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Next generation planar waveguide detection of microcystins in freshwater and cyanobacterial extracts, utilising a novel lysis method for portable sample preparation and analysis

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Highlights

- Sensitive assay for the detection of the most common and toxic microcystin variants
- Detection of free and cell bound microcystin for a true reflection of toxin content
- Novel, highly effective lysis method enabling fast and portable disruption of cells
- Validated to measure microcystins below 1 and 0.1 ng ml^{-1}; free and intracellular
- Next generation planar waveguide biosensor combining quantification and ease of use

Abstract

The study details the development of a fully validated, rapid and portable sensor based method for the on-site analysis of microcystins in freshwater samples. The process employs a novel lysis method for the mechanical lysis of cyanobacterial cells, with glass beads and a handheld frother in only 10 min. The assay utilises an innovative planar waveguide device that, via an evanescent wave excites fluorescent probes, for amplification of signal in a competitive immunoassay, using an anti-microcystin monoclonal with cross-reactivity against the most common, and toxic variants. Validation of the assay showed the Limit of Detection (LOD) to be 0.78 ng ml^{-1} and the CCβ to be 1 ng ml^{-1}. Robustness of the assay was demonstrated by intra- and inter-assay testing. Intra-assay analysis had % C.V.s between 8 and 26% and recoveries between 73 and 101%, with inter-assay analysis demonstrating % C.V.s between 5 and 14% and recoveries between 78 and 91%. Comparison with LC-MS/MS showed a high correlation (R^2 = 0.9954) between the calculated concentrations of 5 different *Microcystis aeruginosa* cultures for total microcystin content. Total microcystin content was ascertained by the individual measurement of free and cell-bound microcystins. Free microcystins can be measured to 1 ng ml^{-1}, and with a 10-fold concentration step in the intracellular microcystin protocol (which brings the sample within the range of the calibration curve), intracellular pools may be determined to 0.1

ng ml^{-1}. This allows the determination of microcystins at and below the World Health Organisation (WHO) guideline value of 1 µg l^{-1}. This sensor represents a major advancement in portable analysis capabilities and has the potential for numerous other applications.

Keywords: Microcystin, planar waveguide, portable biosensor, novel lysis, blue-green algae and cyanobacteria

1. Introduction

Cyanobacteria (Domain Bacteria) originate from the Precambrian era, 3.4 billion years ago and were the first prokaryotes to use water in the fixation of carbon dioxide [1]. They are widespread throughout global waters, both marine and freshwater, encompassing, not only hot tropical to temperate waters, but even the chilly waters of the Antarctic ice shelves [2]. Of the many types of cyanobacteria, *Microcystis aeruginosa* (predominantly freshwater) is the most common species, which produces microcystins. In addition to Microcystis, microcystins are also produced by the following genera: Anabaena, Nostoc, Planktothrix, Anabaenopsis and Hapalosiphon [2]. Microcystins are cyclic heptapeptides with the structure; cyclo-(D-Alanine-X-D-MeAsp-Y-Adda-D-Glutamate-Mdha), where X and Y are variable L-amino acids, MeAsp and Mdha are Methylaspartic acid and Methyldehydroalanine, respectively and Adda is (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-thrimethyl-10-phenyldeca-4(E),6(E)-dienoic acid [3]. There are over 90 variants of microcystins with the most common being Microcystin-LR (MC-LR), which has Leucine at position 2

and Arginine at position 4 [4, 3]. They are hepatotoxic, due to their uptake via the bile acid transport system delivering them into hepatocytes, where they inhibit Serine/Threonine Proteases via Adda, causing over phosphorylation of proteins. Acute toxicosis results in the disruption of the cytoskeleton, loss of cell structure and adhesion, loss of tissue structure and the collapse of sinusoidal capillaries resulting in hepatic haemorrhaging and ultimately death [5–8]. The deaths of 60 patients in a dialysis unit in Brazil, in 1996, have been attributed to the use of contaminated water during dialysis [9]. Lower levels of microcystins, although not acutely lethal, have been shown to promote cancer (Group 2B carcinogen; possibly carcinogenic to humans) and have immunotoxic and genotoxic effects [10–12]. The World Health Organisation (WHO) have set the recommended limit at 1 $\mu g\ l^{-1}$ for drinking water and 20 $\mu g\ l^{-1}$ for recreational waters [13–15]. Microcystins are small (MC-LR being 995.2 Da) and stable compounds, withstanding harsh conditions, such as high temperatures and extreme pH. Removal is usually achieved by activated carbon filtration, ozonation or chlorination; all being commonly utilised in water treatment [16–18].

