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« Protocols for GMO production in novel emerging metazoan, macroalgal and microalgal model organisms available via ASSEMBLE Plus web portal".

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GENERAL DATA

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Participants (names & institutions)	X. Bailly; M. Cock; L. Garczarek, SBR F. Ristoratore; M. Ferrante, SZN H. Escriva; S. Darras; F.Y. Bouget; R. Lamy, OOB D. Ferrier, SOI H. Yasuo, OOV C. Gachon, SAMS C. Brownlee, MBA W. Vyverman, Ghent

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Websites, patent filling, etc.	☐
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Public	☐
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Author(s)	Hector Escriva
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Abstract

The general objectives of this WP:

- **to implement/adapt specific protocols for generation of genetic resources for a panel of emerging/prospective marine model organisms;**
- **to generate a reference set of carefully phenotyped or genotyped genetic resources of different marine organisms ranging from bacteria to metazoans;**
- **to produce and provide access to the phenotypic or genotypic data necessary for the functional description of the genetic resources.**

During these years of the project the different participants in this WP have developed the various activities and experimental approaches that were detailed in the project. Depending on the model, the results of this period are at more or less advanced stages of development, but overall, we can estimate that this WP is progressing correctly and numerous results are expected for the end of the project and for each model studied. Particularly, concerning this deliverable, we have developed or adapted protocols for the deployment of CRISPR/Cas9 system in the different biological models used in this JRA3. This process of genomic modifications is essential if we want generate GMOs for the different biological models.

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1. Introduction

In this deliverable we present a collection of protocols developed by the different partners of JRA3 for the deployment of CRISPR/Cas9 system in model organisms available via ASSEMBLE Plus web portal:

Task1, Functional genomics in marine metazoans

Task 2, Functional genomics in macroalgae

Task 3, Functional genomics in microorganisms

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2. Objective

The objectives of the JRA3 are:

1- To implement/adapt specific protocols for generation of genetic resources for a panel of emerging/prospective marine model organisms
2- To generate a reference set of carefully phenotyped or genotyped genetic resources of different marine organisms ranging from bacteria to metazoans
3- To produce and provide access to the phenotypic or genotypic data necessary for the functional description of the genetic resources.

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3. Protocols for GMO production in novel emerging metazoan, macroalgal and microalgal model organisms available via ASSEMBLE Plus web portal

3.1 CRISPR/Cas9-mediated gene knockout protocol for the ascidian *Phallusia mammillata*

By Céline Hebras (OOV), Rémi Dumollard (OOV), Isa Gomes (OOV) and Alex McDougall (OOV)

1. To prepare the sgRNA we synthesize crRNA and purchase tracrRNA (from IDT: https://eu.idtdna.com/pages). We use the CRISPOR website (http://crispor.tefor.net/; see Note below) to select the DNA sequence from the uploaded *Phallusia mammillata* genome to create the crRNA. This database also indicates the potential offsite targets thus allowing the selection of the most specific sequences. We coinjected the crRNA with tracrRNA (IDT, ref. 1072532) together with Cas9 protein.

2. Cas9 protein is purchased from IDT (ref. 1081058). Dilute 1µl Cas9 protein in 3µl buffer (20 mM Hepes, 150 mM KCl, 5 mM CHAPS, pH7.5, 10 µM final concentration of Cas9). We also synthesized mRNA encoding Cas9 with nuclear localization signals on both N-term and C-term regions flanking the Cas9 coding sequence (a gift from L. Christiaen, NYU, USA). We sub-cloned the entire NLS-Cas9-NLS sequence into pSPE3 to facilitate mRNA production (we used 8 µg/µl final mRNA concentration when mixed with crRNA and tracrRNA) using the mMessage mMachine T3 transcription kit (Ambion).

3. Mix Cas9 protein (10 µM final concentration) or Cas9 mRNA (8 µg/µl final concentration) with tracrRNA/crRNA (13 µM final concentration for both), microinject into unfertilized dechorionated eggs and leave for at least 1 hour for Cas9 protein mixture or 4 hours for Cas9 mRNA mixture before fertilization.

4. Fertilize eggs were left to develop to the tadpole stage at 19°C. Phenotype success rate was scored visually with a binocular microscope. For the *Brachyury* gene, one crRNA out of three gave embryos with short tails (4/6 and 3/4 with the Cas9 protein mixture, or 3/10 and 6/14 with the Cas9 mRNA mixture). For the *tyrosinase* gene, three crRNAs were tested, of which one did not give any phenotype (0/16 embryos) while the other two gave 5/17 and 11/13 embryos with no pigmentation.

5. Transfer one tadpole per Eppendorf and perform PCR (Phusion, ThermoScientific) using primers designed to flank the region targeted by the crRNAs. Briefly, to prepare the CRISPR-injected eggs for PCR we fertilized the eggs and once the embryos reached the tadpole stage (circa 12 hours post fertilization) individual tadpoles were transferred to PCR extraction solution: one tadpole was transferred to an Eppendorf tube containing 50µl Quick DNA Extraction Solution (ref. QE09050, Illumina in approximately 2 to 3µl seawater using a hand-drawn glass micropipette. To fully lyse all cells in Extraction Solution, vortex for 15 secs. and incubate for 2 min. at 98°C then store at -20°C until PCR is performed.

6. Perform PCR using 5 µl of the sample prepared in step 5 using a high-fidelity PCR enzyme and

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sequence product.

Note:
Step 1. Using CRISPOR, copy and paste gene exon sequence using the following instructions (use <2000bp).
Step 2. Select genome (here, *Phallusia mammillata* – white sea squirt – ANISEED MTP 2014).
Step 3. Select "20bp-NGG-Sp Cas9, SpCas9-HF1, eSpCas9 1.1" and Submit.
On results page, click "Exons Only" icon (if desired) to identify Off-targets for 0-1-2-3-4 mismatches. Select strand in first column that has zero or the fewest off-targets.

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3.2 CRISPR/Cas9-mediated gene knockout protocol for the amphioxus *Branchiostoma lanceolatum*

By Stéphanie Bertrand and Hector Escriva, Observatoire Océanologique de Banyuls sur Mer, UMR7232, BIOM

3.2.1 Design and selection of sgRNA:

- Use EuPaGDT online software to find the oligonucleotides required for customized sgRNAs (http://grna.ctegd.uga.edu/).
- Identify the best sgRNA (no off-targets, canonical GG18 if it is possible, best if the 3' region is GC rich)

3.2.2 PCR sgRNA generation

- Oligo sgRNA design

  Example of specific primer design:

  5’TAATACGACTCACTATAGGGGAAGGTTGATTATGCACGTTTTAGAGCTAGAA3’

  T7 promoter is in red.

  CRISPR Binding site in yellow (target site excluding PAM sequence).

  Annealing sequence to the Universal primer in blue.

