ASSEMBLE

ASSOCIATION OF EUROPEAN MARINE BIOLOGICAL LABORATORIES EXPANDED

Acronym: ASSEMBLE Plus

Title: Association of European Marine Biological Laboratories Expanded

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Deliverable 9.1 – JRA3

Protocols for genetic transformation of novel emerging metazoan, macroalgal and microalgal modelorganisms available via ASSEMBLE Plus web portal

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GENERAL DATA

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Dissemination Type

Report	☒
Websites, patent filling, etc.	☐
Ethics	☐
Open Research Data Pilot (ORDP)	☐
Demonstrator	☐
Other	☐

Dissemination Level

Public	☒
Confidential, only for members of the consortium (including the Commission Services)	☐

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Editor(s)	
Version	

Abstract

The general objectives of this WP at the beginning of the project were:

- **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 two first years of the project the different participants in this WP have begun to develop the various activities and experimental approaches that were detailed in the project. Depending on the model, the preliminary 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 within a few months and for each model studied. Particularly, concerning this deliverable, we have developed or adapted protocols to transform the different biological models used in this JRA3. This process of transformation 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 genetic transformation of the different biological models used in the three tasks of the project:

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 genetic transformation of different biological models

3.1 Genetic transformation in ascidian embryo: introducing plasmid DNA by electroporation in the ascidian Phallusia mammillata

By Sébastien Darras, Sorbonne Université, CNRS, Biologie Intégrative des Organismes Marins (BIOM), Banyuls/Mer, France

3.1.1 Before starting

Working temperature: *Phallusia mammillata* embryos can develop at higher temperature (up to 22°C and maybe more) than Roscoff *Ciona intestinalis* (max. 19-20°C). Our experience suggests a large range of temperature (14-22°C).

Material needed:

- dissecting scope
- sea water (SW)
- dechorionation solution: 0.5% NaThioglycolate, 0.1% pronase in SW (mix thioglycolate in SW by shaking, add pronase on top – no mixing! – and keep at 4°C for few hours before use).
- NaOH 2.5 M
- Tris 1M pH 9.5
- scissors and scalpel
- gloves (*Phallusia* blood is a powerful stain!)
- Pasteur pipettes that have soaked in tap water, fire-polished bore (prepare some with large opening for egg collection)
- 15 ml glass centrifuge tubes
- 1% agarose coated Petri dishes
- rotating shaker
- low binding 1.5 ml microtubes
- 4 mm electroporation cuvettes
- BTX ECM830 square electroporator
- 250 µl of electroporation solution made of DNA in 50 µl TE (usually 50 µg, range: 10 to 100 µg) and 200 µl of 0.96 M D-mannitol

3.1.2 Step 1: gamete collections

Open animals with scalpel/scissors between the 2 siphons. *Phallusia* tunic is thick and hard! Don't cut too deep to avoid damaging the animal. Open the tunic by pulling apart with your fingers. Transfer the animal in a dish on its left side (you should see heart beating at the base of the animal, opposite to the siphons). If the animal is on the right side, you should see a large yellowish oviduct full of eggs. Make

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a small cut in the oviduct and collect eggs with a large bored Pasteur pipet. Transfer eggs in 6-well plate dish filled with SW. *Phallusia* individuals normally have enormous amount of eggs (0.5 to 2 ml of eggs). Check egg quality (transparent and uniform egg cytoplasm) and transfer in glass tube for several washes with SW. Eggs can be kept O/N at 14°C spread in a Petri dish with no major decrease in quality.

You should see the spermiduct (nice white duct) underneath the oviduct once you have removed the eggs. Make a small cut and collect sperm into a 1.5 ml tube with a Pasteur pipet. Avoid collecting sea water, debris, blood… Dry sperm is kept active for several days at 4°C.

Usually collect gametes from at least 2 individuals. *Phallusia* is more self-fertile than *Ciona* but we prefer using a mix of sperm for fertilization.

3.1.3 Step 2: dechorionation

Dechorionation is performed before fertilization.

Collect eggs in a glass tube.

'Activate' dechorionation solution by adding 3 drops of NaOH 2.5 M with a Pasteur pipet to 7 ml of dechorionation solution.

Proceed to dechorionation in an agarose-coated dish (around 14 ml of dechorionation solution in a 9 cm diam. dish) placed on a shaker at 70 rpm.

Dechorionation takes 30 to 45 min depending on egg quantity. Regularly check dechorionation status under the scope. Naked eggs gather in the center at the bottom of the dish while non-dechorionated eggs float.

Once most of the eggs are dechorionated, collect them with large bored Pasteur pipet into a glass tube. Wash them extensively before transferring into a new agarose-coated dish. Eggs can be kept O/N at 14°C spread in a Petri dish with no major decrease in quality.

3.1.4 Step 3: fertilization

Sperm activation: three possible activations are efficient (in rare cases, we combine NaOH- and egg-activated sperm for optimal fertilization).

NaOH treatment: add 50µl of Tris 1M pH 9.5 to 1 ml of SW in an 1.5 ml tube, mix and wait for 1 min. Add 5 µl of a mixture of dry sperm. Mix well and wait for 1 min.

Tris treatment: add 2-4 µl of NaOH 1M to 1 ml of SW in an 1.5 ml tube, mix and wait for 1 min. Add 5 µl of a mixture of dry sperm. Mix well and wait for 1 min.

Chorionated eggs treatment: incubate 5 µl of a mixture of dry sperm in 1 ml SW containing 20 µl of chorionated eggs for at least 15 min.

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Basic pH-activated sperm has to be used right away while eggs-activated egg sperm can be kept active at 4°C for several hours.

Fertilization: Add 50-200 µl of activated sperm solution to a dish of eggs (5-6 cm diameter). Mix well, sending the eggs floating in the medium. You should see sperm swimming intensively. Egg deformations (ooplasmic segregation) should be visible within the first 15 min, start washing sperm out.

When eggs are to be fertilized in large quantity, proceed in a glass tube containing the eggs in 10 ml of SW. Add 200-500 µl of each activated sperm and mix well but smoothly. Collect a drop of sea water regularly to check that sperm are swimming and eggs are fertilized. Mix regularly (eggs drop quickly to the bottom of the tube). After 10-15 min, wash extensively to get rid of the sperm.

3.1.5 Step 4: electroporation

- After fertilization and washes of dechorionated eggs, let them collect at the bottom of a glass tube.

- Transfer embryos into 1.5 ml low binding microtube (water level up to 100 µl marked on the tube). Try to put the same amount of eggs for each of your tube.

- In each eggs-containing tube add the DNA solution and transfer the total mix into an electroporation cuvette (proceed for every single tube with a different Pasteur pipet to avoid mixing DNA).

