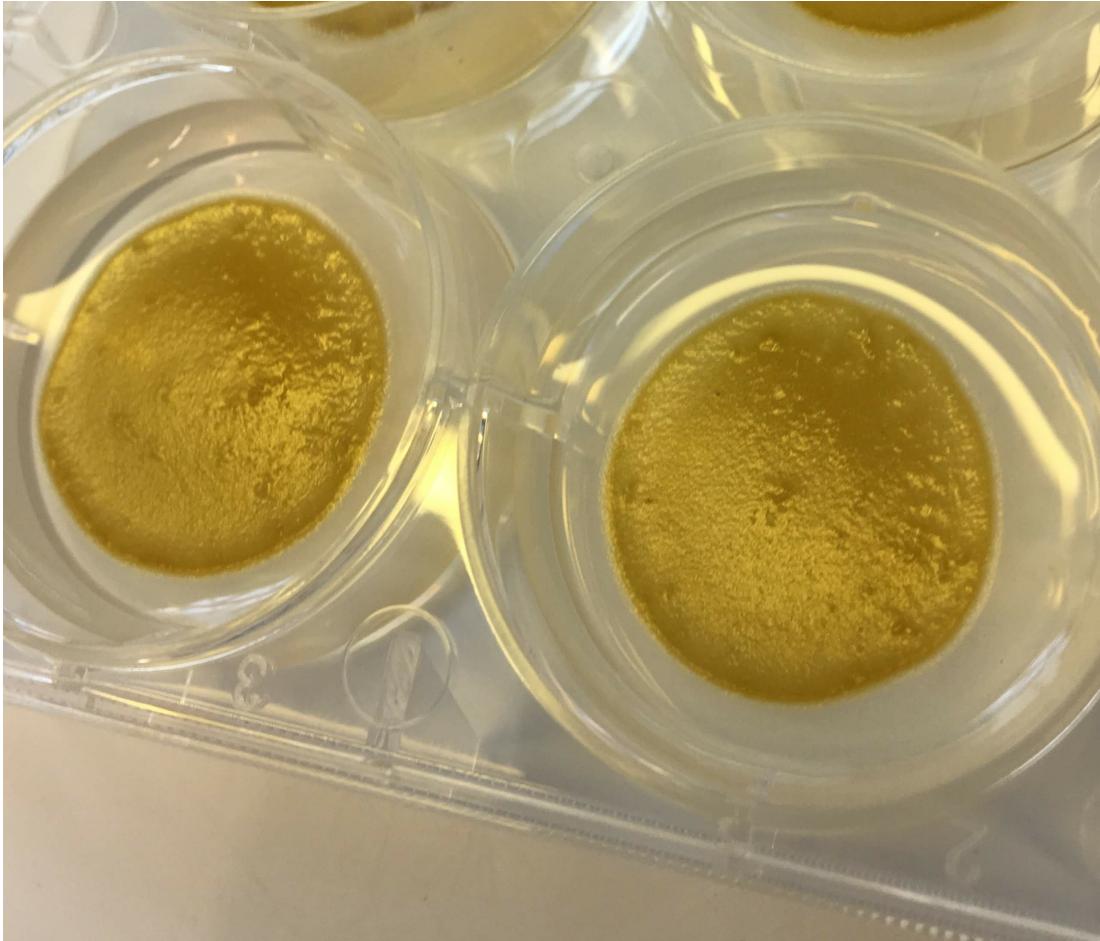


3.2 Spread cells by rotating plate in an orbital motion with hands

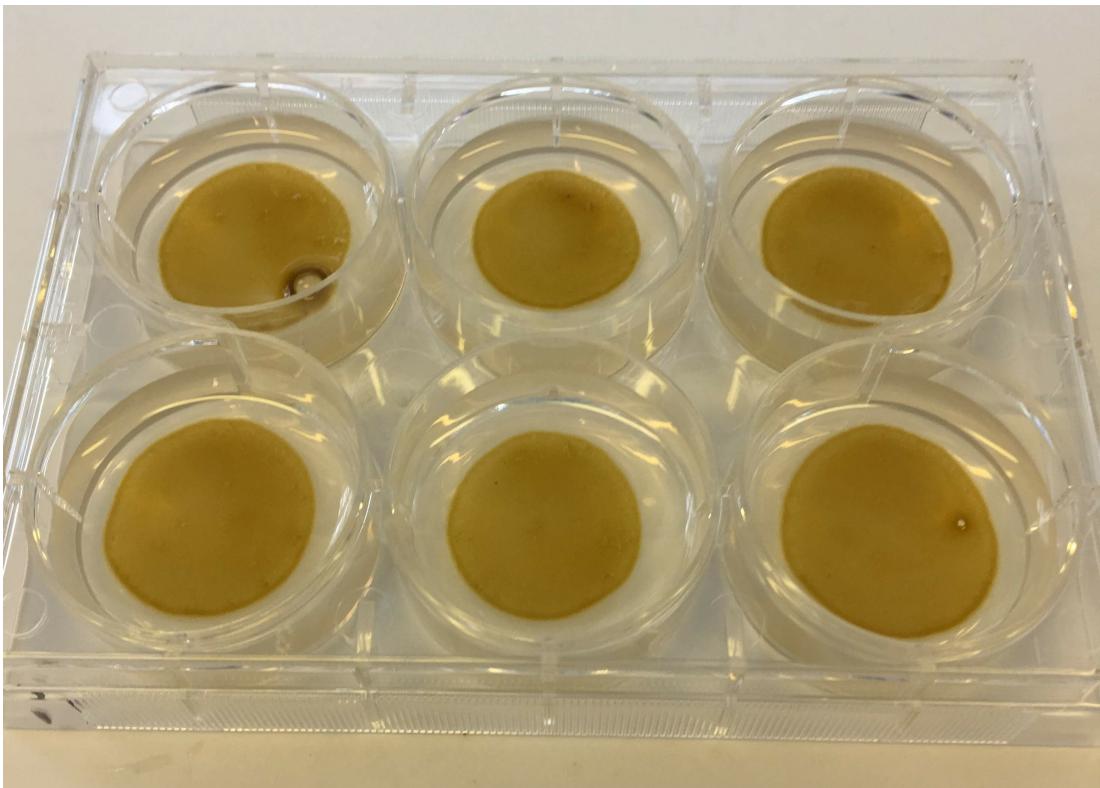
3.3 Dry cells after spreading under PCR hood  **00:20:00**



