



In Vitro Assay for Determining Cyanotoxin Using Serological Methods

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Abstract

Cyanobacteria are free living organisms and the probability of finding them in water body is very high. These are proven to be harmful for humans in the form of hepatotoxins, neurotoxins, or alkaloids (forms of cyanotoxins). Thus, detection of these harmful agents becomes a necessity for a healthy environment at all forms of life. This chapter mainly focuses on the serologic methods of detecting cyanotoxins in blood sera using enzyme-linked immunosorbent assay. Although several other methods under analytical tests like chromatographic techniques can be employed to know the accurate concentration, ELISA on the other hand also furnishes good quality results. The following content will describe the protocol of performing experiments to detect cyanotoxins in their classified form.

Keywords

Serum preparation · Cyanotoxin extraction · Microcystin · Nodularin · Serological analysis · ELISA · Limitations

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34.1 Introduction

Surprisingly, global climate change has its effects deep down onto minute organisms like cyanobacteria as well. The increase in climate change leads to production of toxins released by cyanobacteria, and therefore this leads to contamination of waterbodies and finally affecting us humans because of the quality of water we drink. Molecular nature of these toxins includes cyclic peptides, alkaloids, and organophosphate moiety (Skulberg et al. 1993). There are two ways to detect cyanotoxins present in a sample, analytical and bio-analytical method. Bio-analytical methods include ELISA, Quantitative real-time PCR, colorimetric PPIA, the mouse bioassay, etc. Among the various types of ELISA methods, direct and indirect are mostly employed as illustrated in Fig. 34.1. These methods have low equipment requirements to operate and are therefore easy to operate, produce rapid and effective results, and allow sensitive detection of toxin (Koreivienė and Belous 2012). Serological method ELISA comes in the form of kits as well, and the easy availability of these kits make the test handier because of the convenience and zero labor offered. Other analytical methods which help in cyanotoxin determination are more sophisticated. Other methods are high performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS), and thin layer chromatography (TLC).

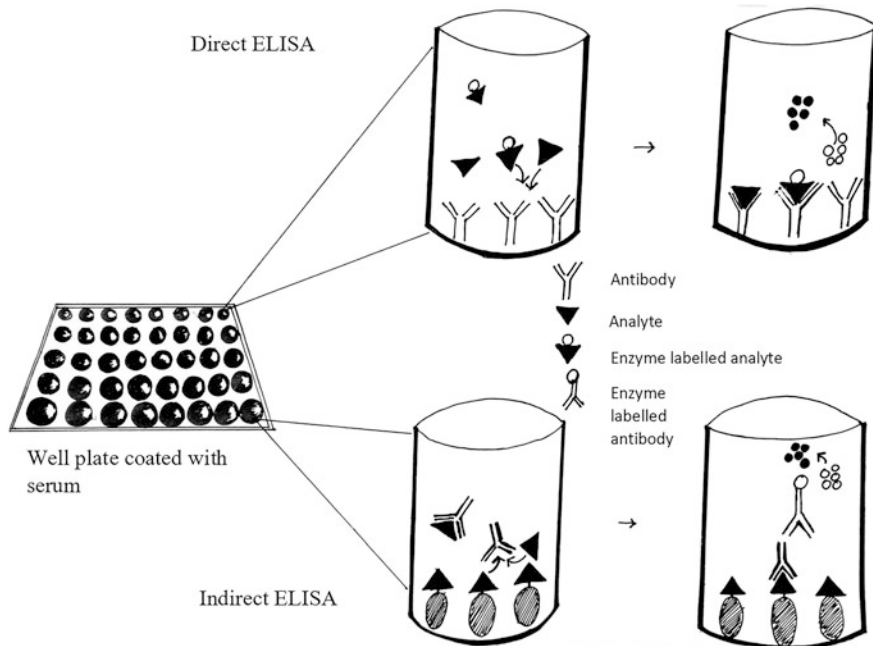


Fig. 34.1 Mechanism of different types of ELISA, direct and indirect

Microcystins are the most common and widely associated toxins of cyanobacteria. These are either freely floating or are bound in human serum. Some common freshwater cyanobacteria are *Anabaena flos-aquae*, *Microcystis aeruginosa*, *Oscillatoria agardhii*, and *Nodularia spumigena* (Sivonen et al. 1990). These cyanobacteria lead to production of harmful toxins, microcystins, and nodularin which have adverse effects on both human and animal life.

