



Detection and Quantification of Small Noncoding RNAs in Marine Diatoms

Alessandra Rogato, Angela Falciatore

► To cite this version:

Alessandra Rogato, Angela Falciatore. Detection and Quantification of Small Noncoding RNAs in Marine Diatoms. *Marine Genomics*, 2498, Springer US, pp.315-326, 2022, *Methods in Molecular Biology*, 10.1007/978-1-0716-2313-8_17 . hal-03870492

HAL Id: hal-03870492

<https://hal.science/hal-03870492>

Submitted on 24 Nov 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Alessandra Rogato^{1,2*} & Angela Falciatore^{3*}

¹Institute of Biosciences and BioResources, CNR, Via P. Castellino 111, 80131
Naples, Italy.

²Stazione Zoologica Anton Dohrn, Integrative Marine Ecology Department, Villa
Comunale, 80121 Naples, Italy

³Laboratoire de Biologie du chloroplaste et perception de la lumière chez les micro-
algues, UMR7141, CNRS, Sorbonne Université, Institut de Biologie Physico-
Chimique, Paris, 75005, France

*Corresponding authors:

Alessandra Rogato: alessandra.rogato@ibbr.cnr.it

Email to: Angela Falciatore: angela.falciatore@ibpc.fr

Detection and quantification of small non-coding RNAs in marine diatoms

Alessandra Rogato^{1,2*} & Angela Falciatore^{3*}

¹Institute of Biosciences and BioResources, CNR, Via P. Castellino 111, 80131 Naples, Italy.

²Stazione Zoologica Anton Dohrn, Integrative Marine Ecology Department, Villa Comunale, 80121 Naples, Italy

³Laboratoire de Biologie du chloroplaste et perception de la lumière chez les micro-algues, UMR7141, CNRS, Sorbonne Université, Institut de Biologie Physico-Chimique, Paris, 75005, France

*Corresponding authors:

Alessandra Rogato: alessandra.rogato@ibbr.cnr.it

Angela Falciatore: angela.falciatore@ibpc.fr

Running Head

Protocols for detection and quantification of sRNAs in diatoms

Abstract

Endogenous small non-coding RNAs (sRNAs) are a large family of essential regulators of gene expression in both eukaryotes and prokaryotes. Various types of sRNAs with different size and mapping to different genome locations have been recently identified

in diatoms, a successful group of phytoplankton in the marine environment. However, their biogenesis and regulatory function are still largely unknown and unexplored in these microalgae, also due to the lack of methods for their experimental analysis. Herein, we present a point-by-point description of the protocols for detection and quantification of sRNAs by Northern-blot analysis and quantitative stem-loop RT-PCR, established in the diatom molecular model specie *Phaeodactylum tricornutum*.

Keywords

Diatom, Small non-coding RNA, Northern blot, Stem-loop qPCR

1. Introduction

In the organisms of the three domains of life, a plethora of small regulatory non coding RNAs (sRNAs) of around 20-32 nucleotide acts as major regulators of gene expression [1]. The most extensively studied sRNAs are the microRNAs (miRNAs) and small interfering RNAs (siRNAs). Both exert gene silencing effects via RNA-induced silencing complex (RISC) pathways [2].

In various organisms, next-generation sequencing (NGS) has provided an unprecedented opportunity to identify and quantify diverse kinds of sRNAs derived from different genomic location and regulating different cellular pathways. These sRNAs differ in their mode of biogenesis and in their function [3]. However, in order to exclude artefactual biases due to the library preparations or sequencing, and to get function insights of novel sRNAs categories identified in less established model organisms, the predicted sRNAs need to be experimentally validated.