Wet cells on plates in need of drying!



Dried cells (evident by loss of sheen on culture surface)



Successfully prepare conjugation transformation plate with dried P.t. cells.

3.4 Cover 6-well plates and seal lid to plate with breath-easy culture tape

3.5 Incubate cells at $\text{at } 18\text{ }^{\circ}\text{C}$ and under medium-intensity light [$\text{M}50\text{ }\mu\text{E m}^{-2}$)

24:00:00

Prepare E. coli for conjugation transformation

4 The night before, grow E. coli cultures in preparation for conjugation

4.1 Inoculate **2 mL** LB media cultures with pipette stab of conjugation-ready E.coli

Ampicillin [$\text{M}100\text{ }\mu\text{g/mL}$

Tetracyclin [$\text{M}10\text{ }\mu\text{g/mL}$

Gentimycin [$\text{M}20\text{ }\mu\text{g/mL}$

$\text{at } 37\text{ }^{\circ}\text{C}$ **16:00:00** shaking

4.2 Ensure to include positive and negative controls

Positive (conjugation episome containing a RFP bacterial expression cassette)

Negative (conjugation episome that confers resistance to nourseothricin antibiotic, in contrast to resistance to phleomycin)

5 Next day, remove E. coli cultures from $\text{at } 37\text{ }^{\circ}\text{C}$



10 conjugation E.coli cultures. The positive control can be seen with a red hue, indicative of stable episomal maintenance in E. coli

5.1 Prepare out-growth E. coli culture media

Prepare **12.5 ml** LB media (Amp-50, Tet-5, Gent-20) for each conjugation and pipette into 50 mL conical tube (one per conjugation reaction)

5.2 Pipette **250 µl** overnight E.coli culture to each 50 mL out-growth tube

Do not seal tube with cap. Rather, place cap on tube loosely and secure with tape

5.3 Shake out-growth cultures at **37 °C** for **05:00:00**

5.4 Measure optical density of out-growth cultures

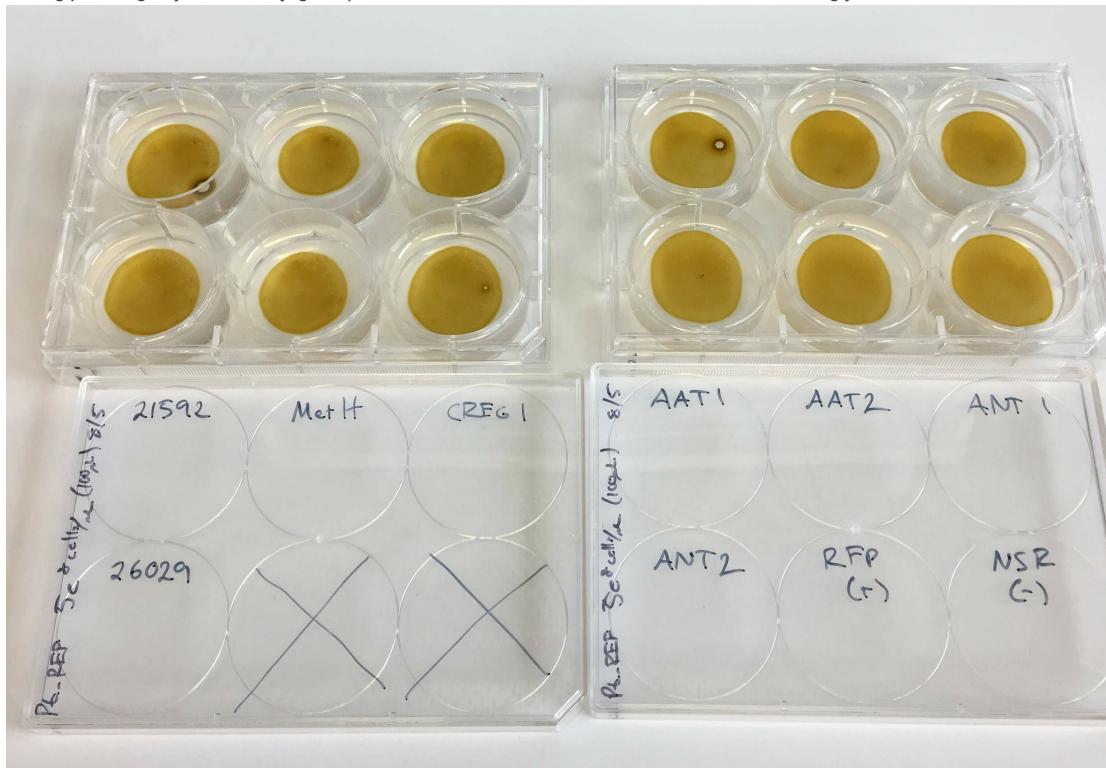
Ideal absorbance reading: OD₆₀₀ = 0.9-1.0

