CARLA - Cellular Activity RNA-based eLisA

For the detection of toxigenic microalgae* in environmental water

*3 different kits available:

Alexandrium CARLA Kit	Cat. #CA-MA-01-00
Dinophysis CARLA Kit	Cat. #CA-MA-02-00
Pseudo-nitzschia CARLA Kit	Cat. #CA-MA-03-00

User Manual

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1. GENERAL DESCRIPTION

CARLA is an RNA-based sandwich hybridization coupled immunoassay for the detection of toxigenic microalgae from environmental water samples in less than 3 hours (incl. sample preparation).

2. INTENDED USE

CARLA allows the identification of presence/absence of living and active target micro-algae (qualitative assay) and/or the estimation of their cellular activity (quantitative assay) from water-extracted genetic material (RNA). The method is easy-to-use and follows standard molecular laboratory procedures. *Microbia Environment* provides basic training to first-time users on demand. A spectrophotometer (ELISA reader) is required for final read-out.

3. TECHNOLOGY

The CARLA test requires the concentration of microorganisms from water samples onto suitable filters and the extraction of total RNA from the collected material.

Step 1 – From total RNA eluates, ribosomal RNA (rRNA) of the target microalgae is recognized and hybridized by a sequence-specific probe attached to a microplate-type solid substrate (= capture probe).
Step 2 – A second free specific probe coupled with a peroxidase-conjugated antibody is used to form the sandwich hybridization triplet (= detection probe).
Step 3 – The addition of a substrate (TMB) for the peroxidase generates a redox colorimetric reaction detectable by absorbance measurement at 450 nm.
The intensity of the signal is proportionally related to the concentration of rRNA of the target present in the sample. The data interpolation is performed from a standard calibration curve.

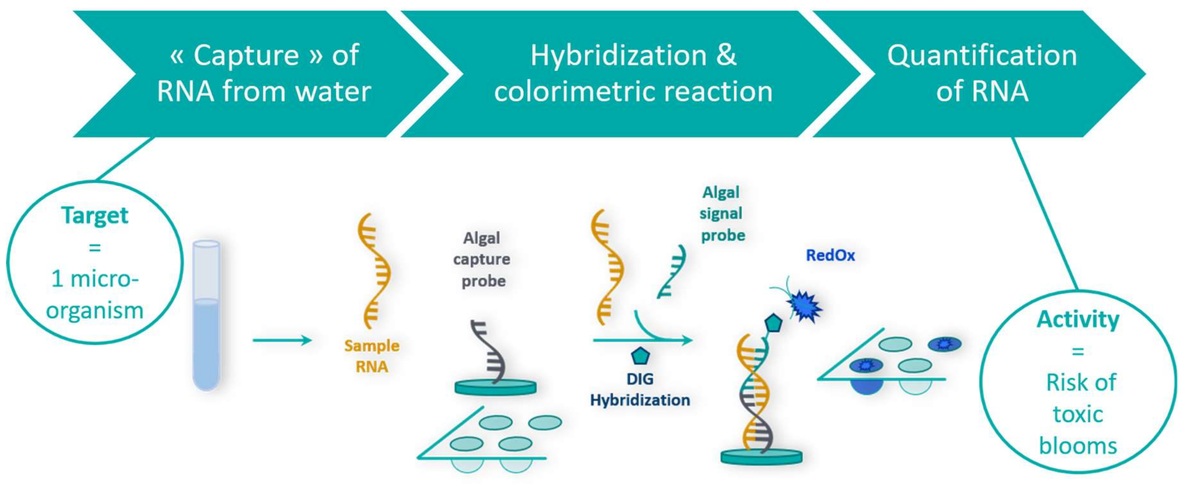

Figure 1 - Schematic view of the CARLA technology

4. KIT CONTENTS AND STORAGE

Upon receipt of the CARLA kit, please open the box and store the different components following the conditions indicated in the table below.