- Place the cuvette into the electroporator cuvette holder.

- Apply an electrical pulse: *37 V for 32 ms*. The pulse should be done towards the end of the first cell cycle (but before cleavage!): at 50-60 min post-fertilisation.

- Let the eggs recover for at least 1 min and transfer them into Petri dishes (usually at least 2 dishes for one electroporation).

- Spread the eggs at low density in agarose coated dishes. Transfer the dishes in incubator at the appropriate temperature (14 to 22°C). Flush SW from the dish with a Pasteur pipet to evenly distribute the eggs. Keep the dishes undisturbed at least during the first two cleavages (otherwise blastomere will fall apart).

- Let the embryos develop until desired stage.

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3.2 Genetic transformation in ascidian embryo: introducing plasmid DNA by electroporation in the ascidian Molgula appendiculata*…*

By Sébastien Darras, Sorbonne Université, CNRS, Biologie Intégrative des Organismes Marins (BIOM), Banyuls/Mer, France

Working temperature: 14-19°C is fine but a wider range may be possible (to be tested).

3.2.1 Material needed

- dissecting scope
- sea water (SW)
- dechorionation solution: 1% NaThioglycolate, 0.05% pronase in SW.
- NaOH 1 and 2.5 M
- scissors and scalpel
- Pasteur pipettes that have soaked in tap water, fire-polished bore
- 15 ml glass centrifuge tubes
- 1% agarose coated Petri dishes
- BTX ECM830 square electroporator and 4 mm electroporation cuvettes
- 250 µl of electroporation solution made of DNA in 50 µl TE (usually 50 µg, range: 10 to 100 µg) and 200 µl of 0.96 M D-mannitol

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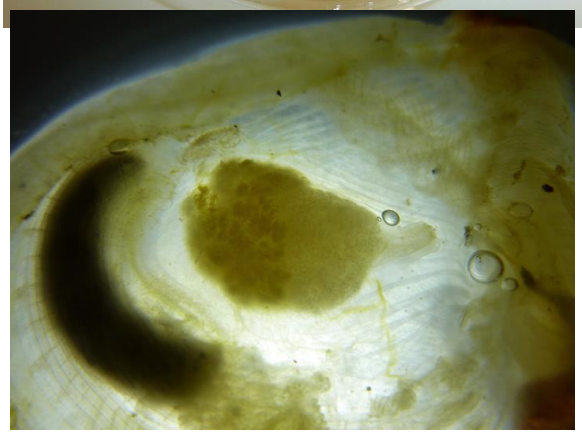
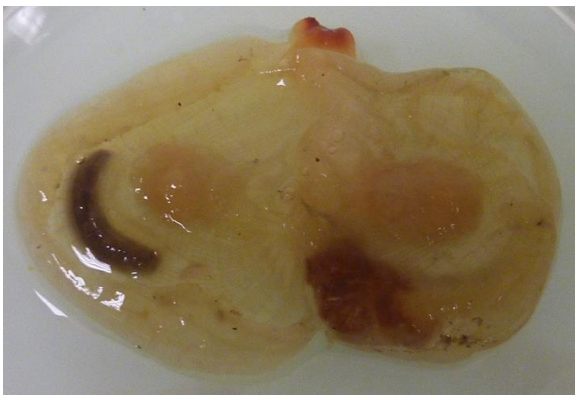
3.2.2 Collection of gametes

Open (2 to 8) animals with scissors starting from the inhaling siphon around the animal all the way to the exhaling siphon (*M. appendiculata* are covered with debris, shells and stones. Use large scissors and make your way around these obstacles). Pull open the tunic and drag the animal still attached by the siphons.



Cut the body wall from siphon to siphon with fine scissors and pull open the animal.



Under the dissecting scope (ie Motic), locate the gonads (one on each side of the animal) under the pharyngeal basket that you remove with tweezers. You should see the short gonoducts pointing towards the siphons, the large ovary covered on one side by the testis.

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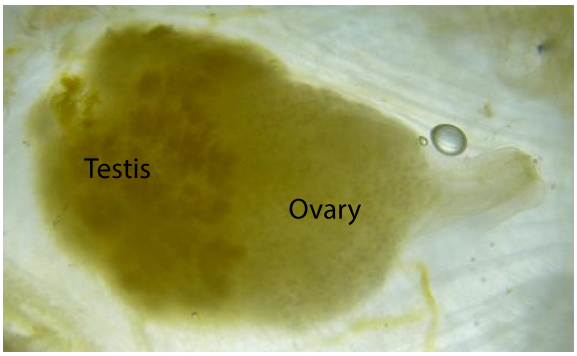

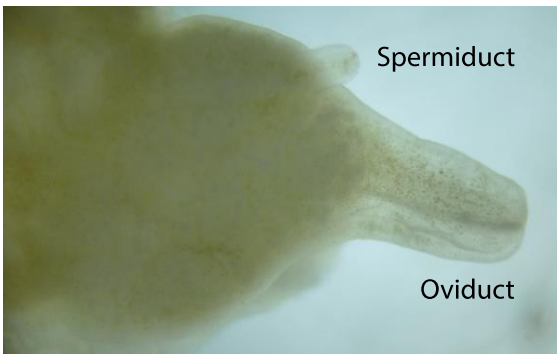

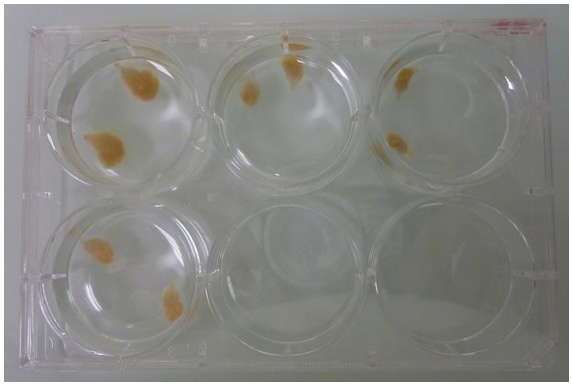

Cut out each gonad out of the animal with scissors and place them in the same well in a 6-well plate filled with SW.

Under the dissecting scope, release oocytes from the ovary with tweezers and transfer the testis to a Petri dish filled with SW (diam. 60). You should see a full range of oocytes (from tiny transparent oocytes to large opaque fully grown oocytes with germinal vesicle (GV)). Oocyte maturation (GVBD) occurs quickly and spontaneously in sea water. Collect all oocytes from one individual (2 ovaries) into a glass tube and wash 3-4 times (GVBD and sperm wash. After all washes are done, collect the eggs from all individuals into a single glass tube.

Testis from all individuals have been collected into the same Petri dish. Release sperm by dissociating each testis with tweezers (discard testis afterwards). Collect the concentrated sperm solution into a glass tube and keep at 4°C until use.

