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Article

A Rapid, Sensitive, and Portable Biosensor Assay for the Detection of Botulinum Neurotoxin Serotype A in Complex Food Matrices

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Abstract: Botulinum neurotoxin (BoNT) intoxication can lead to the disease botulism, characterized by flaccid muscle paralysis that can cause respiratory failure and death. Due to the significant morbidity and mortality costs associated with BoNTs high toxicity, developing highly sensitive, rapid, and field-deployable assays are critically important to protect the nation's food supply against either accidental or intentional contamination. We report here that the B-cell based biosensor assay (CANARY® Zephyr) detects BoNT/A in buffer and various food matrices rapidly in ≤ 40 min, in small volumes ≈ 50 µL, with minimal processing of samples, and is extremely portable (suitcase-sized equipment). BoNT/A was detected at limits of detection (LOD) < 0.075 ng ± 0.02 in assay buffer while milk matrices (non-fat, 2 %, whole milk) increased the LOD to < 0.175 – 0.314 ng. Limits of detection for the assay in complex foods were < 1 ng ± 0.0 (neutralized acidic juices-carrot, orange and apple); < 16.7 ng ± 7.7 (liquid egg); and varied from < 0. 39 – 3.125 ng for solid complex foods (ground beef, green bean baby puree, smoked salmon). These results show that the CANARY® Zephyr assay can be a highly useful tool in clinical, environmental, and food safety surveillance programs.

Keywords: botulinum neurotoxin, biosensor, CANARY®, detection, B-cell based assay, immunoassay, food matrices

Key Contribution: First demonstration using CANARY® technology to detect botulinum neurotoxins in particular serotype A in buffer and multiple food matrices with excellent sensitivity and minimal sample preparation. This technology is fast, uses small volumes, and is portable to the field.

1. Introduction

Clostridium spp. are ubiquitous, gram-positive, anaerobic spore-forming microorganisms that express some of the most potent neurotoxins known to man. Botulinum neurotoxins (BoNTs) cause

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botulism, which is distinguished by flaccid muscle paralysis [1,2]. There are several antigenically and serologically distinct serotypes (A-H); currently, BoNT serotypes A, B, E, and F are known to cause disease in humans [3-7]. These neurotoxins are a public health and safety threat due to their highly toxic nature with a parenteral lethal dose of 0.1- 1 ng/kg and with an estimated oral intoxication dose of 1 μg/ kg. The significant morbidity and mortality associated with such doses of botulinum neurotoxin intoxication necessitates the development of a field-deployable assay capable of detecting toxins at a high sensitivity and specificity that also is compatible with food and environmental samples. Such diagnostics will allow for both the clinical identification of intoxication and the surveillance of consumables for adulteration as a means to start treatment and dispose of contaminated resources.

There are numerous methods (*in vivo*, *ex vivo*, and *in vitro*) that are currently used to detect botulinum neurotoxins and/or *C. botulinum* contamination. The *in vivo* mouse bioassay is considered the "gold standard" because of its high sensitivity (LOD ≅ 20 – 30 pg) [8,9] and reliability to model all aspects of BoNT intoxication [10,11]. However, this assay is time-consuming, expensive, and requires experienced personnel and specialized facilities. Additionally, the *in vivo* toe spread reflex model has been tested for the detection of BoNT in buffer, serum, and milk [10]. Alternative *ex vivo* animal assays, such as the mouse phrenic nerve hemidiaphragm assay, have been developed and are sensitive and faster than the mouse bioassay, but even such alternatives require special equipment and personnel—and they are not compatible for use with complex matrices.

In addition to the *in vivo* and *ex vivo* models described above, a plethora of *in vitro* assays also have been developed and described in the literature. These assays may be divided into seven different categories: (1) immunological and antibody-based assays, (2) nucleic acid-based assays, (3) lateral flow methods, (4) mass-spectrometry based methods, (5) enzymatic based assays, (6) cell-based assays, and (7) antibody and biosensor technologies. Some well-known *in vitro* assays are ELISA, ECL, lateral flow, ENDOPEP-MS, ENDOPEP-ELISA, Spin-Dx, Immuno-PCR, ALISSA, SNAPtide, VAMPtide, and SYNTAXtide. Additionally, newer assays have combined different technologies to improve the sensitivity of detection. Depending on the assay, the detection limits range from sub-picogram to nanogram per mL or attomolar to pM for buffer and some food matrices [12-31].

CANARY® (Cellular Analysis and Notification of Antigen Risks and Yields) is a cell-based biosensor technology. The technology relies on immortal B-cell lines that express antibodies that are specific to a target and also contain aequroin, a calcium-sensitive bioluminescent protein from the *Aequoria victoria* jellyfish. Initial work with CANARY® technology resulted in the detection of pathogens such as *Yersinia pestis*, Vaccinia virus, Venezuelan equine encephalitis virus, *E.coli* O157:H7, and *Bacillus anthracis* with specificity, high sensitivity, rapidity, and small volumes [32]. Recently, the CANARY® Zephyr system was evaluated against a variety of immunoassays (mostly lateral flow) as well as other biological indicator tests using the potential bioterror threats *Bacillus anthracis* and ricin. The study found that the limit of detection of ricin was 3 ng/mL and 10^3 spores/mL for *B.anthracis* [33]. The authors found that compared to all of the other commercially available kits, the CANARY® Zephyr platform was 4 orders of magnitude more sensitive for detecting *B. anthracis* and was the most sensitive for ricin.