5.5 Pellet out-growth cultures

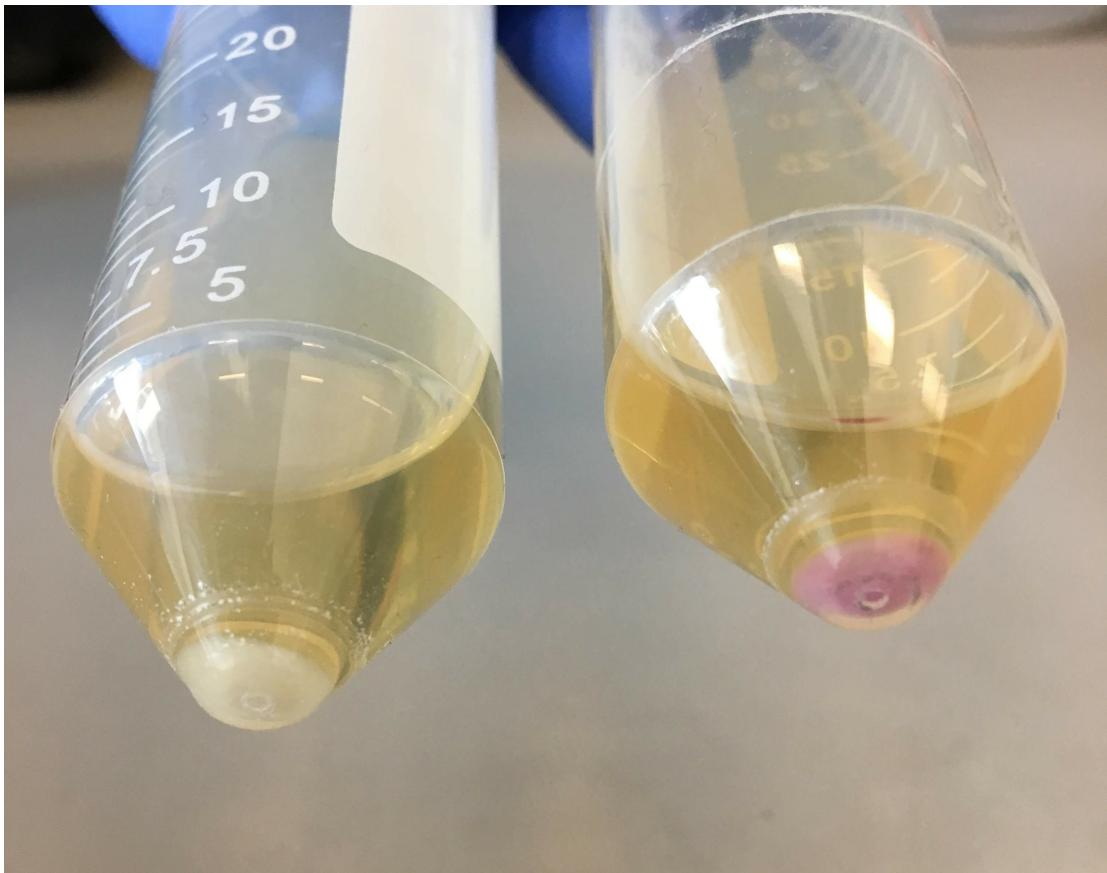
3000 xg

00:15:00

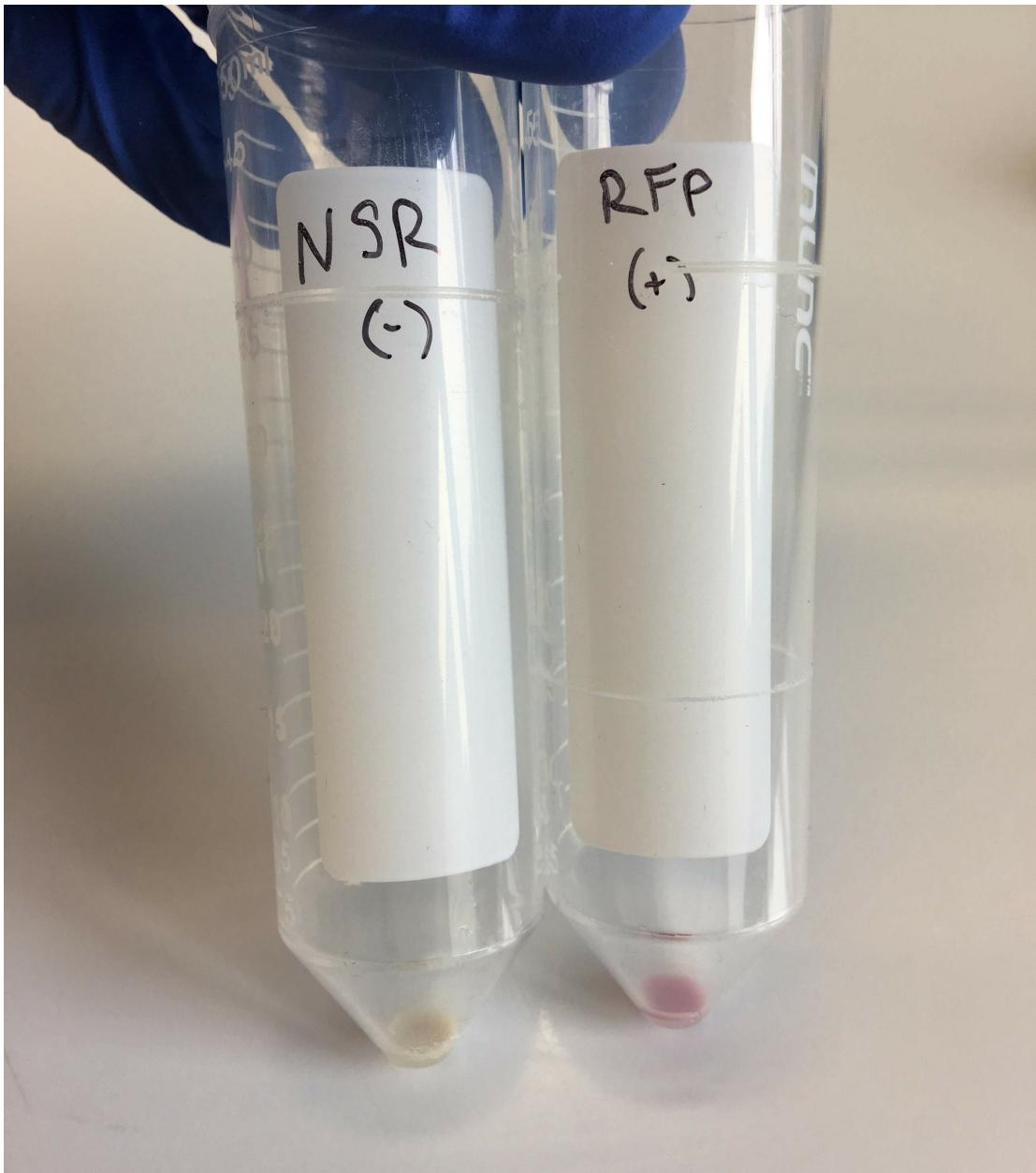
- During pelleting, dry 6-well conjugatin plates under a PCR hood and label each well accordingly



- After pelleting, remove media using vacuum manifold



Pelleted E. coli. Negative control and positive control (RED) shown



After removal of media. Avoid disrupting pellet but remove as much liquid as possible