The ability to detect microcystins is important and many tests exist. The vast majority are laboratory based, with immunoassays enabling high throughput screening for total microcystin concentrations and analytical methods (such as HPLC, Mass Spectrometry[19–21]), although slower, allowing the quantification and identification of individual variants within a sample [22, 3]. Recent attention has turned towards the on-site detection of microcystins. Portable tests have long since focused on the lateral flow format, a major drawback of which is that quantification usually relies on the user's determination. However, the first lateral flow device (LFD) for microcystins, devised by Kim et al (2003) [23], employed a custom made portable-laser fluorescence

scanner, eliminating the subjective nature of results interpretation. For this they used Alexa Fluor 647®, a Cy5 labelled fluorescent probe, as the secondary, detection, antibody, in a competitive assay, whereby a MC-LR conjugate was coated to the surface and an anti-MC-LR monoclonal was used as the primary antibody. This assay format works well for most biosensors, including planar waveguide, a technology, that has been around for decades. Two portable assays, have been developed recently by Long et al (2005) and Herranz et al (2012) [24, 25]. Both utilise modified versions of the aforementioned assay format; Long et al use a Cy5 labelled monoclonal, and Herranz coat the sensor surface with MC-LR as opposed to a MC-LR-conjugate. The biosensors used are the Trace Organic Pollutant Analyser (TOPA) and a commercialised version of the US Naval Research Laboratory (NRL) Array Biosensor prototype, by Long et al and Herranz et al respectively. For the TOPA assay, one analyte was measured at a time, with a cycle time of 20 min, 150 cycles per surface and an LOD of 30 ng l^{-1}. The NRL Biosensor, can detect up to 6 analytes at a time, with 15 cycles per surface, a cycle time of 60 min and an LOD of 16 ng l^{-1}. The present study utilises the next generation of evanescent wave/ planar waveguide detection. This platform has all the advantages related to single use LFDs and portable biosensors yet has the ability to perform the accurate quantification only associated with laboratory based, methods. The unique MBio SnapEsi® LS sensor [26] employed in the present study uses, a patented design, whereby the lens is integrated on a disposable cartridge; a cartridge into which the sample and reagents are added and held. The cartridges are custom made, spotted with (in-house prepared) toxin-protein conjugates, chosen and optimised for the sensitive and selective binding of anti-microcystin monoclonal antibody (also produced in-house). These cartridges eliminate the need for cumbersome and expensive microfluidics often associated with advanced portable

sensors, thus there is no need for pumps, valves, tubing or buffer/waste reservoirs. In the SnapEsi® LS assay there are no concerns about tubing becoming blocked; either by lines drying out due to warm temperatures, air bubbles, or sample particulate blockages. The cartridge can simply be used, read and disposed of. Thus a simple to use, low cost and accurate means of detecting microcystins will be presented.

Further to this, we present a highly novel sample preparation method for the portable lysis of cyanobacterial, *Microcystis aeruginosa*, cells, for the quantification of intra-cellular microcystin levels. Microcystins are inherently endotoxins, therefore, the measurement of freshwater only, fails to detect the majority of toxin present, as has been highlighted by Codd et al (2005) [2] who state that guideline values "should therefore apply to the sum of the intracellular and extracellular microcystin pools"; yet the focus has remained on water testing only. This may be in part due to the difficulties encountered in dealing with cyanobacterial samples in current analytical methods. Cheap, quick and portable lysis usually relies on chemical disruption of cells, using harsh reagents which may interfere with downstream assays, causing matrix interference and thus skewing results. The best cellular disruption occurs via mechanical lysis of the cells, such as that of glass bead beating, which has been demonstrated to achieve full lysis of algal cells, in a quick time of only 10 min [27]. This laboratory based method required a paint shaker to mix the sample/glass bead combination and a centrifuge to separate cells from freshwater samples. To overcome this, centrifugation steps were switched to filtration and the paint shaker was substituted for a, low cost, hand-held, battery operated frother. Other non mechanical methods require filtration of the sample (glass fibre filters, GF/C) followed by slow toxin extraction using solvents which are not compatible with immunoassays.

