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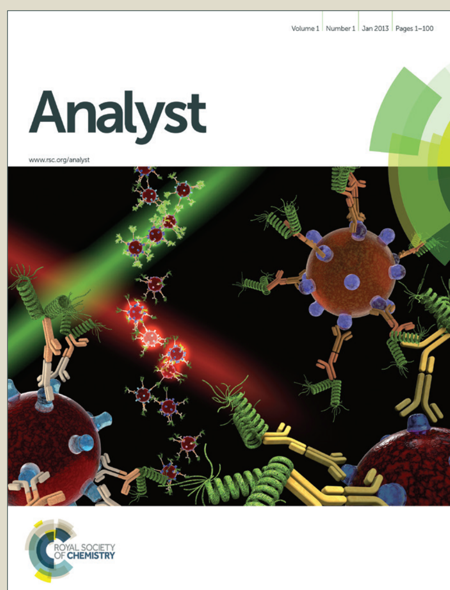

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Development of an ELISA microarray assay for the sensitive and simultaneous detection of ten biodefense toxins.

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Plant and microbial toxins are considered bioterrorism threat agents because of their extreme toxicity and/or ease of availability. Additionally, some of these toxins are increasingly responsible for accidental food poisonings. The current study utilized an ELISA-based protein antibody microarray for the multiplexed detection of ten biothreat toxins, botulinum neurotoxins (BoNT) A, B, C, D, E, F, ricin, shiga toxins 1 and 2 (Stx), and staphylococcus enterotoxin B (SEB), in buffer and complex biological matrices. The multiplexed assay displayed a sensitivity of 1.3 pg/mL (BoNT/A, BoNT/B, SEB, Stx-1 and Stx-2), 3.3 pg/mL (BoNT/C, BoNT/E, BoNT/F) and 8.2 pg/mL (BoNT/D, ricin). All assays demonstrated high accuracy (75-120 percent recovery) and reproducibility (most coefficients of variation < 20%). Quantification curves for the ten toxins were also evaluated in clinical samples (serum, plasma, nasal fluid, saliva, stool, and urine) and environmental samples (apple juice, milk and baby food) with overall minimal matrix effects. The multiplex assays were highly specific, with little cross-reactivity observed between the selected toxin antibodies. The results demonstrate a multiplex microarray that improves current immunoassay sensitivity for biological warfare agents in buffer, clinical, and environmental samples.

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Introduction

Protein toxins, such as the botulinum neurotoxins (BoNT), ricin, shiga toxins (Stx), and staphylococcal enterotoxin B (SEB), are considered to be potential biological threat agents. These toxins pose a threat because of their extreme toxicity, widespread availability, and ease of use. Because of these characteristics, biothreat toxins have been stockpiled for bioweapon use and even used in previous bioterrorism events.[1, 2] In the event of a bioterrorist attack, it may not be obvious which agent was released, although this knowledge is critical for delivery of appropriate medical treatment. Therefore, public health officials require sensitive and specific detection systems that can identify multiple biothreat toxins early enough that appropriate care can be given.

BoNT consists of a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC). The toxin enters the body through ingestion, inhalation, or open wounds and is thereafter transported to cholinergic synapses via circulation.[3] The HC facilitates toxin entry into neurons by specific receptor mediated endocytosis and the LC functions as a metallopeptidase that cleaves soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), thereby inhibiting acetylcholine release and resulting in flaccid muscle paralysis.[4] Seven distinct serotypes (A-G) of botulinum neurotoxin are produced by the bacterium *Clostridium botulinum*; however, only serotypes A, B, E and F have been confirmed to cause botulism in humans. Exceptionally low doses of BoNT are sufficient for poisoning and with an LD_{50} of approximately 1 ng/kg it is the most toxic substance known to man.[1] The mouse bioassay is currently the gold standard for BoNT detection and while this assay is highly sensitive, it is laborious and time-consuming.[5] There are promising alternative approaches to the detection of BoNT that have been reviewed recently which include immunological methods, mass spectrometry, endopeptidase activity assays, and cell based assays.[6-8]

Ricin toxin is derived from the seeds produced by the castor bean plant, *Ricinus communis*. Because of the world-wide distribution of this plant and the ease of toxin purification, ricin is considered a major bioterrorism threat. Ricin consists of two polypeptide chains, a 30 kDa A-chain and a 32 kDa B-chain. The ricin B-chain facilitates toxin entry into cells via receptor-mediated endocytosis.[2, 9] Inside the cell, the ricin A-chain initiates depurination and cleavage of the 28S rRNA subunit leading to inhibition of protein synthesis and cell death.[10-12] The toxicity of ricin has not been extensively studied in humans and varies depending upon the route of exposure. The lethal oral dose for humans is estimated at 1-20 mg/kg and the lethal inhalational dose extrapolated from extensive rodent and primate studies is predicted to be around 5 μg/kg.[13, 14] Currently, the analysis of ricin relies on immunological methods,[13-17] mass spectrometry analysis,[18-20] or functional *in vitro* and *in vivo* assays.[21, 22]