8 Resuspend E.coli pellet

8.1 Pipette **100 µl** S.O.C. media into each tube

8.2 Mix well by vortexing

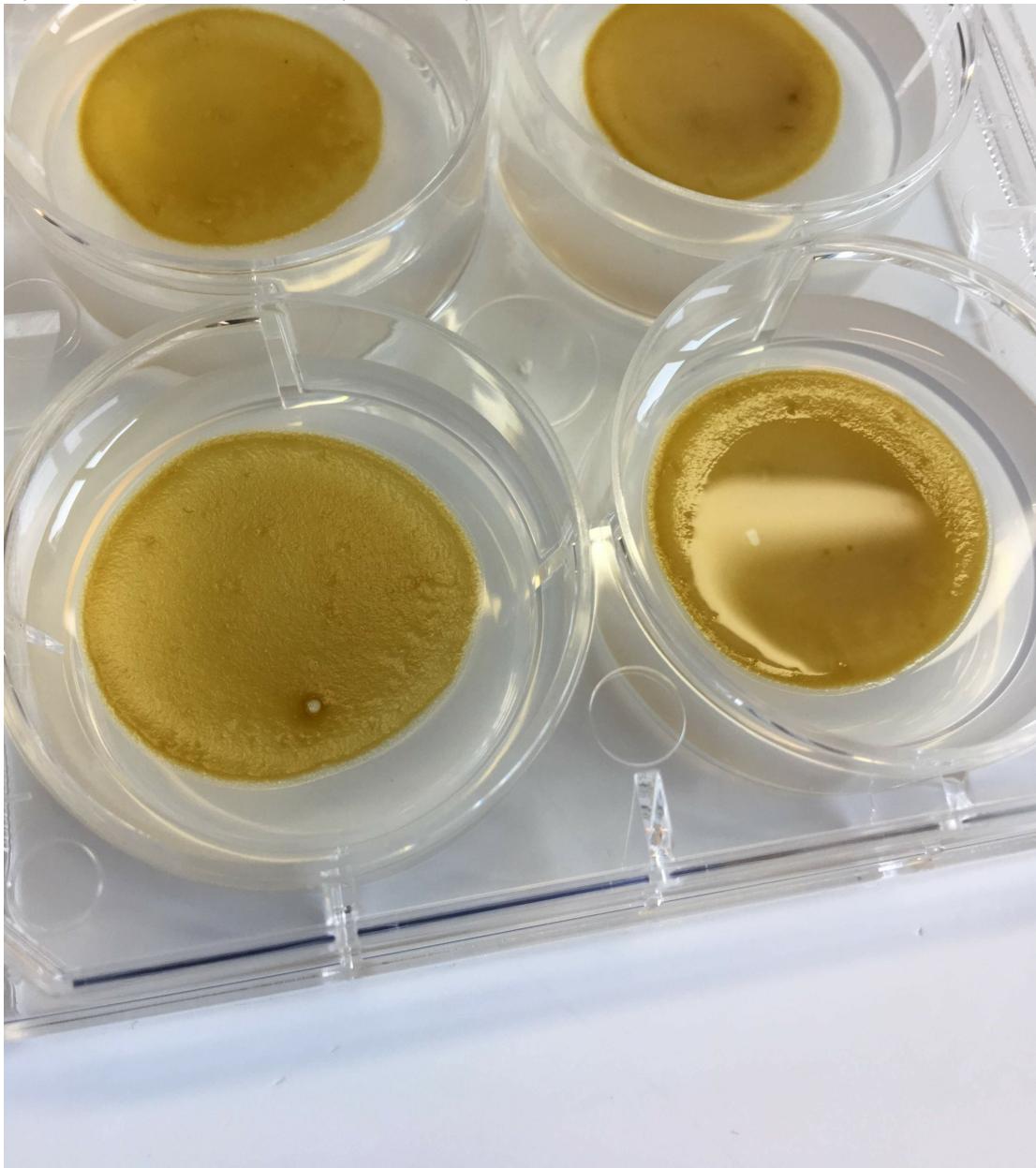
9 Add E. coli to P. tricornutum cells on conjugation plates

9.1 For each conjugation reaction, pipette **100 µl** E. coli culture on top of P. tricornutum cells in the middle of the well.