  Universal primer sequence:
  5’AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC3’

- PCR Reaction mix

Primer Universal:	2µL from 10 µM stock (HPLC quality primer)
Primer Specific:	2µL from 10 µM stock (HPLC quality primer)
Q5 High Fidelity (NEB) buffer 5X	10µL
Q5 High Fidelity Taq pol.	0,5 µL
dNTP 25mM mix	0,5 µL
Nuclease free H20	35µL

- PCR conditions:
  98 °C, 2 min

  30 cycles:

  98 °C, 30 s

  45 °C, 30 s

  72 °C, 30 s

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final step:

72 °C, 5 min

- Purification:
  Use the Promega Wizard SV gel and PCR purification Kit

  Elute in 30µL

  Around 100ng/µL of DNA should be obtained

  Test 1µL of the PCR product (117bp) on a 2% agarose gel

3.2.3 In Vitro Transcription

- Use the T7 flash kit (Epicentre) and prepare the following mix:

  0,9 µl rATP (100 mM)

  0,9 µl rGTP (100 mM)

  0,9 µl rCTP (100 mM)

  0,9 µl rUTP (100 mM)

  1 µl buffer 10X (at RT before adding to the reaction)

  1 µl Enzyme mix

  1µl DTT 100 mM

  0,25 Inh. RNAse

  3,15 µl template PCR

- Incubate at 37°C 6h at least (ideally over night for getting µg of sgRNA).
- Add 0,5 µL DNAse turbo, incubate at 37°C 20 min.
- Add 90 µL RNAse free water.
- Precipitate RNA using 10 µL of sodium acetate 3M and 300 µL EtOH 100%.
- Incubate over night at -20°C or 1-2h at -80 °C.
- Precipitate at max speed (4°C centrifuge) 30 min.
- Wash 2 times with EtOH 70%.
- Resuspend in 50 µL (normally 1-2 µg/µL can be obtained).
- Measure the concentration and load 1µL on a 2% agarose gel.

3.2.4 Egg injection

- Prepare the following mix in 2.5µl:
  sgRNA1 (2µg/µl) + sgRNA2 (2µg/µl) + Cas9 (protein at 5µg/µl) ; the sgRNA and Cas9 are equimolar

- Keep 10 min on ice
- Add 2.5µl of the following mix:
  mCherry mRNA (800 ng/µl) + Glicerol (30%) + Fast green (0.2 mg/mL)

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- Inject the eggs as detailed in the injection protocol. (See deliverable D JRA3.1) and fix the embryos at the desired developmental stage.

3.2.5 PCR on embryos to check for mutations

- Design primers on genomic sequence at around 200bp from the targeted site
- Mix one embryo in a PCR tube with:

dNTP	25mM	1µL
primer Forward (5') 10µM		1µL
primer Reverse (5') 10µM		1µL
Buffer GoTaq2 5X		10µL
MgCl2		4µL
GoTaq2		0,25µL
H20		qsp 50µL

- Use the following program:

94°C	5min	
40 cycles:		
	94°C	30s
	50°C	30s
	72°C	45s
final step:		
	72°C	5min

- PCR products can be sent for Sanger sequencing using the designed primers or cloned using a TA system.

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3.3 Protocol for Transgenesis in Amphioxus

By Lydvina Meister Blanco, Stéphanie Bertrand and Hector Escriva, Observatoire Océanologique de Banyuls sur Mer, UMR7232, BIOM

3.3.1 Material List

- Poly-D-lysine hydrobromide, 5mg, P6407-5MG, Sigma-Aldrich
- Thin-Wall Glass Capillaries, 4 in, OD 1.0 mm, Filament (TW100F-4), WPInstruments
- Next Generation Micropipette Puller P-1000, Sutter Instrument
- Wide Zoom Stereo Microscope - SZX16 - Olympus
- mMESSAGE mMACHINE™ SP6 Transcription Kit – catalog number : AM1340
- Tissue culture dish Easy Grip - A Corning Brand – Ref 353001
- Fast Green FCF – F7232-5G – Sigma Aldrich
- Eppendorf Microloader Tips in racks, Original Eppendorf
- Picospritzer 2 canals - 0 à 100 PSI
- Petri dish 60x15mm with cams – Ref 82.1194.500 - Sarstedt

3.3.2 Preparation of Instruments and Reagents

Poly-D-lysine coated plates

- Prepare 0,125mg/ml poly-D-lysine by adding 40ml of distilled water to the 5g poly-D-lysine.
- Store 30 ml in 10mL aliquots at -20 °C for further coated plates.
- Use the 10ml aliquot immediately and only once to ensure reproducible and robust adhesion of the oocytes to the poly-lysine-coated dish.
- For each 35 mm cell-culture Petri dish, cover the bottom of the Petri dish with 1 ml of the thawed 0.125 mg/ml poly-lysine solution. Incubate at room temperature for 8 min.
- For each 35 mm cell-culture Petri dish, transfer the 0.125 mg/ml poly-lysine solution into another 35 mm cell-culture Petri dish. Incubate at room temperature for 8 min.
- Discard the 0.125 mg/ml poly-lysine solution.
- Let the Petri dishes dry, upside-down at room temperature for 2 hr.
- Store the poly-lysine-coated dish at 4 °C.

Injection needles

- If capillaries are pulled on the type of heating-filament needle puller described in the Materials List, use the following settings: Heat 457, Pull 120, Velocity 50, Time 50, Pressure 300.

Tol II mRNA & mCherry synthesis (Prepare fresh)

- Linearize 5 µg of DNA of interest with the adequate enzyme (usually for 2 hr, at 37 °C). The TolII is inserted in the PCS-TP plasmid. The mCherry is inserted into the pCS2+ plasmid.
- Precipitate the linearized plasmid with 1/10 3M Sodium Acetate and 3/1 of Absolute Ethanol during 20 min a -80°C.
- Centrifuge 20 min at 13,000 x g at 4 °C. Rinse in 70% cold ethanol.
- Centrifuge 10 min at 13,000 x g at 4 °C. Let dry and resuspend in RNase-free water in 10µL.

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- To check the completeness of the digestion, run 1µL of the final volume of the digestion on a 0,8% agarose-TBE gel in TBE buffer at 100 W for 22 min.
- Transcribe 0.5 µg of linearized DNA using an mRNA synthesis kit with the appropriate polymerase (SP6), according to manufacturer's instructions.
- Add 1µL of DNAse TURBO, mix well and incubate for 15min at 37°C.
- Stop the reaction and precipitate the RNA by adding 30 µL Nuclease-free Water and 30 µL of LiCl.
- Mix thoroughly. Chill for ≥30 min at –20°C.
- Centrifuge at 4°C for 15 min at maximum speed to pellet the RNA.
- Carefully remove the supernatant. Wash the pellet once with ~1mL 70% ethanol, and re-centrifuge.
- Carefully remove the 70% ethanol, and resuspend the RNA in 10µL of nuclease free water.
- To check the quality of the synthesis, run 0.2µL of the final volume of the synthesis on a 0,8% agarose-TBE gel in TBE buffer at 100 W for 14 min.
- Determine the RNA concentration and store 2 µl aliquots at -80 °C.

Plasmid linearisation

- Linearize 4 µg of DNA of interest (usually for 1 hr, at 37 °C) with about 30-50U of linearizing enzyme. All the enhancers are inserted in pT2-Min Prom Beta actin – EGFP
- Precipitate the linearized plasmid with 1/10 3M Sodium Acetate and 2/1 of Absolute Ethanol during 20 min a -80°C or overnight at -20°C.
- Centrifuge 20 min at 13,000 x g at 4 °C. Change for 70% cold ethanol.
- Centrifuge 10 min at 13,000 x g at 4 °C. Let dry and resuspend in RNase-free water in 10µL.
- To check the completeness of the digestion, run 1µL of the final volume of the digestion on a 0,8% agarose-TBE gel in TBE buffer at 100 W for 22 min.