Some strains of *Escherichia coli* produce protein toxins that are closely related to Shiga toxin (Stx) from *Shigella dysenteriae*. As a group, these *E. coli* are known as Stx-producing *E. coli* (STEC). STEC are responsible for many outbreaks of hemorrhagic colitis or bloody diarrhea and hemolytic uremic syndrome which can cause kidney failure. There are two major types of Shiga-like toxin, shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2). Stx1 is indistinguishable from the shiga toxin produced by *Shigella dysenteriae* while Stx2 is more divergent. Shiga toxins act to inhibit protein synthesis using essentially the same mechanism as ricin.[12] Stx is composed of a single enzymatic A subunit and a multimer of receptor-binding B subunits, non-covalently associated with the A subunit.[23] Like ricin, A chain of Stx, once it has gained entry into the cytosol of the cell, initiates depurination and cleavage of the 28S rRNA

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subunit, leading to inhibition of protein synthesis and cell death. The Vero cell assay is the gold standard for detection of Stx in clinical and environmental samples; however it is time-consuming and labor intensive.[24] Sensitive and specific PCR-based assays are available for STX-producing organisms; however, they detect the toxin gene sequence, not the toxin itself. Several commercially available immunoassays have been evaluated[25] and other more sensitive immunoassays have been developed.[24, 26, 27]

Staphylococcal enterotoxin B (SEB) is an exotoxin produced by the gram positive cocci *Staphylococcus aureus*. SEB is one of the toxins responsible for staphylococcal food poisoning and it has been stockpiled as a potential biological weapon by some countries. The toxin is a superantigen and intoxication leads to an excessive inflammatory response by the immune system with a release of large amounts of cytokines.[28-30] SEB is extremely toxic with an inhalation exposure LD_{50} of 20 ng/kg and the ability to incapacitate 50% of the population (ED_{50}) with just 0.4 ng/kg.[31, 32] Multiple highly sensitive immunoassays, including immunosensors, point-of-care assays,[33] and protein microarrays,[34] have been developed for the sensitive detection of SEB.[15, 35, 36]

The majority of assays for the detection of toxins have targeted one to two toxins at a time in a test format; however, there is a growing need for assays that analyze multiple toxins simultaneously. Several multiplex toxin platforms have been developed including mass-spectrometry based, [19, 37] suspension array technology, [17, 38-40] and planar protein microarrays. [41-45] Some multiplex assays have been developed to detect toxins in clinical samples[39, 41, 46] or in food samples.[17, 38, 47] Assay sensitivities for the most relevant multiplexed toxin assays are summarized in Supplemental Table 1. For this work we have developed a protein ELISA microarray that detects 10 toxins with high sensitivity and specificity in both clinical and food samples.

Protein microarray technology relies on a miniaturized version of the traditional ELISA whereby multiple antibodies are immobilized onto a solid surface, allowing for the simultaneous analysis of hundreds of antigens within a single experiment. We have expanded upon the ELISA-based protein antibody microarray developed by Varnum et al.[41, 42] to include six botulinum neurotoxins, ricin, Stx1, Stx2, and SEB. Here we employed a sensitive and specific "sandwich" protein ELISA microarray to simultaneously detect all 10 toxins in a number of biological fluids including serum, urine, and saliva and in food samples. This multiplex microarray format allowed us to monitor multiple toxins and screen hundreds of antibodies efficiently and cheaply to determine the most sensitive antibodies. This, together with a powerful yet simple biotin-tyramide amplification system, dramatically increases assay sensitivity producing assays capable of detecting toxins to pg/ml levels in clinical and environmental samples. The assay design can be easily adopted by other research groups, uses relatively inexpensive, commercially available reagents and equipment, while maintaining excellent sensitivity at minimal sample volumes.

Experimental

Assay Reagents

BoNT holotoxins were purchased from Metabiologics (Madison, WI). Ricin toxin (*Ricinus communis* agglutinin II; RCA60) was purchased from Vector Laboratories (Burlingame, CA).

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Nontoxic recombinant SEB was acquired from BEI resources (Manassas, VA). Shiga toxin was purchased from List Biological Laboratories (Campbell, CA). Antibodies used as capture or detection antibodies were either obtained from the lab of Dr. James D. Marks at the University of California-San Francisco[48-52] or were commercially available. In order to test all combinations of possible antibody pairs for our sandwich ELISA, an aliquot of the antibodies were labeled with biotin using EZ-Link NHS-Chromogenic-Biotin (Pierce, Rockford, IL). The manufacturer's protocol was followed to biotinylate about 130 µg of protein to be used as detection antibody. Biotin-NHS was purchased from Peirce (Rockford, IL) and biotinyltyramide was prepared as previously described.[53] HRP-streptavidin and Cy3-streptavidin was obtained from Jackson ImmunoResearch (West Grove, PA).

Slide preparation and microarray production

The ELISA microarray was carried out as previously described.[41, 42, 54] In brief, capture antibodies specific to each toxin were prepared at concentrations of 1 mg/ml in phosphate buffer saline (PBS, pH 7.2) and printed as 4 replicate spots in each well on the slide. Initially, spotting was done with a NanoPlotter 2.1 (GeSim, Germany); however, current arrays are contract printed at ArrayIt (Arrayit Corp., Sunnyvale, CA). Custom made aminopropylsilane-coated glass slides (Erie Scientific, Portsmouth, NH) were used with a stamped grey hydrophobic barrier that defined 16 individual wells in an 8x2 grid. Following printing, slides were air-dried and blocked for one hour in 1% casein in PBS (Bio-rad). Slides were then washed with PBS containing 0.05% Tween 20 (PBS-T) before proceeding with the ELISA microarray assays or stored dry at -20°C.