From here, the procedure is very similar to Ciona intestinalis.

3.2.3 Dechorionation

Dechorionation can be performed before or after fertilization (naked eggs fertilize well).

Collect eggs in a glass tube.

« Activate » dechorionation solution by adding 3 drops of NaOH 2.5 M with a Pasteur pipet to 3.5 ml of dechorionation solution.

Add the activated solution to the eggs.

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Dechorionation takes 10-12 min. Regularly check dechorionation status under the scope and mix deeply but softly (naked eggs are fragile) by pipetting. Avoid air bubbles. Dechorionation is more difficult to follow than in *Ciona* : while follicle cells are quickly lost, the vitelline membrane is more difficult to see (look close, change scope mirror orientation, the presence of test cells on the egg is a good indication that embryos are not fully dechorionated).

Once most of the eggs are dechorionated, stop the reaction by removing most of the solution and filling up with SW. Wash extensively 3-4 times (do not hesitate to get rid of floating debris).

3.2.4 Fertilization

Sperm activation:

Add 8 µl of NaOH 1 M to 1 ml of sperm solution.

Fertilization: Add 200 µl of activated sperm solution to eggs (either in a Petri dish (5-6 cm diameter) or in a glass tube, 10-15 ml). Mix well, sending the eggs floating in the medium. You should see sperm swimming intensively. Egg deformations (ooplasmic segregation) and polar body emission should be visible within the first 10-15 min, start washing sperm out.

3.2.5 Electroporation

Proceed as for *Ciona* and *Phallusia.*

20V, 32 ms

Culture

Spread the eggs at low density in agarose coated dishes. Transfer the dishes in incubator at the appropriate temperature. Flush SW from the dish with a Pasteur pipet to evenly distribute the eggs. Keep the dishes undisturbed at least during the first two cleavages (otherwise blastomere will fall apart). *Molgula* develop VERY fast (late tailbud in 12hrs at 19°C).

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3.3 Amphioxus (Branchiostoma lanceolatum) oocyte microinjection…

(Protocol adapted from Estelle Hirsinger and Le Petillon et al (1))

By Hector Escriva *Sorbonne Université, CNRS, Biologie Intégrative des Organismes Marins (BIOM), Banyuls/Mer, France*

3.3.1 Equipment, reagents and consumables

Item	**Catalog ref**	**Details**
Consumables		
Petri dishes 100 mm diam	ATGC #BP93B-15	Bacterial with arrow
Petri dishes 60 mm diam		Bacterial with arrow
Petri dishes 35 mm	Falcon # 353001	Culture-treated
Filtration unit (Stericup 1L)	Fisher # W21719	
Spin-X	Costar # 8160	0,22 micron filtration
Microloader	Dutscher # 034903 or Eppendorf # 5242 956.003	
Needle storage jar for 1,2 mm diam needles	WPI # E212	http://www.wpi-europe.com
Needles	World precision instruments TW100F-4	
Cristal plastic cups		
Fine forceps		Dumont n°5
Reagents		
Glycerol	SIGMA # G2025	
Fast Green	SIGMA #F7252	
H2O Dnase, Rnase-free	Gibco # 10977-035	
Polylysine	SIGMA # P9155	Poly-L-Lysine hydrobromide
DNA, mRNA of interest		
Equipment		

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200x fluorescent binocular scope	Olympus SZX16	
Micromanipulator	NARISHIGE MN-151	
Injector with accessories	Picospritzer II or III	From SERCOM (http://www.sercom-france.fr)
Needle puller	Sutter P1000	

3.3.2 Spawning induction

All experiments described here detail the protocol for the amphioxus species *Branchiostoma lanceolatum*. For other amphioxus species, the protocol should be adapted (particularly water temperature). The amphioxus used here are collected near the Racou beach (Argelès-sur-Mer, France) from May to July, during their natural spawning season when the gonads are mature (2).

1- Keep ripe animals at 17°C in tanks with sand in a sea water open circuit. Feed the animals 2 to 3 times per day (30,000-80,000 cells/ml/day of mixed algae (1/3 of *Dunaliella tertiolecta*, 1/3 of *Isochrysis galbana*, and 1/3 of *Tetraselmis suecica*) and with a 14/10 hours' day/night cycle for a period of time ranging from two weeks for animals collected early during the season (early May) to 3-5 days for animals collected later (June and July).
2- In order to induce the spawning, place the amphioxus at 23°C in a FSW tank without sand, keeping the day/night cycle during the afternoon (two to three hours before the light is off).
3- 24 hours later (two to three hours before the light is off), separate the animals individually in small plastic cups with 20-30 ml of FSW (without sand). Place the plastic cups on the black background in a quiet room at 19°C with the light on and turn off the light when the night is supposed to start in the previous day/night cycle.
4- Around one hour after the light is turned off, the first animals start to spawn. Use a portable red light to check for spawning. Then, transport the plastic cups with the animals and their gametes in another room and leave the animals that did not spawn yet in the dark. Most likely, they will spawn latter.
5- Recover the sperm with a pipette and keep it diluted in the sea water at 4°C to use it for several hours.
6- Transfer gently the eggs in a scratched Petri dish.

Fertilize the eggs with 60-70µl of sperm/FSW per Petri dish or proceed to microinjection prior fertilization, then check the efficiency of the fertilization by looking a few minutes later if the fertilization membrane has swelled up.

3.3.3 Microinjection protocol

Reagents stocks:

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10x Fast green: stock solution: 5 mg/mL in DNAse, RNase-free water
Filtered sterile on SPIN-X 0,22 micron (Costar # 8160) - 3-5 min at 13000 rpm.
Stored at 4°C.

Glycerol 99 %: SIGMA # G2025

On the previous day:

a- Polylysine-coated dishes:

Polylysine : Poly-L-Lysine hydrobromide SIGMA #P9155
Commercial bottles stored at -20°C
Dissolve 5 mg of polylysine in 40 mL dH2O
Aliquot by 10 mL
Store at -20°C
Avoid thaw-freeze cycles. Avoid re-using Polylysine.