Injection mix and loading of the injection needles

- Just before starting the injections, make a 3 µl injection mix in RNase- and DNase-free water with final concentrations of 100 ng/µL of plasmid linearized, 100 ng/µL of TolII, 250ng/µL of mCherry, 15% glycerol, 15% Fast Green.
- NOTE: The Fast Green colors the solution, which allows monitoring of the injection efficiency and the identification of successfully injected embryos. Glycerol favors mRNA diffusion within the oocyte.
- Centrifuge 4 min at 18,000 x g to pellet crystals. Keep on ice until use.
- With a 10 µl microloader, collect 0.3 µl of the injection mix, avoiding the bottom of the tube, where the crystals have been pelleted and fill the injection needle. Let the injection mix slowly travel to the tip of the injection needle during 5min in order to reach the tip of the micropipette.

Preparation of oocytes for injection

- Deposit the oocytes along a vertical line on the polylysine-coated dish containing filtered sea water to carry out injections.

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- Leave the poly-D-lysine coated plates for 5 minutes without touching in order to let the eggs stick properly to the plate.
- Inject small numbers (30 oocytes) to minimize the exposure time of oocytes to poly-lysine, which tends to deform developing embryos (data not shown). Follow the injection protocol in Deliverable D JRA3.1.

3.3.3 Fertilization, selection of injected embryos and embryo culture

- Fertilize the oocytes as soon as a series has been injected. Add 1-5 drops of sperm to the oocytes and swirl the dish to detach the embryos from the polylysine-coated dish. NOTE: The fertilization envelope should become apparent on the embryos after about 1 min.
- Transfer the embryos fertilized by spilling the volume contained into the coated dish into a scraped Petri dish. Remove the embryos from the polylysine-coated dish as soon as possible. To make sure that no embryos are left in the box, perform this step twice with filtrated sea water.
- NOTE: In case of prolonged exposure to poly-lysine, the embryos tend to warp.
- At the gastrula stage (around 12hours), select with a fluorescent dissecting scope with DSR filter the successfully-injected embryos, i.e., those with a normal morphology that exhibit a red fluorescent signal (embryos expressing the mCherry injected).
- Keep the embryos in culture in filtered sea water at 19 °C until the desired stage for in vivo imaging.

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3.4 CRISPR/Cas9-mediated gene knockout protocol for *Clytia hemisphaerica*

By Tsuyoshi Momose (OOV), Julie Uveira (OOV)

TIMELINE

1 (1 week)
- guide RNA design (evalaton of microhomology and off-target)
- Genotyping PCR primer design
- sgRNA synthesis

2 (< 1 week)
- Test injection (100~ embryos/gRNA)
- Extract genomic DNA from 3 day old planulae and PCR (wild type control needed)
- Efficiency test by TIDE analysis

3 (< 1 week)
- Injection of 1~2 selected sgRNAs. 250~1000 injection/target to get several slides for both male and female (1 hour)
- Metamorphosis (3days after injection)

4 (1~2 months)
- Colony growth and maintenance

5
- Check phenotype (if possible).
- PCR genotyping
- TIDE analysis

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3.4.1 sgRNA from design to preparation

Making sgRNA

1) Linearize cloned sgRNA plasmid
    a. Cut 5 µg of the plasmid with DraI in 1x FastDigest Buffer (200 µL scale)
        i. 20 µL FD buffer (10x)
        ii. H2O
        iii. Plasmid DNA
        iv. 2 µL DraI
    b. Check with gel electrophoresis. 1.8 kbp and 0.3kbp band should be observed.
    c. Purify by phenol/chloroform and chloroform extractions
        i. Add equal volume of phenol chloroform, vortex and centrifuge (14000 rpm for 1 min)
        ii. Take upper layer and add equal volume of chloroform, vortex and centrifuge (14000 rpm for 1 min)
        iii. Take upper layer
    d. Ethanol precipitation
        i. Add x1/10 volume of NaOAc (20 µl)
        ii. Add 2.5 volume of Ethanol (500 µl)
        iii. Incubate for 1 hour at -20°C
        iv. Centrifuge for 15 min at maximum speed.
        v. Remove supernatant and add 200 µl of 70% ethanol
        vi. Centrifuge for 2 min at maximum speed
        vii. Remove supernatant completely
        viii. Dry (5~10 minutes)
    e. Dissolve into H2O to 0.5 µg/µl concentration
2) RNA synthesis
    a. Use MEGAshortscript T7 kit (AM1354M Ambion/ThermoFisher) for sgRNA synthesis. Make following mixture:

10x reaction buffer	2 µl
H2O	8-x µl
ATP solution (75 mM)	2 µl
CTP solution (75 mM)	2 µl
GTP solution (75 mM)	2 µl
UTP solution (75 mM)	2 µl
Linearized pDR274 construct	1~2 µg (x µl < 8 µl)
T7 enzyme mix	2 µl

    b. Incubate 3~4 hours at 37°C
    c. Add 1 µl Turbo DNase and incubate for 15 min at 37°C
    d. Add 115 µl H20 and 15 µl NH4Ac stop solution (in the kit) you can stop here (-20°C)
3) Purification
    a. Phenol extraction and chloroform extraction

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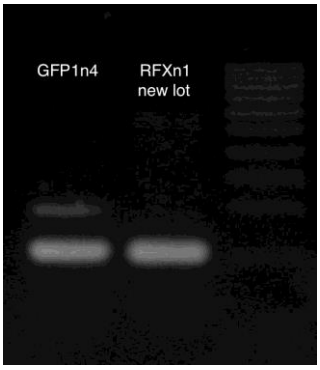
i. Add equal volume of phenol chloroform (150 µL), vortex and centrifuge (14000 rpm for 1 min)
ii. Take upper layer and add equal volume of chloroform (150 µL), vortex and centrifuge (14000 rpm for 1 min)
iii. Take upper layer

b. Ethanol precipitation and rinse with 70% ethanol
   i. Add 2.5 volume of Ethanol (370 µl)
   ii. Incubate for 1 hour at -20°C :you can stop here (-20°C)
   iii. Centrifuge for 15 min at maximum speed.
   iv. Remove supernatant and add 200 µl of 70% ethanol
   v. Centrifuge for 2 min at maximum speed
   vi. Remove supernatant completely
   vii. Dry (5~10 minutes)
c. Dissolve into 50 µl H2O
d. Further purify with probe quant G50
e. Take 1 µl to measure OD260 and estimate yield
f. Ethanol precipitation (using NaOAc pH 4.5) followed by rise with 70% ethanol
   i. Add x1/10 volume of NaOAc (5 µl)
   ii. Add 2.5 volume of Ethanol (125 µl)
   iii. Incubate for 1 hour at -20°C: you can stop here (-20°C)
   iv. Centrifuge for 15 min at maximum speed.
   v. Remove supernatant and add 100 µl of 70% ethanol
   vi. Centrifuge for 2 min at maximum speed
   vii. Remove supernatant completely
g. Air dry max. 5 min. (Avoid over-dry that makes hard to dissolve RNA precipitation.)
h. Dissolve into H2O at 80 µM (=2.8 µg/µl)

4) Check on the gel
   a. Do electrophoresis. Usually you will see a band at 100 bp with “normal” agarose gel. It seems not a big problem to have dimer band at 200 bp.