Serum samples are analyzed using ELISA—enzyme-linked immunosorbent assay. Free microcystins are directly analyzed using ELISA and an indirect method is also employed for determination of protein bound microcystins using GC/MS to find the amount of MMPB (2-methyl-3-methoxy-4-phenylbutyric acid) derived from microcystins as a result of chemical oxidation. This represents the total amount of microcystins present (Hilborn et al. 2007). Microcystins are categorized under hepatotoxins and liver being their target organ. Microcystin-leucine arginine is a common cyanobacterial toxin released by cyanobacteria which results in harmful effects on the reproductive organs leading to reproductive toxicity.

34.2 Protocol

34.2.1 Using a Commercially Available ELISA KIT from Enviro Logix, Inc. Portland, ME, USA

1. Take the human blood samples that need to be identified as intoxicated by microcystins.
2. Separate the serum from the blood cells.
3. Freeze the separated serum at -70°C .
4. The serum sample is thawed.

Sample Preparation

5. In a 15 mL corex tube made of glass, add 1 mL aliquot of the serum sample followed by 10 mL of methanol. Multiple such tubes can be set up.
6. These are then centrifuged for 30 min at 9000 rpm.
7. In a 20 mL scintillation vial decant the supernatant.
8. Treat the pellet with 5 mL of methanol and allow it to suspend.
9. This is once again centrifuged at 9000 rpm for about 30 min and added to a scintillation tube.
10. Add 5 mL of hexane and vortex it.
11. Discard the hexane layer only and retain the methanol layer.
12. Wash the methanol layer with 5 mL hexane and repeat this for at least three times.
13. In a Speed VAC concentrator, dry the samples under vacuum at 40°C , this is then taken up in 2 mL of 5% HOAc.
14. The solution should be passed through the Oasis HLB solid phase extraction cartridge.

15. The cartridge should be conditioned with 1:1 column volume of methanol and water, respectively.
16. The cartridge is then washed with 5 mL of methanol in water 30% (v/v).
17. In order to elute the microcystin, the samples are treated with 5 mL of methanol followed by drying.
18. Resuspend the samples in 10% (v/v) of methanol in water in a volume of 1 mL.
19. Centrifuge this for 3.5 h at 10,200 rpm through a YM-10 molecular weight cut off filter.
20. Divide these treated serum samples into wells of the ELISA KIT- EnviroLogix, Inc. Portland, ME, USA.
21. Preferred purchase of standard toxins is extractions from cultures of 98% *Microcystis aeruginosa*. (Wright State University).

ELISA procedure

22. Free microcystins are detected by direct competitive ELISA tests. These contain a rabbit antibody that is conjugated to bovine serum albumin against microcystin-LR. The analyte competes for the antibody-binding site against the microcystin-LR peroxidase (microcystin enzyme conjugate). On the addition of a substrate, there is an induction of color change which is read at a wavelength of 450 nm by ELISA plate reader.
23. Lighter coloration indicates higher concentration of the toxin and darker coloration shows lower concentration of the toxin.
24. All the reagents and standards for performing the assay are included in the kit.
25. Run the serum samples in triplicate.
26. Add 125 μL of assay diluent to each of the wells.
27. 20 μL of the negative control and 20 μL of calibrator is added to their respective vials.
28. And the serum samples of 20 μL should be added to their respective wells.
29. Mix the contents of the wells for a good 20–30 s.
30. Cover the well plate with parafilm.
31. Incubate the setup for 30 min at room temperature.
32. Now add about 100 μL of the microcystin-enzyme conjugate to each of the wells in the well plate.
33. Now cover the plates with parafilm and again incubate it for 30 min at room temperature after mixing the contents prior to incubation.
34. For washing, flood the contents of each well with the PBS solution for about 4 cycles (four times) followed by shaking to empty the well each time using a multi-reagent plate washer.
35. Now add about 100 μL of the substrate provided.
36. Mix the contents in the wells and follow it up with parafilm covering.
37. Incubate the plate at room temperature for 30 min.
38. Add 100 μL of stop solution provided in the kit, and thoroughly mix the contents in each well.