Thaw an aliquot
Spread 1,2 mL in 35 mm cell-culture Petri dish (5 dishes total, Falcon #353001)
Incubate on the bench for 5 min
Transfer the Polylysine into another 35 mm Petri dish (5 dishes total)
Incubate on the bench for 5 min
Discard the Polylysine
Make sure to remove droplets and let dry, upside-down on the bench for 1h30-2h
Store at 4°C in Saran to avoid contamination

Best to use the Polylysine-coated dishes the next day

b- Filtered artificial sea water (about 1L) on 0,22 µm for spawning, injection and imaging

c- Injection needles

World precision instruments TW100F-4 (borosilicate glass with filament, OD/ID Tolerance: ±0.1 mm, length 10 mm)

Pulled on a Sutter P-1000 with the following settings:

Heat 457, Pull 120, Vel 60, Time 50, Pressure 300

On the day:

Take 50-500 eggs to perform injection

Fertilize right away the rest as control fertilization and also to fix embryos at different stages

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Fertilize the uninjected eggs at the end of the injection session to assess their health at the latest time. Co-inject always 500ng of mRNA coding for mCherry for checking injected *vs* non injected eggs one day later

a- Injection mix:

Glycerol: roughly 15% final concentration
Fast green 1x
DNA ou mRNA of interest (RNA final concentration around 1 µg/µL)
mCherry mRNA (1 µg/µL)
qsp H2O RNAse, DNase free

Ex:	1,5 µL mRNA (1,6 µg/µL)	final concentration: ~1 µg/µL
	0,5 µL mRNA mCherry (1 µg/µL)	final concentration: 170ng/µL
	0,5 µL Fast green	final concentration: ~0,5 %
	0,5 µL Glycerol 99%	final concentration: ~16 %
	Total: 3 µL	

Centrifuge 4 min at 13000 rpm to pellet crystals
Keep on ice

Load at least two needles with 1-1,5 µL of injection mix each (avoiding the bottom of the tube) with Microloader tips (long ones, Dutscher # 034903 or Eppendorf # 5242 956.003) and install the needle in the storage jar (WPI #E212; with liquid at the bottom to prevent evaporation of the injection mix)

Left-over injection mix can be put back at -80°C and re-used on another day

Load the needle ahead of time (when the first male spawns) so that the injection mix has time to travel to the tip of the needle, there are fewer bubbles and the needle can be cut thinner

Cut the tip of the needle very fine (in the curve)

Injection *per se*:

Binocular used for injection: Fluorescent to monitor injection live

~ 200x magnification (Olympus SZX16)

Transfer oocytes with a P20 micropipette onto the polylysine-coated dishes in filtered-sterile ASW. Spit them out along a line so that injection can be carried out in an ordered way.

Make small injection series (~ 30-50 embryos) so that they do not stay too long under the scope and on the polylysine

Illumination: dark field but not all the way, so that the embryos are as translucid as possible

Pulses of 120 msec with pressure between 0-10 psi

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The angle between the needle and the horizontal should be around 50°
Too shallow pushes the oocyte around; too steep makes you go through the oocyte too fast.

Go to the core of the oocyte (not too superficial: the dye comes out right away; not too deep: the oocyte explodes)

Inject (1 or 2 pulses)

Move the needle up and down a little 'to make room' and to uncouple the needle from the injected bubble

Pull the needle out swiftly so that the injected bubble does not come along

With Fast Green, one can monitor the injection live (but the color fades away fast).

Fertilise as soon as the series has been injected

Let the embryos detach from the polylysine while injecting another series

Remove from the polylysine plate as soon as possible; if embryos stay too long, they tend to develop into very densely-packed blastulae flattened on the side that was against the dish. They may then develop into mild monsters.

Transfer injected embryos into a scratched Petri 3cm dish and let them develop in the dark at 19°C.

3.3.4 References:

1- Yann Le Petillon, Stéphanie Bertrand and Héctor Escrivà. Spawning induction and embryo micromanipulation protocols in the amphioxus *Branchiostoma lanceolatum*. Chapter 19 in « Brain Development: Methods and Protocols, Methods in Molecular Biology", vol. 2047, Springer Nature 2019, In press
2- Fuentes, M., M. Schubert, D. Dalfo, S. Candiani, E. Benito, J. Gardenyes, L. Godoy, F. Moret, M. Illas, I. Patten, J. Permanyer, D. Oliveri, G. Boeuf, J. Falcon, M. Pestarino, J. G. Fernandez, R. Albalat, V. Laudet, P. Vernier and H. Escriva (2004). "Preliminary observations on the spawning conditions of the European amphioxus (*Branchiostoma lanceolatum*) in captivity." J Exp Zoolog Part B Mol Dev Evol 302(4): 384-391.

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3.4 Selection of stable transformants in Ostreococcus tauri*,* Ostreococcus lucimarinus *and* Bathycoccus prasinos *…*

By Francois-Yves bouget, Laboratoire d'Oceanographie Microbienne. Banyuls sur Mer
Works for medx.doi.org/10.17504/protocols.io.zj2f4qe
Protist Research to Optimize Tools in Genetics (PROT-G)

3.4.1 Inclusion of cells in semi-solid agarose medium

1- Autoclave a solution of 2.1% low melting agarose in H2O. Keep at 65-90 °C in a water bath.

2- For each transformation prepare 8 Petri dishes (55 mm diameter) and 8 x 15 ml tubes each containing 9 ml of ASW plus the required selection. (G418 at 1 mg/ml).

3- Add 1 ml of LMP agarose to the 9 ml in one of the tubes. Close the tube, and mix gently by inverting.

4- Add 0.5 ml of overnight transformed cells (see protocol on transient transformation), quickly mix and gently pour into the plate. pay attention to avoid bubles . Repeat this process for all tubes.

5- Let the plates dry open in the flowhood for about one hour, so that the agarose solidifies.

6- Close the plates and transfer them to large square Petri dishes. Add wet paper towel to keep the chamber humid. Seal the square plates with medical tape. Place the square plates in the incubator for about 10 days.

3.4.2 Selection of transformants

7- Transformant colonies should appear after 10 to 21 days. Pick colonies using with cut-off yellow tips. Suck out the green colony. Take care not to include any cells from neighboring colonies.

8- Transfer the cells to 0,2 ml of ASW medium containing the selection (G418), in 96 wells microplate. Allow cultures to grow for 7 days in the culture incubator.

9- After one week transfer to 20 ml culture flasks and grow for 7 to 10 days. Stable integration into the genome by random insertion or homologous recombination can be detected using PCR (see Lozano et al, Plant Journal 2014). When the transgene containsa luciferase reporter, a first screening can be performed directly by measuring luminescene in a microplate luminometer.

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3.5 Transient transformation of Ostreococcus species (OTTH595, RCC809 and RCC802) and Bathycoccus

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Works for medx.doi.org/10.17504/protocols.io.g86bzze
Protist Research to Optimize Tools in Genetics (PROT-G)

3.5.1 Cell preparation

1) Starting from a culture of Ostreococcus tauri, RCC809 or Bathycoccus in stationary phase, innoculate cultures at 1 million cells/ml as determined by flow cytometry (Accuri C6 BD) in 200 ml plastic flasks in Artificial Seawater supplemented with Keller medium supplement (trace metals, vitamins, nitrate and Phosphate as described in Djouani Tahri et al., PloS ONE 2011). For each transformation (including control), you should plan on using 50 ml de culture in exponential phase.