Component	Quantity	Container	Storage	T°C
Specific components				
96-well microtiter plate with pre-coated wells (capture probes) filled with conservation buffer	12 x 8 breakable strips	Alum pouch	6 months	4°C
Standard solutions	5 x 0.6 mL	Clear 2-mL microtubes	6 months	4°C
Hybridization Solution (signal probe + hybridization buffer)	2 x 1.2 mL	Clear 2-mL microtubes	6 months	4°C
Non-specific components				
Fragmentation Buffer	1 x 0.4 mL	Clear 0.5-mL microtube	6 months	4°C
STOP Fragmentation Solution	1 x 0.4 mL	Clear 0.5-mL microtube	6 months	4°C
Solution A = horseradish peroxidase-antibody conjugates	1 x 1.6 mL	Amber 2-mL microtube	6 months	4°C
Solution A dilution buffer	1 x 8.4 mL	Clear 10-mL flask	6 months	4°C
Solution B = peroxidase substrate (TMB)	1 x 10 mL	Amber 10-mL flask	6 months	4°C
STOP Solution = sulfuric acid	1 x 5 mL	Clear 5-mL flask	6 months	RT
Wash Buffer 5X	1 x 35 mL	Clear 60-mL flask	6 months	RT

5. ADDITIONAL MATERIALS REQUIRED

The following materials and/or equipment are NOT provided in the CARLA kit but are necessary to successfully conduct the experiment:

Sample preparation (see 6.2)

- Peristaltic pump
- Filters
- Tubes with beads and buffer (for cell lysis)*
- RNA extraction materials*

* Available within the *Microbia Environnement* - Sample Preparation Kit (50 prep.), cat. #ME-SP-50

Colorimetric test (see 7.)

- Microplate reader able to read absorbance at 450 nm.
- Incubator or Thermoshaker at 60±1°C
- Bench-top centrifuge
- Bench-top vortex
- Bead beater (optional)
- Orbital shaker (optional)
- Micro-pipettes with capability of measuring volumes ranging from 1 to 1000 µL and disposable plastic tips (1-1000 µL)
- Multi-channel pipettes (50-300 µL) or automated microplate washer
- RNase-free, sterile 1.5-2 mL microtubes
- 96-well PCR–type microplate frame (optional)
- Disposable reagents reservoir
- Cover tape for microplate
- Molecular biology water
- Molecular biology Ethanol (95-100%)
- Container with 200 mL capacity (for dilution of Wash Buffer 5X)
- Graduated cylinder
- Timer

6. BEFORE THE ASSAY

Before starting the assay, (1) identify the protocol suitable for your objective, (2) plan and proceed with the water sampling in accordance with your monitoring program, and (3) prepare RNA extracts from your water samples.

6.1. Test planification

	QUALITATIVE ASSAY	QUANTITATIVE ASSAY
Objectives	Rapid detection of active target microalgae	Determination of toxicity risk classes based on activity level of target microalgae
Applications	One-shot analyses Screening activities	Early warning system, when used for monitoring (repeated analyses)
Results		Cell activity Low High
Protocol (see section 7.)	**PROTOCOL A** No need of sample dilution	**PROTOCOL B** Need of 3 sample dilutions

Figure 2 - Protocol options for use of the CARLA kit

6.2. Water sampling and storage

- Collect 1 L of water per sampling point.
- To concentrate microorganisms, gently mix the collection flask/tube and immediately filter water through polycarbonate or nylon filters with a porosity of 10 µm (recommended for commodity, use lower porosity of 3-10 µm if relevant for your case study). Alternatively, store samples for up to 4 hours at room temperature with protection from direct light before filtration.
- Filter the water until clogging. Use several filters if necessary. A pre-filtration using filters with a porosity of 100-µm is recommended for water rich in coarse materials.

The *Microbia Environment* water sampler SOFiA has been designed for optimal filtration to run the test and is highly recommended. As a replacement, use a pump with the same characteristics (www.microbia-environnement.com/en/brochures-protocols).

- Fold and transfer each filter into a 2-mL tube filled with beads* (recommended ∅1.5 to 5-mm stainless beads or ∅1-mm glass beads).
- Immediately add **1 mL of RNA Lysis buffer***. The filter should be completely immersed in the buffer. Use one single tube for each filter. Filter(s) used during pre-filtration must be processed in the same conditions. Filters immersed in lysis buffer can be stored at room temperature for up to 72 hours. Beyond that, they must be stored at -20°C.