Diatoms are prominent marine unicellular algae [4]. Although recent diatom genome analyses have highlighted the presence of unique combinations of genes, recruited during their history as secondary endosymbionts, as well as by horizontal gene transfer [5], very little is known about the mechanisms of regulation of gene expression in these algae and on the processes controlling adaptive responses to changing environment. In 2014, Rogato and colleagues [6] and Lopez-Gollom and co-workers [7] identified a plethora of sRNAs in three diatom species for which complete genome sequences have been assembled, annotated and published: *Phaedactylum tricornutum* [5], *Thalassiosira pseudonana* [8] and *Fragilariopsis cylindrus* [9]. By the high-throughput sequencing of short RNA fragments extracted from diatoms grown under different conditions of light and nutrients, different sRNA populations of different size (19-35

nucleotide) have been described [6,7]. These sRNAs map different genome localization such as tRNAs, Transposable Elements (TEs), intergenic region and small nuclear RNA (snRNAs). Canonical miRNAs were not found while TEs marked by DNA methylation represent the majority source of sRNAs. Comparative analysis of genome composition in diatoms, revealed strong variation in transposable element dynamics suggesting that TEs play a key role in genome evolution and gene function [10]. Rogato and co-workers suggested that the TE-derived sRNAs, differentially expressed in the different tested growth conditions, may affect TE transcripts by sequence complementarity, contributing to the regulation of stress responses. Furthermore, other sRNAs mapping tRNAs, intergenic regions and snRNAs were highly expressed and have been experimentally detected and validated. These sRNAs are likely implicated in the regulation of still uncharacterized genetic processes.

Here we describe in detail the optimized protocols for the detection and quantification of sRNAs expression by Northern-blot analysis and quantitative stem-loop RT-PCR [11] in the molecular model specie *P. tricornutum* exposed to different stress conditions. These protocols represent a useful support to further characterize the biogenesis of these sRNAs, and understand their molecular tasks and functions in diatoms and other phytoplanktonic organisms [6].

2. Materials

2.1 Diatom culture and medium

1. Cell culture flasks with vented filter cap.
2. Wild-type *P. tricornutum* culture.
3. F/2 medium containing: deionized sterile water, 20 g/l Sea salts, 1X F/2 solution [12], without silicate.

4. Artificial Sea Water (ASW): 410 mM NaCl, 20 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM KCl, 3 mM KNO_3 , 9.9 mM CaCl_2 , 0.03 mM Na_2EDTA , 3 mM glycylglycine, 1.9 μM Fe- NH_4 -citrate, 8.1 μM H_3BO_3 , 1.04 μM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.41 μM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.49 μM Na_2MoO_4 , 0.42 μM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 6.5 μM $\text{Na}_2\text{tartrate} \cdot 2\text{H}_2\text{O}$, 21.6 nM O_2Se , 33.6 μM KBr, 3.75 μM $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, 10 μM NaF, 0.65 μM $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 9.8 nM Na_3VO_4 , 0.4 nM $\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 8.25 mM Tris, pH was adjusted before autoclaving to 8.0 by adding HCl.

After sterilization add: 0.3 mM K_2HPO_4 , Vitamins: 0.1 $\mu\text{g/l}$ thiamine-HCl, 50 $\mu\text{g/l}$ B12, 50 $\mu\text{g/l}$ biotin, [13], without silicate.

2.2 RNA extraction

1. RNase Surface Decontaminant.
2. Ice cold Phosphate Buffer Saline 1X (PBS): 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , and 2 mM KH_2PO_4 .
3. Trizol Reagent (*see Note 1*).
4. Chloroform, molecular biology grade.
5. Isopropanol, molecular biology grade.
6. 75% ethanol RNase free.
7. Absolute ethanol, molecular biology grade.
8. DEPC-treated water.
9. 50 mL conical tubes.
10. Refrigerated centrifuge.