Mix the E. coli culture very well with a pipette prior to dispensing in well.

Spread E. coli in an orbital motion (refer to video) for each well (do not wait until all 6 wells have E. coli to spread, spread after every dispensing)

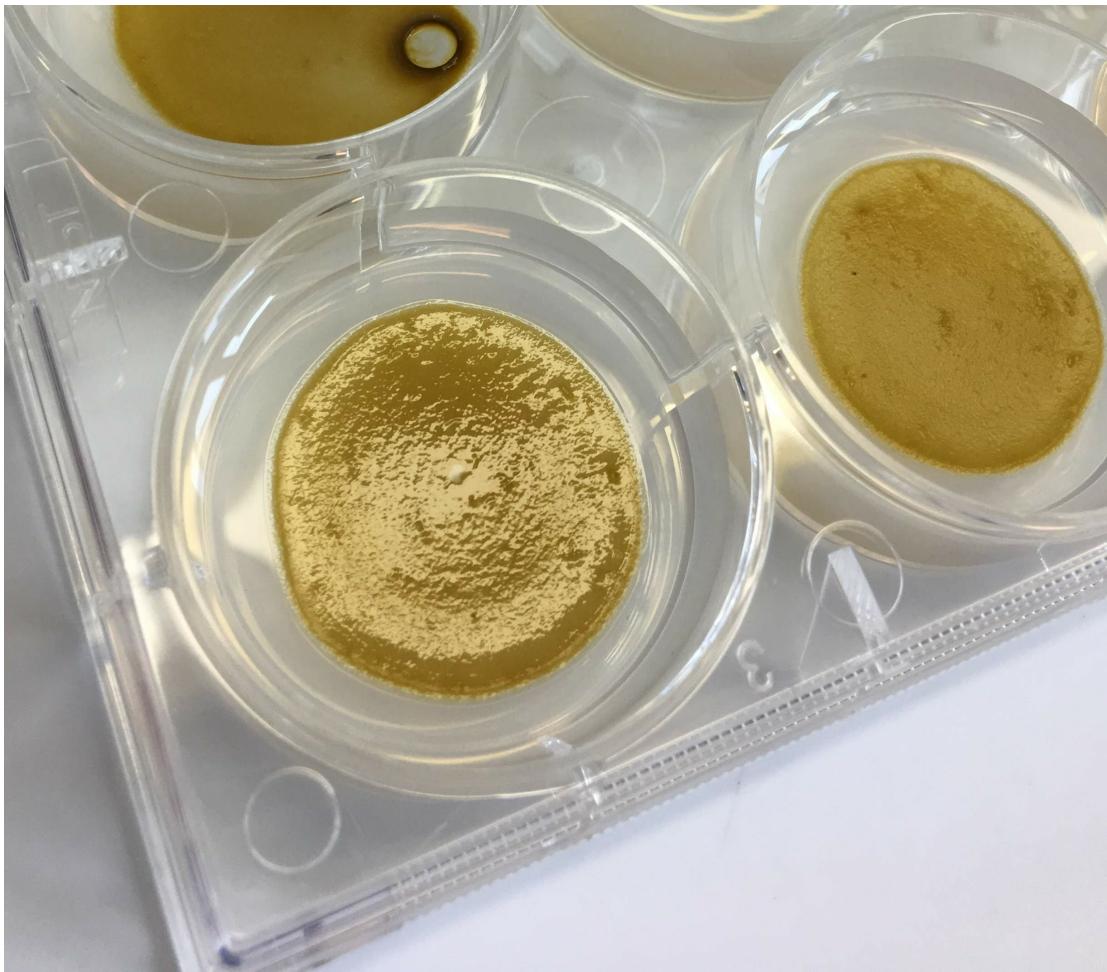
10 Dry E. coli on top of P. tricornutum cells (10-20 minutes)