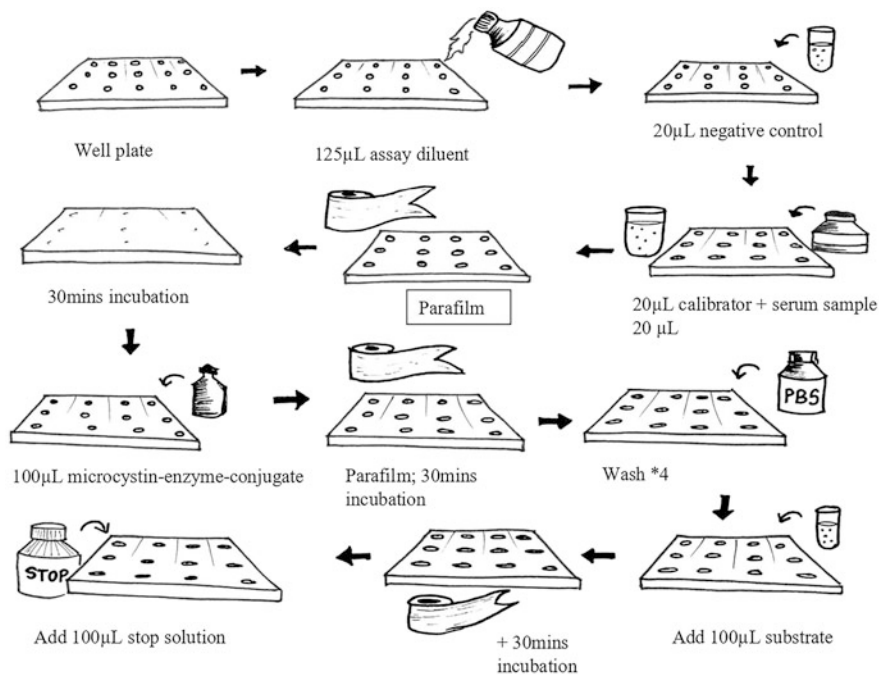


Fig. 34.2 Commercial ELISA kit protocol flowchart

39. Now read the plate at 450 nm wavelength (Hilborn et al. 2005). Figure 34.2 shows a detailed diagrammatic representation of the above-described procedure.

34.2.1.1 Observation

In order to estimate the amount or concentration of the cyanotoxin—microcystin in the tested serum sample, the plate with serum sample must be read for the intensity of color through a colorimetric method at a wavelength of 450 nm. This value should be estimated on the basis of the standard concentration of microcystin used whose concentration is already known. In general, the serum sample set up that has higher concentrations of the toxin will show lighter coloration whereas the serum sample having lower concentrations of the toxin will show deeper coloration.

34.2.2 Direct Competitive ELISA and Indirect ELISA

34.2.2.1 Materials

- Standard microcystins MCYST-LR, MCYST-RR, MCYST leucine-alanine (MCYST-LA), MCYST leucine-tyrosine (MCYST-LY).
- Ethylenediamine (EDA)-modified BSA.

- ELISA microtiter plates and minisorp RIA tubes from Nunc (Roskilde, Denmark).
- N-hydroxysuccinimide; N,N'-dicyclohexylcarbodiimide; dimethylformamide.
- Sodium phosphate buffer; Phosphate-buffered saline.

Sigma Chemical Co. (St. Louis, Mo.) provided with

- Bovine serum albumin BSA (RIA grade).
- Poly-L-lysine (MW = 58,000).
- N-acetylmuramyl-L-alanyl-Disoglutamine (NAMAG).
- 30% hydrogen peroxide; ortho-phenylenediamine tablets (OPD)
- Female albino rabbits were purchased from Smith's Rabbitry (Seymour, Wis.) (free of *Pasteurella* spp.).