Sandwich ELISA

Each standard curve consisted of 12 points spanning the full range of the assay, including an assay blank of 0.1% casein in PBS. Toxins were diluted into the appropriate sample diluent. Twenty microliters of diluted toxin was applied to each well of the microarray and incubated for up to 16 hours on a gently rotating orbital shaker. Three washes were performed after each incubation step with PBS containing 0.05% Tween 20 (PBS-T). The slides were then incubated with the appropriate mix of biotinylated detection antibodies and incubated for 2 hours. The signal was enhanced using the biotinyltyramide amplification system.[54, 55] The slides were incubated with 1 µg/mL HRP-conjugated Streptavidin for 30 minutes, followed by incubation for 10 minutes with 1 µg/ml biotinyltyramide. Finally, the slides were incubated with 1 µg/ml Cy3-conjugated streptavidin for 30-60 minutes in the dark with gentle rocking followed by a final wash and then rinsed with distilled water and dried. Experiments in the shortened assay series were incubated as indicated in the text. Cy3 fluorescence was detected by scanning slides on an LS Reloaded (Tecan, Switzerland) microarray scanner (laser: 532nm; filter: 575 nm).

Analysis of complex clinical and environmental samples

Milk (2% reduced fat), apple juice, and baby food (Gerber Beef and Beef Gravy) were purchased from a local grocery store. Apple juice was neutralized to pH 7.0 using 1N sodium hydroxide. Toxins were spiked directly into undiluted apple juice (after neutralization), milk, and baby food

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samples. Milk samples, with exogenous toxin, were centrifuged at 5000 x g for 5 minutes and the interphase layer, between the fat layer on top and the sediment, was saved for analysis. Baby food samples were incubated with toxin for the indicated time and then PBS-T was added at a 1:1 w/v ratio (i.e. 1 gram baby food: 1 mL PBS-T). The mixture was vortexed for 30 seconds and then centrifuged at 1000 x g for 5 minutes at room temperature. The supernatant was again centrifuged at 5000 x g for 10 minutes and the supernatant from this final spin was saved for analysis.

Urine, saliva, serum, and plasma were from an anonymous female donor (Golden West Biologicals, Inc., Temecula, CA). These samples were centrifuged at 12,000 x g for 15 minutes, aliquoted, and stored at -80°C until used. Nasal specimens were collected by rotating a sterile swab against the anterior nasal mucosa for about 3 seconds and repeating for the other naris. The swab was immediately placed in a 1.5 ml tube with 1 ml assay buffer (0.1% casein solution in PBS) and vortexed for 1 min, aliquoted, and stored at -80°C. Stool was collected by an anonymous donor, mixed with equal volume to weight of gelatin phosphate buffer (0.2% bovine gelatin and 0.4% Na_2PO_4, pH 6.4), and centrifuged at 2500 x g for 30 minutes at 4°C. The supernatant was stored at -80°C until use.[56, 57] For experiments with clinical samples, toxins were spiked into undiluted nasal, urine and saliva samples. Centrifuged stool samples were diluted 1:4 in 40% fetal bovine serum in PBS-T, while serum and plasma were diluted 1:4 in assay buffer before toxin addition.

Assay validation

Specificity was determined by incubating individual toxin antigens, at a concentration of 1250 pg/mL, with microarrays containing all ten toxin capture antibodies. The assays were then incubated with a complete mix of detection antibodies. Cross-reactivity was expressed in percent of the analyte concentration divided by the concentration of the specific antigen and multiplied by 100.

Assay precision for both within- (intra) and between- (inter) runs was evaluated by spiking toxins in assay buffer at two concentrations within the linear portion of each calibration curve and measuring them at least three times each day over three days with four replicates per concentration. Using the predicted concentrations the coefficient of variation (%CV) was determined as the ratio of the standard deviation to the mean for three values of a given concentration.

The limit of detection (LOD) was determined as the tested concentration of analyte that produced a mean signal intensity greater than 3 times the standard deviation of the antigen-free blank. Assay accuracy was determined by the recovery rate expressed as a percentage of the expected concentration divided by the predicted concentration calculated in ProMAT.

Data analysis

Fluorescence data were quantified using ScanArray Express software (Perkin-Elmer). Data analysis was performed using ProMAT Calibrator and the Protein Microarray Analysis Tool (ProMAT).[58, 59] This publicly available software normalizes data to a control protein, then fits the data to standard curves and predicts protein concentrations, in addition to performing statistical analysis specifically for ELISA microarray data. GFP protein (Millipore, Billerica, MA) was added as a calibrant at a concentration of 100 pg/ml to standard and spiked toxin

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mixtures which were made new for each assay. Biotinylated detection anti-GFP (Rockland Inc., Gilbertsville, PA) was used at a concentration of 25 ng/ml. ProMAT Calibrator normalized spot intensities relative to GFP in order to minimize inherent variability due to slide and reagent processing within and between assays.[58, 60] Standard curves were generated using the adjusted values in ProMat.