Presented here is an assay, proven to be capable of detecting microcystins in both free and intracellular states, to a level of 1 ng ml^{-1} for free and 0.1 ng ml^{-1} for intracellular microcystins. This rapid, semi-quantitative test, has an assay time of only 15 min for free microcystins, and under 30 min for intracellular microcystins, inclusive of sample preparation.

2. Materials and Methods

2.1. Reagents and Chemicals

Microcystin-LR was purchased from Enzo Life Sciences, through Alpha Technologies Ltd, Larne Northern Ireland. Bovine Serum Albumin (BSA), Phosphate buffered saline (PBS) tablets, Tween-20 and apo-Transferrin (bovine) were purchased from Sigma-Aldrich, Gillingham, UK. Cyanobacterial cultures were obtained from the Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland and the Laboratory of Ecotoxicology, Genomics and Evolution (LEGE) at the Centre of Marine and Environmental Research (CIIMAR), Porto, Portugal. From CCAP *M. aeruginosa* strains 1450/3 (non-toxic) and 1450/6 (toxic) were purchased and from CIIMAR, strains LEGE 91093, LEGE 91094, LEGE 91095 and LEGE 91096 were received. Jaworski's and BG11 media were purchased from CCAP, Oban, Scotland. Alexa Fluor 647 goat anti—rabbit IgG and Alexa Fluor 647 goat anti—mouse IgG antibodies were purchased

from Invitrogen Ltd, Paisley, Scotland. The anti-microcystin, 5C4, monoclonal was prepared in-house and details will be published elsewhere.

2.2. Apparatus

For cell lysis a hand held frother was purchased from Argos Direct, Stafford, UK, while the paint shaker, a Minimix standard shaker, was purchased from Merris Engineering, Berkshire, UK. Swinnex, 25 mm filter holders, 25mm gaskets and 25 mm MF-Millipore (mixed cellulose esters, hydrophilic, 0.45 µm and black gridded) membranes, together with Millex-HA (0.45 µm, mixed cellulose) filters were purchased through Premier Scientific Ltd, Belfast, Northern Ireland. The SnapEsi® LS System [28] was supplied by MBio Diagnostics Inc, Boulder, Colorado, USA, as were the microarray cartridges.

2.3. Preparation of Toxin Protein Conjugate (TPC); MC-LR-Transferrin

MC-LR, 0.25 mg, was reconstituted in 50 µl Dimethyl sulfoxide (DMSO). To this was added 50 µl *N*-Hydroxysuccinimide (NHS) (130 mM) and 100 µl N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (39 mM), both dissolved in 50 mM 2-(N-Morpholino) ethanesulfonic acid (MES), pH 4.7. This was stirred for 30 min, protected from light, at room temperature. The active ester solution was then added, dropwise to a vial containing 1 mg of Transferrin dissolved in 1 ml PBS pH 7.4. This was stirred at room temperature, for 2 hours, again protected from light. The TPC was dialysed against PBS pH 7.4 and tested by ELISA. The TPC was sent to mBio Diagnostics for microarray printing, at 100 µg ml^{-1}.

193

194 ## 2.4. Microarray Printing

195 Microarrays were printed using a Bio-Dot AD3200 robotic arrayer. Briefly, spots were
196 produced, with a diameter of 0.5 mm, using a Bio-Jet print head that dispensed 20 nl.
197 Four replicates of the TPC were printed onto a grid with 1 mm centres. Microarrays
198 were then blocked with a protein-based blocking agent (0.5% casein in PBS with
199 Proclin300 antimicrobial) prior to spin-drying. Microarrays were then assembled into
200 an injection moulded cartridge, which contained a 5 mm wide fluidic channel (max
201 volume 30 µl) with a single inlet port for the addition of sample and reagents. Assays
202 were carried out on a rack, angled for optimum flow rate, enhancing passive fluid flow,
203 which is aided by capillarity due to the narrow fluidic channel.