2) Grow cells for 4 to 5 days depending on the light conditions, until they reach densities of 30 to 40.106 cells/ml.

3) Count cells by flow cytometry. Check by SyBR Green II straining that bacterial contamination is below 2%.

4) Transfer lcultures to 50 ml Falcon tubes.

5) Centrifuge at 8000g for 10 min at 4°C.

6) Remove the supernatant, resuspend the cell pellet in 1 ml de sorbitol 1M (pH 7.5) in H2O MQ, at 4°C.

7) Transfer the cell suspension to 1.5 ml eppendorf.

8) Centrifuge at 8000g for 10 min at 4°C.

9) Remove 900 µl of supernatant

10) Resuspend cells by gently pipetting.

3.5.2 Electroporation of the transgene

1) Add 5µg of transgene DNA to cell suspension. Keep on ice for 5 minutes. The transgene consists of the high affinity phosphate promoter fused to the firefly luciferase (see Djouani Tahri et al., PloS one 2011).

2) Transfer cells to a 2 mm electroporation cuvette (Biorad).

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3) Apply an electric field

For Ostreococcus tauri (OTTH595): capacitance: 25µF, resistance 600 Ώ, voltage 1.35KV.

For Ostreococcus sp RCC809: capacitance: 25µF, resistance 600 Ώ, voltage 1.4KV.

For Bathycoccus (RC4222): capacitance: 25µF, resistance 600 Ώ, voltage 1.5KV.

For Ostreococcus lucimarinus RCC802: capacitance: 25µF, resistance 600 Ώ, voltage 1.2KV.

4) Add 1ml of fresh culture Medium to resuspend the cells.

5) Add 40 ml of culture medium and transfer to a culture flask.

6) Incubate at 20°C overnight in a light incubator.

At this stage, transient transgene expression can be measured or stable transformants can be selected (see relevant protocols).

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3.6 Transient luciferase expression in Ostreococcus (OTTH595, RCC809) and Bathycoccus

By Francois-Yves Bouget, Valérie Vergé et Jean-Claude Lozano, Laboratoire d'Océanographie Micrbienne, Banyuls sur Mer.
Works for medx.doi.org/10.17504/protocols.io.hcib2ue
Protist Research to Optimize Tools in Genetics (PROT-G)

1) Prepare CCLR Buffer

Solution stock
Final concentration
Potassium phosphate 1 M, pH 7.8
100 mM
EDTA 500 mM, pH 8
1 mM
Triton X 100
1%
Glycérol 50%
10%
Cool the buffer on ice.

2) Start from the 40 ml transformation (see protocol on Transient transformation). Transfer cells in a 50 ml falcon tube

3) Centrifuge at 8000g for 10 min at 4°C

4) Discard the supernatant and resuspend cells in 300 µl of CCLR in 2ml eppendorf

5) Add 2 tungsten beads.

6) Break the cells in a tissue Lyser (2 x 45 sec. at 30 Hz).

7) Centrifuge for 5 minutes à 4°C, 6000g.

8) Transfer the supernatant in a new Eppendorf

9) Centrifuge for 20 minutes at 4°C, 13000gu

10) Save the supernatant and Keep on ice.

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3.6.1 Protein dosage using the bicinchoninic assay (BCA)

Principle: Proteins reduced Cu(II) to Cu(I) under alkaline conditions. Bicinchoninic acid is highly specific for Cu (I), which forms a purple complex that can be colorimetrically quantified at a 562 nM. Absorbance is proportional to the amount of proteins.

1) Mix 1 ml of bicinchoninic acid with 20 µl of copper sulfate at 4% (W/v) for each sample

2)For each sample, add 20 µl of protein extract to the 1m BCA reagent in 1.5 ml eppendof.

3) incubate tubes at 37°C for 30 min (you should include a BSA standard between 0 and 2 mg/ml).

4) Transfer samples in cuvettes for measurement by spectrophotometry of absorbance at 562 nm.

3.6.2 Luciferase assay

1) Prepare Luciferase assay reagent (LAR)

Stock solution
Final concentration
Tricine 1M pH 7.8
100mM
MgCl2 500mM
5 mM
EDTA 500mM pH8
100µM
DTT 1M
3.3mM
CoA sodium salt hydrate MM 767.53
270µM 2.07mg/10ml
D Luciférin 10mM
500µM
ATP dissodium salt hydrate MM 551 275.5 mg/ml
500µM

2) Keep LAR on ice in the dark until use

3) Transfer cell extracts in 96 wells white microplates (Nunc)

4) Place themicroplate in a luminoter (such as Berthold Centro)

5) Read the luminescence 5 secondes after automated injection of LAR

3.6.3 Quantification of luciferase expression

Normalize the luminescence value (RLU) by the amount of protein. You should include a background control (no cell extract).

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3.7 Mutagenesis protocols for Kordia algicida (heterotrophic bacterium)

(These protocols are inspired by Zhu et al. 2017 and currently adapted for *Kordia algicida*)

By West N, Larocque R, Thomas F, Jeanthon C, Lami R

3.7.1 Targeted genes: quorum sensing luxR

The objective is to check whether the Flavobacteria *Kordia algicida* can be subjected to similar mutagenesis protocols as those developed by Zhu et al. (2017) on *Zobellia galactanivorans*. The authors involved in this reference study are associated to these experiments.

3.7.2 Experimental procedures

Bacterial strains: *Kordia algicida* was the wild type strain used in this study. The donor strain of E. coli used for conjugative transfer of plasmids was S17-1 λ pir.

3.7.3 Chemicals and substrate materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich. Sodium alginate samples with M/G ratios of 0.5 and 2.0 were provided by Danisco (Landerneau, France). The G content was confirmed by ^{1}H-NMR spectroscopy to be 66% and 33% respectively (data not shown).