Results and discussion

Optimization of antibody microarrays

In order to develop sensitive assays, over 70 antibodies were screened in a microarray format, including commercially available antibodies and high-affinity antibodies produced by the Marks lab. Initially, all possible capture antibodies specific to a single toxin were printed in a single chip. Each specific toxin chip was exposed to a serial dilution of the corresponding toxin and probed with all possible relevant detection antibodies. The capture and detection antibody pair that produced the best standard curve based on sensitivity, high reproducibility, and R^2 value was chosen for further analysis in the multiplex assay. Optimal antibody pairs are listed in Table 1 and were previously established for the six BoNT.[41] The optimal detection antibody concentrations were established by analyzing the signal-to-noise ratio for each assay at varying concentrations of detection antibody concentration as described previously.[41, 61] The detection antibodies and their corresponding optimal concentration are listed in Table 1.

Calibration curves and performance of the multiplex toxin assay

Following assay optimization, the ten capture antibodies were printed on one chip for multiplexed detection of all ten toxins simultaneously within one assay. Figure 1 shows the standard curves for all ten toxins over a concentration range from 0 to 5000 pg/ml in assay buffer (blue line). The LODs in assay buffer were: 1.3 pg/mL for BoNT/A, BoNT/B, SEB, Stx-1, and Stx-2; 3.3 pg/mL for BoNT/C, BoNT/E, and BoNT/F; and 8.2 pg/mL for BoNT/D, and Ricin (Table 2). All curves had an excellent linear correlation fit (R^2) of at least 0.98. For all ten toxins, the dynamic range of the assay covered between two to three orders of magnitude of concentration above the LOD (Figure 1).

Assay accuracy was evaluated in a series of recovery studies by spiking assay buffer with three known concentrations of antigen within the linear portion of the standard curve. Toxin recovery varied from 75% to 120% (Table 3).

Assay precision was determined at two toxin concentrations by calculating intra- and inter-assay variability and is summarized in Table 4. The percent CV was calculated either for 12 test replicates run at the same time on the same day (intra-assay) or from test samples assayed on 3 separate days (inter-assay). The intra-assay variability ranged from 2% to 20% except for BoNT/E which had a higher variability of 43% at the lower concentration of 51 pg/mL. The inter-assay variability ranged from 4% to 20% except for Stx-1 which was 29% at 320 pg/mL. These results demonstrate that the multiplexed ELISA microarray is highly accurate, precise and has great potential for the simultaneous detection of even low concentrations of multiple bioterrorism toxin agents.

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Assessment of reagent interference between arrays

Because all ten toxins are measured simultaneously, it is important to test the specificity of each assay. To demonstrate that the assays are specific for each toxin, experiments were undertaken to determine potential cross-reactivity between assays. This involved testing each antigen singly, at a high concentration (1250 pg/mL), on the completed multiplexed microarray consisting of all ten capture antibodies immobilized on the slides and a mixture of all detection antibodies. All of the ten toxins were specifically detected with most antigens having less than 2% cross-reactivity with their non-specific capture antibodies (Table 5). A minor cross-reactivity was detected between BoNT/C toxin and BoNT/D capture antibody. This cross-reactivity has been previously documented and shown to be specific to a cross-reaction between the BoNT/D capture antibody and BoNT/C toxin and not due to interactions between the BoNT/D capture antibody and a BoNT detection antibody.[41] It has been found recently that the antibodies generated against BoNT/D were raised to a mosaic strain of BoNT/D-C.[62] The D-C mosaic strain consists of BoNT/D amino acid sequence in the light chain, but with amino acid sequence similar to BoNT/C in the heavy chain. For whatever reason, it is likely that the BoNT/D capture antibody recognizes a conserved epitope, likely within the heavy chain, shared between BoNT/C and BoNT/D. Nevertheless, the observed signal from the BoNT/D capture antibody and the BoNT/C antigen was minor (<15% of the BoNT/C assay with all 10 toxins) and does not have an impact on the assay's ability to distinguish the BoNT serotypes C and D.

Analysis of multiplex toxin assay in complex clinical and food matrices

To assess the potential use of the toxin ELISA microarray in real-world samples, we spiked the toxins into both clinical (serum, plasma, nasal fluid, urine, saliva, and stool) and food samples (milk, apple juice and beef baby food). The detection of multiple biodefense toxins in clinical samples can provide a powerful diagnostic tool for use by medical personnel in the event of a bioweapon event. While a majority of the toxin assays only experienced a slight decrease in assay sensitivity, a few of the assays were affected more significantly (Table 2). The sensitivity of BoNT/D detection is slightly reduced in plasma samples to 21 pg/mL, and is further reduced to 128 pg/mL in nasal, saliva, urine, and serum samples and 800 pg/mL in stool. Similarly, the LOD of ricin was reduced when measured in specific clinical fluid matrices. However, the toxin microarray assay detects ricin in serum, plasma, nasal and saliva samples with a sensitivity of greater than 2 orders of magnitude required to detect a lethal dose in an average weight human (Table 6).