3.4.2 Preparing IDT crRNA/tracrRNA

Dissolve crRNA and tracrRNA at 200 µM (200 pmol/µl) into 1x Duplex buffer provided by IDT

Mix /

crRNA (200 µM)	3 µl
tracrRNA (200 µM)	3 µl
1x Duplex buf	4 µl

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Denature@95°C for 5 min then cool down to 25°C at -0.1°C/sec

(Folder TSUYO, PROGRAM ANNEAL)

Store crRNA/tracrRNA (60 µM) at -20°C.

3.4.3 Injection

About 30 min before spawning prepare:

Cool down centrifuge to 12°C

Mix

10 µM Cas9 2 µl

60~80 µM guide RNA 0.5 µl (x1.2~2 molecules of Cas9)

Incubate for 10 min at RT

Centrifuge for 10 min at 12°C~

Inject

3.4.4 Genotyping

Design Primer

PCR product size should be 500~800 bp. It is OK even if longer than that as long as PCR works. The target must be > 200 bp away from the both sides. (100 bp away in the worst case). 24~27 bp range is recommended for the primer length for *Clytia* due to the low GC contents. (Tm 63°C using Primer3 program on the web or Geneious Pro.)

Primer pair should be validated in advance to make sure that PCR works well using genomic DNA extracted from 2~3 polyps as a starting material (see below for the protocol). Most primer works well genomic DNA extracted from 500 planulae but some does not with that from 2~3 polyps.

There should be no indel polymorphisms between sequence primer and the target. (Clean sequencing result if one of the two PCR primer is used for direct sequencing of PCR product).

Confirm single band PCR product, or you need to cut individual bands for genotyping.

More detail see

https://www.deskgen.com/landing/tide.html

3.4.5 Genome DNA extraction

Use Qiagen DNeasy tissue and blood kit and follow the attached protocol with following additional recommendations.

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- Start from 2~3 polyp (or 50~100 planula larvae in case of sgRNA evaluation). Put them into an Eppendorf tube.
- Remove sea water as much as possible. For swimming planulae, place the on ice and wait for a few minutes. Centrifuge 2~3 seconds using table top toy centrifuge then remove sea water carefully under stereomicroscope. (Freeze sample here if necessary.)
- Freeze sample here if necessary
- Incubation (after adding buffer ATL and proteinase K) is for 3~6 hours at 60°C
- Elution is only once with 200 µl distilled water.

3.4.6 PCR

Use Phusion DNA polymerase (Thermo Scientifique M0530S/L, also from NEB)

Genomic DNA solution	12 µl
5x HF buffer	6 µl
Primer (10 µM)	0.6 µl each
dNTP (10 mM each)	0.6 µl
H2O	9.9 µl
Phusion DNA polymerase	0.3 µl
Total 30 µl	

Consider making premix for everything except for genomic DNA solution.

98°C 2 min, (98°C 10 sec, 63°C 20 sec, 72°C 30 sec/kb, minimum 30 sec) x 40 cycles, 72°C 10 min. store at 4°C.

3.4.7 Post-PCR process

1. Take 5 µl and do electrophoresis to check PCR.
2. Purify the remaining PCR product by "Wizard® SV Gel and PCR Clean-Up System" (**A9281** or **A9282**). Follow the manufacturer's protocol except for the elution with 30 µl distilled water instead of 50 µl.
3. Repeat necessary if amount of PCR products is not enough for sequencing. If there are multiple bands only for mutant candidates, most likely one of them can be very long deletion. In this case, purify each band by cutting gel after agarose electrophoresis.
4. Send PCR products for sequencing, using either of PCR primers as a sequence primer.

3.4.8 TIDE analysis

https://tide.deskgen.com

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3.5 Protocol for the generation of targeted CRISPR-Cas9-based gene knockouts in the model brown alga Ectocarpus

Note: This protocol is taken from the following manuscript in preparation:

Targeted CRISPR-Cas9-based gene knockouts in the model brown alga *Ectocarpus*

Yacine Badis1,2, Delphine Scornet1, Minori Harada3, Celine Caillard1, Morgane Raphalen1, Claire M.M. Gachon2,4, Susana M. Coelho1, Taizo Motomura3, Chikako Nagasato3 & J. Mark Cock1

1Roscoff Biological Station, Place Georges Teissier, 29680 Roscoff, France; 2The Scottish Association for Marine Science, Scottish Marine Institute, Oban, Argyll PA37 1QA, United Kingdom; 3Muroran Marine Station, Hokkaido University, Hokkaido 060-0808, Japan; 4Museum National d'Histoire Naturelle, UMR 7245 Molecules de Communication et Adaptation des Micro-organismes, CP 54, 57 rue Cuvier, 75005 Paris, France.

3.5.1 gRNA design and in vitro validation of RNP complexes

The CRISPOR tool (http://crispor.tefor.net/), with the *Ectocarpus* species 7 strain Ec32 genome as reference, was used to select the highest scoring crRNAs for exons near the 5' end of target genes. Purified crRNAs, tracrRNA and Cas9 protein (HiFi Cas9 Nuclease 3NLS) were ordered from IDT and assembled into RNP complexes following the manufacturer's instructions with a Cas9:gRNA ratio of 1:1. RNP *in vitro* cleavage assays were performed on purified PCR amplicons containing the target sites following the New England Biolabs online protocol (https://international.neb.com/protocols/2014/05/01/in-vitro-digestion-of-dna-with-cas9-nuclease-s-pyogenes-m0386). Briefly, 90 fmol of purified PCR amplicon were added to 900 fmol of preassembled RNP complex in NEB 3.1 buffer for a final volume of 30 µL. The reaction was incubated for one hour at 37^{o}C and the digestion was then terminated by addition of 1 µL Proteinase K (NEB) and incubation for 10 minutes at 65^{o}C. The DNA digestion products were analysed on a 1.5% agarose gel.

3.5.2 Biological Material

This study used the male *Ectocarpus* species 7 strain Ec32, for which a reference genome is available (Cock *et al.*, 2010; Cormier *et al.*, 2017). Strain Ec32 was previously referred to as *E. siliculosus* (Peters *et al.*, 2004) but a recent study (Montecinos *et al.*, 2017) indicates that it belongs to a distinct, at present unnamed, species, which is referred to as *Ectocarpus* species 7. *Ectocarpus* species 7 strain Ec32 was cultured in sterilized natural seawater enriched with half strength Provasoli solution (half-strength PES; Starr and Zeikus, 1993). The culture conditions were 13°C with a light:dark cycle of 12h:12h (20 µmol photons m-2 s-1) and daylight-type fluorescent tubes (Coelho *et al.*, 2012). All manipulations were performed under sterile conditions in a laminar flow hood.