204

205 ## 2.5.Culturing

206 CCAP cultures were maintained in Jaworski's Medium, while CIIMAR LEGE cultures
207 were maintained in BG11 Medium. Culturing was done in glass Erlenmeyer flasks
208 (wide necked) with cotton wool plugs and foil lids and incubated at 20 °C with a light
209 intensity of 116 µmol m^{-2} s^{-1}. All consumables were sterilised by autoclaving at 121 °C
210 for 15 min. Culturing work was carried out in a pre-sterilised (via 30 min UV
211 exposure) UV3 HEPA PCR cabinet. Cultures were maintained in log phase, with fresh
212 cultures being seeded from denser cultures (nearing stationary phase); 40 ml dense
213 culture added to 160 ml fresh media. Culture growth was monitored by cell counting
214 using Lugol's stain and a haemocytometer under x200 magnification.

215

2.6. Sample Lysis

A 25 mm swinnex filter (in a 25 mm Swinnex Filter Holder) was used to filter 50 ml of sample/culture. The filter membrane was then transferred to a 7 ml bijou and the captured cells resuspended with 5 ml PBS pH 7.4. To 5 ml of sample/culture, 1 g of 0.1 mm glass beads was added to a small glass beaker (15 ml), whose diameter was just large enough to accommodate the frother whisk, whilst limiting the space for beads/cells to accumulate in. The frother was then switched on and allowed to 'whisk' the sample/bead mixture for 10 min. Once finished the sample was twice filtered (0.45 µm Millipore), to ensure complete removal of glass beads/fragments; once should be enough, but a second filtration was included in case the first filter was perforated. A negative toxin producer (CCAP 1450/3) was treated in the same way to produce the matrix in which calibrants are prepared.

2.7. Assay

Reagent/Sample Preparation

Assay Buffer

The buffer was PBS pH 7.4 containing 0.05% Tween-20, 0.45 µm filtered.

Antibody Solutions

The monoclonal antibody, 5C4 was diluted (v/v), 1 in 100, from the stock solution of approximately 0.88 mg ml^{-1}. The diluent was Assay Buffer containing 1% BSA, the BSA acting as a stabiliser. The working stock was maintained at 4 °C and diluted again, with Assay Buffer (without the added BSA) to a final concentration of 1 in 10000, for use in the assay. The 5C4 monoclonal antibody was shown to have cross-

reactivity for the most common microcystin variants, as follows: MC-RR, 108%; MC-YR, 68%; MC-LA, 69%; MC-LW, 71%; MC-LF, 68%; and Nodularin, 94%.

The detection antibody, Alexa Fluor 647 goat anti-mouse IgG, was used to prepare a detection antibody working stock solution, also stored at 4 °C (without added BSA), protected from light. The stock solution was prepared by adding 100 µl of each antibody to 2 ml of Assay Buffer. For use in assay conditions, a further 1 in 10 dilution (with Assay Buffer) was required to get to the final 10 µg ml^{-1} working solution.

Calibrants

Calibrants were prepared from a 1 µg ml^{-1} solution of MC-LR, to yield concentrations of 200, 50, 20 and 0 ng ml^{-1}, with dilutions made using Assay Buffer. These were then used to spike 950 µl of matrix, using 50 µl per spike, to get final solutions with the following concentrations: 10, 2.5, 1 and 0 ng ml^{-1}.

Assay Conditions

Equal volumes of 5C4 antibody and sample/calibrant were mixed and 150 µl immediately applied to the cartridge. After 7 ½ min, 150 µl of detection antibody was added. After 7 ½ min (15 min total time) the cartridge was read using the SnapEsi reader.

2.8.Liquid Chromatography Tandem- Mass Spectrometry (LC-MS/MS) Validation

Freeze-drying and solid phase extraction (SPE) were used to prepare and extract microcystins from Microcystis aeruginosa samples for LC-MS/MS detection, as reported elsewhere; using modified methods from Kim et al. (2009), Lawton et al. (1994), Msagati et al. (2006) and Mooney et al. (2011) [29–31, 20]. Briefly, 50 ml samples were freeze-dried and resuspended in, 5 ml, 75% methanol before enrichment and purification with OASIS HLB cartridges, after the methanol content was diluted to 15%. Microcystins were eluted with 6 ml methanol, containing 0.1% Trifluoroacetic acid, dried under nitrogen, resuspended in 80% methanol and analysed by LC-MS/MS; using a Waters Acquity UPLC and a Quattro Premier XE Mass Spectrometer, run in electrospray positive mode (ESI).