3.7.4 Growth conditions for genetic manipulations

Kordia algicida strains were grown at 30°C in Cytophaga medium (DSMZ medium 172) which consisted of 1.0 g L^{-1} yeast extract, 1.0 g L^{-1} tryptone, 24.7 g L^{-1} NaCl, 0.7 g L^{-1}KCl, 6.3 g L^{-1} $MgSO_4 \cdot 7H_2O$, 4.6 g L^{-1} $MgCl_2 \cdot 6H_2O$, 1.2 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.2 g L^{-1} $NaHCO_3$, pH 7.2. Marine conjugation medium consisted of 1.0 g L^{-1} yeast extract, 1.0 g L^{-1} tryptone, 5 g L^{-1} NaCl, 0.35 g L^{-1} KCl, 3.15 g L^{-1} $MgSO_4 \cdot 7H_2O$, 2.3 g $L^{-1}$$MgCl_2 \cdot 6H_2O$, 0.6 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.1 g L^{-1} $NaHCO_3$ (pH 7.2). Marine conjugation agar was identical except for the addition of 15 g agar L^{-1}. Marine conjugation medium has salt concentrations that both *E. coli* and *K. algicida* tolerate and was developed to allow conjugative transfer of DNA from *E. coli* into marine bacteria. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Antibiotics were used at the indicated concentrations when needed: ampicillin, 100 µg ml^{-1}; chloramphenicol, 20 µg ml^{-1}; erythromycin, 50 µg ml^{-1}; kanamycin, 35 µg ml^{-1}; tetracycline, 20 µg ml^{-1}.

3.7.5 Conjugative gene transfer into K. algicida

E. coli S17-1 λ *pir* strains containing mobilizable plasmids were grown overnight in LB at 37°C and *K. algicida* was grown overnight in Cytophaga medium at 30°C. *K. algicida* cells (10–20 ml) were harvested (3,700 × g, 10 min) and washed once with Cytophaga medium. *E. coli* cells (5–10 ml) were harvested (3700 × g, 10 min) and washed once with LB medium. *K. algicida* and *E. coli* cells were suspended in marine conjugation medium and mixed together (approximately 1:1 ratio; approximately 100 µl final volume), and spotted on 0.45 µm nitrocellulose filter membrane (Merck Millipore, MA, USA) that was overlaid on marine conjugation agar and allowed to dry. The membranes prevented *K. algicida* from penetrating into the agar and thus allowed maximum recovery of cells. Following overnight incubation

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at 30°C, cells were scraped off the filter membranes, diluted in Cytophaga medium and plated on Cytophaga agar containing erythromycin. Plates were incubated for 3 to 4 days at 30°C.

3.7.6 Deletion of K. algicida luxR (protocol currently tested in our lab – not optimized)

A 2 kb fragment including the first 54 bp of *luxR* and its upstream region was amplified using primers KALG0001 (introducing a PstI site) and KALG0002 (introducing a SalI site). The fragment was digested with PstI and SalII and ligated into pYT313, which had been digested with the same enzymes, to generate pKA1. A 2kb fragment including the last 36 bp of *luxR* and the downstream region was amplified using primers KALG0003 (introducing a SalI site) and KALG0004 (introducing a BamHI site). The fragment was digested with SalI and BamHI and ligated into pKA1, which had been digested with the same enzymes, to generate the deletion construct pKA3 (Fig. **1**). Preliminary experiments were performed to test if plasmid pKA3 could be introduced into *K. algicida* by conjugation using the *E. coli* strain S17-1 λ *pir* previously transformed with pKA3. Colony screening revealed the absence of *K. algicida* mutants and only *E. coli* colonies were detected. Future experiments will be aimed at optimising the liquid and solid media compositions to improve *K. algicida* growth thus improving the chances of a successful conjugation.

3.7.7 Construction of vectors for integrative complementation in K. algicida

Because the first conjugation attempts were not successful, subsequent experiments will aim to test the ability to deliver constructs into *K. algicida*, using a modified version of the pYT362 integrative plasmid (Zhu et al. 2017) that confers green fluorescence to successfully conjugated target cells. This plasmid contains the super folder *gfp* gene (sf*gfp*) under control of an *ompA* promoter region from *F. johnsoniae* and a 2.1 kbp fragment of the *Z. galactanivorans* genome. The pYT362 vector had to be modified to replace this latter region by a so-called Safe Harbor Site (SHS) in the *K. algicida* genome. SHS are regions where new genetic elements can be inserted without disrupting the regulation or expression of adjacent genes. A potential *K. algicida* SHS was identified by using the ICEfinder online tool to find regions with putative integrative and mobilizable elements. One such region containing a putative relaxase and integrase gene was amplified by primers incorporating BamHI and NheI restriction sites to create a 2.4 kb fragment. To facilitate insertion of the SHS fragment into pYT362, an NheI restriction site was first inserted upstream of the ompA promoter using the Quikchange Lightning Multi Site-Directed Mutagenesis kit (Agilent) to create pKA6. pKA6 was digested with BamHI and NheI and gel purified to remove the *Z. galactanivorans* region and subsequently ligated to the *K. algicida* SHS fragment, digested with the same enzymes to create plasmid pKA7. Following conjugation tests with pKA7, suicide vectors to test complementation of the luxR mutants will be created by replacing the sfGFP gene in pKA7 by the wild type promoter-less luxR genes by digestion with XbaI and SphI and ligation. In each case insertion of the suicide vector at the SHS will be confirmed by PCR.

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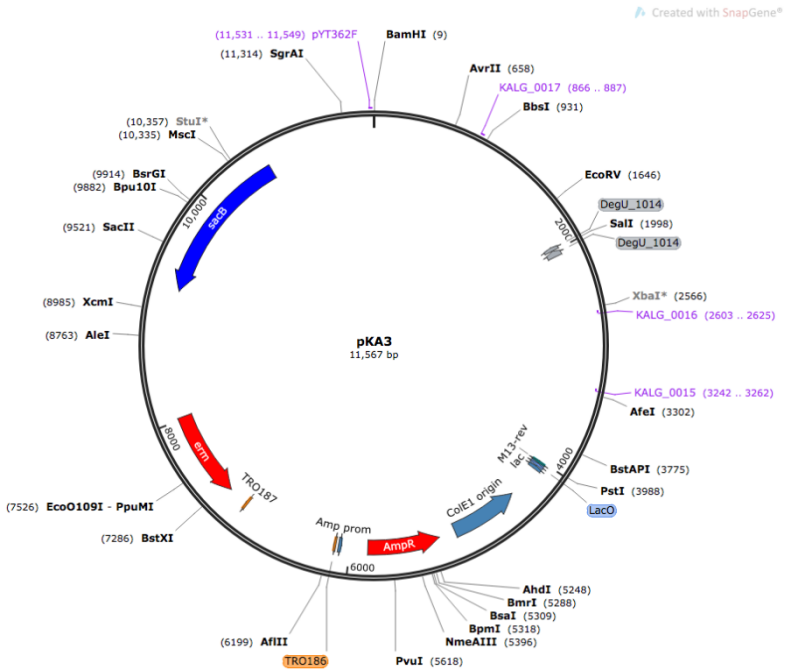

Fig 1. Map of pKA3 showing *K. algicida* lux R deletion construction. Note that this luxR gene was annotated as DegU by the Prokka bacterial genome annotation software (Seemann, 2014).