34.2.2.2 Methodology

Building Immunity

- MCYST-LR-EDA-BSA:

Conjugation of MCYST-LR to EDA-BSA to form an immunogen 5 mg MCYST-LR is dissolved in 0.08 mL ethanol and diluted with 0.32 mL of distilled water, pH 5 → Solution A 20 mg in 0.4 mL of water with pH 5.0 from the EDA-BSA solution → Solution B.

Solution A and B with a few drops of 0.2 N NaOH added dropwise to Solution C. Solution C, EDPC solution is made by dissolving 1.32 g of EDPC in 1.5 mL of water with pH 5. The entire reaction is carried out at room temperature for 5 h, with a constant maintenance of pH 5. Post completion the entire solution undergoes dialysis 0.1 M NaCl (2 L) at 6 °C for 24 h and later lyophilized.

- MCYST-LR-PLL for indirect ELISA:

1.8 mg of MCYST-LR is added to a solution, this solution contains 0.28 mg of N-hydroxysuccinimide dissolved in 0.28 mL of dimethylformamide and 0.5 mg of N,N'-dicyclohexylcarbodiimide is again dissolved in 0.5 mL of dimethylformamide solvent. The final solution is incubated at room temperature for 30 min. Activated succinimide ester is added dropwise to PLL solution (2.7 mg in 2 mL of 0.13 M freshly prepared bicarbonate) and stirred for an hour. The reaction mixture later undergoes dialyzation against 0.01 M sodium phosphate buffer (2 L) with pH 7.5 for 3 days, and in the end lyophilized (Chu et al. 1989).

Antisera Extraction

Once the toxins are prepared, they are induced into the host (rabbit) and antisera from rabbits are extracted and converted to a saturated form. The extraction of antisera is chronologically shown in Fig. 34.3.

34.2.2.3 Performing ELISA

Direct competitive ELISA:

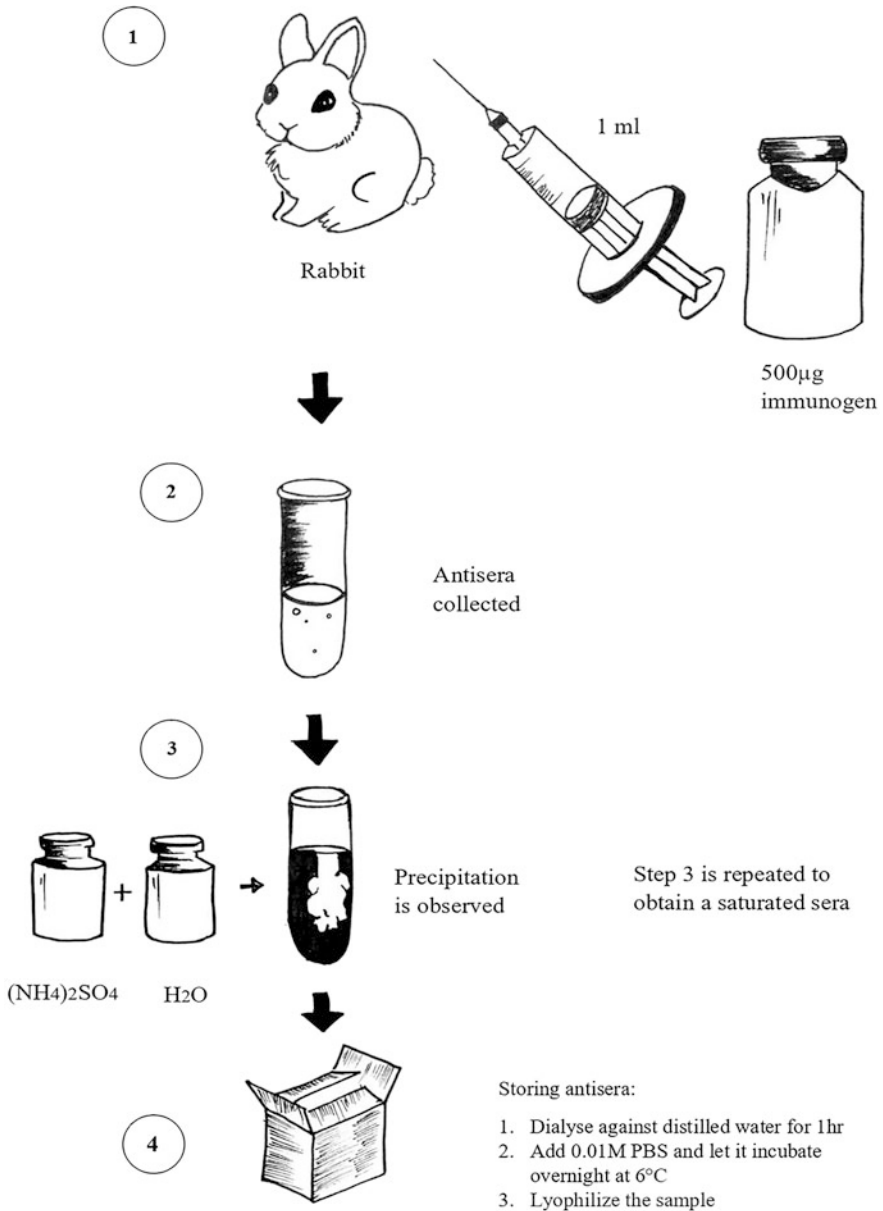


Fig. 34.3 Extraction of antisera from rabbit

1. ELISA plate used in this experiment is provided from Dynatech Immunlon I, or equivalent.
2. 0.1 mL of antiserum coating is spread in each well after diluting antibody with 0.01 M PBS and left for overnight incubation (6 °C).

3. Wash each well with 0.35 mL PBS-tween.
4. Add 0.17 mL BSA-PBS in each well and let the plate incubate for 30 min (37 °C).
5. Wash the plate four times by following the same procedure.
6. Now add diluted variants of microcystins in individual wells in the volume 0.05 mL.
7. Add a blank buffer with MCYST-LR-HRP (marker) of 0.1 mL volume and let the plate incubate for 60 min (37 °C).
8. Again, wash the plate four times following the same procedure.
9. Add 0.1 mL of OPD substrate and let the plate incubate for 10 min (RT).
10. End the procedure by adding 0.1 mL of stopping reagent and observe for the samples at 490 nm (Chu et al. 1987).

Indirect ELISA:

1. 0.1 mL of MCYST-LR-PLL (antigen) is added to each well and left for incubation overnight (6 °C).
2. Wash the plates with 0.16 mL and 0.35 mL of PBS-tween buffer twice individually.
3. Add 0.17 mL of 1% gelatin in PBS and incubate the plate for 30 min (37 °C).
4. Wash the plate with 0.35 mL of PBS-tween four times.
5. 0.1 mL of anti-MCYST-LR-antiserum is added in each well and mixed well. Allow the plate to incubate for an hour (37 °C).
6. Wash the plate four times by following the same procedure.
7. Add 0.1 mL of goat anti-rabbit IgG-HRP conjugate (marker) at 1:20,000 dilution in PBS.
8. Incubate the plate for 60 min (37 °C) and wash it.
9. Add 0.1 mL of OPD substrate and leave the plate in dark at RT.
10. In the end add 0.1 mL of 1 N HCl and observe the ELISA plate at 490 nm in automatic reader (MR 600; Dynatech Industries, Inc., Alexandria, Va.) (Fan et al. 1984).

34.2.2.4 Radioimmunoassay (RIA) Method

This method is performed to determine antigen concentration with the help of antibodies present in antisera. Antigens are radiolabeled with markers which bind to specific antibody and increase the radioactivity thereby indicating the presence of antigen. Radioimmunoassay uses ammonium sulfate precipitation method to separate free (not radiolabeled) and bound (radiolabeled) toxins (antigen). 50 µL of radioactive marker ligand is incubated with 0.15 mL of antiserum solution of different dilutions in PBS for 30 min (RT) and then for 1 h (6 °C) or longer. Separation of bound and free ligand takes place by ammonium sulfate precipitation method. This method provided a clear conclusion on the free and bound toxins present in antiserum. Antibody titer is the reciprocal of the total amount of antiserum (mL) required to give 50% binding of tritiated microcystin (reduced MCYST-LR). In the experiment performed, total amount of antiserum used is 50 µL and marker ligand used in antibody titer is reduced MCYST-LR. Radioactivity was determined

using liquid scintillation spectrometer (model LS-5801; Beckman Instruments, Inc., Fullerton, Calif.) in 4.5 mL volume of Ecolume (ICN Biochemicals, Inc., Irvine, Calif.).