2.9. Surface Plasmon Resonance (SPR) Assay

CM5 research grade chips were used on a Biacore Q instrument. CM5 chips were coated with MC-LR using the method devised by Vinogradova et al [32]. The flow rate was 20 µl min^{-1} for 4 min per cycle, with the 5C4 monoclonal antibody used at a 1 in 1000 dilution (v/v) using the 0.88 mg ml^{-1} stock solution. 5C4 was mixed with sample prior to injections using a blend of 30% antibody and 70% sample. The calibration curve consisted of six points; 10, 5, 2.5, 1, 0.5 and 0 ng ml^{-1} MC-LR. Calibrants were prepared in matrix (*M. aeruginosa* CCAP 1450/3 lysate) to normalise for matrix effects. Regeneration of the chip surface was achieved by injecting 75 mM Sodium hydroxide and 10% Acetonitrile (in deionised water) for 1 min at 20 µl min^{-1}.

3. Results and Discussion

3.1.Milk Frother Validation

Validation of the frother required testing samples lysed by both the laboratory based paint shaker [27] and portable frother by SPR. This was due to the observation that disintegration of the glass beads occurred upon mixing, which resulted in the appearance of small fragments, which could be mistaken for *M. aer* cells when viewed microscopically, See Fig 1 for images. To overcome this, identical samples were tested by SPR. For one sample, the paint shaker and 0.5 mm beads were employed and for the other the frother and 0.1 mm beads were used. The ratio of 1 g per 2 ml of sample was maintained, thus 2.5 g of 0.1 mm glass beads were added to the 5 ml sample. The same mixing time was used for both, 10 min. The final samples were then tested by SPR to determine the toxin concentrations present. The concentration as determined by the paint shaker method was 9.0 ng ml^{-1}, while that of the frother method was 9.25 ng ml^{-1}, resulting in a 103 % recovery of microcystin.. This confirmed that the lysis procedure was as effective as that of the paint shaker.

3.2.Assay Format

The format of the assay was competitive inhibition, whereby the more microcystins that were present in a sample/calibrant, the less anti-microcystin, 5C4, monoclonal there was available to bind to the surface bound TPC. Thus the more microcystins present in a given sample, the lower the signal would be. This is shown in Fig 2, showing the fluorescence recorded for the four identical microcystin spots, on cartridges with calibrants added.

3.3.Assay Validation

The limit of detection (LOD) was determined by taking the mean response for 20 negative (toxin free) samples and subtracting 3x standard deviations (S.D.) as is standard for inhibition assays [33]. The response was then converted to concentration via the calibration curve and derived as 0.78 ng ml^{-1} (see Table 1). This level was used as a guide to estimate the detection capability (CCβ), which was 1 ng ml^{-1}, equivalent of the 1 µg l^{-1} WHO recommended level. Taking into consideration the 10-fold concentration step in the sample preparation method for intra-cellular measurement, this brings samples containing 0.1 ng ml^{-1} into the measureable range of the calibration curve. No sample preparation method, other than filtration (0.45 µm) was included for free microcystin measurement, as this would only serve to reduce the speed of analysis. As can be seen from Fig 3, there was no overlap between the 20 x 1 ng ml^{-1} spiked samples and the 20 negative samples from the LOD calculation (to 1x S.D.). Populations were also shown to be significantly different with a p value < 0.0001; 2-tailed, unpaired t-test. The assay had a dynamic range (IC_{10} to IC_{90}) of 0.22 to 5.12 ng ml^{-1}.

Repeatability and reproducibility (intra- and inter-run robustness) were demonstrated by spiking at 3 different toxin levels, with 4 replicates per spiking level, across 2 batches of cartridges and repeating this over 3 days. The data was then analysed per day to determine repeatability of the assay and then across the 3 days to determine the reproducibility of the assay. The 3 spiking levels chosen were CCβ, the midpoint (IC_{50}) and the level at which 75% inhibition of signal was achieved (IC_{75}), which were 1, 1.27 and 2.78 ng ml^{-1} respectively. Inter-run analysis showed that spiking level recoveries all lay between 78 and 91% with % C.V.s lower than 15% (see Table 2). For intra-run

analysis, recoveries were between 73 and 96 % with % C.V.s below 22% (see Table 2). This demonstrated a high degree of repeatability for what is a semi-quantitative assay, and providing values that could be expected with a fully quantitative, laboratory based assay,

Final validation of the assay was performed by testing samples by the SnapEsi method and comparing them to values determined by LC-MS/MS analysis. Table 3 shows the calculated concentrations of the 5 *M aeruginosa* strains tested. The two microcystin pools were combined to give a total microcystin content of each sample. These were then compared to the concentrations derived by LC-MS/MS analysis and as can be seen from the data in Table 4 the difference between the calculated concentrations is between 0.8 to 1.3-fold, with an average of a 1.04-fold difference. Differing sample preparation methods in addition to the differing detection methods generally result in much greater variation in data generated. The R^2 value, when the calculated concentrations were plotted against each other, Fig 4, was 0.9954, demonstrating the reliability and accuracy of the method.

