CARLA - **C**ellular **A**ctivity **R**NA-based e**L**is**A**

For the detection of toxigenic cyanobacteria* in environmental water

*Four different CARLA kits are available:

Microcystis	for *Microcystis sp*. (genus)	# CA-CY-01-01
Planktothrix	for *Planktothrix sp*. (genus)	# CA-CY-02-01
ADA	for *Anabaena-Dolichospermum-Aphanizomenon* (clade)	# CA-CY-03-01
Cylindrospermopsis	for *Cylindrospermopsis sp*. (genus)	# CA-CY-04-01

User Manual

Version 1.1
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GENERAL DESCRIPTION

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

INTENDED USE

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

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 cyanobacteria 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 target rRNA present in the sample. The data interpolation is performed from a standard calibration curve.

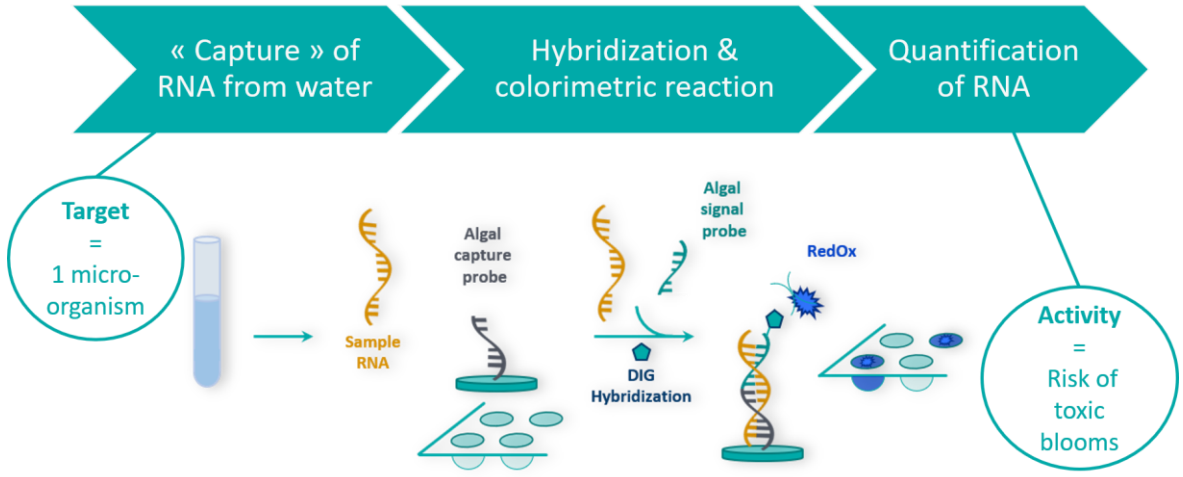


Figure 1 - Schematic view of the CARLA technology

TEST PLANIFICATION

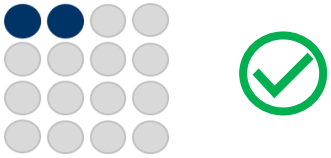
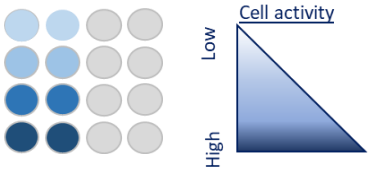
	QUALITATIVE ASSAY	QUANTITATIVE ASSAY
Objectives	Rapid detection of active target cyanobacteria	Determination of toxicity risk based on activity level of target cyanobacteria
Applications	One-shot analyses Screening activities	Early warning system, when used for monitoring (repeated analyses)
Results		
Sample dilution	No need of sample dilution	Sample dilutions required in case of saturated signal

Figure 2 - Protocol options for use of the CARLA kit

KIT CONTENTS AND STORAGE

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

Important! In case you plan to use different CARLA kits at once (to target different cyanobacteria groups = Multiple assay, see APPENDIX B), please note that some components are specific to one target cyanobacteria group and should be used exclusively for its detection, while other components are non-specific and can be used, mixed and prepared as bulk with no particular attention.

Component	Quantity	Container	Storage	T°C
Specific components				
96-well microtiter plate with pre-coated wells (capture probes)	12 x 8 breakable strips	Alum pouch	6 months	4°C
Standard solutions	5 x 1,25 mL	Clear 2-mL microtubes	6 months	4°C
Signal probe (in Hybridization buffer)	2 x 1.75 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 1.7 mL	Amber 2-mL microtube	6 months	4°C
Solution A dilution buffer	1 x 8.5 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 6 mL	Clear 5-mL flask	6 months	RT
Wash Buffer 5X	1 x 50 mL	Clear 60-mL flask	6 months	RT

Component	Concentration (ng/L)		
	Microcystis sp.	***Planktothrix sp.***	***Anabaena-Dolichospermum-Aphanizomenon sp.***
Standard 0	0	0	0
Standard 1	5	5	5
Standard 2	10	10	25
Standard 3	25	25	50
Standard 4	75	75	125

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

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

* The *Microbia Environment* water sampler SOFiA has been designed for optimal filtration to run the test and is highly recommended (see APPENDIX C). As a replacement, use a pump with the same characteristics.