SEB detection in serum, plasma and saliva demonstrate a considerable loss in sensitivity. Our initial experimental assays showed a high background signal and subsequent decreased LOD for SEB in these fluids. Earlier studies made similar observations [43, 63-65] where it was noted that the interference was attributed to pre-existing SEB antibodies in blood.[66, 67] We attempted, unsuccessfully, to remove antibodies using protein A/G and L columns (data not shown). Therefore, SEB detection was not performed in serum, plasma, or saliva samples. However, SEB was detected with good sensitivity in urine, nasal, and stool with LODs of 3.3, 51 and 8.2 pg/mL respectively (Table 2 and 6). These LODs are well below the clinically relevant detection level of 280 pg/mL (LD_{50} of 20 ng/kg).[32]

All ten assays were also performed in stool samples. Initially, low signal was detected for all of the toxins, despite thorough centrifugation, filtration, and dilution. This drastic

interference was also seen by Dezfulian et al,[68] in stool samples from infants with botulism. Further studies confirmed these interfering factors to be caused by proteolytic activity in fecal specimens which release the capture antibodies from the slide surface.[69] The addition of 40% fetal bovine serum (FBS) was able to sufficiently block the interference when it was used as a fecal diluent at a 1:4 ratio. Therefore, stool samples were analyzed following dilution with 40% FBS. With this modification, most of the toxins had an LOD of less than 51 pg/mL except BoNT/D (800 pg/mL), ricin (800 pg/mL), and Stx2 (128 pg/mL).

Because bacterial and plant toxins have been the cause of both accidental and intentional poisonings, there is a need to detect them in food matrices to assure a safe food supply. Previous studies have detected multiple toxins in food matrices with good to excellent LODs, however these studies have measured a small set of 5 or fewer toxins[17, 19, 38, 39, 70, 71] or have focused on the measurement of toxins in a single food matrix, such as milk.[40, 44] Therefore, we tested our toxin microarray assay for the detection of the 10 toxins spiked into milk, apple juice and baby food. As shown in Tables 2, the fluorescence signal and subsequent LODs did decrease in food matrices, particularly for all ten toxins spiked into baby food samples. This is likely due to fluorescence quenching due to some component in the baby food matrix as has been shown in previous work. [17] Despite the reduced LOD, the toxin assay was sufficiently sensitive to detect the 10 toxins in food samples well below that of an oral lethal dose (Tables 2 and 6). For the majority of the assays, the limit of detection is at least two orders of magnitude lower than the oral LD_{50}.

Rapid assay

In order to produce a more rapid assay, the incubation times for specific steps in the assay, including incubation with antigen and detection antibody, were reduced. Figure 2 shows the calibration curves for selected assays (BoNT/A, ricin, Stx-1) of the developed rapid assays with total assay times of either 2- or 4-hours (additional calibration curves shown in Supplemental Figure 1). The 2 hour assay was performed with the following incubation times: antigen (1 hour); detection antibody (25 minutes); HRP-conjugated Streptavidin (15 minutes); biotinyltyramide (10 min); and Cy3-conjugated Streptavidin (10 minutes); whereas the 4-hour assay used these incubation times: antigen (2 hours); detection antibody (1 hour); HRP-conjugated Streptavidin (20 minutes); biotinyltyramide (10 min); and Cy3-conjugated Streptavidin (15 minutes). A comparison of the detection limits of the two rapid assays and our standard 12 hour assay is shown in Table 7. As the assay time is reduced there is a corresponding increase in the detection limit of the assay. However, most of the assays still display excellent sensitivity limits even with a 2 hour total assay time. For instance, BoNT/A, BoNT/B, Stx-1, and Stx-2 all have LOD of either 3.3 or 8.2 pg/mL. The LOD for BoNT/C and BoNT/E are reduced (51 pg/mL); however, these LOD are still comparable with the mouse hemidiaphragm assay. While the LOD for ricin and SEB are reduced even further, the assay sensitivity is still greater than the relevant clinical detection levels required for either oral or inhalational exposure (Table 6) by greater than two orders of magnitude for ricin and by 2-fold for SEB.

Conclusion

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Using high-affinity capture and detection antibody pairs we developed a highly sensitive ELISA protein microarray assay capable of simultaneously detecting ten biodefense toxins, BoNT/A – F, ricin, SEB, Stx1, and Stx2. These toxins were not only sensitively detected in buffer but also in complex clinical and environmental matrices at levels in the low pg/mL range and with a minimal sample volume of 20 µL. These LODs are among the lowest reported for the multiplexed detection of protein toxins (Supplemental Table 1) and, to our knowledge, one of only a few multiplexed toxin assays verified in both clinical and food samples. Many multiplex assays currently exist that can detect and quantify several biological toxins. [15, 19, 38, 43, 44, 63, 64, 72] To date, the multiplex ELISA-based protein antibody microarray presented here demonstrates an excellent assay that is able to achieve some of the lowest detection limits and maintain sensitivity below the reported LD_{50} in a wide range of biological fluids. The assay uses relatively inexpensive and commercially available reagents along with the powerful biotintyramide amplification system which can be easily adopted by other laboratories. Most notably, the simple microarray format can be readily developed for high-throughput analysis of numerous biological toxins in complex clinical and environmental samples.