One well (bottom right) with wet E. coli dispensed and spread



Almost dry well....



Completely dry well with E. coli on top of P. tricornutum

Conjugation Transformation

11 The conjugation mechanistically operates when the E. coli are exposed to an optimal growth temperature and for a certain duration.

P. tricornutum is most competent to conjugation at **30 °C 01:30:00**

11.1 After all E. coli have dried, wrap each plate with breath-easy tape

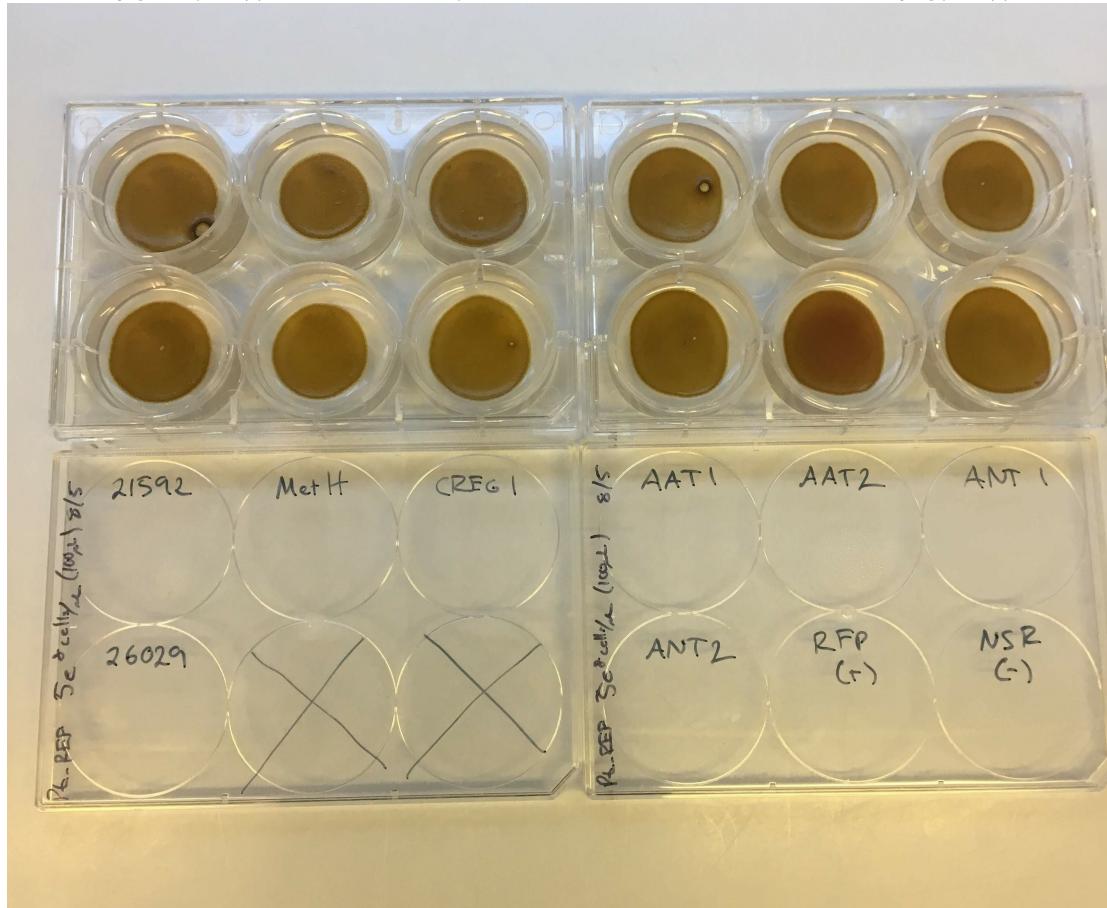
11.2 Incubate 6-well plate at **30 °C 01:30:00** and in the dark

11.3 After incubation, place conjugation plates at **18 °C [M]50 uE m-2** for **48:00:00**

Scrape *Phaeodactylum* cells off conjugation plates

12 After 48 hrs, collect *P. tricornutum* cells in 2 mL tubes

Remove conjugation plate(s) from incubation and put them under a PCR hood, take off lid to start drying plate(s)