Though there are multiple technologies that are used to detect botulinum neurotoxins in buffer and complex matrices, each of the technologies have their strengths and weaknesses. The cons may

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be due to the time required for experimentation, cost, expert personnel, specialized facilities, expensive and bulky equipment, sensitivity, or incompatibility with complex matrices such as sera, milk, juices, ground meat, eggs, and smoked fish. Therefore, the reality is that multiple technologies may be required for specific conditions to make rapid determinations, especially in clinical, food safety, and environmental settings. In this study, we sought to evaluate the feasibility of using the CANARY® Zephyr system to detect botulinum neurotoxin serotype A in buffer as well as in 10 complex matrices. Limits of detection and specific sample preparation protocols will be determined.

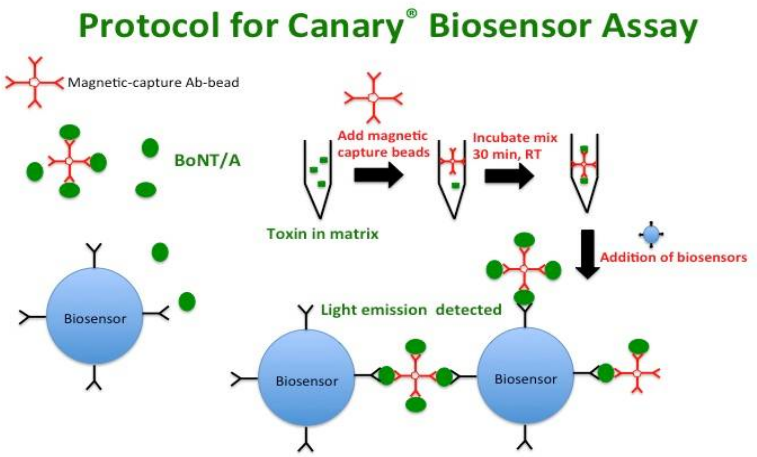
2. Results

2.1. CANARY® Zephry B-cell based assay can detect botulinum neurotoxin serotype A with high sensitivity

Figure 1A depicts a schematic of the CANARY® biosensor assay. Immunomagnetic capture beads specific to BoNT/A were incubated with toxin in buffer or matrix for 30 min at room temperature to allow for the toxin:immunomagnetic bead complex to form a multi-valent epitope. Biosensors expressing membrane-bound antibodies that are specific to a different epitope of BoNT/A than those used on the magnetic beads were then added to the reaction. The binding of the multi-valent epitope on the magnetic beads by the antibodies on the biosensors' surface leads to antibody clustering or "crosslinking," which results in an intracellular calcium influx that activates the aequorin molecules and, hence, luminescence. The luminometer detects the light output, which is expressed as relative light units (RLU) over time (120 sec, read every second).

Assay sensitivity for BoNT/A was first determined in buffer provided by the manufacturer. Serial dilutions of toxin in assay buffer were made and the luminescent signal was measured. Figure 1B shows that there is high sensitivity for BoNT/A and the relative light unit detected (RLU) is concentration-dependent. The Zephyr software depicts the RLU detected by the luminometer as a graph in real-time (left) and then the sample is determined to be either positive or negative based on a propietary algorithm (right table). As one can determine from this representative experiment, 0.0625 ng of BoNT/A was the last positive sample in the experiment while 0.0312 ng did not register as positive, even though there was some RLU above the zero toxin buffer control (left table).

A



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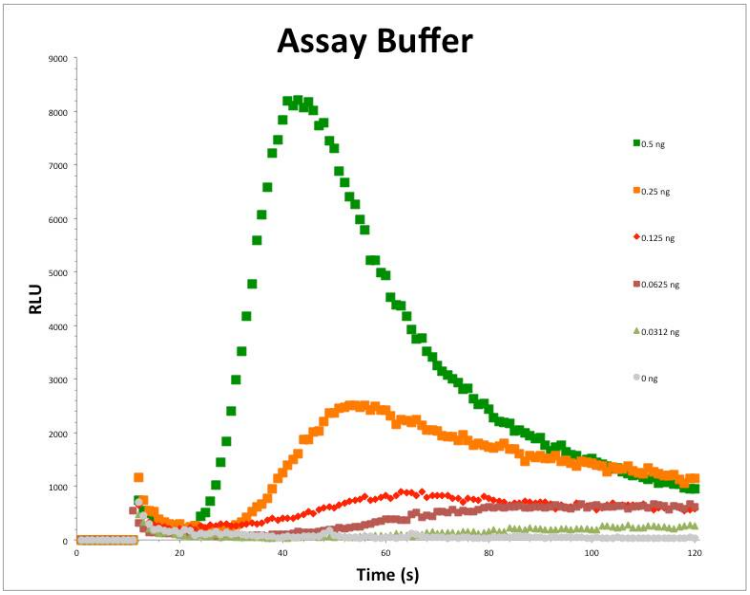
B



BoNT/A (ng)	Read Out
0.5	Positive
0.25	Positive
0.125	Positive
0.0625	Positive
0.0312	Negative
0	Negative

Figure 1. CANARY® biosensor assay detects BoNT/A in assay buffer with high sensitivity in a concentration-dependent manner. (**A**) Schematic of CANARY® biosensor assay. (**B**) A representative graph depicting the relative light unit (RLU) detected by a luminometer as the concentration of the BoNT/A: immunomagnetic bead complex is bound to the biosensors. One representative data set is presented.