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Table 1. Optimal antibody sets used for multiplex toxin protein ELISA microarray.

Antigen	Capture Antibody	Source	Detection Antibody	Source	Detection Antibody concentration (ng/mL)
BoNT/A	mAb AR4	1	mAb RAZ1	1	100
BoNT/B	IgY AHcB	2	mAb 1B10.1	1	100
BoNT/C	mAb AF5425	3	mAb 1C1	1	100
BoNT/D	mAb 8DC1	1	mAb 8DC2	1	25
BoNT/E	mAb 3E6.2	1	mAb 3E4.1	1	100
BoNT/F	mAb 6F8	1	mAb 6F5	1	100
Ricin	mAb AB-RIC-MAB2	4	mAb R2031-52H	7	150
SEB	sheep anti-SEB, SLBI202	5	sheep anti-SEB, SBBC202	5	150
Stx1	mAb MBS311734	6	mAb STX1-10D11	5	150
Stx2	mAb MBS311736	6	mAb STX2-BB12	5	150

Antibody sources: 1-James Marks laboratory, UCSF; 2-Gallus Immunotech, Fergus, ON; 3-R& D Systems, Minneapolis, MN; 4-Critical Reagents Program, Frederick, MD; 5-Toxin Technology, Sarasota, FL; 6-My BioSource Inc., San Diego, CA; 7-US Biological, Swampscott, MA

Table 2. Limits of detection (pg/mL) of toxins in buffer and other complex matrices with the ELISA microarray.

Sample	LOD (pg/mL)									
	BoNT/A	BoNT/B	BoNT/C	BoNT/D	BoNT/E	BoNT/F	Ricin	SEB	Stx-1	Stx-2
Buffer	1.3	1.3	3.3	8.2	3.3	3.3	8.2	1.3	1.3	1.3
Nasal	8.2	8.2	8.2	128	21	51	51	51	21	8.2
Plasma	1.3	1.3	21	51	3.3	8.2	21	NA	1.3	8.2
Saliva	3.3	1.3	21	128	3.3	320	320	NA	8.2	8.2
Serum	8.2	3.3	3.3	128	8.2	8.2	21	NA	8.2	1.3
Stool	51	51	51	800	51	21	800	8.2	51	128
Urine	3.3	8.2	3.3	128	3.3	3.3	1.3	3.3	1.3	1.3
Milk	8.2	8.2	128	320	8.2	8.2	8.2	0.5	3.3	0.5
Baby food	21	21	51	51	21	51	51	51	21	21
Apple juice	51	128	3.3	320	3.3	51	51	1.3	8.2	3.3

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Table 3. Percent recovery for toxins in buffer.

Toxin	pg/mL		
	51	128	320
BoNT/A	111	97	88
BoNT/B	108	99	96
BoNT/C	109	96	97
BoNT/D	98	104	88
BoNT/E	109	83	99
BoNT/F	105	103	95
Ricin	109	101	99
SEB	116	106	103
Stx-1	100	90	75
Stx-2	120	107	105

Table 4. Precision of the toxin microarray ELISA expressed as intra- and inter-assay CVs.

Toxin	Intra-assay %CV[1]		Inter-assay %CV[2]	
	51	320	51	320
BoNT/A	13	12	8	7
BoNT/B	3	19	17	14
BoNT/C	17	11	20	7
BoNT/D	17	2	7	11
BoNT/E	43	13	10	18
BoNT/F	16	13	8	4
Ricin	20	19	9	4
SEB	19	17	17	19
Stx-1	11	15	16	29
Stx-2	16	7	11	8

[1] Intra-assay %CVs were calculated from the average values from three samples analyzed simultaneously

[2] Inter-assay %CVs were calculated from the average values from three samples analyzed on three consecutive days

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Table 5. Percent cross-reactivity of antigens and capture antibodies.

Toxin	Capture Antibody									
	A	B	C	D	E	F	Ricin	SEB	Stx-1	Stx-2
BoNT/A[1]		<2	<2	<6	<2	<2	<2	<5	<2	<2
BoNT/B	<2		<2	<6	<2	<2	<2	<2	<2	<2
BoNT/C	<2	<2		<15	<5	<2	<2	<2	<2	<2
BoNT/D	<2	<2	<2		<5	<5	<5	<2	<2	<2
BoNT/E	<2	<2	<2	<6		<2	<2	<2	<2	<2
BoNT/F	<2	<2	<2	<5	<2		<2	<2	<2	<2
Ricin	<2	<2	<2	<5	<2	<2		<2	<2	<2
SEB	<5	<2	<2	<5	<2	<10	<2		<2	<2
STX-1	<2	<2	<2	<10	<2	<5	<2	<2		<10
STX-2	<2	<2	<2	<5	<2	<2	<2	<2	<2	

[1]Percent cross-reactivity is determined as the median (n=3) relative fluorescence units corresponding to the non-specific capture antibody divided by the median relative fluorescence units of the specific capture antibody.