*Tubes with beads and lysis buffer provided within the Microbia Environnement - Sample Preparation Kit (50 prep.), cat. #ME-SP-50, see 6.3.2.

6.3. RNA sample preparation

6.3.1. Cell lysis

- Place samples at 60°C for 10 min. Shake each tube for 2 min (or vortex at max speed).
- Centrifuge for 30 s, collect the supernatant in a new tube and add **1 mL** of ethanol (95-100%) [1:1]$_{VOL}$. Mix well.

6.3.2. RNA extraction

We recommend to use the Microbia Environnement - Sample Preparation Kit (50 prep.), (cat. #ME-SP-50) and the following protocol adapted from *Quick*-RNA Miniprep Kit from *Zymo Research* (cat. # R1054-1055), for guaranteed final data interpretation (see 10.).

Perform all steps at RT and centrifugation at min 10,000 g for 30 s, unless specified.

- Transfer the cell lysate into a **Zymo-SpinTM IIICG Column** (green).
- Due to the column capacity (800 µL), proceed with 3 rounds of centrifugation/reload to pass the whole volume. Discard the flow-through each time and keep the column.
- Add **400 µL** of RNA Prep Buffer, centrifuge and discard the flow-through.
- Add **700 µL** of RNA Wash Buffer, centrifuge and discard the flow-through.
- Add **400 µL** of RNA Wash Buffer, centrifuge for 1min to ensure complete removal of the buffer and transfer the column into a 2-mL nuclease-free tube.
- For elution, add **100 µL** of DNA/RNA-free water (Molecular Biology Grade water) directly to the column matrix and centrifuge.

The eluted RNA can be used immediately or stored frozen at -20°C.
It is recommended to measure the total RNA concentration, e.g. by measuring the absorbance at 260 nm with a spectrophotometer.

7. ASSAY PROCEDURE

In this section, the complete protocol for performing the CARLA test using a **100-µL RNA eluate** with a duplicate read is presented. A short-version illustrated protocol including sample preparation is available on the *Microbia Environment* website. A calculation table is provided to facilitate the preparation of multiple samples and the use of replicates.
www.microbia-environnement.com/en/brochures-protocols

If not familiar with RNA handling, please refer to specific laboratory procedures (see 10.)

7.1. Preparation of reagents

All supplied solutions should be removed from storage one hour before use to allow them to reach room temperature (18-25°C).

Standards, Fragmentation buffer, STOP Fragmentation solution, Hybridization solution, Solution B and STOP solution are ready-to-use and do not require any further dilutions. Mix them gently and spin down in order to ensure homogeneity.

Solution A and Wash buffer must be diluted before use.

Preparation of 'working' Solution A: In sterile conditions, add the content of the stock Solution A (1.6 mL) to the dilution buffer (8.4 mL).

Preparation of 'working' Wash buffer 1X: In sterile conditions, add the content of the stock Wash buffer 5X (35 mL) in 140 mL of Molecular Biology Grade Water.

Label as appropriate. After use, store the solutions at +2-8°C.

7.2. Preparation of the microplate

In addition to the wells to be used for the standards, determine the total number of wells required based on the number of samples to be tested (Figure 3).

For both standards and samples, we recommend to work with at least duplicates.

Take the required number of strips from the pouch. Unused strips should be returned to the pouch and stored at +2-8°C.
Remove the storage liquid from selected strips by vigorously tapping the plate holder upside down (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells.

Important: The standards must be measured with each new test. Never use values of standards from an old test even carried out under the same conditions.