2.2. Zephyr detects BoNT/A in whole milk, 2% milk, and non-fat milk

Based on reports with other toxins, the detection of BoNT/A in assay buffer with a high level of sensitivity is an excellent, and expected, result. However, detection assays should be flexible enough to detect BoNT/A in various complex matrices that may be from clinical, food, and environmental settings, since these would be the types of samples that would be evaluated from governmental, diagnostic, and pharmaceutical laboratories. Therefore, three different milk matrices (whole milk, 2% milk, and non-fat milk) were spiked with toxin and serially diluted in matrix; then, the biosensor assay proceeded in the same fashion as for assay buffer. Figure 2 shows the live graph results using whole milk as well as the table read-out. Similar to assay buffer, RLU is concentration-dependent and the LOD was < 0.0625 from this one experiment. However, the final LOD for whole milk incorporating multiple experiments show some inter-assay variability perhaps due to matrix, toxin stock, etc. (Table 1).

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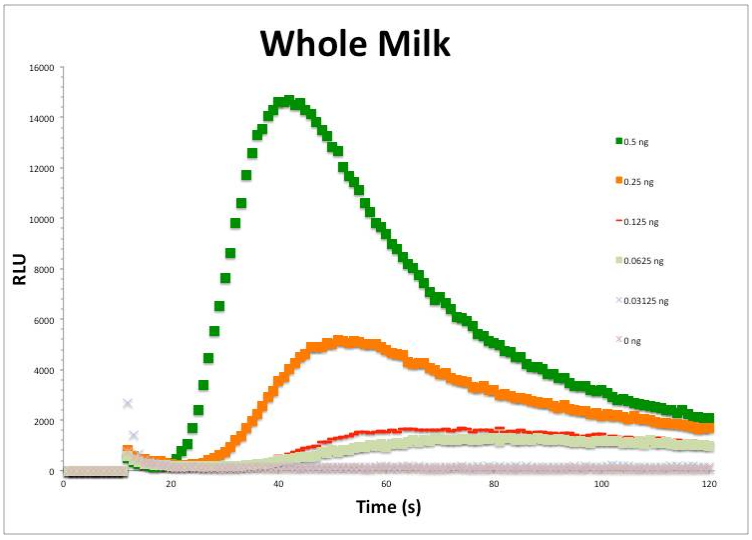

BoNT/A (ng)	Read Out
0.5	Positive
0.25	Positive
0.125	Positive
0.0625	Positive
0.0312	Negative
0	Negative

Figure 2. Whole milk has no effect on the detection of BoNT/A using CANARY®. A toxin concentration-dependent signal similar to assay buffer was detected in the whole milk matrix. Two independent experiments were performed and one representative data set is presented.

Table 1 shows the estimated limits of detection for the CANARY® assay for BoNT/A in assay buffer and the three milk matrices. The limit of detection was determined to be the average of the last positive sample by the Zephyr program using a proprietary algorithm based upon previous work [32]. Assay buffer shows the highest sensitivity with a LOD of < 0.075 ng ± 0.02. The three milk matrices had slightly elevated LODs in comparison to assay buffer but was not statistical significant (two-tailed unpaired Student's *t*-test, $p > 0.05$). The higher LOD in 2% milk as compared to whole milk or non-fat milk is most likely to due to inter-assay variability.

Table 1. Milk matrices have sensitive detection limits in the CANARY® assay.

Matrix	Detection Limits (ng)
Assay Buffer	< 0.075 ± 0.02
Whole Milk	< 0.185 ± 0.18
2% Milk	< 0.314 ± 0.4
Non-fat Milk	< 0.175 ± 0.11

Various milk matrices containing different fat content were spiked with BoNT/A in order to evaluate the ability of the CANARY® biosensor assay to detect the toxin. Samples were determined to be either positive or negative by the Zephyr program using a proprietary algorithm. Two to three independent experiments were performed for each milk matrix and assay buffer. The detection limit was calculated using the average of the last positive read out for each experiment ± SD. There was no statistical significance as determined by two-tailed unpaired Student's *t*-test, $p > 0.05$ for all conditions compared with assay buffer.