4. Conclusions

Due to the true and accurate level of microcystins in a water sample only being ascertained if both free and cell-bound levels are quantified, a method has been developed, and fully validated, to allow the calculation of the total microcystin content of a sample. To allow for the development of a rapid and portable assay, a novel method of cell lysis was also developed and validated, whereby a frother was used to vigorously agitate a sample containing, 0.1 mm glass beads, mechanically lysing the

cyanobacterial cells, without the need for harsh chemicals, in just 10 min. The planar waveguide assay, using a SnapEsi® LS sensor, is rapid, taking only 15 min and using an anti-microcystin monoclonal antibody (5C4), that detects the more common, and toxic, variants of microcystin; MC-LR, MC-RR, MC-YR, MC-LA, MC-LW and MC-LF. The assay can detect free microcystins to 1 ng ml^{-1} and intracellular microcystins to 0.1 ng ml^{-1}. The difference being due to a 10x concentration step that was included in the intracellular sample preparation method; no sample preparation method was required in the measurement of the free microcystin fraction. In addition to this, a single, cheap, cartridge is used per sample that is simply discarded after reading, to give an assay that is not only rapid and portable, but has quantitative capabilities that many semi-quantitative laboratory based methods fail to reach.

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442

443 List of Figures:

444 Fig 1. Image a (x 200 magnification) shows 0.1 mm glass beads after 10 min mixing

445 with the milk frother. The majority of beads disintegrate forming small fragments that

446 may be mistaken for cyanobacterial cells. Image b (x 200 magnification) shows CCAP

1450/6 *M. aeruginosa* cells; which would be difficult to distinguish amongst the bead fragments in image a.

Fig 2. Images of microcystin spots as treated with calibrants: a, 10 ng ml^{-1}; b, 2.5 ng ml^{-1}; c, 1 ng ml^{-1}; and d, 0 ng ml^{-1}.

Fig 3. Determination of CCβ as 1 ng ml^{-1}, as shown by the 0 ng ml^{-1} and 1 ng ml^{-1} populations not overlapping (to 1 S.D.)

Fig 4. Comparison of mBio and LC-MS/MS results, showing a good correlation, whereby $R^2 = 0.9954$.

List of Tables:

Table 1. Determination of LOD as 0.78 ng ml^{-1}

Table 2. Measured concentrations of spiked samples, along with S.D., % C.V.s and mean recoveries, for repeatability and reproducibility analysis of assay.

Table 3. Microcystin content of *M. aeruginosa* cultures as calculated by mBio SnapEsi.

Table 4. Comparison of mBio and mass spec concentrations for toxin producing *M. aeruginosa* samples.

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Response (Normalised Signal)					Calc Conc (ng ml^{-1})
Mean	**S.D.**	**%C.V.**	**3x[S.D.]**	**LOD**	
1060.9	133.8	12.6	401.4	659.5	0.78

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	Spiking Level (ng ml^{-1})	Measured Concentration (ng ml^{-1}) ± S.D.	C.V. (%)	Mean Recovery (%)
Day 1	1	0.95 ± 0.13	14.0	95.1
	1.27	0.99 ± 0.28	21.9	78.2
	2.78	2.03 ± 0.39	19.3	73.1
Day 2	1	0.86 ± 0.13	15.5	85.9
	1.27	1.06 ± 0.28	26.3	83.3
	2.78	2.02 ± 0.36	17.6	72.6
Day 3	1	0.91 ± 0.10	10.5	90.9
	1.27	1.29 ± 0.11	8.4	101.3
	2.78	2.49 ± 0.44	17.8	89.4
Overall (Days 1 to 3)	1	0.91 ± 0.05	5.0	90.6
	1.27	1.11 ± 0.15	13.9	87.6
	2.78	2.18 ± 0.27	12.2	78.4