Remove lids in PCR hood to start drying cells. The Cas9 negative control (RFP) conjugation still has a red color

12.1 Obtain ASW media supplemented with both NO₃ and NH₄

Prepare 2mL tubes to collect scraped cells

I recommend using a hockey stick styled cell scraper. It allows more control of scraping in a small well area



Pictured are all materials required for cell scraping: P1000, filtered tips, NH4 ASW media, 2mL tubes (1 for each conjugation) and conjugation plate with cells ready for scraping

12.2 For each well...

Pipette **500 µl** media and gently scrape off cells with a hockey-stick shaped cell spreader

***** DO NOT USE A LOOP *****

Collect cells with a P1000 pipette

Repeat once

12.3 Mix collected cells very well with pipette

Place tubes at $\Delta 18\text{ }^{\circ}\text{C}$ [W]50 $\mu\text{E m}^{-2}$ for $\text{O}01:00:00$



2mL tubes with scraped cells are incubated at 18C, under irradiant white light (50 $\mu\text{E/m}^2$) and shaking on an orbital shaker. This allows any adherent cells that formed chunks to separate and homogenize prior to selection

Select transformed *Phaeodactylum tricornutum* cells

13 During 1 hr incubation, dry *P. tricornutum* selection plates

13.1 Selection Plates:

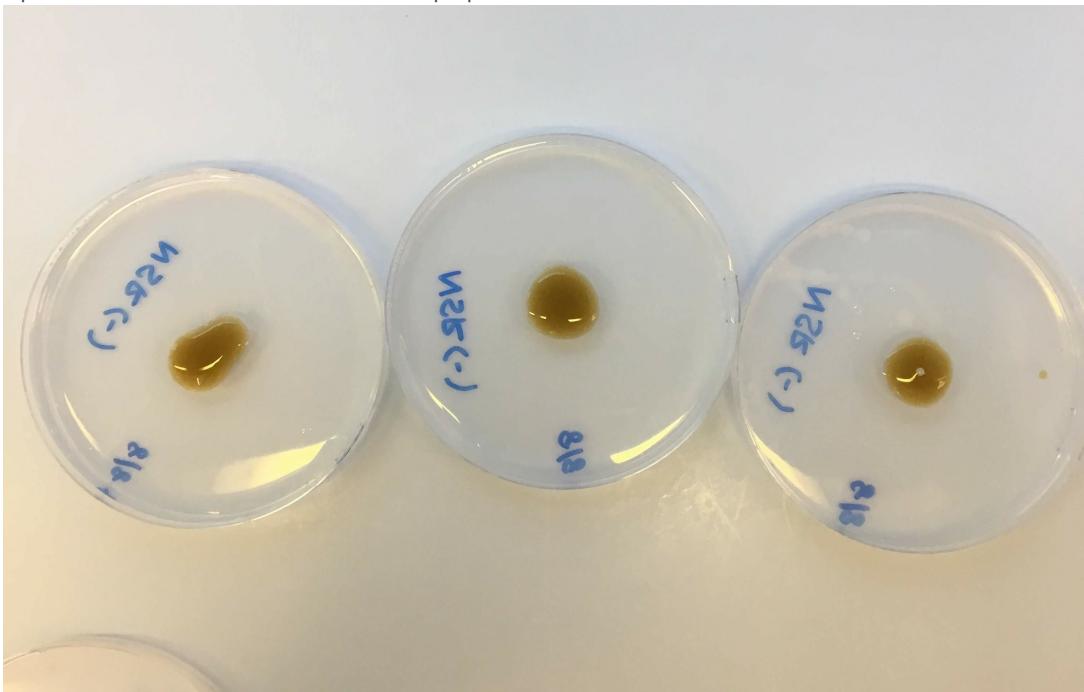
For each conjugation reaction, prepare 3 selection plates (100mm x 15mm culture plates) with 35 mL media each

Ex: Prepare **650 ml** of plate selection media (enough for 1 6-well conjugation plate, 18 plates)

1. Autoclave 325 mL 1.6% agarose
2. Incubate at $\Delta 50\text{ }^{\circ}\text{C}$ after sterilization
3. Incubate **325 ml** ASW supplemented with **163 μl** NaNO_3 and **163 μl** NH_4Cl , also at $\Delta 50\text{ }^{\circ}\text{C}$
4. Thaw Phleomycin stock (100mg/mL) and chloramphenicol (20 mg/mL)
5. Pour agarose into ASW flask and mix
6. Add **325 μl** Phleomycin (50 $\mu\text{g/mL}$ final concentration) and **325 μl** Chloramphenicol (10 $\mu\text{g/mL}$ final concentration) when temperature of ASW/agarose falls below $\Delta 50\text{ }^{\circ}\text{C}$
7. Pipette **35 ml** selection media into each culture plate
8. Dry under PCR hood for $\text{O}00:20:00$
9. Store at $\Delta 4\text{ }^{\circ}\text{C}$ until needed