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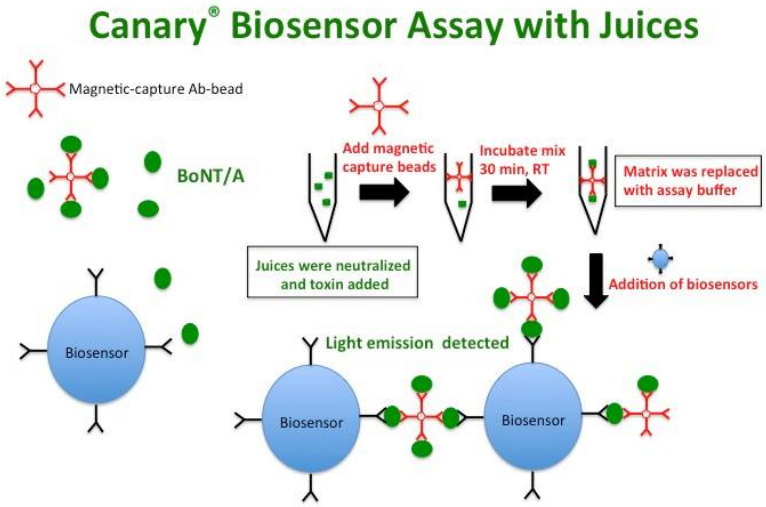
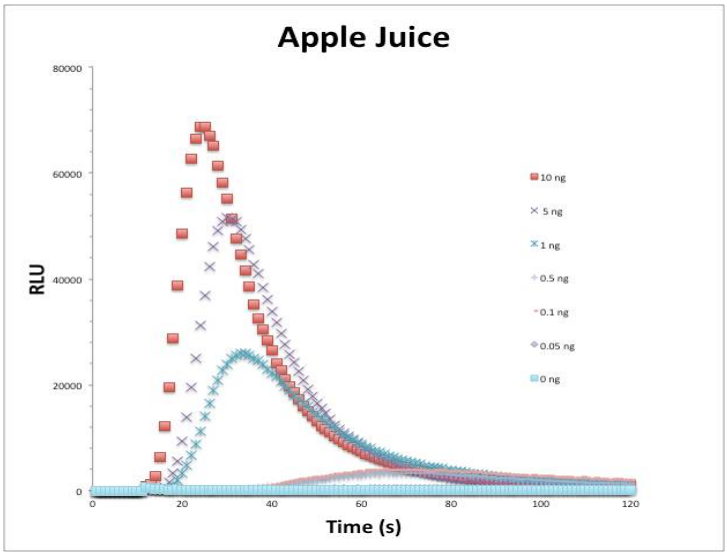
2.3. Detection of BoNT/A in acidified juices requires neutralization

Acidic juices such as apple, carrot, and orange have been used as liquid matrices for study by many BoNT detection platforms. We wanted to validate the use of the biosensor assay against these three commonly tested matrices. It was determined that the original CANARY® biosensor assay protocol had to be modified at two key steps (Figure 3A). Acidic juices were first neutralized with 5 M Tris pH 8.0 (10% final volume) and then toxin was spiked into matrix. Serial dilutions from this dose were made sequentially into neutralized juice matrices before adding magnetic capture beads. After incubation with the capture beads, a magnetic separator was used to capture the toxin matrix:immunomagnetic bead complex; removal of matrix, and then replacement with an equal volume of assay buffer. Biosensors were then added and luminescent signal was captured. As seen in Figure 3B, detection of BoNT/A in apple juice was possible but, as compared to assay buffer and milk matrices, the sensitivity (< 1 ng) was decreased.

A



B



BoNT/A (ng)	Read Out
10	Positive
5	Positive
1	Positive
0.5	Negative
0.1	Negative
0	Negative

Figure 3. Acidic juices require neutralization before usage with the CANARY® biosensor assay. (**A**) Schematic of the CANARY® biosensor assay used with acidic juices. Two key modification steps were added to the protocol: 1) neutralization of the acidic juices with Tris buffer before the addition of BoNT/A and 2) utilization of a magnetic bead separator to capture the toxin:immunomagnetic bead complex to remove matrix and then replacement with an equal volume of assay buffer. (**B**) A toxin concentration-dependent increase in signal is detected. Three independent experiments were performed and one representative data set is presented.

Table 2 shows the estimated limits of detection for the CANARY® assay in detection of BoNT/A in neutralized orange, apple, and carrot juices. The three juice matrices had a much higher elevated LOD (< 1.0 ng ± 0.00) that was statistically significant ($p < 0.05$) as compared to assay buffer (< 0.075 ng ± 0.02).

Table 2. Detection limits of CANARY® biosensor assay in neutralized acidic juices.

Matrix	Detection Limits (ng)
Orange Juice	< 1.0 ± 0.00
Apple Juice	< 1.0 ± 0.00
Carrot Juice	< 1.0 ± 0.00

Acidic juices first were neutralized and then spiked with BoNT/A in order to evaluate the ability of the CANARY® biosensor assay to detect the toxin. Samples were determined to be either positive or negative by the Zephyr program using a proprietary algorithm. Two to three independent experiments were performed for each neutralized matrix. The detection limit was calculated using the average of the last positive read-out for each experiment. Statistical significance was determined by using the two-tailed unpaired Student's *t*-test, $p < 0.05$ for all neutralized acidic juices compared with assay buffer.