3.5.3 Biolistic delivery of CRISPR-Cas9 RNP complexes

To obtain gametes for biolistic delivery, 2 mm gametophyte germlings were transferred to individual 140 mm petri dishes (ten gametophytes per dish) containing 70 mL of culture medium. For each biolistic delivery, about a hundred fertile gametophytes bearing plurilocular gametangia were collected after four weeks in culture and induced to release gametes (Coelho *et al.*, 2012) in 60 mm petri dishes containing 5 mL of solidified (1.5% agar) culture medium by adding 10 mL of ice-cold sterile natural seawater. The gametophytes were removed after 6 hours and the released gametes left to settle overnight on the agar substrate. Subsequent steps were performed using the same culture dishes as settled gametes attach firmly to the agar substrate. One hour before the experiment, the seawater medium was removed by delicate inversion, and the agar plates were left to dry for 10 min under a laminar flow-hood. Microparticle RNP coating and microcarrier preparation were performed

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as described by Serif et al. (2018). If not otherwise stated, 3 mg of gold nanoparticles (0.6 µm diameter, Bio-Rad) and 8 µg of total RNP complexes were used for each biolistic shot using a membrane rupture pressure of 1800 psi, a vacuum of 27 Hg and a target distance of 6 cm. Initial tests, carried out to verify delivery of nanoparticules to different cell types were carried out using a mixture of 3 mg of gold nanoparticles (0.6 µm diameter, Bio-Rad) that had been pre-incubated in a 8mM solution of AF488-labelled 10 kDa Dextran (Invitrogen). Bombardments were carried out using a Bio-Rad PDS-1000/He system. After bombardment, 15 mL of fresh culture medium was added and the gametes allowed to recover for 48h prior to the application of selection, for example by addition of 20 mM 2-fluoroadenine (Sigma Aldrich). For droplet digital PCR detection of TM events, biolistic delivery was performed on 2 week old immature gametophytes (5 to 6 individuals per test) spread on the same solidified medium.

3.5.4 Droplet Digital Polymerase Chain Reaction Assays to Detect Cas9 induced indel mutations

Indel detection was carried out using between four and 125 ng of total DNA on a QX200 Droplet Digital PCR System with 5(6)-carboxyfluorescein (FAM) and hexachloro-fluorescein (HEX) labelled oligonucleotide probes (Bio-Rad, Hercules, CA). Oligonucleotide primers and probes (Fig. 2a) were obtained from Bio-Rad. ddPCR reactions were carried out by Ingenierie et Analyse en Genome Editing (IAGE, Montferriez sur lez, France). A QX200 Droplet Generator (Bio-Rad) was used to distribute PCR components to individual reaction vessels. Droplets were generated by combining 70 µl of droplet generation oil with 22 µl of the PCR mix and 40 µl of resulting droplet reaction was subjected to amplification. The cycling conditions for the PCR reaction included an initial incubation for 10 min at 95 °C followed by 40 cycles of 94 °C for 30 s and 55 °C for 60 s. Amplified plates were transferred to a Droplet Reader (Bio-Rad) and the digital PCR data were analysed with the provided Quanta Soft analytical software suite.

3.5.5 References

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3.6 Proteolistic-mediated RNP transformation of *Cylindrotheca closterium*

This protocol has been adapted from Serif *et al.,* (2018) to *Cylindrotheca closterium* in collaboration with SZN.

By Sien Audoor[1], Darja Belisova[1], Anna Santin[2], Monia Russo[2], Mariella Ferrante[2] and Wim Vyverman[1]

[1]Universiteit Gent, Ghent, Belgium; [2]Stazione Zoologica Anton Dohrn, Naples, Italy

3.6.1 Design gRNAs against target genes

Use following link to design guides with possibility to upload a custom genome

http://grna.ctegd.uga.edu/

3.6.2 In vitro test of gRNAs

Prepare the gRNA

- Resuspend each RNA oligo (Alt-R CRISPR-Cas9 crRNA, tracrRNA, sgRNA) in IDTE buffer to a stock concentration of 100 µM.
- Mix the crRNA and tracrRNA oligos in equimolar concentrations in a sterile microcentrifuge tube to a final duplex concentration of 10 µM: 1 µl 100 µM crRNA, 1 µl 100 µM tracrRNA, 8 µl NF duplex buffer
- Heat the duplex at 95°C for 5 min.
- Remove from heat and allow to cool to room temperature (15–25°C).

Create the RNP complex

- Combine the guide RNA and Cas9 enzyme in equimolar amounts.
- 10 µl crRNA:tracrRNA duplex, 1.6 µl Cas9 enzyme (62 µM stock), 88.4 µl PBS
- Incubate at room temperature for 5–10 min for optimal formation of the RNP complex.

Perform the in vitro digestion reaction

- Assemble the reaction at room temperature (15–25°C): 1 µl PBS, 1 µl 1 µM Cas9 RNP, 1 µl 50 nM DNA substrate, 7 µl NF water
- Incubate the reaction at 37°C for 60 min.
- Add 1 µL Proteinase K (20 mg/mL) to the reaction, then incubate the mixture at 56°C for 10 min to release the DNA substrate from the Cas9 endonuclease.

Visualise cleaved products

Analyse digestion through agarose gel electrophoresis (load enough material!).

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3.6.3 In vivo CRISPR

Reagents:

- Alt-R® CRISPR-Cas9 crRNAs (IDT, 2 nmol, keep at -20°C once in solution)
- Alt-R® CRISPR-Cas9 tracrRNA (IDT, 5 nmol, keep at -20°C once in solution)
- PBS 1X depc
- Usual equipment for biolistic
- 2-fluoroadenine (2-FA) 2500x
- Adenine (ADE, Sigma, ref A2786)

Before - Design your experiment

- Include all of the appropriate controls, comprising a negative control by transforming cells with Cas9 alone

Prepare diatom cultures

You need an exponentially growing ***Cylindrotheca*** culture (around 1.0-2.0 x 10^6 cells mL^{-1}). 1.5 x 10^8 cells for shoot / 1.5 x 10^6 cell/ml = 100 ml for shoot

100 ml for shoot * *5* (*4* shoots and *1* control) = *500* ml total of culture needed

Day 1: Assemble the crRNA::tracrRNA complexes

- Work in an RNAse-free environment. Use filter tips. Wash bench-top with MQ H2O and 60% EtOH. Spread some RNAse Zap.

- Resuspend the lyophilised crRNA (-20°C) and tracrRNA (-20°C) with NF IDT Duplex buffer to a final concentration of 100µM. Mix up and down.

  If you bought 5 nmol of tracrRNA, add 50µL NF IDT Duplex buffer. If you bought 2 nmol of tracrRNA, add 20µL NF IDT Duplex buffer to get a stock solution at 100µM

- Store the solution on ice or at -20°C for longer periods.