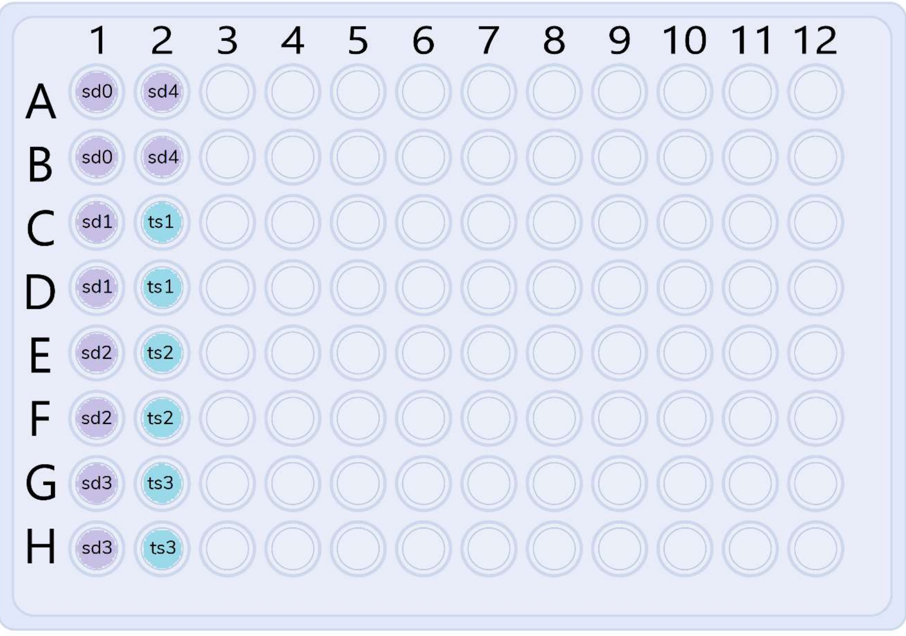

Figure 3- Example of microplate working scheme for 1-sample assay in duplicates (protocol B with 5 standards; sd0-sd4: standards for calibration curve, ts1-ts3: test sample dilutions)

7.3. Protocol A (QUALITATIVE)

If possible, all incubation steps should be performed on an orbital shaker to homogenize solutions.

7.3.1. RNA fragmentation

- Add **4 µL** of Fragmentation buffer to each 100-µL RNA eluate.
- Mix gently and place at 70°C for 10 min.
- Add **4 µL** of STOP Fragmentation solution.

Place tubes of fragmented RNA on ice or at +2-8°C while preparing the next step. If not processed immediately, keep samples frozen at -20°C.

7.3.2. Pre-hybridization

- Add **70 µL** of hybridization buffer + **30 µL** of Molecular Biology Grade Water to each tube of fragmented RNA (final volume: 200 µL).
- Mix well by pipetting up and down, place at 60°C for 10 min.

If many samples are run in parallel, prepare a large volume of pre-hybridization mix.
Example: for 5 samples in duplicates (10 units), prepare 500 µL of pre-hybridization mix (700 µL hybridization solution + 300 µL water), distribute 100 µL to each unit.

7.3.3. Transfer to the microplate > Hybridization

- Start filling the first 4 wells of the microplate with duplicates of standard solutions STD0 (negative control) and STD4 (positive control). Pipette directly 100 µL per well.
- Transfer and duplicate each pre-hybridized sub-sample in the following wells (100 µL per well).
- Cover the microplate (or strips on the support) with an adhesive film or an aluminium foil and incubate at 60°C for 10 min.

> Note: from here, indications are given for the full stripped microplate (or strips on the support). Volumes are given per well (no distinction between STD, samples, dilutions)

7.3.4. First wash

The washing technique is critical for the performance of the assay, it is therefore recommended to proceed with caution.

- After incubation, remove as much excess liquid from the wells as possible.
- Wash the wells 3 times with Wash buffer X1 taking care to completely fill and empty the wells at each wash cycle.
- Completely remove the remaining liquid from the wells by vigorously tapping the plate holder upside down (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells.

7.3.5. Antibody addition

- Pipette 100 µL of working Solution A into each well.
- Incubate the strips/plate at room temperature for 15 min.

7.3.6. Second wash

- Repeat the 3x washing cycles as intensively as described in 7.3.4.

7.3.7. Colorimetric reaction

- Pipette 100 µL of Solution B into each well.
- Incubate the strips/plate in the dark for 15 min at room temperature.
  The liquid in the wells will turn blue.
- Stop the reaction by adding 50 µL of STOP solution to each well.
  The color will change to yellow.
- Measure immediately the optical density (OD) in a microplate reader using a 450 nm filter.