**Available within the Microbia Environnement - Sample Preparation Kit (see APPENDIX C).

Colorimetric assay

- Microplate reader able to read absorbance at 450 nm.
- Incubator or Thermoshaker at 60±1°C and 30±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

SAMPLE PREPARATION

Water sampling, filtration and storage

1. Collect 20 mL of water per sampling point for each target cyanobacteria group (for volume adjustments to Multiple Assay, see APPENDIX B).

2. To concentrate microorganisms, gently mix the collection flask/tube and immediately filter water through filters with a porosity of 1 µm (polycarbonate or nylon filters).

   > Alternatively, keep water samples at ambient temperature with protection from direct light before filtration, e.g., in an isothermal box (< 4 hours max). Do not use a cool box!

3. Filter the water until clogging. Use several filters if necessary. A pre-filtration using filters with a porosity of 10 to 100-µm is recommended for water rich in coarse materials.

4. Fold and transfer each filter into a 2-mL tube filled with RNA Lysis or conservation 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. Store sample following manufacturer protocol.

   > *Available within the *Microbia Environnement* - Sample Preparation Kit (See APPENDIX C)

RNA extraction

5. Perform cell lysis and RNA extraction following manufacturer protocol.

   > We recommend to use the *Microbia Environnement* - Sample Preparation Kit (See APPENDIX C) and the following protocol adapted from *NucleoSpin RNA* Kit from *Macherey-Nagel* (cat. # 740955.50) (see APPENDIX A), for guaranteed final data interpretation.

6. Elute RNA in a final volume of 100 µL of Molecular Biology Grade water for each target cyanobacteria group (see APPENDIX B). The eluted RNA can be used immediately or stored frozen at -20°C for 48h max.

7. Estimate the total RNA concentration, e.g., by measuring the absorbance at 260 nm with a spectrophotometer (not mandatory).

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).
Solution A and Wash buffer must be diluted before use. The others solutions are ready-to-use, mix them gently in order to ensure homogeneity.

Preparation of 'working' Solution A

8. In sterile conditions, add the content of the stock Solution A (1.7 mL) to the dilution buffer (8.5 mL). After use, store the solution at +2-8°C.

Preparation of 'working' Wash buffer 1X

9. Add the content of the stock Wash buffer 5X (50 mL) in 200 mL of Molecular Biology Grade Water. After use, store the solutions at RT.

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. 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.

10. Take the required number of strips from the pouch. Unused strips should be returned to the pouch and stored at +2-8°C.
11. Add 100 µL of Washing buffer 1X in the wells. Wait for ca. 1 min and remove the buffer from selected strips by vigorously tapping the plate holder upside down against absorbent paper to ensure complete removal of liquid from the wells.

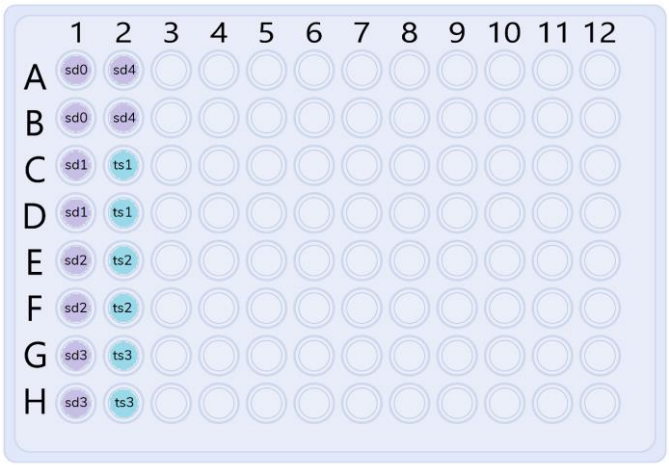


Figure 3- Example of microplate working scheme for 1-sample assay in duplicates with 5 standards; sd0-sd4: standards for calibration curve, ts1-ts3: test sample dilutions.