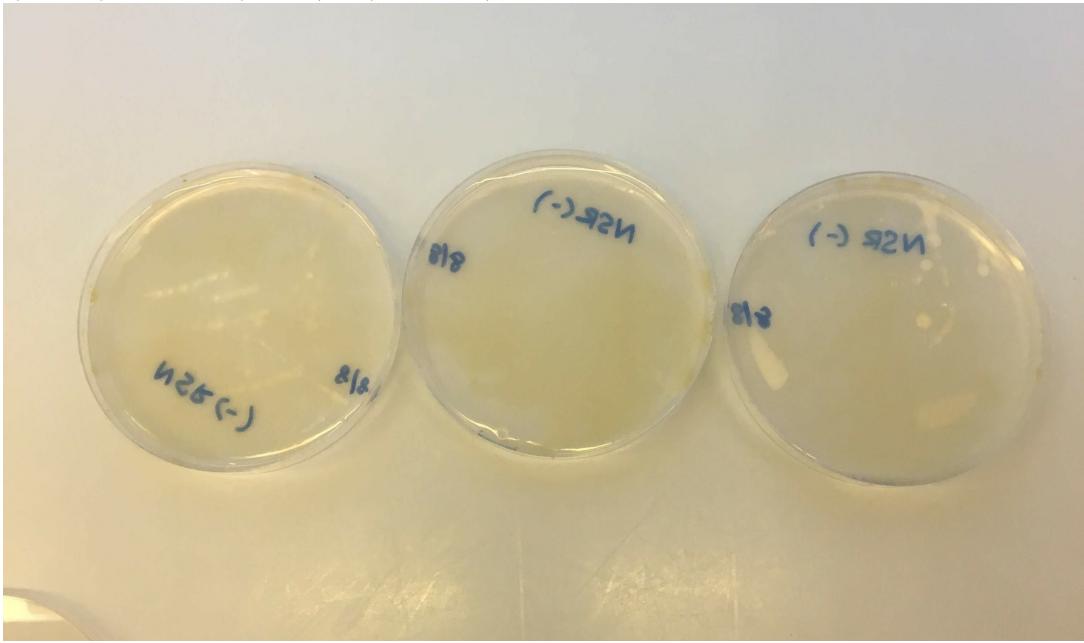
13.2 Dry selection plates under a PCR hood for at least 00:20:00

13.3 Pipette 300 uL of collected *P. tricornutum* cells per plates



cells on selection plate (300uL, 300uL, ~200uL (remaining volume))

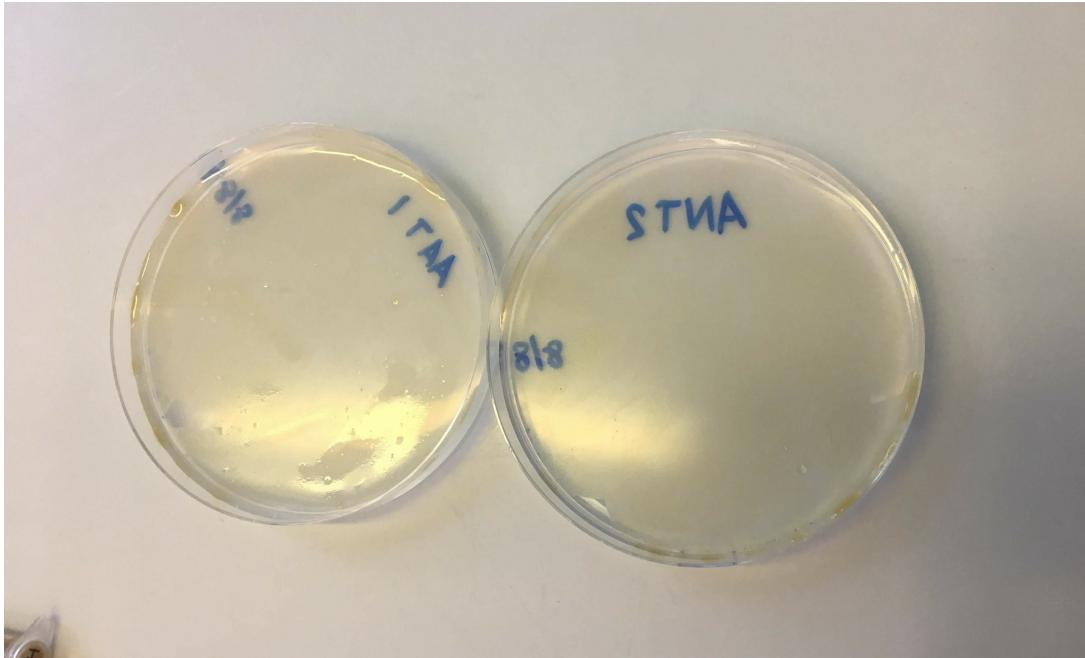
13.4 Spread on plate with cell spreader (or loop is fine here)



cells spread on selection plate

13.5

Dry cells on selection plates and seal plates with breath-easy tape



cells ought to completely dry on selection before sealing. Pictured are two plates. I use reflection of near-by lighting to determine dryness. The left plate is almost dry with patches of wet (lower right corner of plate) and the right plate is completely dry (less reflective than wet area)

14

Incubate selected *P. tricornutum* at 18°C [M]50 $\mu\text{E m}^{-2}$ for at least 10 days or until dark circular colonies begin forming.



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