2.4. Detection of BoNT/A in liquid egg, ground beef, green bean baby food, and smoked salmon

Since the biosensor assay can detect BoNT/A in assay buffer, milk matrices, and acidic juices with varying levels of sensitivity, more complex matrices consisting of liquid egg, ground beef, green bean baby food, and smoked salmon were spiked with toxin and the LOD for each complex matrix was determined. The estimated limits of detection for the CANARY® assay in the detection BoNT/A in liquid egg, ground beef, green bean baby puree, and smoked salmon are presented in Table 3. These complex matrices required minor modifications to the biosensor assay in terms of sample preparation. Liquid egg was diluted 1:10 into assay buffer and toxin was spiked into each tube with the 1:10 liquid egg/assay buffer mixture with specific concentrations instead of serially diluting previously with liquid matrices. 0.025 g of ground beef, green bean baby food, and smoked salmon were weighed out and put in reaction tubes with specified toxin doses and assay buffer to a volume of 250 µL. The samples were incubated at room temperature for 30 minutes before centrifugation at 10,000 x g for 5 minutes. 50 µL of the cleared supernatant was used with the magnetic capture beads for the continuation of the assay. Amongst these matrices, liquid egg diluted 1:10 matrix gave the highest sensitivity of < 16.7 ng ± 7.7; it is at this concentration after a 1:10 dilution indicating that the concentration of BoNT/A actually present in the undiluted matrix is much higher. BoNT/A was detected by the assay in both ground beef and green bean baby food, thus indicating that these two types of matrices are amenable to the assay. Smoked salmon had the lowest level of sensitivity (<3.125 ng ± 0.0) compared to assay buffer and all nine matrices..

Table 3. Detection limits of CANARY® biosensor assay for liquid egg, ground beef, green bean baby food, and smoked salmon.

Matrix	Detection Limits (ng)
Liquid egg	< 16.7 ± 7.7
Ground beef	< 0.39 ± 0.22
Green bean baby food	< 0.91 ± 0.6
Smoked salmon	< 3.125 ± 0.0

Liquid egg matrix was diluted 1:10 with assay buffer before toxin was added. Ground beef, green bean baby food, and smoked salmon at 0.025 g were added to assay buffer and toxin was added to a final volume of 250 µL. After incubation for 30 minutes at room temperature, samples were centrifuged at 10,000 x *g* for 5 minutes. Cleared supernatants were used for the assay. Samples were determined to be either positive or negative by the Zephyr program using a proprietary algorithm. Two to three independent experiments were performed for each matrix. The detection limit was calculated using the average of the last positive read-out for each experiment. Statistical significance as determined by the two-tailed unpaired Student's *t*-test, $p < 0.05$ for all conditions except for green bean baby food ($p = 0.07$) compared with assay buffer.

3. Discussion

BoNTs are among the most poisonous substances known to man and cause the disease botulism, which is distinguished by flaccid muscle paralysis that can lead to respiratory failure and death. Botulism occurs through three routes: foodborne, wound, and infant botulism. BoNTs are considered Tier 1 Select Agents (CDC) and pose a public health and food safety concern due to the their potential use by bioterrorists. Assays to detect BoNTs must have high sensitivity and

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specificity, be compatible with food and environmental samples, and they also should be field-deployable to be of use to a variety of people including first-responders, clinical and diagnostic technicians, and food inspectors.

BoNT detection assays utilize multiple methods including antibody-based, mass-spectrometry, nucleic acid-based, cell-based, and enzymatic assays, as well as *in vivo* and *ex vivo* mouse assays in buffer and some matrices [8-29]. All of the current technologies have advantages and disadvantages. Advantages are: high sensitivity, faster time, cost-effectiveness, smaller volumes, complex sample compatibility, and multiplex capability. However, the disadvantages are notable and include long experiment times; high costs associated with testing, including the need for expensive (and unwieldy) equipment; the need for expert personnel to conduct the experiments and specialized facilities in which to conduct them; and sensitivity or incompatibility with complex matrices such as sera, milk, juices, ground meat, eggs, and smoked fish. No single technology is adequate for use in clinical, food safety, or environmental settings; thus, all available and new technology platforms should be assessed for their ability to detect BoNTs.

The CANARY® Zephyr system utilizes a B-cell based biosensor system coupled with immunoprecipation of the toxin complex to detect BoNT/A. This study has found that the assay is rapid: (< 40 min) from the addition of immunomagnetic capture beads to the reaction matrix to the read out of luminescence and final determination of positive or negative for the sample tested, which is better than most traditional methods. The CANARY® Zephyr system is also portable, suitable for field-deployable because it consists of a laptop, a small centrifuge, and a small luminometer that can fit in a suitcase [33]. Yet, its portability does not sacrifice utility, as the assay also uses small volumes (50 µL) to facilitate multiple sample analysis for precious samples.