3.7.8 References

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. Bioinformatics, 14: 2068-9. doi: 10.1093/bioinformatics/btu153

Zhu, Y. , Thomas, F. , Larocque, R. , Li, N. , Duffieux, D. , Cladière, L. , Souchaud, F. , Michel, G. and McBride, M. J. (2017). Genetic analyses unravel the crucial role of a horizontally acquired alginate lyase for brown algal biomass degradation by *Zobellia galactanivorans*. Environ Microbiol, 19: 2164-2181. doi:10.1111/1462-2920.13699

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3.8 Genetic transformation of the diatom Cyclotella cryptica

(Adapted from Dunahay et al., 1995)

By Angela Falciatore, Sorbonne Université, Centre National de la Recherche Scientifique, Institut de Biologie Paris-Seine, Laboratory of Computational and Quantitative Biology, F-75005, Paris, France

3.8.1 Specific material:

Cyclotella cryptica CCMP332 culture, grown in 12h photoperiod, 30µE, 20°C, in L1 50% artificial sea water medium, +Si.
Tungsten M17 microcarriers (Bio-Rad)
PDS-/He Biolistic Particle Delivery System Stopping screens (Bio-Rad)
Rupture discs 1,550 psi (Bio-Rad)
Macrocarrier (Bio-Rad)
Plasmid DNA carrying a selective marker
CaCl2 2.5 M
Spermidine (Invitrogen)
Nourseothricin

3.8.2 Procedure:

Cells:

- Harvest cells in exponential phase (~3,6x10^5 cells/ml) by centrifugation (3000 g, 10 min).
- Remove supernatant and resuspend cells to have a concentration 1.4x10^8 cell/ml.
- Spread 500 µl of the cell suspension (~7x10^7 cells) on L1 50% artificial sea water +Si, 1% agar. Allow the plate with the cell suspension to dry. Store the plate at 18°C, 30 µmol m^{-2} sec^{-1} until the shoot.

Particle preparations:

- Tungsten M17 microcarrier particles (Bio-Rad), are prepared as indicated in the Bio-Rad PDS-/He Biolistic Particle Delivery System Instruction Manual, with 2 µg of plasmid DNA carrying selective marker; 50 µl 2.5 M CaCl2 and 1 µl 1 M spermidine.

Bombardment:

- Use 1550 psi rupture discs and follow Bio-Rad PDS-/He Biolistic Particle Delivery System Instruction Manual.
- Using the Heptadaptor, the DNA-coated particles are divided on the seven macrocarriers placed on the macrocarrier holder.
- Plate containing the cells is placed on the target shelf at 2 cm below the stopping screen, and the vacuum level is set to 25 psi
- Right after the shot, recover the cell by washing them off the plate with 1 ml of L1 50 % sea water +Si, and resuspend them in 25 ml of L1 50 % sea water +Si.
- Let the cells recover for 2 days in 12h photoperiod, 30 µmol m-2 sec-1, 18°C.

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Selection:

- Mix 1.6 ml of melted 4% agar solution with 40°C-prewarmed 25ml of L1 50 % sea water +Si, in order to have L1 50 % sea water +Si, 0.25 % agar, and add Nourseothricin antibiotic at a final concentration of 100 µg/ml. Keep the mixture at 37°C,
- Count the cell number of the suspension to add to the above L1-0.25% agar mixture $6x10^6$ cells (concentrate the suspension if you have to add more than one ml).
- When ready take the mixture out of the waterbath, let it cool down to ~33 °C. Add the cells; gently invert for mixing.
- Poor the L1 50 % sea water +Si, 0.25 % agar, Nourseothricin, mixed with the cell into plates.
- Place the plates in 12h photoperiod, at 30 µmol m-2 sec-1, 18°C.
- Colonies should start appearing after 3 weeks.
- Colonies can be picked up by pipetting them out of the matrix, resuspended in flasks in 1ml L1 50 % sea water +Si for molecular characterization.

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3.9 Transformation of Synechococcus by conjugation

By Morgane Ratin, Laurence Garczarek, Théophile Grébert, Charles Halouze, Station Biologique, Roscoff, France, in Transformation of *Synechococcus* by conjugation. **protocols.io** https://protocols.io/view/transformation-of-synechococcus-by-conjugation-7fvhjn6

3.9.1 Bacterial strain and plasmids [1]

- E. coli MC1061 is used as donor in conjugation
- pRK24: conjugal plasmid derivative of RK2. It contains the following genes: tra, trf and par and an oriT and is tetracycline and ampicillin resistant [2],
- pRL528: helper plasmid; carries mob, chloramphenicol resistant [3],
- pMUT100: integrative plasmid, suicide vector consisting of pBR322 containing kananamycin resistance cassette from pUC4K in PstI site, tetracycline and kananamycin resistant
- pRL153 replicative plasmid, RSF1010 derivative consisting of bp 680–2516 of Tn5 ligated to bp 2118–7770 of RSF1010, kananamycin resistant

3.9.2 Plasmids construction

- For deletion: plasmid derivative from pMUT100 containing an 800bp homologous region of the targeted gene, inserted for instance between BamHI and SphI sites. After conjugation and a simple crossing over event, the plasmid is integrated into the genome. *Synechococcus* sp. mutant are selected based on their kanamycin resistance.
- For complementation: plasmid derivative from pRL153, containing the targeted gene as well as the upstream and downstream conserved regions. *Synechococcus* sp. complemented clones are selected based on their kanamycin resistance.

3.9.3 Preparation of *Synechococcus* cells

- Grow cells in 500mL PCR-S11 in low light 20µE.m-2.s-1, 24°C
- Add pluronic to 0.005% final
- Spin down the cells at 4500g for 10 min, discard supernatant and carefully resuspend in a few mL of fresh PCR-S11
- Spin down again the cells and carefully resuspend in PCR-S11 to a final volume of 150µL

3.9.4 Preparing the E. coli cells

- Day -2: If starting from -20°C glycerol stock of MC1061 strain (carrying pRK24 + pRL528 + pMUT100 or pRL153 derivative plasmid), streak the conjugative strain on a selective LB Petri dish and grow overnight.
- Day -1: Grow a 50mL culture of MC1061 strain transformed with your plasmid in liquid LB with antibiotics (ampicilline 100µg/mL, chloramphenicol 34µg/mL, antibiotic corresponding to plasmid resistant) overnight at 37°C with vigorous shaking.
- Spin down the cells to get rid of all the culture medium, carefully resuspend into 1mL of LB (no antibiotics), spin down the cells again
- Repeat the previous step
- Carefully resuspend in PCR-S11 to a final volume of 250µL