7.4. Protocol B (QUANTITATIVE)

If possible, all incubation steps should be performed on an orbital shaker to homogenise solutions.

7.4.1. RNA fragmentation

- Add **4 µL** of Fragmentation buffer to each 50 µL RNA eluate.
- Mix gently and place at 70°C for 10 min.
- Add 4 **µL** of STOP Fragmentation solution.

Place tubes of fragmented RNA on ice or at +2-8°C while preparing the next step. If not processed immediately, keep samples frozen at -20°C.

7.4.2. Dilutions and Pre-hybridization

- Prepare 3 separate clean tubes and proceed with coupled sample pre-hybridization and dilutions as follows:

  Please, respect the sequential order and keep tubes on ice while pipetting.

	Tube 1	Tube 2	Tube 3
1. Fill tubes with hybridization solution	70 µL	70 µL	70 µL
2. Add Molecular Biology Grade Water	80 µL	105 µL	120 µL
3. Add RNA eluate from step 7.4.1	50 µL	25 µL	10 µL
Resulting sample dilution (Final volume 200 µL)	1/2	1/4	1/10

- Mix well by pipetting up and down, and place at 60°C for 10 min.

7.4.3. Transfer to the microplate > Hybridization

- Start filling the first 10 wells of the microplate with duplicates of the 5 standard solutions (STD0 to STD4). Pipette directly **100 µL** per well.
- Transfer and duplicate each pre-hybridized sub-sample in the following wells (**100 µL** per well).
- Cover the microplate (or strips on the support) with an adhesive film or an aluminium foil and incubate at 60°C for 10 min.

Note: from here, indications are given for the full stripped microplate. Volumes are given per well (no distinction between STD, samples, dilutions)

7.4.4. First wash

The washing technique is critical for the performance of the assay, it is therefore recommended to proceed with caution.

- After incubation, remove as much excess liquid from the wells as possible.
- Wash the wells 3 times with Wash buffer X1 taking care to completely fill and empty the wells at each wash cycle.
- Completely remove the remaining liquid from the wells by vigorously tapping the plate holder upside down (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells.

7.4.5. Antibody addition

- Pipette 100 µL of working Solution A into each well.
- Incubate the strips/plate at room temperature for 15 min.

7.4.6. Second wash

- Repeat the 3x washing cycles as intensively as described in 7.3.4.

7.4.7. Colorimetric reaction

- Pipette 100 µL of Solution B into each well.
- Incubate the strips/plate in the dark for 15 min at room temperature.
  The liquid in the wells will turn blue.
- Stop the reaction by adding 50 µL of STOP solution to each well.
  The color will change to yellow.
- Measure immediately the optical density (OD) in a microplate reader using a 450 nm filter.

8. INTERPRETATION OF RESULTS

Results are expressed as optical density (OD) measurements using a microplate reader.

8.1. Protocol A (QUALITATIVE)

8.1.1. Assay acceptance criteria

Based on mean values for negative control (Standard 0) and positive control (Standard 4)

OD_{450} STD 0	< 0.150
OD_{450} STD 4	> 0.500

If the values of STD 0 and STD 4 do not meet these criteria, the test is not considered valid and must be repeated.

8.1.2. Determination of positive results

- Subtract the mean OD value of STD 0 to each OD reading.
- Samples with a final $OD > 0.05$ are considered positive, which means that the target is present and active in the water collected at the sampling point. If $OD < 0.05$, the sample is negative and the organism is not detectable.

$$OD_{450} \text{ sample} - OD_{450} \text{ STD } 0 \geq 0.1$$

8.1.3. Consideration of replicates

- In the context of screening studies or one-shot analyses, carefully consider the results giving one of the duplicates as positive and not the other. For more accuracy, repeat the test or follow Protocol B to quantify the activity of the targeted microalgae.
- If you used more than 2 replicates per sample, you may consider whether the mean OD value is acceptable (standard deviation < 10%) to possibly exclude outliers.

8.2. Protocol B (QUANTITATIVE)

Create a standard curve by plotting the OD reading values for each standard solution against the corresponding concentrations (refer to labels on tubes). Estimate the best fit regression.