- In a PCR tube, mix the various components as follows, scaling-up volumes based on your needs:

Component	Volume [µL]	Final quantity [pmol]
Alt-R CRISPR crRNA (100 µM)	3	300
Alt-R CRISPR tracrRNA (100 µM)	3	300
IDT Duplex buffer	4	
Total volume	10	

NB: this will give a crRNA::tracrRNA duplex solution containing 30 µM duplex

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- In a thermocycler, incubate 5 min at 95°C and immediately remove from the block NB: Do not let the tubes cool down within the block!
- Cool down at RT on the bench
- Store on ice for immediate usage or at -20°C for longer periods (stable for months)

Day 1: Plate the diatom cultures for proteolistic

- Use an exponentially growing ***Cylindrotheca*** culture (around 1.0-2.0 x 10^6 cells mL^{-1})
- Collect the required culture volume to get 1.5 x 10^8 cells in one or more 50 mL conical tubes.
- Centrifuge at 3000 rpm, 18°C for 5 min.
- Discard supernatant.
- Resuspend pellet with the bit of supernatant left (around 200 µL).
- Spread the cells on an NEPC agar plate, the spread has to be circular and around 4 cm in diameter.
- Let dry under the hood, close the plate and place it in the growth chamber for 2-3 hours

Day 1: Assemble the RNP complexes

- Calculate the total volume of Cas9 you need. Dilute the corresponding amount of IDT HiFi Cas9 v3 protein from a first stock solution at 10 $g.L^{-1}$ (i.e. 65.5 µM, kept at -20°C, to a second stock solution at 2.5 $g.L^{-1}$ (i.e. 15.6 µM) using NF H2O. This step will facilitate downstream work because the solution at 10 $g.L^{-1}$ is viscous and hard to manipulate.
  NB: MCas9=160 kDa= 1.6 x 10^5 $g.mol^{-1}$

  If you need 25 µg Cas9 protein total: take 2.5 µL HiFi Cas9 v3 stock at 10 g L^{-1}, add 7.5 µL NF H2O and you get 10 µL HiFi Cas9 v3 solution at 2.5 g L^{-1}

- For each RNP complex to assemble, mix the various components as follows, scaling-up volumes based on your needs:

Component	**Volume [µL]**	**x 9 [µL]**	**Final quantity [pmol]**
crRNA::tracrRNA duplex (at 30 µM)	0.4	3.6	48
Alt-R Cas9 enzyme (at 15.6 µM)	0.8	7.2	49
PBS 1X	0.8	7.2	
Total volume	2	18	

NB2: the molar ratio of gRNA:Cas9 is 1:1

For the negative control, with only Cas9, we need the same amount of enzyme of the other shots (8 µg each):

Component	**Volume [µL]**	**x 2 [µL]**
Alt-R Cas9 enzyme (at 15.6 µM)	3.2	6.4

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PBS 1X	4.8	9.6
Total volume	8	16

- Incubate at room temperature for 10-20 min
- Store on ice or at 4°C in the fridge for longer periods of time (stable for months)

- Prepare one tube for each proteolistic shoot and combine the required RNP complexes, using 1-4 µL of the RNP preparation for each complex (maximum total volume 8 µL on the macrocarrier).

- Keep the mixes on ice until use.

Name RNP mix	**Volume [µL]**
gRNA + Cas9 Cc APT1	*2*
gRNA + Cas9 Cc APT2	*2*
gRNA + Cas9 Cc target1	*2*
gRNA + Cas9 Cc target2	*2*
Total volume RNPs	*8*

Day 1: Load the mixed RNP complexes onto gold particles

- Take a 50 µL gold particles aliquot (containing 3 µg gold particles, diameter 0.6 µM; kept at -20°C).
  NB1: it is sufficient for 5 proteolistic shoots.

- Thaw at RT.
- Centrifuge at max speed, RT, for 1 min.
- Remove the supernatant with a pipette, resuspend the pellet with 50 µL PBS 1X.
- Centrifuge at max speed, RT, for 1 min.
- Carefully remove the supernatant with a pipette.
- Repeat the washing step once.
- Resuspend the pellet with 50 µL PBS 1X. Vortex.
- Store the washed gold particles on ice.
- Turn the laminar flow hood on, wipe it with MQ H2O and 60% EtOH.
  NB: the following steps have to be performed under the hood

- Dip the macrocarrier holders and the macrocarriers into 100% ethanol, let them dry.
- Place each macrocarrier into a macrocarrier holder.
- Resuspend the gold particles by flickering and mild vortexing (slowly).
- Put on ice.

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- Transfer **10 µL of the homogenous gold particles** solution into each RNP mixture.
- Mix by pipetting up and down.
- Spread the solution evenly at the center of the macrocarrier.
  NB: try to deposit it continuously while doing concentric circles with the pipette

- Indicate the corresponding proteolistic shoot number next to each loaded macrocarrier.
- **Let dry under the hood for 2 or 3 hours.** NB: of course, let the air flow on!

Day 1: Proteolistic shoots

Similar procedure to classical biolistic: 1550 psi rupture discs, wash the apparatus carefully between shots (MQ H2O and 60% EtOH).

After proteolistic shoots, put all the cells of the same shoots in one flask:

- a flask 400 ml NEPC without selection with the cells of 4 shots
- a flask 100 ml NEPC without selection with the cells of negative control.

Day 2: Replating

After 24h from proteolistic shoots, put the cells in 10 flasks with 50 ml NEPC + selection 2-FA and 10 plates with 30 ml NEPC + agar 2% + selection 2-FA

If **CcAPT** was targeted as the endogenous selection marker, use plates containing NEPC medium, 2% agar, 20 µM 2-FA (from a 2500X stock solution, in DMSO, kept at 4°C; stable over months) and 5 mg L^{-1} ADE (from a 100X stock solution prepared).

2-FA: We have a stock solution of *1.5 mg/ml* and we need a final concentration of 20 µM PM (2-FA) = 152.12 g/mol

1.5 mg/ml=*1.5* g/l, so *1.5*g/152.12g/mol = *0.0098* mol in 1L = *9.8* mmol/l = *9.8* mM
CiVi=CfVf, so *9.8* mM · X = 20 µM · 30 ml (in 1 petri dish)

X = *61.2* µl per petri

61.2 µl per petri * *8* petri (*2**2 shoots *PtAPT* + *2* control + *2* control Pt Parigi) = *480* µl total

ADE: We have a stock solution 100X

In 1 petri with 30 ml of medium: 300 µl of ADE

So 300 µl of ADE * *8* petri (*2**2 shoots *PtAPT* + *2* control + *2* control Pt Parigi) = *2.4* ml total

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3.7 Proteolistic-mediated RiboNucleoProtein transformation

By Monia Teresa Russo1, Anna Santin2 and Mariella Ferrante2.

1Department of Research Infrastructures for marine biological resources, Stazione Zoologica Anton Dohrn, Naples, Italy

2 Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Naples, Italy

➢ *Pseudo-nitzschia multistriata*

This protocol has been adapted to *P. multistriata* from Serif et al. (2018). It has to be further optimized, but some crucial steps have been fixed:

- Inclusion of *P. multistriata* genome in the CRISPOR database (http://crispor.tefor.net/; Concordet and Haeussler, 2018) for RNA guides design
- Identification of the endogenous genes used for selection [*Adenine Phosphoribosyl Transferase* (APT) and *Uridine Monophosphate Synthase* (UMPS); Serif et al. (2018)] in the *P. multistriata* genome
- Experimental validation of the APT sgRNAs activity through *in vitro* assay; UMPS sgRNAs need further analyses
- Determination of the concentration of the drugs (5-Fluoroorotic acid (5-FOA), 100 µg/ml and 2-Fluoroadenine (2-FA), 20 µM) to be used for selection

