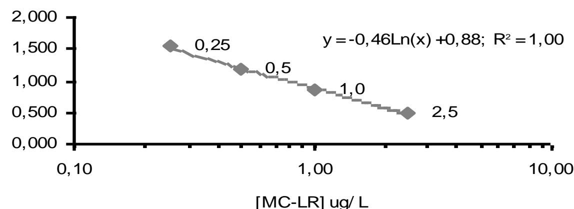
E. Calculations and Graphic Representation of Results

****Note:** A worksheet to calculate the results is available from Abraxis free of charge. Please, contact us for further information.

1 Obtain a standard curve by plotting standards absorbance at 405 nm in the *y axis* and concentration of Microcystin-LR in a logarithmic *x axis*. Draw a standard curve. An example of standard curve is shown below:



2 The concentration of microcystins in the sample is calculated by interpolating the calibration curve or using the following equation:

$$y = a\,Lnx + b \qquad \mathbf{x = EXP(y\text{-}b/a)}$$

Where "***x***" value is concentration of microcystin-LR equivalents in the sample and the "***y***" the absorbance at 405 nm.

F. REFERENCES

1. An, J., and W.W. Carmichael. 1994. Use of a colorimetric protein phosphatase assay and enzyme linked immunoassay for the study of microcystins and nodularins. Toxicon. 1994 Dec;32(12):1495-507.
2. McElhiney, J., and Lawton, L.A. Detection of the cyanobacterial hepatotoxins microcystins. Toxicol Appl Pharmacol. 2005 Mar 15;203(3):219-30.
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4. Bouaicha N, Maatouk I, Vincent G, Levi Y. A colorimetric and fluorometric microplate assay for the detection of microcystin-LR in drinking water without preconcentration. Food Chem Toxicol. 2002 Nov;40(11):1677-83.

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031317

Microcystins/Nodularins PP2A, Microtiter Plate

Test for the Detection of Microcystins and Nodularins In Water

Product No. 520032

1. General Description

Microcystins/Nodularins PP2A Kit is an enzymatic test for the detection of microcystins and nodularins in water. A simple and rapid method that allows to quantify whether the toxin concentration is over the maximum allowed levels (1 µg/L, OMS 1998).

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Microcystins in solution. Avoid contact of standard and stopping solutions with skin and mucous membranes. If these reagents come in contact with the skin, wash with water. Recommended: Polypropylene material should be avoided throughout sample collection, conservation and treatment, since loss of toxins has been shown to occur.

3. Storage and Stability

The Microcystins/Nodularins PP2A Kit should to be stored in the refrigerator (4–8°C) prior to use and protected from light. The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box.

4. Test Principle

Microcystins/Nodularins PP2A Kit is based on the phosphatase activity inhibition by microcystins. Under normal conditions the phophatase is able to hydrolyse a specific substrate that can be detected at 405 nm. Samples containing microcystins will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. The concentration of the toxin in the sample can be calculated using a standard curve.

5. Limitations of the Microcystins ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in water samples, test interferences caused by matrix effects can't be completely excluded.

Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit, wrong pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C). The assay procedure should be performed away from direct sun light.

As with any analytical technique (GC, HPLC, etc…..) positive results requiring some action should be confirmed by an alternative method.

Working Instuctions

A. Materials Provided

1. Microtiter plate
2. 4 vials of Phosphatase
3. Standards Microcystins (4): 0.25, 0.50, 1.00, 2.50 ppb
4. 1 vial Chromogenic Substrate
5. 1 vial Phosphatase Dilution Buffer
6. 1 vial Stop Soultion

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the assay buffer, substrate and stop solutions in order to equalize the incubations periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

SOLUTIONS

All reagents must be allowed to reach room temperatura (23 ± 3ºC) before starting the assay.

1. Phophatase Solution**:** Add 3 mL of Phosphatase Dilution Buffer to one of the Phosphatase vials and mix carefully by inversion. Gently shake the solution at room temperature (23 ± 3 ºC) for 60 minutes (or manually several times during that period) to ensure that the enzyme is fully hydrated. This solution must be stored under refrigeration if not used immediately after dissolution. Do not use the Phosphatase Solution for following days.

Each enzyme vial contains enough volume of phosphatase for 24 wells. If more than one vial is needed, dissolve each vial as described above, make a pool with the content of those vials and mix gently by inversion before use.