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 calculation table is provided to facilitate the preparation of multiple samples in parallel and/or of simultaneous use of different CARLA kits as Multiple assay (see APPENDIX B).
*If not familiar with RNA handling, please refer to the **PRECAUTIONS** section. If possible, all incubation steps should be performed on an orbital shaker to homogenise solutions.*

RNA fragmentation

12. Add **4 µL** of Fragmentation buffer to each 100 µL RNA eluate.

    > Attention! Do not miss to multiply accordingly for more than 1 target cyanobacteria group (see APPENDIX B).

13. Mix gently and place at 70°C for 10 min.
14. Add **4 µL** (same volume) of STOP Fragmentation solution to each 100 µL RNA eluate.
15. Place tubes of fragmented RNA on ice or at +2-8°C while preparing the next step.

RNA dilution

16. For each fragmented RNA tube, prepare two new 1.5 mL tubes and perform dilutions 1/10 and 1/100 with Molecular Biology Grade Water

    > This dilution step is recommended but not mandatory. It provides more accuracy to determine the linearity of the signal and to calculate the final rRNA concentration with the best fit to the calibration curve. However, to lighten the experimental procedure, it is possible to read non-diluted samples only and to decide – if the signal is too high – for a second read of diluted samples afterwards.

Attention! Specific kit components are used from this step. If you perform a multiple assay, the volume of fragmented RNA needs to be divided by the number of target cyanobacteria groups. Each sub-sample should be mixed with the corresponding specific signal probe (in hybridization solution) as described in the next step.

Pre-hybridization

17. Pipette **90 µL** from each of the 3 tubes resulting from previous step (non-diluted, dilution 1/10, dilution 1/100) into 3 new tubes.
18. Add **70 µL** of hybridization buffer with **50 µL** of Molecular Biology Grade Water to each tube (Final volume ≈ 210 µL).
19. 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.
    > Run pre-hybridization in a PCR-type plate.

Hybridization

20. 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.
21. Transfer and duplicate each pre-hybridized sub-sample in the following wells (100 µL per well).
22. Cover the microplate (or strips on the support) with an adhesive film or an aluminium foil and incubate at 60°C for 10 min.

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

First wash

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

23. After incubation, remove as much excess liquid from the wells as possible.
24. Wash the wells 3 times with 200 µL of Wash buffer X1 taking care to completely fill and empty the wells at each wash cycle.
25. 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.

Antibody addition

26. Pipette 100 µL of working Solution A into each well.
27. Incubate the strips/plate at 30°C for 15 min.

Second wash

28. Repeat the 3x washing cycles as intensively as described in 21-23.

Colorimetric reaction

29. Pipette 100 µL of Solution B into each well. Incubate the strips/plate in the dark at 30°C for 15 min. The liquid in the wells will turn blue.
30. Stop the reaction by adding 50 µL of STOP solution to each well. The colour will change to yellow.
31. Measure immediately the optical density (OD) in a microplate reader using a 450 nm filter.

INTERPRETATION OF RESULTS

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

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	> 1.000
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.

Determination of the cellular activity level

- Subtract the mean OD value of STD 0 to each OD read.

  > If an experimental blank has been processed (e.g., use of pure water in place of a test sample), it is recommended to rather subtract this OD value to each OD reading.

- Samples with a final OD > 0.100 are considered positive, which means that the cyanobacteria target is present and active in the water collected at the sampling point. If OD < 0.100, the sample is negative and the organism is not detectable.

OD_{450} sample – OD_{450} STD 0* ≥ 0.100 (*or blank)

- Use the table below to determine the cellular activity level taking into account the dilution factors of your sample. Consider if the signal shows a certain degree of linearity between dilutions for relevance.

	OD_{450} sample – OD_{450} STD 0* (*or blank)			
Dilution 1	< 0.100	> 0.100		
Dilution 1/10	< 0.100		> 0.100	
Dilution 1/100	< 0.100			> 0.100
Activity	NO	LOW	MODERATE	HIGH

Quantification of the cellular activity

- The evaluation of the assay can be performed using the calculation shit provided by *Microbia Environnement* (available on demand).

- Calculate the mean absorbance value for each of the standards and subtract the STD0 value. Construct a standard curve by plotting the OD value for each standard on the y-axis versus the corresponding RNA concentration (ng/L) on the x-axis, and define the correlation coefficient (a). Standard concentrations are indicated directly on the tube labels. Samples showing a lower OD than standard 1 should be reported as containing <5 ng/L. Samples showing a higher concentration than standard 4 (75 or 125 ng/L depending on cyanobacteria target) must be diluted to obtain accurate results.
- For each sample, the cellular activity expressed in ng RNA/L of water, is determined by dividing the mean blank-corrected OD relative to each dilution factor by the correlation coefficient a. To normalise the RNA concentration in the microplate well to the actual RNA concentration in filtered water sample, a coefficient of 0.0054 is finally applied.

$$\bar{X}\left(\frac{OD * dilution\ factor}{a}\right) * 0.0054$$

with, OD : sub-sample mean absorbance, a : calibration correlation coefficient, and dilution factors of 2.33 (dilution 1), 23.33 (dilution 1/10), 233.33 (dilution 1/100).