3.7.1 Reagents:

- Alt-R® CRISPR-Cas9 crRNAs (IDT, 2 nmol or 20 nmol scale, keep at -20°C once in solution)
- Alt-R® CRISPR-Cas9 tracrRNA (IDT, 20 nmol or 100 nmol scale, keep at -20°C once in solution)
- Alt-R® S.p. HiFi Cas9 Nuclease V3 (IDT, 100 µg or 500 µg, keep at -20°C)
- PBS 10x
- Usual equipment for biolistic transformation (macrocarriers, 1550 psi rupture disks…)
- 5-fluoroorotic acid (5-FOA, fisher scientific, ref 10609920, keep at 4°C)
- Uracile (URA, Sigma, ref U1128)
- 2-fluoroadenine (2-FA, FluoroChem, ref 005193)
- Adenine (ADE, Sigma, ref A2786)
- Dimethyl sulfoxide for cell culture (DMSO, Dutscher SAS, ref P60-36720100)
- RNase AWAY (Sigma, ref 83931)

3.7.2 Before - Design the crRNAs

1. IMPORTANT: amplify and sequence the candidate gene in your *Pseudo-nitzschia* strain since its sequence may significantly differ from that reported in the reference genome, particularly pay attention to the single nucleotide polymorphisms (SNPs) patterns between both alleles within your strain.
2. Use CRISPOR (http://crispor.tefor.net/) to identify potential Cas9 target sites within your candidate gene, following a few criteria:
    - Sequence of the format "NNNNNNNNNNNNNNNNNNNNNGG" (N_{20}NGG)
    - Situated within an exon (https://protists.ensembl.org/Pseudonitzschia_multistriata/Info/Index)
    - Situated within an active site encoding region, if the aim is to generate KOs
    - Situated close to the 5' end of the gene (exon 1), pay attention to cryptic ATGs

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- No off-targets predicted by CRISPOR within *Pseudo-nitzschia* genome
- As high Moreno-Mateos score as possible (>50)
- Absolutely avoid target sites including SNPs between alleles, as you may end up preferentially targeting one allele over the other
- If using 2 guides simultaneously for a given gene, the resulting deletion should generate a frameshift

NB1: Pick at least 2 independent targets per gene, preferentially 3
NB2: CRISPOR gives suggestions using the format "CAACGAAGCGGGTAAAAACTTGG", corresponding to 20 nucleotides (nt) of target sequence and 3 nt of Protospacer Adjacent Motif (PAM) sequence (red). You will only input the protospacer sequence when ordering your cRNAs
(DO NOT INCLUDE THE PAM)

Pm_APT1: GGTTTCGTGCAACTTAGATT
Pm_APT2: GTAGAGCGTTACCAAGCGAT
PM_UMPS1: GGCTTGTAGCAGGCAGCGTA
Pm_UMPS3: TCGCAAAGCTGGCGCAGCAA

3. Order the required Alt-R CRISPR crRNAs to IDT.
   NB: a 2 nmol scale is sufficient for dozens of individual shoots
   Using the above example, I would order 2nmol of an ALT-R crRNA with the following protospacer sequence: CAACGAAGCGGGTAAAAACT
4. Upon reception, store the crRNAs and tracrRNA at – 20°C until usage.

3.7.3 Before - Design your experiment

Here are a few key points for your design:

- Include all of the appropriate controls, comprising a negative control by transforming cells with Cas9 alone and a positive control with RNPs targeting the endogenous selection marker alone (PtAPT, PtUMPS…)
- Avoid using more than 20 µg RNP per proteolistic shot, which already represents an important volume of liquid to load onto the macrocarrier and takes a lot of time to dry (overnight drying required)

3.7.4 Before - Assemble the crRNA::tracrRNA complexes

- Work in an RNAse-free environment. Use filter tips. Wash bench-top with MQ H_2O and 60% EtOH. Spread some RNAse Zap.
- Resuspend the lyophilized crRNA (-20°C) and tracrRNA (-20°C) with NF IDT Duplex buffer to a final concentration of 100 µM. Mix up and down.
  If you bought 2 nmol of (tra)crRNA, add 20 µL NF IDT Duplex buffer to get a stock solution at 100 µM
- Store the solution on ice or at -20°C for longer periods.
- In a PCR tube, mix the various components as follows, scaling-up volumes based on your needs:

Component	**Volume [µL]**	**Final quantity [pmol]**

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Alt-R CRISPR crRNA (at 100 µM)	3	300
Alt-R CRISPR tracrRNA (at 100 µM)	3	300
IDT Duplex buffer	4	
Total volume	10	

NB: this will give a crRNA::tracrRNA duplex solution containing 30 µM duplex

- In a thermocycler, incubate 5 min at 95°C and immediately remove from the block
  NB: Do not let the tubes cool down within the block!
- Cool down at RT on the bench
- Store on ice for immediate usage or at -20°C for longer periods (stable for months)

3.7.5 Friday – Diluition of the diatoms culture

You need an exponentially growing *Pseudo-nitzschia* culture (around 1.0-2.0 x 10^5 cells mL^{-1}).

3 x 10^7 cells for shot / 1.5 x 10^5 cell/ml = 200 ml for shot

200 ml for shot * 6 (4 shots and 2 control) = 1200 ml total of culture needed

NB: on Friday dilute the culture at 0.2-0.3 x 10^6 cell/ml, so as to have about 1.5 x 10^6 cell/ml on the following Monday.

3.7.6 Monday - Plate the diatom culture for proteolistics

- Use an exponentially growing *Pseudo-nitzschia* culture (around 1.0-2.0 x 10^5 cells mL^{-1}; grown under 12 h:12 h day:night cycles, 100 µmol photons m^{-2} s^{-1} white light during the day, 20 °C; shaking 90 rpm).
- NB: you will plate 3 x 10^7 cells per petri dish (corresponding to 1 shot), so for 10 shots you will need 3 x 10^8 cells corresponding to a 1 L culture at 1.5 x 10^5 cells mL^{-1}.
- Collect the required culture volume to get 3 x 10^7 cells in one or more 50 mL conical tubes.
- Centrifuge at 112 x g, 18°C for 10 min.
- Discard supernatant.
- Resuspend pellet with the bit of supernatant left (around 200 µL).
- Spread the cells onto an F/2 agar plate (Sigma F/2 solution, 20 g L^{-1} Sigma sea salt), the spread has to be circular and around 4 cm in diameter.
- Let dry under the hood, close the plate and place it in the growth chamber for 2-3 hours (12 h:12 h day:night cycles, 100 µmol photons m^{-2} s^{-1} white light during the day, 20°C).

3.7.7 Tuesday - Assemble the RNP complexes

- Calculate the total volume of Cas9 you need. Dilute the corresponding amount of IDT HiFi Cas9 v3 protein from a first stock solution at 10 g L^{-1} (i.e. 65.5 µM, kept at -20°C) to a second stock solution at 2.5 g L^{-1} (i.e. 15.6 µM) using NF H_2O. This step will facilitate downstream work because the solution at 10 g L^{-1} is viscous and hard to manipulate.
  NB: M_{Cas9}=160 kDa= 1.6 x 10^5 g mol^{-1}
  If you need 25 µg Cas9 protein total: take 2.5 µL HiFi Cas9 v3 stock at 10 g L^{-1}, add 7.5 µL NF H_2O and you get 10 µL HiFi Cas9 v3 solution at 2.5 g L^{-1}
- For each RNP complex to assemble, mix the various components as follows, scaling-up volumes based on your needs:

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Component	Volume [µL]	Final quantity [pmol]
crRNA::tracrRNA duplex (at 30 µM)	1.6	48
Alt-R Cas9 enzyme (at 15.6 µM)	1.6	49
2.5x NEB 3.1 buffer	3.2	
Total volume	8	

NB1: this will give a RNP solution containing the equivalent of 1 µg μL^{-1} Cas9
NB2: the molar ratio of gRNA:Cas9 is 1:1

- Incubate at room temperature for 10-20 min
- Store on ice or at 4°C in the fridge for longer periods of time (stable for months)
- Prepare one tube for each proteolistic shot and combine the required RNP complexes, using 1-4 µL of the RNP preparation for each complex (maximum total volume 8 µL on the macrocarrier).
- Keep the mixes on ice until use.