MCYST-LR (0.5 mg) → reduced MCYST-LR

MCYST-LR was treated with below mentioned reagents in sequential order:

3.4 μmol of NaBH_4 in 1mL redistilled 2-propanol; 0.85mL ethanol; 0.15mL of diluted acetic acid (0.1% glacial acetic acid in ethanol).

This reduced toxin is used in RIA as a marker for detection of specificity and determining antibody titers.

34.3 Conclusion

RIA titer values of MCYST-LR-EDA-BSA were the highest, 300–2500. Therefore, rabbits immunized with MCYST-LR-EDA-BSA were further studied (3 rabbits) and others were not.

The three rabbits ELISA and RIA reports showed nearly similar titer values. Production of antibody is observed as early as 4 weeks of time.

Direct ELISA is more versatile as it is less time consuming than indirect ELISA method (Msagati et al. 2006).

34.4 Advantages and Disadvantages of ELISA

Advantages	Disadvantages
Do not require radioactive compounds	Variable cross-reactivities hence may underestimate concentration of cyanotoxins
Evaluates only total value of cyanotoxin in a sample	Does not identify individual isoforms
Less equipment requirements, rapid and effective results	Does not assess the toxicity

- ELISA method helps in the determination of only free microcystins. Microcystins usually target the liver and mostly stay bound to the proteins. Therefore, the total count of microcystins can be detected only by Gas Chromatography/ Mass Spectroscopy.
- Limit of detection of ELISA for polyclonal antibody is 2.5 mg/L, and for monoclonal antibody—0.1 mg/L (Singh et al. 2012).

References

Chu FS, Fan TS, Zhang GS, Xu YC, Faust S, McMahon PL (1987) Improved enzyme-linked immunosorbent assay for aflatoxin B₁ in agricultural commodities. *J Assoc Off Anal Chem* 70(5):854–857

- Chu FS, Huang XUAN, Wei RD, Carmichael WW (1989) Production and characterization of antibodies against microcystins. *Appl Environ Microbiol* 55(8):1928–1933
- Fan TS, Zhang GS, Chu FS (1984) An indirect enzyme-linked immunosorbent assay for T-2 toxin in biological fluids. *J Food Prot* 47(12):964–967
- Hilborn ED, Carmichael WW, Yuan M, Azevedo SM (2005) A simple colorimetric method to detect biological evidence of human exposure to microcystins. *Toxicon* 46(2):218–221
- Hilborn ED, Carmichael WW, Soares RM, Yuan M, Servaites JC, Barton HA, Azevedo SMFO (2007) Serologic evaluation of human microcystin exposure. *Environ Toxicol* 22(5):459–463
- Koreivienė J, Belous O (2012) Methods for cyanotoxins detection. *Bot Lith* 18(1):58–65
- Mtagati TA, Siame BA, Shushu DD (2006) Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins. *Aquat Toxicol* 78(4):382–397
- Singh S, Srivastava A, Oh HM, Ahn CY, Choi GG, Asthana RK (2012) Recent trends in development of biosensors for detection of microcystin. *Toxicon* 60(5):878–894
- Sivonen K, Carmichael WW, Namikoshi M, Rinehart KL, Dahlem AM, Niemelä SI (1990) Isolation and characterization of hepatotoxic microcystin homologs from the filamentous freshwater cyanobacterium *Nostoc* sp. strain 152. *Appl Environ Microbiol* 56(9):2650–2657
- Skulberg OM, Carmichael WW, Codd GA, Skulberg R (1993) Taxonomy of toxic Cyanophyceae (cyanobacteria). In: *Algal toxins in seafood and drinking water*, pp 145–164