In this study, we have established that CANARY® can detect BoNT/A with a LOD of < 0.075 ng ± 0.02. Milk matrices (non-fat, 2 %, whole milk) slightly increased the LOD to < 0.175 – 0.314 ng, which was not statistically significant compared to assay buffer. Similar to previous work with other technologies such as ELISA and ECL [19], sample preparation of acidic juice (orange, apple, carrot) required neutralization of pH for the CANARY® assay. Neutralized acidic juice samples were not centrifuged to remove particulate matter as used previously with other technologies but a matrix removal step was added using a magnetic bead separator before biosensors were added. All neutralized acid juices (orange, apple, carrot) had a LOD < 1 ng ± 0.0. Diluted liquid egg 1:10 with assay buffer before spiking with BoNT/A and subjected to CANARY® gave a LOD < 16.7 ng ± 7.7. Experiments with undiluted liquid egg were unsuccessful. Solid complex foods also are known to be problematic because of the particulate matter, and so experimental protocols have used the cleared supernatant of ground beef as the matrix to spike toxin into for detection assays [31]. However, in this study, toxin was added directly to the ground beef with assay buffer. After incubation to allow for the dispersal of toxin in the matrix, the mixture was centrifuged to remove the particulate matter before proceeding with the biosensor assay. Using this sample preparation, the limit of detection was < 0. 39 ng ± 0.22. The same sample extraction protocol was applied for both green bean baby food as well as smoked salmon. Green bean baby food had an LOD of < 0.91 ng ± 0.6. The highest LOD from all ten complex matrices was smoked salmon, with a LOD of < 3.125 ng ± 0.0. The LODs determined from assay buffer and complex matrices using CANARY® Zephyr are within the range of LODS determined using the different detection technologies (sub-picogram to nanogram per mL or attomolar to pM concentrations).

In this study, the first reported study using the CANARY® Zephyr biosensor assay system to detect BoNTs, we have shown that CANARY® Zephyr is a useful platform that can be applicable in surveillance, clinical, and environmental testing of BoNT/A with good sensitivity, short time < 40

minutes, and reliability. This technology rapidly detects BoNT/A as compared to the extended time periods for other detection methods such as ELISAs (hours) and the mouse bioassay (days). Lateral flow devices, while simple and fast, have complex matrix and sensitivity issues in comparison to CANARY® Zephyr. Further optimization of sample extraction and biosensor protocol may yield improvements in the assay's sensitivity. Development of new BoNT/A or utilizing known BoNT/A monoclonal antibody sequences to generate new immunomagnetic capture beads or biosensors can improve the assay. Additionally, this platform could be further developed into rapid detection kits for the other BoNT serotypes and subtypes.

4. Materials and Methods

4.1 Reagents

Botulinum neurotoxin serotype A (holotoxin and toxin complex) were obtained from Metabiologics (Madison, WI). CANARY® Zephyr BoNT/A kit (25 rxns) containing assay buffer, reconstitution buffer, negative control, positive control, B-cell biosensors, microcentrifuge tubes, and immunomagnetic capture beads were obtained from PathSensors, Inc. (Baltimore, MD). The CANARY® detection system consists of a laptop, a small microcentrifuge, and a luminometer. Whole milk, 2% milk, non-fat milk, orange juice (no pulp), carrot juice, apple juice, green bean baby food, 80:20% ground beef, and smoked salmon were bought from a local supermarket.

4.2 Biosensor assay protocol for assay buffer and milk matrices

Botulinum neurotoxin serotype A at 1 mg/mL was diluted in phosphate gelatin buffer 1:100 to 10 µg/mL and allowed to disperse for 30 minutes at room temperature. From the 10 µg/mL stock, toxin was diluted into assay buffer or milk matrices to generate the highest concentration in a total volume of 200 µL. The sample was incubated for 30 minutes at room temperature before serial dilutions down were made in the respective matrices. 50 µL of diluted toxin in matrix was added to CANARY® Zephyr microcentrifuge tubes and then 10 µL of immunomagnetic capture beads was added. The reaction was placed on a rotator for 30 minutes at room temperature. At the end of the reaction time, 20 µL of the B-cell biosensor cells (in reconstitution buffer for at least 30 minutes at room temperature in the dark) was added to the top of the microcentrifuge tubes containing either all of the 50 µL (original concentration) or 11 µL (1:5 of the original concentration) of the toxin matrix:immunomagnetic bead complex. Initiation of the Zephyr software will cause the reaction tube to be centrifuged for 5 seconds before placement into the luminometer to be read immediately. Luminescence was recorded every second for a total of 120 seconds and displayed live in a graph. At the end of the run, the software uses a proprietary algorithm to determine if the sample was positive or negative. The algorithm that determines positive or negative responses is based on the signal-to-background noise ≥ 3 and the luminescent peak occurrence over a particular period of time after initiation of the luminometer run. Detection limits were determined by average of the last positive read out for each buffer/matrix from at 2-3 independent experiments.

4.3 Modified CANARY® protocol for acidic juices: carrot, apple, and orange juice

The protocol is similar to the one used for assay buffer and milk matrices. However, there are two key modifications: 1) Acidic juices were neutralized with 5 M Tris pH 8.0 (10% final volume) [19] before spiking and serial dilution with toxin. 2) After the 30 minute reaction to allow for binding of toxin:matrix with immunomagnetic capture beads; a magnetic separator was used to capture the complex, removal of the matrix, and replacement with an equal volume of assay buffer. All the subsequent steps following were the same as for *4.2* (assay buffer and milk matrix).

4.4 Assay protocol for liquid egg, ground beef, green bean baby food, and smoked salmon

The protocol is similar to 4.2 with some modifications. Of note, the toxin was spiked directly into each reaction matrix instead of serial dilution as with assay buffer, milks, and acidic juices. Liquid egg (egg yolk and egg white mixed) was diluted 1:10 into assay buffer before the toxin was added into the 1:10 liquid egg/assay buffer mix to give the indicated concentrations. 0.025 g of ground beef (80:20%), green bean baby food, and smoked salmon were added to each tube along with assay buffer and the indicated toxin dose to a volume of 250 µL. The ground beef, green bean baby food, and smoked salmon with toxin and assay buffer were incubated at room temperature for 30 minutes. The samples were vortexed vigorously for 1 minute before centrifugation at 10,000 x g for 5 minutes to pellet the insoluble particulates. 50 µL of the cleared supernatants were then used for the CANARY® reaction with immunomagnetic beads.