- If the initial volume of filtered water (FILT Volume) differs from the volume indicated at step 1 of the protocol (PR Volume), i.e., 20 mL / target cyanobacteria, apply a correction as follows.

$$\overline{X}\left(\frac{OD * dilution\ factor}{a}\right) * 0.0054 * \frac{FILT\ Volume}{PR\ Volume}$$

The data acquisition can be integrated in monitoring programs to track trends in target cyanobacteria activity over time. Usually, analyses one or twice a week provide sufficient time resolution to anticipate blooms (c.a. 5-7 days ahead a peak). The colored-scale of activity level can be used as *a priori* risk classes, but adjustments are necessary depending on the category of risk to be assessed (bloom an/or toxicity) and use cases of the investigated water resource (e.g., bathing water, recreative water, drinking water).

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

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.

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.

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.

APPENDIX A

Lysis and RNA extraction Protocol

Sample Preparation Kit (50 prep.)	**#ME-SP-50**

Follow step 1-4 (Sample Preparation) to collect, filter and store samples.

Cell lysis

1. Insert each filter into a 2-mL tube filled with beads.
2. 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.
3. Place samples at 60°C for 10 min. Shake each tube for 2 min (or vortex at max speed).
4. Centrifuge for 30 s, collect the supernatant in a new tube and add 1 mL of ethanol 70% [1:1]$_{VOL}$. Mix well.

RNA extraction

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

5. Transfer the cell lysate into a NucleoSpin RNA Column (blue).
6. Due to the column capacity (750 µL), proceed with 3 rounds of centrifugation/reload to pass the whole volume. Discard the flow-through each time and keep the column.
7. Add 200 µL of Buffer RAW2, centrifuge and discard the flow-through.
8. Add 600 µL of Buffer RA3, centrifuge and discard the flow-through.
9. Add 250 µL of Buffer RA3, centrifuge for 2 min to ensure complete removal of the buffer and transfer the column into a 2-mL nuclease-free tube.
10. For elution, add 100 µL of DNA/RNA-free water (Molecular Biology Grade water) directly to the column matrix and centrifuge for 2 min at maximum speed.

> It is recommended to use the eluted RNA immediately. In case of impediment, it is possible to store it frozen at -20°C for 48h max.
> ***For long-term storage, eluted RNA should be stored at -80°C.***
>
> The total RNA concentration can be estimated, e.g., by measuring the absorbance at 260 nm with a spectrophotometer (not mandatory).

APPENDIX B

Multiple Assay

For 1 sampling point		1 target	2 targets	3 targets
1 tube for all	**Water filtration**	20 mL	40 mL	60 mL
	RNA extraction/elution	100 µL	200 µL	300 µL
	Fragmentation	+ 4 µL buffer + 4 µL STOP	+ 8 µL buffer + 8 µL STOP	+ 12µL buffer + 12 µL STOP
3 tubes / target	**Dilution series 1/10 and 1/100**	Resuspension 10 µL in 90 µL water	Resuspension 20 µL in 180 µL water	Resuspension 30 µL in 270 µL water
	Hybridization	Use of 90 µL of RNA per dilution + 70 µL Signal probe in hybr. solution + 50 µL water (210 µL final volume to separate in 2 x 100 µL and transfer to the microplate)		

APPENDIX C

Ordering Information

Product		Content	Cat. No.
Sample Preparation Kit		For 50 prep: Isolation of total RNA from filtered water samples	#ME-SP-50
SOFiA *Système Opérationnel de Filtration Autonome Operational System for Autonomous Filtration		Peristaltic pump with a filtration system for rapid and standardized sampling of surface water	SOFiA
CARLA Kits	**Microcystis**	Sequence-specific hybridization biosensors for the ribosomal RNA (rRNA) of *Microcystis sp.* cyanobacteria	# CA-CY-01-01
	Planktothrix	Sequence-specific hybridization biosensors for the ribosomal RNA (rRNA) of *Planktothrix sp.* cyanobacteria	# CA-CY-02-01
	ADA	Sequence-specific hybridization biosensors for the ribosomal RNA (rRNA) of *Anabaena/Dolichospermum/Aphanizomenon (ADA clade)* cyanobacteria	# CA-CY-03-01
	Cylindrospermopsis	Sequence-specific hybridization biosensors for the ribosomal RNA (rRNA) of *Cylindrospermopsis sp.* cyanobacteria	# CA-CY-04-01

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