Table 6. Established oral and inhalational LD_{50} values

Toxin	Oral			Inhalation		Reference
	LD_{50} (µg/kg)	Relevant clinical detection level[1] (ng/mL)	Relevant detection level in 100 mL food (µg/mL)	LD_{50} (µg/kg)	Relevant clinical detection level[1] (pg/mL)	
BoNT	1	14	0.7	0.003	42	1
Ricin	10,000	140,000	7000	3	42,000	73,74
SEB[2]	-	-	-	0.02	280	32
Stx-1[3]	8	112	5.6	8	112,000	75
Stx-2[3]	0.29	4	0.2	0.29	4000	76

[1]Relevant clinical detection level calculated as the minimal concentration of toxin to detect an exposure equivalent to the LOD assuming an average weight person (70kg) with 5L of blood in circulation
[2]For SEB, the oral LD_{50} value has not been reported
[3]For Stx-1 and Stx-2, the oral and inhalational LD_{50} values are based upon the intraperitoneal LD_{50}

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Table 7. Limits of detection (pg/mL) for toxin microarray assay with shortened assay times.

Assay	**Total assay time (hours)**		
	12	**4**	**2**
BoNT/A	1.3	1.3	3.3
BoNT/B	1.3	8.2	8.2
BoNT/C	3.3	21	51
BoNT/D	8.2	320	320
BoNT/E	3.3	21	51
BoNT/F	3.3	8.2	128
Ricin	8.2	128	128
SEB	1.3	128	128
Stx-1	1.3	3.3	8.2
Stx-2	1.3	1.3	8.2

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Figure 1. Standard curves for the simultaneous detection of the toxins in buffer, milk and plasma using an ELISA protein microarray. Error bars refer to the standard deviations of four microarray replicates.

Figure 2. Standard curves for the simultaneous detection of select toxins in buffer using a shortened ELISA protein microarray. Error bars refer to the standard deviations of four microarray replicates.

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Figure 1.