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Culture	Cell bound Concentration (ng ml^{-1})	Free Concentration (ng ml^{-1})	Total Concentration (ng ml^{-1})
1450/06	7.9	21.2	29.2
91093	63.6	16.5	80.1
91094	38.7	21.6	60.3
91095	128.2	461.5	589.7
91096	72.5	18.2	90.7

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Culture	Calculated Concentration (ng/ml)		Difference (x-fold)
	mBio	Mass Spec	
1450/06	29.2	23.7	0.8
91093	80.1	108.1	1.3
91094	60.3	64.5	1.1
91095	589.7	536.2	0.9
91096	90.7	98.9	1.1

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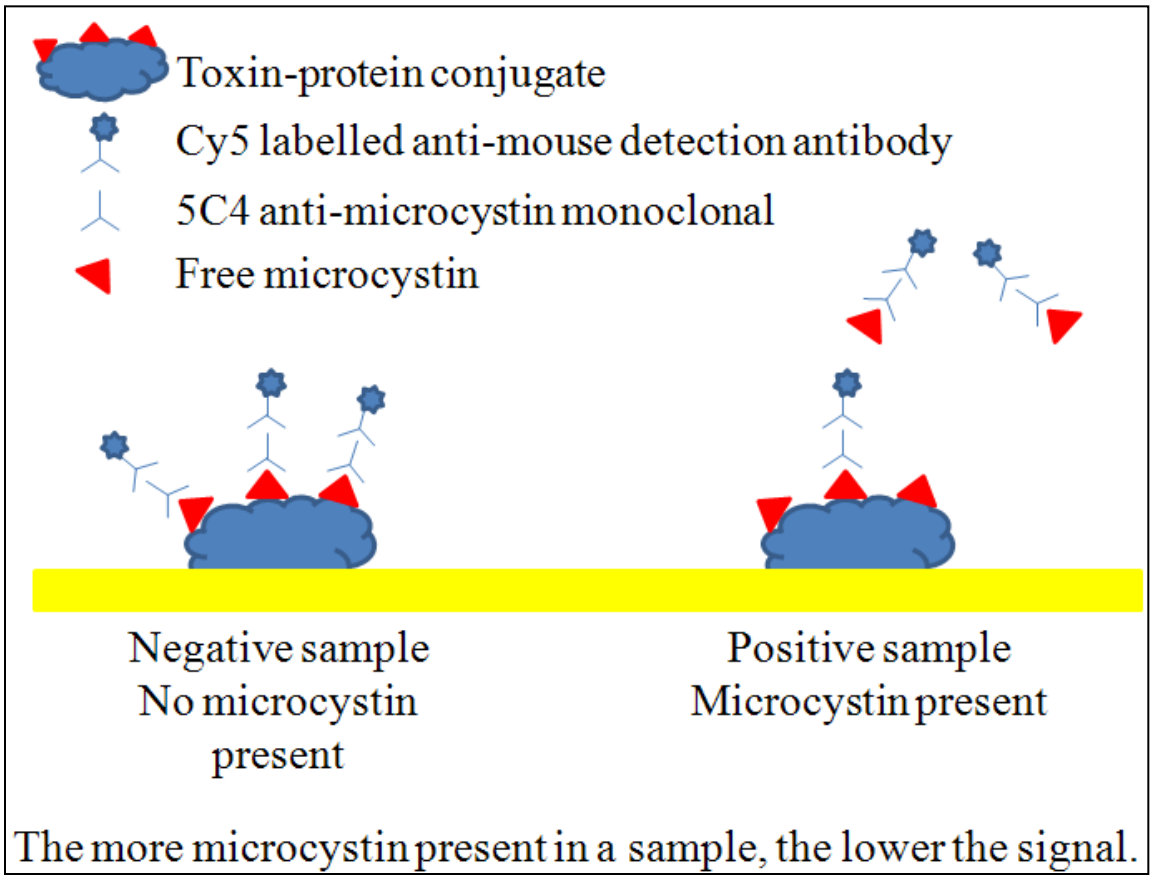
*Graphical Abstract (for review)

1. Sample Filtration → 2. Resuspension of cells → 3. Sample Lysis



4. Sample filtration ← 5. Application of sample ← 6. Read Signal