11. Centrifuge tubes (2 and 1,5 mL).
12. Micropipettes.
13. Sterile filter pipette tips.
14. Vortex mixer.
15. Spectrophotometer.
16. Heat block.
17. Agarose gel: molecular biology Agarose and 0,5X TAE buffer (Tris-acetate-EDTA) RNase-free obtained from a 50X TAE buffer stock containing: 50 mM EDTA sodium salt, 2 M Tris-HCl (adjust pH to 8.0) and 1 M acetic acid.
18. RNA Gel-loading dye: 50% glycerol, 50 mM Tris HCl (adjust pH to 7.7), 5 mM EDTA, 0,03% Bromophenol Blue.
19. UV Transilluminator.

2.3 RNA precipitation to enrich the small RNA fraction (not mandatory)

1. Total RNA.
2. 5-10% Polyethylene glycol (PEG) 200.
3. 0.5 M NaCl.
4. Absolute ethanol, molecular biology grade.
5. RNase-free water.
6. Microfuge tubes.
7. High-speed microcentrifuge.

2.4 Northern blot analysis of low molecular weight RNAs

1. Acrylamide Gel: 6 M Urea; 40% Acrylamide 19:1; 10X TBE Buffer (Tris Borate EDTA) RNase-free stock containing 0,02 M EDTA sodium salt, 1 M Tris-HCl (adjust pH to 8.0) and 1 M boric acid; Ammonium persulfate (APS); TEMED (*N'*-tetramethylethylenediamine).
2. N⁺ membranes.
3. Transfer Semi-dry system.
4. UV Crosslinker.
5. Hybridization oven.
6. Glass hybridization tubes.
7. Rectangular Pyrex dishes.
8. Orbital shaker.
9. Plexiglas shield.
10. Geiger counter.
11. Long metal forceps.
12. X-ray cassettes.
13. Phosphorimager and image eraser device.
14. Low Range RNA ladder.

2.5 Isotope labelling of oligo DNA probes and washing buffer (*see Note 2*)

1. γ -³²P-ATP.
2. Reverse complement oligonucleotide (20 to 32 nt) to the predicted sRNA, diluted to working concentration (10 μ M).
3. T4 Polynucleotide Kinase (T4 PNK).
4. 10X T4 Polynucleotide Kinase reaction Buffer.

5. Ready-to-use hybridization buffer for Northern (*see Note 3*).
6. Microfuge tubes.
7. Vortex.
8. High-speed microcentrifuge.
9. Sterile distilled water.
10. Wash Buffer: 2X SSC (Saline-sodium citrate) buffer, RNase-free obtained from a 20X SSC stock containing: 3 M Sodium Chloride, 0.3 M Sodium Citrate, (adjust pH to 7) and Sodium Dodecyl Sulfate (SDS).
11. U6sRNAs loading control oligo probe sequence: GCTAATCTTCTCTGTATC.

2.6 Reverse transcriptase reactions

1. Thermocycler.
2. Microfuge tubes.
3. PCR tubes.
4. Micropipettes.
5. Sterile filter pipette tips.
6. DNase (stored at -20 °C).
7. 10X DNase Buffer (stored at -20 °C).
8. Reverse Transcriptase (RT) (stored at -20 °C).
9. 10X Reverse Transcriptase Buffer (stored at -20 °C).
10. 2 mM dNTPs (obtained from a stock of 10 mM) (stored at -20 °C).
11. RT-loop Primer 1 μ M (obtained from a stock of 10 μ M) (stored at -20 °C).
12. DTT (DL-Dithiothreitol) to stabilize enzymes (stored at -20 °C).
13. Recombinant Ribonuclease Inhibitor RNaseOUT.