Name RNP mix	Volume [µL]	* n^ shots	Final mix volume [µL]
gRNA + Cas9 PmAPT1	4	2	8
gRNA + Cas9 PmAPT3	4	2	8
gRNA + Cas9 PmUMPS1	4	2	8
gRNA + Cas9 PmUMPS2	4	2	8

NB: Do different combinations, so for example, take 4 µL RNP mix (gRNA + Cas9 PmAPT1) and mix with 4 µL RNP mix (gRNA + Cas9 PmAPT3) = 8 µL of total volume, if you want to target the PtAPT loci for direct selection on 2-FA containing medium.

Name 1^ mix	[µL]	Name 2^ mix	[µL]	[µL] on macrocarrier	Replicates
gRNA + Cas9 PmAPT1	4	gRNA + Cas9 PmAPT3	4	8	2
gRNA + Cas9 PmUMPS1	4	gRNA + Cas9 PmUMPS2	4	8	2

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Perform *in vitro* assay to experimentally validate the activity of CRISPR guide RNA before practical application

- Amplify a genomic fragment of about 500 bp encompassing the sgRNA region
- Purify the fragment
- Assemble the Cas9 RNP-DNA complex with 10:1 molar ratio (DNA final concentration 50 nM) at RT in 10 µl final volume with 1x PBS
- Incubate the reaction at 37°C for 60 min
- Add 1 µl proteinase K (20 mg/ml)
- Incubate at 56°C for 10 min
- Analyze the digestion by agarose gel electrophoresis

3.7.8 Tuesday - Load the mixed RNP complexes onto gold particles

- Preparation of gold particles: stock aliquots of gold particles can be prepared and stored at -20°C. Procedure: place 60 mg of the dry gold particles in 1 ml of 100% ethanol in a microfuge tube. Vortex on high for 1-2 min. Repeat 3x. Pellet particles in microfuge (1 min); wash 2x in 1 ml DEPC water. Suspend in 1 ml DEPC water and aliquot 50 µl into sterile microfuge tubes (vortex continuously while pipetting).
- Take a 50 µL gold particles aliquot (containing 3 µg gold particles and sufficient for 5 proteolistic shots) and thaw at RT.
- Centrifuge at 3824 x g, RT, for 1 min.
- Remove the supernatant with a pipette, resuspend the pellet with 50 µL 1x PBS.
- Centrifuge at 3824 x g, RT, for 1 min.
- Carefully remove the supernatant with a pipette.
- Repeat the washing step once.
- Resuspend the pellet with 50 µL 1x PBS.
- Store the washed gold particles on ice.
- Turn the laminar flow hood on, wipe it with MQ H_2O and 60% EtOH.
  NB: the following steps have to be performed under the hood
- Deep the macrocarrier holders and the macrocarriers into 100% ethanol, let them dry.
- Place each macrocarrier into a macrocarrier holder.
- Resuspend the gold particles by flickering and mild vortexing.
- Transfer 10 µL of the homogenous gold particles solution into each RNP mixture.
- Mix by pipetting up and down.
- Spread the solution evenly at the center of the macrocarrier.
  NB: try to deposit it continuously while doing concentric circles with the pipette
- Indicate the corresponding proteolistic shot number next to each loaded macrocarrier.
- Let dry under the hood for 2 or 3 hours.

3.7.9 Tuesday - Proteolistic shots

- Similar procedure to classical biolistics: 1550 psi rupture discs, wash the apparatus carefully between shots (MQ H_2O and 60% EtOH).
- Seal the petri dish with Parafilm after each shot.
- Place the petri dish in the growth chamber, cells facing down, as usual.
  NB1: culture conditions as usual, i.e. 12 h:12 h day:night cycles, 100 µmol photons m^{-2} s^{-1} white light during the day, 20 °C

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3.7.10 Thursday - Replating

After 48h from the proteolistics shots, resuspend the cells with 100 mL sterile F/2 + Si medium, one for every shot.

- If **PmUMPS** was targeted as the endogenous selection marker, use plates containing 20 g L^{-1} Sigma sea salts, 10 g L^{-1} agar, 1x Sigma F/2 solution (from a 50x stock solution kept at -20°C), 300 µg mL^{-1} 5-FOA (from a 1000x stock solution, in DMSO, kept at 4°C; prepared less than a month before) and 50 µg mL^{-1} URA (from a 1000x stock solution prepared in 1N NaOH, can be stored over months at 4°C)
    a) FOA: We have a stock solution of 60 mg/ml and we need a final concentration of 100 µg/ml:
    $C_iV_i=C_fV_f$, so 60 mg/ml · X = 100 µg/ml · 100 ml (in 1 flask)
    X = 166 µl per flask
    166 µl per flask * 3 flask (2 shots PmUMPS + 1 control) = 498 µl total
    b) URA: We have a stock solution 1000x
    In 1 flask with 100 ml of medium: 100 µl of URA
    So 100 µl of URA * 3 flask (2 shots PmUMPS + 1 control) = 300 µl total

- If **PmAPT** was targeted as the endogenous selection marker, use plates containing 20 g L^{-1} Sigma sea salts, 10 g.L^{-1} agar, 1x Sigma F/2 solution (from a 50x stock solution kept at -20°C), 10 µM 2-FA (from a 1000x stock solution, in DMSO, kept at 4°C; stable over months) and 5 mg L^{-1} ADE (from a 1000x stock solution prepared).
    c) 2-FA: We have a stock solution of 3 mg/ml and we need a final concentration of 20 µM
    PM (2-FA) = 152.12 g/mol
    3 mg/ml=3 g/l, so 3g/152.12g/mol = 0.0196 mol in 1L = 19.6 mmol/l = 19.6 mM
    $C_iV_i=C_fV_f$, so 19.6 mM · X = 20 µM · 100 ml (in 1 flask)
    X = 105 µl per flask
    105 µl per flask * 3 flask (2 shots PmAPT + 1 control) = 315 µl total
    d) ADE: We have a stock solution 100x
    In 1 petri with 30 ml of medium: 300 µl of URA
    So 300 µl of URA * 3 flask (2 shots PmAPT + 1 control) = 900 µl total

Resistant colonies should appear after to 3-4 weeks.

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4. Conclusion

Here we present the first protocols for GMO production (both transgenesis and KO) on different biological models that have been developed by the different partners of the project. Obviously, we still have other protocols that we do not present here because they are not completely developed and they will be included in the list in a close future.

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5. Appendices

5.1. Appendix 1

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5.2. Appendix 2

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