> **Attention:** this reagent is blue and becomes brownish when dissolved. If the phosphatase turns to brownish colour before the hydratation, please discard as this reagent could be damaged.

2. Standards: the standards are ready to use. They are provided in vials containing a total volume of 1.2 mL.

C. Assay Procedure

1. Add 50 µL of each Microcystin-LR standard in duplicate (i. e.: wells A1 and A2, 0.25 µg/L; wells B1 and B2, 0.50 µg/L; wells C1 and C2, 1.00 µg/L; wells D1 and D2, 2.50 µg/L). We recommend using duplicates or triplicates.

2. Add 50 µL of each sample in duplicate into the remaining wells of the microtiter plate.

3. Add 70 µL of the Phosphatase Solution to each well.

4. Add 90 µL of Chromogenic Substrate to each well and mix gently. The substrate contains solid in suspension. Do not mix the reagent prior to use and avoid taking any solid.

5. Put the adhesive film on wells and Incubate the plate for 30 minutes at 37 ºC.

6. Add 70 µL of Stop Solution to each well. Mix gently.

7. Read the absorbance of samples and standards at 405 nm. Use an empty well as blank, if necessary.

D. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 µL)
2. Multi-channel pipette (50-250 µL) or stepper pipette with plastic tips (10-250 µL)
3. Microtiter plate reader (wave length 405 nm)
4. Timer
5. Tape or Parafilm
6. Glass vials with Teflon-lined caps
7. Distilled or deionized water
8. Vortex mixer
9. Heater at 37 +/- 2 °C

E. Sample Preparation

1. Drinking water (water treated in drinking water stations):

Sample preparation is not required. Following the procedure described in section **C (Assay Procedure)** the content of dissolved microcystins will be determined. Please, pay attention to the note mentioned below.

2. Water from reservoirs, rivers, etc

The content of dissolved microcystins, intracellular microcystins and total microcystins can be determined (see Scheme below). Please, pay attention to the note mentioned below.

2.1 Dissolved microcystins: Sample preparation is not required. Following the procedure described in section C**,** dissolved microcystins content will be determined.

*****If any interferences are suspected (i.e. high concentration of heavy metals, turbidity), please contact Abraxis for technical assistance.*****

2.2 Intracellular microcystins:

a) Take 200 mL of sample and filer in vacuum through a 0.8 µm nylon membrane (i.e. Whatman Nylon Membrane Filters, ref.: 7408-004). Reserve the filtrate for further determination of total microcystins, as it is explained in paragraph 2.3.

b) Take the membrane with the residue and place in a glass flask. The membrane can be cut into pieces to improve the extraction step.

c) Add 10 mL of 80% MeOH in water with 0.1% TFA and 0.1% Tween 20. Incubate at room temperature for 30 minutes with gentle stirring and in absence of light.

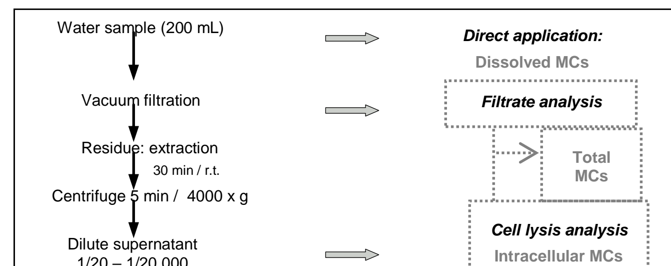
d) Centrifuge at 4000 g for 5 min.

e) Take the supernatant and dilute it 20 times (dilution 1/20) with distilled water. At this point, the sample is ready to continue the assay as is shown in Section **C**. This way, the content of intracellular microcystins is determined. If the concentration of MC-LR equivalents exceeds 2.5 µg/L, we advice to perform the assay on a range of supernatant dilutions of 1/20, 1/200, 1/2000,…

2.3 Total microcystins:

Use the filtrate obtained in 2.2.a. and perform the assay described in **section C**. Total microcystins contained in the sample are calculated by adding the concentration of microcystins found in the filtrate plus the intracellular microcystins (2.2.e).

NOTE: Presence of thiosulphate (or strong oxidizing reagents) may interfere in the assay, therefore collecting samples in bottles with this chemical or adding it to the simple prior to testing should be avoided.



Scheme procedure for water samples from reservoirs, rivers, etc.