2.7 Stem-loop qPCR

1. Micropipettes.
2. Sterile filter pipette tips.
3. Real-time PCR thermal cycler.
4. Microcentrifuge.
5. PCR tubes.
6. Plates: 96 or 384 well plates.
7. Plate seals.
8. SYBR Green mix.
9. cDNA template.
10. 10 μ M Forward and reverse primers.
11. PCR grade water.
12. Oligos for Stem Loop qPCR :

Universal Reverse PCR primer: AGTGCAGGGTCCGAGGTATTC

SnoR85 sequence: GTGCATTCAAAAGCCCTTACATGT

3. Methods

3.1 Diatom cultures conditions

1. Start a *P. tricornutum* culture, growing it at 18 °C under 12h:12h day:night cycles. For Northern blot analysis shown in Figure 1, cells were grown under different conditions [6] Low Light (LL) (30 μ mol photons $\text{m}^{-2} \text{s}^{-1}$), Normal Light (NL) (80 μ mol photons $\text{m}^{-2} \text{s}^{-1}$); High Light (500 μ mol photons $\text{m}^{-2} \text{s}^{-1}$) for 5

hours (HL), dark for 60 hours (D) and under iron starvation (-Fe), either in F/2 media or ASW medium (see section 2 Materials).

2. Prepare F/2 liquid medium and for the iron starvation, growth cultures of *P. tricornutum* at low light, in ASW medium, containing 5 nmol/l Fe-NH₄-citrate as final concentration.
3. When the cells have reached the exponential phase of growth, collect 100 mL of culture. Per each condition, collect cell pellets by centrifugating the cultures in two conical tubes of 50 mL at 1300 x g at 4 °C for 10 min. Discard large part of the supernatant.
4. Transfer the cell pellet into 2 mL tubes, and spin again at 12000 x g at 4 °C for 3 min.
5. Remove the residual supernatant and wash quickly the cell pellet with 1 mL of ice-cold PBS 1X, from PBS Buffer 10X RNase-free stock, to remove salts
6. Spin at 12000 x g at 4 °C for 3 min.
7. Discard supernatant and freeze the cells with liquid N₂, then proceed with the extraction or store the cell pellet at -80 °C for months.

3.2 RNA extraction (see Note 4)

1. Lyse cells with 1,5 mL Trizol Reagent by repetitive pipetting.
2. Incubate the homogenized sample for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.
3. Centrifuge to remove cell debris.
4. Transfer the supernatant to new 2 mL tube.
5. *Phase separation*: Add 0.3 mL of chloroform per 1,5 mL of Trizol Reagent. Cap sample tubes securely. Vortex samples vigorously for 15 s and incubate them at room temperature for 15 min. Centrifuge the samples at no more than 12000 x g

for 15 min at 4 °C. Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Transfer upper aqueous phase carefully without disturbing the interphase into fresh 1,5 mL tube (*see Note 5*).

6. *RNA Precipitation*: Precipitate the RNA from the aqueous phase by adding isopropyl alcohol. Use 0.75 mL of isopropyl alcohol per 1,5 mL of Trizol Reagent used for the initial homogenization. Cap the tubes and invert several times to mix it. Incubate samples at Room Temperature (RT) for 10 min and centrifuge at 12000 x g for 10 min at 4 °C. Remove the supernatant completely. The RNA precipitate forms a gel-like pellet on the side and bottom of the tube.
7. *RNA wash*: Wash the RNA pellet once with 75% ethanol, adding 1,5 mL of 75% ethanol per 1,5 mL of Trizol Reagent used for the initial homogenization (*see Note 6*). Mix the samples by inverting the tubes and centrifuge at 12000 x g for 5 min at 4 °C. Repeat above washing procedure once. Remove all leftover ethanol.
8. *Redissolving RNA*: Air-dry or vacuum dry RNA pellet for 5-10 min. Dissolve RNA in RNase free water by passing solution a few times through a pipette tip.
9. *Spectrophotometric Analysis*: Take OD at 260 nm and 280 nm to determine sample concentration and purity. The A260/A280 ratio should be above 1.6.

3.3 Northern blot analysis

Perform Northern blot analysis of low molecular weight RNA, with 20 µg of total RNA (**Figure 1**).

1. *Sample preparation*: Start with 20 µg (up to 40 µg for low expressed sRNAs) of total RNA and fill it up with H₂O to get the same volume each tube. Then add 1

vol of Formamhhyde 100%, denaturate at 95 °C for 5 min and put in ice for another 5 min. Spin down and add ¼ volume of Gel-loading dye.