4.5 Determination of limits of detection and statistical significance

The limit of detection was determined to be the average of the last positive sample determined by the Zephyr program using a proprietary algorithm based upon previous work [32]. Statistical significance was determined by two-tailed unpaired Student's *t*-test, for all conditions compared with assay buffer with significance being $p < 0.05$.

Author Contributions: L.W.C., A.R.F., and C.C.T conceived and designed the experiments; C.C.T. performed the experiments; L.W.C., A.R.F., and C.C.T. analyzed the data; L.W.C., A.R.F., and C.C.T. wrote the paper.

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Conflicts of Interest: A.F is an employee of PathSensors Inc.

References

1. Arnon, S.S.; Schechter, R.; Inglesby, T.V.; et al. Botulinum toxin as a biological weapon: Medical and public health management. *JAMA* **2001**, *285*, 1059-1070.

2. Center for Disease Control and Prevention, 2015 Annual Report of the Federal Select Agent Program. *Center for Disease Control and Prevention; Atlanta, GA, USA:* **2016**.

3. Rossetto, O.; Pirazzini, M.; Montecucco, C. Botulinum neurotoxins: Genetic, structural and mechanistic insights. *Nat Rev Microbiol* **2014**, *12*, 535-549.

4. Rummel, A. The long journey of botulinum neurotoxins into the synapse. *Toxicon* **2015**, *107*, 9-24.

5. Tighe, A.P.; Schiavo, G. Botulinum neurotoxins: Mechanism of action. *Toxicon* **2013**, *67*, 87-93.

6. Hill, K.K.; Xie, G.; Foley, B.T.; Smith, T.J. Genetic diversity within the botulinum neurotoxin-producing bacteria and their neurotoxins. *Toxicon* **2015**, *107*, 2-8.

7. Barash, J.R.; Arnon, S.S. A novel strain of clostridium botulinum that produces type b and type h botulinum toxins. *J Infect Dis* **2014**, *209*, 183-191.

8. Ferreira, J.L.; Eliasberg, S.J.; Edmonds, P.; Harrison, M.A. Comparison of the mouse bioassay and enzyme-linked immunosorbent assay procedures for the detection of type a botulinal toxin in food. *J Food Prot* **2004**, *67*, 203-206.

9. Wictome, M.; Newton, K.; Jameson, K.; Hallis, B.; Dunnigan, P.; Mackay, E.; Clarke, S.; Taylor, R.; Gaze, J.; Foster, K., *et al*. Development of an in vitro bioassay for clostridium botulinum type b neurotoxin in foods that is more sensitive than the mouse bioassay. *Appl Environ Microbiol* **1999**, *65*, 3787-3792.

10. Pellett, S. Progress in cell based assays for botulinum neurotoxin detection. *Current topics in microbiology and immunology* **2013**, *364*, 257-285.

11. Thirunavukkarasu, N.; Johnson, E.; Pillai, S.; Hodge, D.; Stanker, L.; Wentz, T.; Singh, B.; Venkateswaran, K.; McNutt, P.; Adler, M., *et al*. Botulinum neurotoxin detection methods for public health response and surveillance. *Frontiers in Bioengineering and Biotechnology* **2018**, *6*, 80.

12. Sharma, S.K.; Ferreira, J.L.; Eblen, B.S.; Whiting, R.C. Detection of type a, b, e, and f clostridium botulinum neurotoxins in foods by using an amplified enzyme-linked immunosorbent assay with digoxigenin-labeled antibodies. *Appl Environ Microbiol* **2006**, *72*, 1231-1238.

13. Ching, K.H.; Lin, A.; McGarvey, J.A.; Stanker, L.H.; Hnasko, R. Rapid and selective detection of botulinum neurotoxin serotype-a and -b with a single immunochromatographic test strip. *J Immunol Methods* **2012**, *380*, 23-29.

14. Babrak, L.; Lin, A.; Stanker, L.H.; McGarvey, J.; Hnasko, R. Rapid microfluidic assay for the detection of botulinum neurotoxin in animal sera. *Toxins* **2016**, *8*, 13.

15. Koh, C.Y.; Schaff, U.Y.; Piccini, M.E.; Stanker, L.H.; Cheng, L.W.; Ravichandran, E.; Singh, B.R.; Sommer, G.J.; Singh, A.K. Centrifugal microfluidic platform for ultrasensitive detection of botulinum toxin. *Anal Chem* **2015**, *87*, 922-928.

16. Rasooly, R.; Stanker, L.H.; Carter, J.M.; Do, P.M.; Cheng, L.W.; He, X.; Brandon, D.L. Detection of botulinum neurotoxin-a activity in food by peptide cleavage assay. *Int J Food Microbiol* **2008**, *126*, 135-139.