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3.9.5 Conjugation

- Spot 5 50µL drops of MC1061 on a 0.6% washed agar Petri dish.
- Add on each drop successively 10, 20, 30, 40 and 50µL of *Synechococcus* cells, mix gently by pipetting.
- Split each of these 5 mixes into smaller drops (<10µL) on the agar.
- Wrap the plates with venting/micropore tape and place them at 30°C and low white light (~10µE.m-2.s-1) for 2 days

3.9.6 Plating

- Scrap the spots on the plates using a flame sterilized metal spatula and place them into PCR-S11 to have a 1020µL final volume. The spots should be thick and sticky. Let them sit in PCR-S11 for at least 30 min before carefully resuspending them.
- Prepare 6 small glass bottles of 0.3% autoclaved washed agar, let cool down in a water bath at 37°C.
- Let bottles stand for about 2 min at room temperature, add antibiotics and 170µL of cells, then pour into the Petri dish (repeat 5 times)
- Incubate at low light 20µE.m-2.s-1 and 24°C until colonies appear

3.9.7 References

[1] Brahamsha, B. 1996. A genetic manipulation system for marine *Synechococcus*. App. Environ. Microbiol. 62:1747-1751
[2] Meyer, R., Figurski, D. and Helinski, D.R. 1977. Physical and genetic studies with restriction endonucleases on the broad host range plasmid RK2. Mol. Gen. Genet. 152:129–135.

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3.10 Transformation of Synechococcus by electroporation

By Morgane Ratin, Laurence Garczarek, Théophile Grébert, Charles Halouze, Station Biologique, Roscoff, France, in Transformation of *Synechococcus* by electroporation. protocols.io https://protocols.io/view/transformation-of-synechococcus-by-electroporation-7fnhjme

3.10.1 DNA preparation

- Prepare 5 µg of purified DNA at 1 µg/µL
- Desalt DNA by drop dialysis on nitrocellulose membrane against clean water

3.10.2 Preparing *Synechococcus*

- Grow *Synechococcus* in 50 mL of PCR-S11 in low light (25 µE.m-2.s-1) until mid-log phase
- Add 25 µL of Pluronic 10%
- Centrifuge at 9000g and16°C for 7min, and re-suspend in 1 mL of resuspension buffer (Sorbitol 1M, 0.005% pluronic)
- Repeat 3 times
- Resuspend in 40 µL of resuspension buffer

3.10.3 Electroporation of the cells

- Put the 40 µL of *Synechococcus* in 1ml electroporation cuvette
- Add DNA
- Electroporation: Capacitor 25µF, Resistance 600Ω, Pulse 2.5kV (12.5kV/cm)
- Immediately after electroporation, add 1ml of PCRS11on the cells directly into the cuvettes.

3.10.4 Liquid Inoculation

- In culture tubes, add 250 µL of electroporated *Synechococcus* in 5 mL of PCR-S11 without Antibiotics
- Keep the cells overnight in low light
- The next day, in a deepwell, inoculate 12 different volumes (1, 2.5, 5, 10, 15, 20, 30, 40, 50, 70, 90, 100 µl) of electroporated *Synechococcus* in 1,5mL of PCR-S11 + antibiotic
- Keep the deepwell under low light (25 µE.m-2.s-1)

3.10.5 Plating

- Prepare 6 small glass bottles of autoclaved 0.3% washed agar, let them cool down in a water bath at 37°C.
- Let bottle at room temperature for 2 min, add antibiotics and electroporated cells (test different dilutions) and pour in the Petri dish (repeat 5 times for each electroporation)
- Incubate under low light 20µE at 24°C until colonies appear

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3.11 C. closterium *transformation*

By Wim Vyverman, Laboratory of Protistology and Aquatic Ecology, Department of Biology, Faculty of Science, Ghent University, Gent, Belgium.

3.11.1 Preparation of diatom cells

- Grow cells to a cell density of around 1x10^6 cells/ml
- Centrifuge 1x10^8 cells per agar plate to be transformed (~ 3,000 g, 10 min)
- Resuspend cells in 250-300 µl liquid culture medium per agar plate
- Using a wire loop spread the diatoms in a 5.2 cm diameter circle in the middle of the agar plate
- Allow the cells to dry onto the agar plate.

3.11.2 Coating of W-particles with plasmid DNA

a- Washing the W-particles:

- Resuspend 10 mg W-particles in 500 µl Ethanol
- Centrifuge 30 sec (Important to not over-centrifuge particles)
- Resuspend pellet in 250 µl Ethanol
- Vortex for 1-2 min
- Centrifuge 30 sec
- Wash pellet 3 times with 250 µl sterile water; following the last centrifugation resuspend in 150 µl H_20
- Divide the suspension in 50 µl aliquots

b- Coating the W-particles:

- To each 50 µl aliquot, **under constant vortexing add (prepare pipets!)**
  - 5 µl 1 µg/ul Plasmid DNA (if co-transformation, add 5 µg of each plasmid; adapt H_2O volume)
  - 50 µl 2.5 M $CaCl_2$
  - 20 µl 0.1 M Spermidine (free base, Sigma-Aldrich is the best one)
- Vortex continuously for 3 min
- Centrifuge 10 sec, remove supernatant
- Resuspend pellet in 250 µl Ethanol
- Vortex for 1 min
- Centrifuge for 10 sec, remove supernatant
- Resuspend pellet in 50 µl Ethanol
- Store on ice until use

3.11.3 Transformation:

- Once the diatoms have dried onto the agar plate transfer the DNA coated W-particles to the microcarrier: place a 10-20 (~15) µl drop of the suspension (mix very well!) onto the centre of

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the microcarrier (which is already placed in the microcarrier holder) and allow to air dry (about 10 min).

- Shoot the diatoms at 1550 psi –the vacuum inside the chamber has to be at the absolute maximum (~28).
- Transfer the diatoms from the agar plate into liquid culture. Transfer all of the cells from one agar plate into 300 ml liquid medium and combine the cells from the different shooting conditions into the one culture (without antibiotic).
- Incubate cells for 24 h under constant illumination.
- Determine the cell density
- Centrifuge sufficient cells to plate 0.5 x 10^7 cells per agar plate (containing 200 µg/ml ClonNAT – plates should only be made 1-2 days before use)
- Streak cells onto agar plate (use ~200 µl cells, avoid clumps of cells). Incubate plates for 8-14 days with constant light (not upside down)
- Transfer single colonies to 1.5 ml liquid culture (200 µg/ml ClonNAT) in a micro-titer plate.

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4. Conclusion

Here we present the first protocols 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.

Generally, we are now at a point where most if not all of the proposed biological models in the different tasks of the JRA3 can be transformed with more or less efficacy, and these advances let us now work on the development of the production of GMOs (both transgenesis and KO) in the different biological models.

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5. Appendices

5.1. Appendix 1

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5.2. Appendix 2

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