2. Separate the RNA extracted from cells on 14% acrylamide gel, by using a vertical gel electrophoresis system. Use combs of 1.5 mm and also corresponding 1.5 mm glass
3. Remove Urea in each well and pre-run at 100V for 30 min.
4. Remove again Urea in each well and load the samples.
5. Migrate the samples for 4 h at 80Volt in 0,5X TBE.
6. Transfer the RNA on a N⁺ membrane using a semi-dry system for 1 h at 80 Volt- 300mA.
7. *Probe preparation:* 9 µL water (RNase free), 3 µL of oligonucleotide complement to the sRNA at 10 µM, denaturate at 95 °C for 5 min and put in ice for another 5 min, add 2 µL of 10X PNK buffer, 5 µL of γ-³²P-ATP (50 µCU), then add 1 µL T4 PNK and put at 37 °C for 45-60 min.
8. After transfer, put the membrane on 2X SSC for 10 min.
9. Cross link in a UV Stratalinker. Turn on the UV Stratalinker. Place the membrane with RNA side up into the chamber. Close the door. Press Auto Cross Link. Press Start
10. Pre-hybridization with 20 mL of Hybridization buffer for at least 20 min at 42 °C
11. Load the probe into the hybridization buffer and hybridize the membrane over night with oligonucleotide complementary to the target sRNA sequence. The temperatures for the hybridization depending on the T_m of the probe (*see Notes 7, 8*).

12. *Washing*: wash 2 times for 20 min with 2X SSC, 0,2% SDS at 40 °C. If the radioactivity is still high performed another washing 2X SSC, 1% SDS for 10 min (*see Note 9*).
13. Remove blot from hybridization tube, wrap securely with Saran wrap and expose to Phosphoimaging screen or X-ray screen.
14. Scan screen after 5-10 h exposure and then expose overnight or several days for signal optimization
15. As reference loading control hybridized with radiolabeled probes recognizing the U6 snRNA gene. U6 snRNA is a spliceosome core component (**Figure 1**).

3.4 Stem-loop Reversed Transcription reaction

Because of their small size and possible low expression levels, detection of the small RNAs by traditional methods is technically demanding. The Stem-loop real-time reverse transcriptase polymerase chain reaction (qRT-PCR) can be used to quantify sRNA expression in diatoms, with a protocol adapted from [11]. The primers for these reactions required the design of a Stem-loop RT primer, and a set of primers complementary to the sRNA for the subsequent quantitative PCR amplification. The stem-loop RT primers bind the 3' portion of sRNA molecules, initiating reverse transcription of the sRNA and provide more specificity and sensitivity than linear primers owing to the base stacking and spatial constraints of the stem-loop structure.

The stem-loop RT primer is used to reverse transcribe the sRNA molecule using total RNA isolated from *P. tricornutum*. The qPCR is carried out with a forward primer of 21 nt specific for the sRNA target sequence and a universal reverse primer complementary to the sequence of the loop portion of an RT stem-loop primer (*see Note 10*) (**Figure 2**).

1. *Starting materials*: Total RNA obtained by Trizol extraction (see section 3.2), and suspended in RNase-free water.
2. *DNase treatment*: Prepare a mix as follow: 12 µg of RNA (x µL); DNase Buffer 10x (1 µL); RNase OUT (0,1 µL); DNase (2 µL) H₂O up to 10 µL. Incubate at 37 °C for 50 min. Add 2 µL Stop solution and incubate at 65 °C for 10 min, followed by 5 min on ice. The RT reactions contained 1 µg/µL of total RNA.
3. *Stem-loop reverse transcriptase reactions (see Note 14)*: Prepare Mix A as follow: 2 mM dNTPs (2 µL); 1µM RT-loop primer (1 µL); H₂O 10.4 µL. Heat at 70 °C for 5 min and then place the samples on ice for at least 5 min.
4. Prepare Mix B as follow: RT Buffer; RT enzyme; 0.1 M DTT; RNaseOUT.
5. Assemble MixA+MixB and add 1 µL of DNase-treated RNA (1 µg). Final volume 20 µL.
6. Perform the Stem-loop reverse transcription reaction as follows: 16 °C for 30 min, 30 °C for 30 sec, 42 °C for 30 sec, 50 °C for 1 sec for 60 cycles 85 °C for 5 min and holding at 4 °C (*see Note 11*).