17. Bagramyan, K.; Kaplan, B.E.; Cheng, L.W.; Strotmeier, J.; Rummel, A.; Kalkum, M. Substrates and controls for the quantitative detection of active botulinum neurotoxin in protease-containing samples. *Anal Chem* **2013**, *85*, 5569-5576.

18. Cheng, L.W.; Stanker, L.H.; Henderson, T.D., 2nd; Lou, J.; Marks, J.D. Antibody protection against botulinum neurotoxin intoxication in mice. *Infect Immun* **2009**, *77*, 4305-4313.

19. Cheng, L.W.; Stanker, L.H. Detection of botulinum neurotoxin serotypes a and b using a chemiluminescent versus electrochemiluminescent immunoassay in food and serum. *J Agric Food Chem* **2013**, *61*, 755-760.

20. Scotcher, M.C.; Cheng, L.W.; Stanker, L.H. Detection of botulinum neurotoxin serotype b at sub mouse ld(50) levels by a sandwich immunoassay and its application to toxin detection in milk. *PLoS One* **2010**, *5*, e11047.

21. Stanker, L.H.; Merrill, P.; Scotcher, M.C.; Cheng, L.W. Development and partial characterization of high-affinity monoclonal antibodies for botulinum toxin type a and their use in analysis of milk by sandwich elisa. *J Immunol Methods* **2008**, *336*, 1-8.

22. Stanker, L.H.; Scotcher, M.C.; Cheng, L.; Ching, K.; McGarvey, J.; Hodge, D.; Hnasko, R. A monoclonal antibody based capture elisa for botulinum neurotoxin serotype b: Toxin detection in food. *Toxins (Basel)* **2013**, *5*, 2212-2226.

23. Bagramyan, K.; Barash, J.R.; Arnon, S.S.; Kalkum, M. Attomolar detection of botulinum toxin type a in complex biological matrices. *PLoS One* **2008**, *3*, e2041.

24. Bagramyan, K.; Kalkum, M. Ultrasensitive detection of botulinum neurotoxins and anthrax lethal factor in biological samples by alissa. *Methods Mol Biol* **2011**, *739*, 23-36.

25. Barr, J.R.; Moura, H.; Boyer, A.E.; Woolfitt, A.R.; Kalb, S.R.; Pavlopoulos, A.; McWilliams, L.G.; Schmidt, J.G.; Martinez, R.A.; Ashley, D.L. Botulinum neurotoxin detection and differentiation by mass spectrometry. *Emerging Infectious Diseases* **2005**, *11*, 1578-1583.

26. Wang, D.; Baudys, J.; Hoyt, K.M.; Barr, J.R.; Kalb, S.R. Further optimization of peptide substrate enhanced assay performance for bont/a detection by maldi-tof mass spectrometry. *Analytical and Bioanalytical Chemistry* **2017**, *409*, 4779-4786.

27. Kalb, S.R.; Baudys, J.; Wang, D.; Barr, J.R. Recommended mass spectrometry-based strategies to identify botulinum neurotoxin-containing samples. *Toxins* **2015**, *7*, 1765-1778.

28. Liu, J.; Gao, S.; Kang, L.; Ji, B.; Xin, W.; Kang, J.; Li, P.; Gao, J.; Wang, H.; Wang, J., *et al.* An ultrasensitive gold nanoparticle-based lateral flow test for the detection of active botulinum neurotoxin type a. *Nanoscale Res Lett* **2017**, *12*, 227.

29. Orlov, A.V.; Znoyko, S.L.; Cherkasov, V.R.; Nikitin, M.P.; Nikitin, P.I. Multiplex biosensing based on highly sensitive magnetic nanolabel quantification: Rapid detection of botulinum neurotoxins a, b, and e in liquids. *Anal Chem* **2016**, *88*, 10419-10426.

30. Dunning, F.M.; Ruge, D.R.; Piazza, T.M.; Stanker, L.H.; Zeytin, F.N.; Tucker, W.C. Detection of botulinum neurotoxin serotype a, b, and f proteolytic activity in complex matrices with picomolar to femtomolar sensitivity. *Applied and Environmental Microbiology* **2012**, *78*, 7687-7697.

31. Worbs, S.; Fiebig, U.; Zeleny, R.; Schimmel, H.; Rummel, A.; Luginbuhl, W.; Dorner, B.G. Qualitative and quantitative detection of botulinum neurotoxins from complex matrices: Results of the first international proficiency test. *Toxins (Basel)* **2015**, *7*, 4935-4966.

32. Rider, T.H.; Petrovick, M.S.; Nargi, F.E.; Harper, J.D.; Schwoebel, E.D.; Mathews, R.H.; Blanchard, D.J.; Bortolin, L.T.; Young, A.M.; Chen, J., *et al.* A b cell-based sensor for rapid identification of pathogens. *Science* **2003**, *301*, 213-215.

436 33. Bartholomew, R.A.; Ozanich, R.M.; Arce, J.S.; Engelmann, H.E.; Heredia-Langner, A.; Hofstad, B.A.;
437 Hutchison, J.R.; Jarman, K.; Melville, A.M.; Victry, K.D., *et al.* Evaluation of immunoassays and general
438 biological indicator tests for field screening of bacillus anthracis and ricin. *Health Secur* **2017**, *15*, 81-96.

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