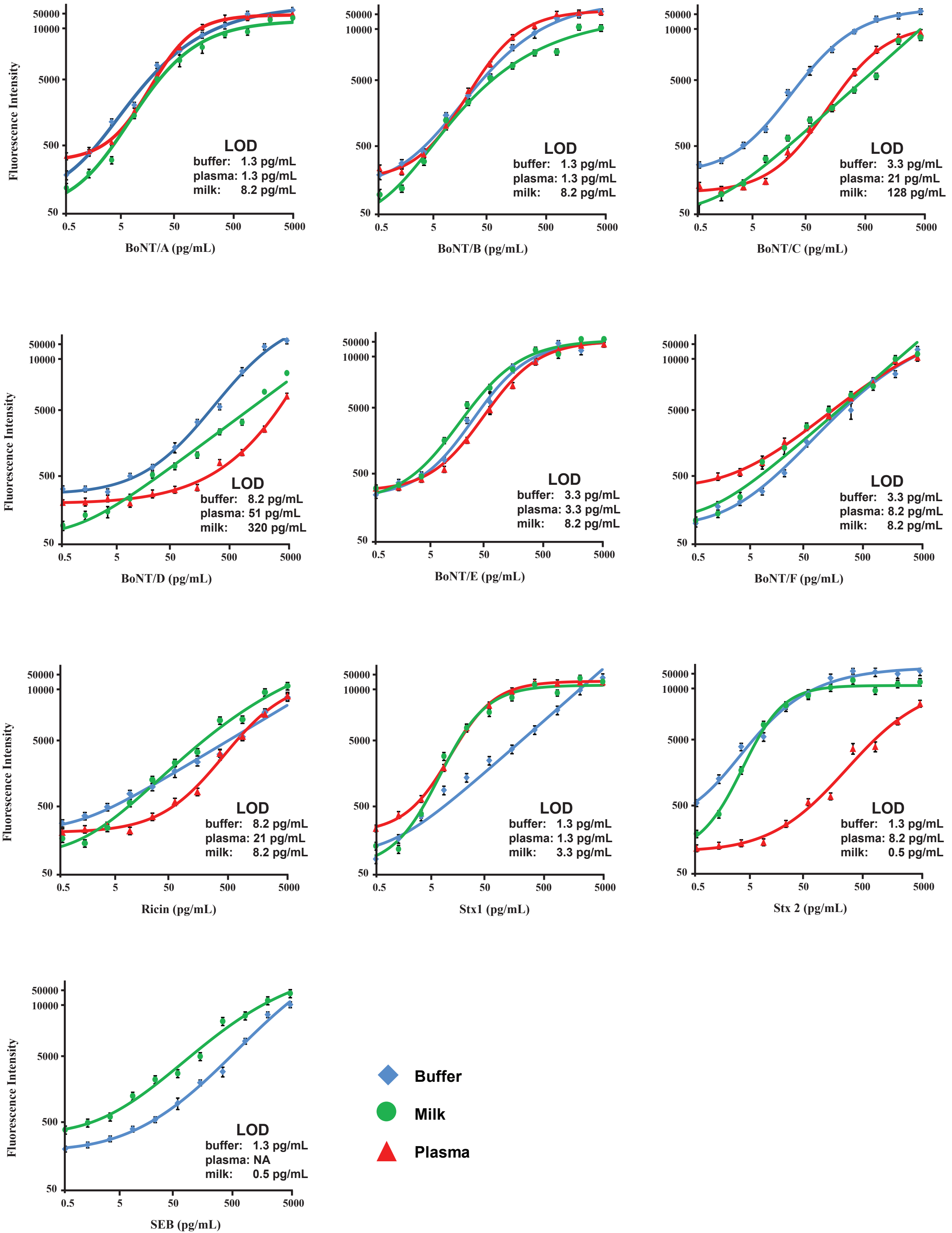

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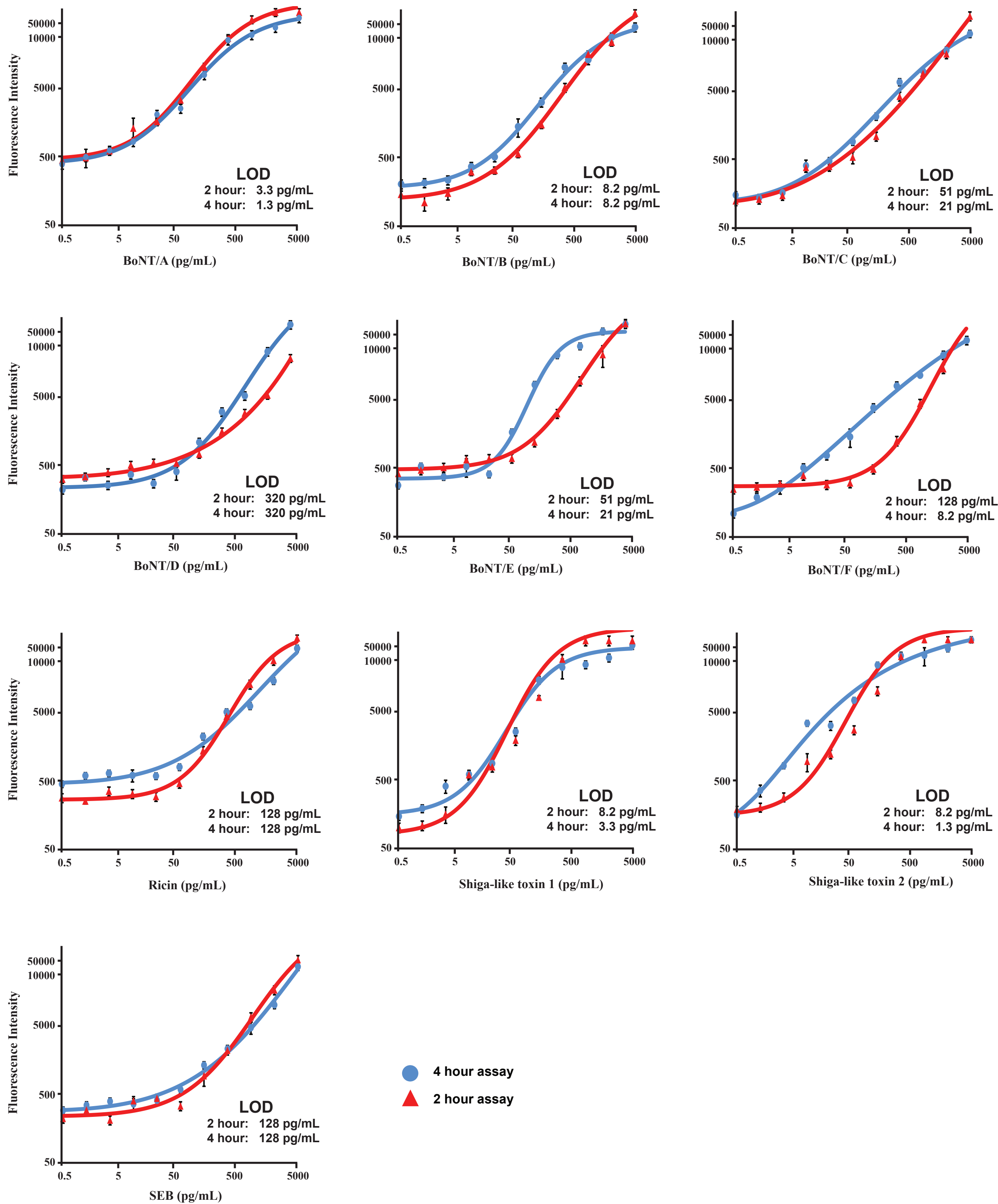
Figure 2.



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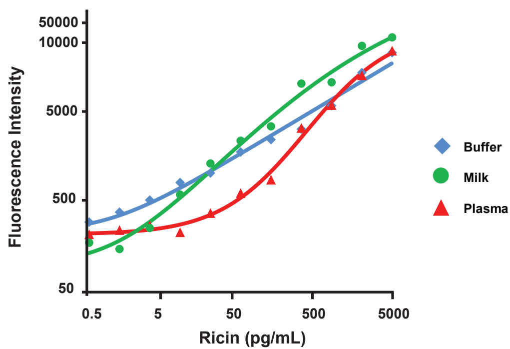
An ELISA-based protein microarray was developed for the sensitive and simultaneous detection of 10 biodefense toxins.



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