3.5 Stem-loop qPCR

The 20 µL of the obtained cDNAs were used for the qRT-PCR, performed as described:

1. Prepare the SYBR Green master mix as follow (*see Note 12*): volume per single reaction 10 µL. 2X SYBR Green 1X Final 5 µL; (2.5 µM) Specific Forward Primer + Universal Revers Primer 2 µL; PCR grade H₂O 2 µL. Mix gently and spin down the solution.
2. Carefully aliquot 9 µL of master mix into each plate well, add 1 µL of specific

cDNA (*see* **Note 13**).

3. Mix reactions and spin.
4. Seal the PCR plate and label (according to instrument requirements).
5. Run samples as per instrument manufacturer recommendations using following protocol: initial denaturation 95 °C for 10 min; 45 cycles 95 °C for 15 sec, 60 °C for 20 sec, 72 °C for 40 sec. Perform a melting curve at the end of the program to check the amplification efficiency by steps of 1 °C (from 95 °C to 50 °C).

Run all samples in triplicate. Analyse data using the $2^{-\Delta\Delta C_t}$ method for relative expression [14], with the small nucleolar RNA SnoR85 as endogenous reference because not susceptible to silencing factors.

4. Notes

1. Always use gloves and eye protection. Trizol may cause severe damage, use it in a chemical hood. Avoid contact with skin or clothing, and avoid breathing vapor.
2. Always use gloves, eye protection and plexiglas shield. Avoid contact with skin or clothing.
3. This material was provided by Sigma Aldrich. The Sigma's PerfectHyb Plus is a ready-to-use hybridization buffer, up to 20x more sensitive than conventional buffers. In particular, it is recommended to use this buffer for low expressed sRNAs. A small amount of precipitate that has formed in the solution can be dissolved at 30-35 °C.
4. Work in an RNase-free environment, wash bench-top with MilliQ H₂O and 60% ethanol, spread RNase Surface Decontaminant.

5. Measure the volume of the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol Reagent used for homogenization.
6. The RNA precipitate can be stored in 75% Ethanol for 1 week at 4 °C, or at least 1 year at -20 °C.
7. The hybridization buffer containing the probe can be stored at -20 °C for 2 weeks and re-used.
8. Include the appropriate controls, by using the U6 probe.
9. Use 20 mL of washing solution.
10. The stem loop RT primer is specific to the individual sRNA, with a six-nucleotide extension at the 3' end that is reverse complement of the last six nucleotides at the 3' end of the sRNA. Forward primers are specific to the sRNA sequence but exclude the last six nucleotide at the 3' end of the sRNA. A 5' extension of 5-7 nucleotides is added to each primer to increase the melting temperature; these sequences are chosen randomly and are GC-rich.
11. For this step, prepare 1 sample extra to cover pipetting errors.
12. The 20 µL of cDNA obtained can be used for the stem-loop qPCR immediately or stored at least 1 week at 4 °C.
13. As control, include negative RT 1 µL and RNase free water 1 µL samples.
14. U2 sRNAs oligo probe sequence: GTACACGGTCAAGGCGGTGAGTG.
15. Oligos for Stem Loop qPCR:

U2- 5' mature sequence: ATTGGCTTTGATCTTGTGTAG

Forward U2-5' primer: CCGGCGATTGGCTTTGATCTT

RT-loop primer U2-5' sRNA:

GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCT

References

1. Alexander Hüttenhofer, Jörg Vogel, Experimental approaches to identify non-coding RNAs, *Nucleic Acids Research*. 2006;34,2:635–646.
2. Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. *Cell*. 2009;136,4:642-655.
3. Guleria P, Mahajan M, Bhardwaj J, Yadav SK. Plant small RNAs: biogenesis, mode of action and their roles in abiotic stresses. *Genomics Proteomics Bioinformatics*. 2011;9,6:183-199.
4. Falciatore A, Jaubert M, Bouly JP, Bailleul B, Mock T, Diatom Molecular Research Comes of Age: Model Species for Studying Phytoplankton Biology and Diversity, *The Plant Cell*. 2020;32,3:547–572.
5. Bowler C, Allen AE, Badger JH, Grimwood J, Jabbari K, Kuo A, Maheswari U, Martens C, Maumus F, Otillar RP, Rayko E, Salamov A, Vandepoele K, Beszteri B, Gruber A, Heijde M, Katinka M, Mock T, Valentin K, Verret F, Berges JA, Brownlee C, Cadoret JP, Chiovitti A, Choi CJ, Coesel S, De Martino A, Detter JC, Durkin C, Falciatore A, *et al*: The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature* 2008, 456:239–244.
6. Rogato A, Richard H, Sarazin A, Voss B, Cheminat Navarro S, Champeimont R, Navarro L, Carbone A, Hess WR, Falciatore A. The diversity of small non-coding RNAs in the diatom *Phaeodactylum tricornutum*. *BMC Genomics* 2014,15: 698.
7. Lopez-Gomollon S, Beckers M, Rathjen T, Moxon S, Maumus F, Mohorianu I,

- Moulton V, Dalmay T, Moch T. Global discovery and characterization of small non-coding RNAs in marine microalgae. *BMC Genomics* 2014,15:697.
8. Armbrust EV, Berges JA, Bowler C, Green BR, Martinez D, Putnam NH, Zhou S, Allen AE, Apt KE, Bechner M, Brzezinski MA, Chaal BK, Chiovitti A, Davis AK, Demarest MS, Detter JC, Glavina T, Goodstein D, Hadi MZ, Hellsten U, Hildebrand M, Jenkins BD, Jurka J, Kapitonov VV, Kroger N, Lau WW, Lane TW, Larimer FW, Lippmeier JC, Lucas S, *et al.* The genome of the diatom *Thalassiosira pseudonana*: ecology, evolution, and metabolism. *Science* 2004, 306:79–86.
 9. Mock, T., Otilar, R., Strauss, J. *et al.* Evolutionary genomics of the cold-adapted diatom *Fragilariopsis cylindrus*. *Nature* 2017, 541: 536–540.
 10. Maumus F, Allen AE, Mhiri C, Hu H, Jabbari K, Vardi A, Grandbastien MA, Bowler C. Potential impact of stress activated retrotransposons on genome evolution in a marine diatom. *BMC Genomics* 2009, 22;10:624.
 11. Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods*. 2007, 3:12.
 12. Guillard RRL. Culture of Phytoplankton for Feeding Marine Invertebrates, In: Smith, W.L. and Chanley, M.H. (eds.) 1975, *Culture of Marine Invertebrate Animals*, pp. 29–60 Springer US, Boston, MA
 13. Vartanian M, Desclés J, Quinet M, Douady S, Lopez PJ. Plasticity and robustness of pattern formation in the model diatom *Phaeodactylum tricornutum*. *New Phytol.* 2009, 182: 429-442

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001, 25,4:402-8.

Acknowledgments

This work was supported by funding from the European Assemble plus (Association of European Marine Biological Research Laboratories Expanded, H2020-INFRAIA-1-2016-2017), the Fondation Bettencourt-Schueller and the “Initiative d’Excellence” program (Grant “DYNAMO,” ANR-11-LABX-0011-01) to A.F.