8.2.1. Acceptance criteria

Based on mean values for negative control (standard 0) and positive control (standard 4)

OD_{450} STD 0	< 0.150
OD_{450} STD 4	> 0.500
R^2	> 95%

If the values of STD 0, STD 4 and R^2 do not meet these criteria, the test is not considered valid and must be repeated.

8.2.2. Determination of activity-based toxicity risk classes

- Subtract the mean OD value of STD 0 to each OD reading.
- Samples with a final OD > 0.05 are considered positive, which means that the target is present and active in the water collected at the sampling point. If OD < 0.05, the sample is negative and the organism is not detectable.

$$OD_{450} \text{ sample} - OD_{450} \text{ STD } 0 \geq 0.1$$

- Use the table below to determine the toxicity risk class taking into account the dilution factors of your sample.

	OD_{450} sample – OD_{450} STD 0			
Dilution 1/2	< 0.1	> 0.1		
Dilution 1/4	< 0.1		> 0.1	
Dilution 1/10	< 0.1			> 0.1
Toxicity risk	ABSENCE	LOW	MODERATE	HIGH

8.2.3. Quantification of the cellular activity

- Multiply each value by the dilution factor, and calculate the mean value from the different dilutions.
- Calculate the mean value between duplicates (or replicates), and carefully examine acceptance criteria (standard deviation < 10%) to possibly exclude outliers.

- Use the standard curve equation to normalize the value and estimate the amount of RNA from your sample. Standard concentrations are indicated directly on the tube labels.
  Note: as the standard solutions are prepared from synthetic nucleic acid sequences, and not directly from environmental or microalgae culture RNA, the final RNA concentration must be expressed in Equivalent-Units (RNA Eq.U).

The data acquisition described with Protocol B can be integrated in monitoring programs to track trends in target microalgae activity over time. Usually, analyses once or twice a week provide sufficient time resolution to anticipate toxic blooms (c.a. 7 days ahead a peak).

Important precaution: repeat standard calibration for each new experiment and normalize data against each new set of standard lots.

9. SAFETY

- The CARLA kit should be handled by persons trained in laboratory techniques and used according to the principles of good laboratory practice.

- The reagents provided in the CARLA kit may be harmful if swallowed, inhaled or absorbed through the skin. Despite low volume/concentration, please take special precaution with the Solution B and the STOP Solution:

  The Solution B contains 3,3',5,5'-Tetramethylbenzidine (TMB). Avoid contact with skin or mucous membranes. Wash off immediately with large amount of water if the solution comes in contact with skin or mucous membranes.

  The STOP Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear appropriate eye, hand and face protection when handling this material. When the experiment is complete, be sure to rinse the plate with copious amounts of running water to dilute the STOP Solution before discarding the plate.

10. PRECAUTIONS

- If you are unfamiliar with RNase-free laboratory conditions, please take the following basic precautions to avoid contamination from human hands, dust, or bacterial origin:
  - Treat the benches and clean the pipettes with an RNase decontamination solution (such as 0.1M NaOH or specific commercial solutions)
  - Wear gloves throughout the test and change them each time after touching skin, door handles, and common surfaces.

- Reagents are supplied at fixed working concentration. Optimum sensitivity and specificity will be reduced if reagents are modified or not stored under the recommended conditions.
- Do not mix different lots of reagents.
- Avoid microbial contamination of opened reagent bottles.
- Make sure that no cross contamination occurs between the wells.
- It is essential for the correct performance of the test that solution A doe not contaminate other reagents and equipment.
- Make sure that kit components are not exposed to temperatures > 40°C.

11. ASSAY RESTRICTIONS

- The CARLA kit is intended for research use only, not for diagnostic or clinical procedures in human patients.
- The materials included in the CARLA kit should not be used after the expiration date indicated on the kit label.
- The reagents or substrates included in the CARLA kit must not be mixed or replaced by reagents or substrates from other kits.
- Variations in pipetting technique, washing technique, operator's laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.
- The test is designed to eliminate interference and background by non-target RNA sequences or factors present in any biological samples. However, the possibility of background noise cannot be fully ruled out until all factors have been tested using the test kit.