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CRISPR-Cas9 mutagenesis in Phaeodactylum tricornutum, particle bombardment

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Mark Moosburner[1]

[1]Scripps Institution of Oceanography

JCVI West Protocols

Mark Moosburner
Scripps Institution of Oceanography, J. Craig Venter Institu...

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We use this protocol and it's working

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Abstract

Particle bombardment mediated transformation of CRISPR-Cas9 components to the diatom Phaeodactylum tricornutum

Materials

MATERIALS

- Ultra Pure Carrier 5.5 Grade Helium, Size 300 Cylinder, CGA-580 **Airgas Catalog #**HE UPC300
- Rupture Disks **Bio-Rad Laboratories Catalog #**1652330
- PDS-1000/He Hepta System **Bio-Rad Laboratories Catalog #**1652258
- Macrocarriers **Bio-Rad Laboratories Catalog #**1652335
- Tungsten M-10 Microcarriers **Bio-Rad Laboratories Catalog #**1652266

Phaeodactylum cell preparation

1 Plate wild-type Phaeodactylum in preparation for particle bombardment

1.1 Calculate the cell concentration of a culture of Phaeodactylum tricornutum that is grown axenically.

Calculate the volume required for 1×10^8 cells total.

Pellet at 2000 x g for 20 minutes at 17^{o}C.

1.2 Dry growth agar plate under a PCR hood while cells are pelleting

Agar Plate = 15mL 1.6% agarose + 15mL NO_3-ASW (artificial sea water)

1.3 Remove media from pellet and resuspend in 200uL ASW

Spread on a plate evenly

Allow cells to completely dry on plate while under PCR hood

Seal plate w/ breath-easy tape

1.4 Grow cells on plate for 4 days

Cas9 and sgRNA plasmid preparations

2 Prepare plasmids for particle bombardment

*** start 2 days prior to bombardment ***

2.1 2 days prior to bombardment....

Obtain sequence verified expression plasmids for Cas9 and sgRNA from frozen bacterial stocks.

Grow up 15 mL of each plasmid OVN

2.2 Extract and purify all plasmid the next day by performing 3 5mL mini-preps for each 15mL culture.

*** at the end of all mini-preps, elute the 3 columns pertaining to one plasmid into a single collection epitube ***

2.3 Measure the plasmid concentration

Calculate the volume required for 8-ug plasmid DNA for each plasmid

Particle Bombardment

3 Prepare plasmids

3.1 Pipette 8ug of each plasmid into one 1.5mL epitube.

Use a speed-vac to concentrate tdNA to a total of 5-10uL

4 Prepare particle bombardment materials

- Turn on helium tank to pressure = 200psi
- Obtain vacuum pump and set up with PDS-1000
- Re-suspend tungsten beads in 50% glycerol (vortex to mix)
- Obtain a plat-form vortex
- Prepare 2.5M $CaCl_2$
- Prepare 0.1M Spermidine
- Prepare 70% EtOH
- Obtain 100% EtOH
- Obtain Isopropanol
- 1 rupture disk
- 7 macrocarrier disks

- Place Phaeodactylum plate in PCR with the PDS-1000 system to allow the plate to dry before bombardment

4.1 Mix tungsten bead by vortex

Pipette 50uL beads into 1.5 epitube.

Platform vortex for 5 minutes

During the 5 minutes, sterilize the 7 macrocarrier disks.

- pour 70% EtOH into a petri dish
- wash each disk using tweezers by shaking the disk in the 70% EtOH
- dry on other half of petri dish

4.2 After 5 min on plate vortex...

Pipette DNA into tungsten beach

Pipette 50uL CaCl2 (2.5M), vortex

Pipette 20uL Spermidine (0.1M) to hybridize DNA to beads

Mix by flicking tube with finger MANY TIMES.

Plate vortex for 3 minutes

4.3 During 3 minutes....

Place 7 dried macrocarrier disks into the carrier disk manifold. Ensure disks are secured under the lip of each macrocarrier position.

4.4 After 3 minutes....

Pellet tungsten beads in a table-top centrifuge (fixed rotor)

Centrifuge at 8000 rpm for 10 seconds (beads will pellet very easily)

Remove supernatent

Resuspend in 140uL 100% EtOH, **MIX THOROUGHLY** by pipette

Repeat pelleting and resuspension 2X

On last resuspension, add only 100uL 100% EtOH and **MIX THOROUGHLY**

MAINTAIN HOMOGENY BY FLICKING TUBE

4.5 Pipette 10uL of beads onto each macrocarrier disk

Allow beads to dry completely on disks

4.6 While drying....

Briefly wash rupture disk in isopropanol

Insert "wet" rupture disk into rupture manifold and screw manifold into the bombardment system

Secure the metal mesh screen between the two plates of the carrier disk manifold.

Insert manifold into square metal fitting and insert into the appropriate slot of the bombardment system.

(The rupture manifold should be suspended directly above the carrier disk manifold and aligned with the 7 holes)

4.7 Place the Phaeodactylum plate below and in line with the carrier disk manifold

4.8 Shut door to the bombardment system

Turn on the vacuum manifold. Allow the vacuum pressure to build to 25 inch Hg and hold.

Press and hold the "FIRE" button. The pressure will build until ~1300 psi when the rupture disk ruptures. There will be a loud bang! Once the loud bang occurs, "VENT" the bombardment system to remove the vacuum pressure.

4.9 Place the lid on the Phaeodactylum plate

Wrap the plate in tin foil

Incubate the plate at 18^{o}C for 48hrs

Select for transformed Phaeodactylum cells

5 After 48hrs in the dark...

Scrape cells off plate and resuspend in a 2mL epitube with ASW.

5.1 Prepare selection plates

Each plate: 15mL 1.6% agarose + 15mL ASW (w/ appropriate N-substrate) + Phleomycin antibiotic (20mg/mL)

Dry plates under PCR hood before plating cells

5.2 For each transformation, dry 3 selection plates.

Plate all cells on the 3 plates and spread (maximum 400uL per plate)

Allow cells to completely dry under PCR hood.

*** Phleomycin resistant colonies appear after 3-4 weeks***