Figures captions

Figure 1. Low molecular weight Northern blot analysis for the validation of the size and expression of sRNAs derived from the U2 snRNAs of *P. tricornutum* (chr5: 696.774–696.999). Total RNAs was extracted from cell cultures grown under different conditions: High Light (HL), Normal Light (NL), Low Light (LL), Dark (D) and iron starvation (-Fe). sRNAs mapping the U2-3' were detected using a 24 nt long oligonucleotide complementary to the sRNA (see **Note 14**). The U6 sRNAs were used as loading control. Two sRNAs of diverse sizes were detected, whose intensity varies in the different tested growth condition. The higher molecular weight band corresponds to the U2 sRNA precursor (Pre-sRNA), whose intensity remains unaltered across the samples. M, microRNA Marker. Modified from [6].

Figure 2. Schematic showing the stem-loop RT-PCR method for the relative transcript levels of low expressed sRNAs in *P. tricornutum*. (a) A stem-loop RT primer binds to the 3' portion of the sRNA, initiating reverse transcription in the presence of RT. (b) The RT product is amplified using an sRNA-specific forward primer and the universal reverse that is reverse complement of the last six nucleotides at the 3' end of the sRNA (c) The quantification is achieved by qPCR through SYBR Green (F) incorporation during amplification. (d) The quantification is achieved by measuring the fluorescence of the distinct reactions. The fluorescence increases at the level of the amplification products. (e) For the analysis performed in *P. tricornutum*, we have successfully used this approach to quantify a sRNAs called U2-5', mapping the 5' region of the chr5: 696.774–696.999 (modified from Figure 3C in [6]), not detected by Northern blot (see **Note 15**), contrary to the sRNAs U2-3' shown in Figure 1. The expression values of the U2-5' sRNA was normalized to the snoRNA.

FIG1

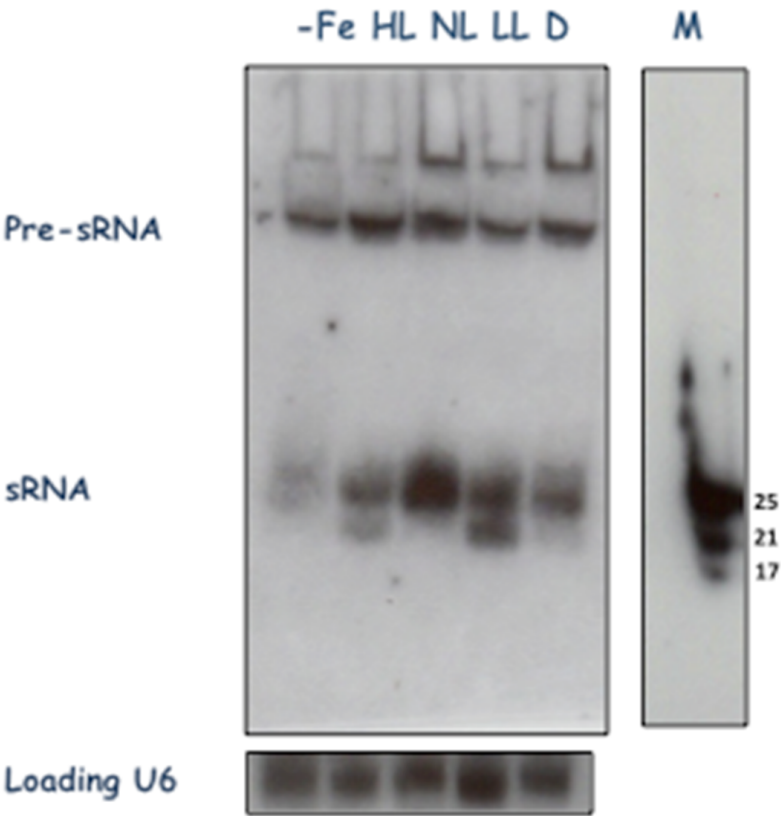


FIG2

