Video Article

Isolation and Quantification of Botulinum Neurotoxin From Complex Matrices Using the BoTest Matrix Assays

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Abstract

Accurate detection and quantification of botulinum neurotoxin (BoNT) in complex matrices is required for pharmaceutical, environmental, and food sample testing. Rapid BoNT testing of foodstuffs is needed during outbreak forensics, patient diagnosis, and food safety testing while accurate potency testing is required for BoNT-based drug product manufacturing and patient safety. The widely used mouse bioassay for BoNT testing is highly sensitive but lacks the precision and throughput needed for rapid and routine BoNT testing. Furthermore, the bioassay's use of animals has resulted in calls by drug product regulatory authorities and animal-rights proponents in the US and abroad to replace the mouse bioassay for BoNT testing. Several *in vitro* replacement assays have been developed that work well with purified BoNT in simple buffers, but most have not been shown to be applicable to testing in highly complex matrices. Here, a protocol for the detection of BoNT in complex matrices using the BoTest Matrix assays is presented. The assay consists of three parts: The first part involves preparation of the samples for testing, the second part is an immunoprecipitation step using anti-BoNT antibody-coated paramagnetic beads to purify BoNT from the matrix, and the third part quantifies the isolated BoNT's proteolytic activity using a fluorogenic reporter. The protocol is written for high throughput testing in 96-well plates using both liquid and solid matrices and requires about 2 hr of manual preparation with total assay times of 4-26 hr depending on the sample type, toxin load, and desired sensitivity. Data are presented for BoNT/A testing with phosphate-buffered saline, a drug product, culture supernatant, 2% milk, and fresh tomatoes and includes discussion of critical parameters for assay success.

Video Link

The video component of this article can be found at http://www.jove.com/video/51170/

Introduction

Botulinum neurotoxins (BoNTs) are the deadliest substances known, with intravenous human lethal doses estimated at 1-3 ng/kg[1,2]. Seven structurally similar serotypes of BoNT, labeled A through G, exist, each consisting of a heavy chain domain responsible for cell binding, uptake, and translocation into the cytosol and a light chain that encodes a zinc endopeptidase[3-5]. The exquisite toxicity of BoNT results from, in part, its specific binding and entry into motor neurons at the neuromuscular junction[6]. Once inside the neuron, the light chain endopeptidase specifically cleaves one or more of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins required for vesicle fusion, inhibiting neurotransmitter release and leading to flaccid paralysis[7-14]. Commonly known as the disease "botulism," paralysis of the diaphragm and intercostal muscles by BoNT ultimately results in respiratory failure and death unless early diagnosis and treatment are received.

Human foodborne botulism is most commonly associated with BoNT serotypes A, B, E, and F (BoNT/A, BoNT/B, *etc.*) and usually results from the ingestion of contaminated food[15,16]; although, several cases of wound botulism were reported amongst intravenous drug users[17,18]. In the United States, infant botulism resulting from the ingestion of *Clostridium* spores by children under the age of one is the most common form of botulism[19-21]. However, foodborne BoNT outbreaks resulting from improper home canning and food processing were reported in both the United States and abroad. Between 2000-2009, at least 338 cases of foodborne botulism were reported worldwide including six fatalities[22]. The ability to rapidly and sensitively detect foodborne botulism outbreaks is a critical indication that could aid early diagnosis[23,24]. Furthermore, detection methods that allow cost-effective and routine food testing will lead to improved food security.

BoNT's neuronal specificity and long biological half-life also makes it a potent therapeutic. In the United States, BoNT-based drugs are approved by the Food and Drug Administration for the treatment of cosmetic conditions and neuromuscular-related disorders including glabellar lines, cervical dystonia, migraine headaches, overactive bladder, and strabismus. Numerous "off-label" applications are documented, including high-dose treatments for severe muscle dysfunction[25-28]. Accurate toxin quantification is critical for correct dosing, as underdosing may lead to ineffective treatment while overdosing puts patients at risk of potentially harmful side effects. Unfortunately, no standardized potency assay protocol is shared across manufacturers, resulting in unit definition inequalities between BoNT-based drug products[29-31].

The standard test for BoNT is the mouse bioassay in which BoNT-containing samples are injected intraperitoneally into mice and the numbers of deaths recorded over 1-7 days[16,32,33]. The mouse bioassay is very sensitive with limits of detection (LOD) of 5-10 pg BoNT/A[34]; however, ethical concerns over animal use, the high cost of training personnel and maintaining animal facilities, long assay times, and the lack of standardized

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protocols resulted in calls to develop standardized, animal-free BoNT testing and quantification methods[35-39]. Recently, several alternate BoNT quantification methods were developed that offer mouse or near-mouse bioassay sensitivity[40-49]. These methods commonly use fluorescence, mass-spectrometry, or immunological methods and offer assay times considerably shorter than the mouse bioassay without animal use. Mass-spectrometry approaches combined with immunological techniques were shown to detect and quantify BoNT contained in food and other complex samples; however, personnel training requirements and specialized equipment limit these assays[50-55]. Most other alternate assays are not readily applicable to complex sample testing or lack the throughput required for routine BoNT testing. The highly variable nature of food sample viscosity, pH, salt content, and matrix constituents presents an especially difficult challenge when trying to develop *in vitro* assay methods with sensitivity to match the extreme potency of BoNT. Furthermore, even simple and relatively benign buffer systems, such as those resulting from resuspension of BoNT-based drug products, contain salt, albumin, and sugar stabilizers (*i.e.* excipients) that significantly impact *in vitro* BoNT potency[56]. Toxin purification is required for accurate activity testing of all but the simplest of samples[56-59].

The BoTest Matrix assays were designed for rapid, high-throughput, and consistent quantification of BoNT from highly complex samples using equipment commonly found in research laboratories[56,60]. These assays use paramagnetic beads covalently linked to serotype-specific anti-BoNT antibodies to bind and sequester BoNT out of a sample and then remove interfering matrix compounds by washing. Following washing, bound BoNT proteolytic activity is then quantified in an optimized reaction buffer using a reporter compatible with the BoNT serotype being tested. These reporters are fluorogenic proteins consisting of a N-terminal cyan fluorescent protein (CFP) moiety and a C-terminal yellow fluorescent protein derivative (Venus) moiety linked by a BoNT substrate, SNAP25 residues 141-206 or synaptobrevin residues 33-94 constituting the BoTest A/E or B/D/F/G reporters, respectively[45]. Reporter cleavage by BoNT is monitored using Förster resonance energy transfer (FRET). When the reporter is intact, excitation of CFP results in FRET to Venus, quenching CFP emission and exciting Venus emission. Cleavage of the reporter by BoNT prevents FRET, leading to an increase in CFP emission and decrease in Venus emission. BoNT activity can then be quantitatively measured using the ratio of the CFP and Venus emissions. LOD below 3 pg are possible from a wide range of foods using a high-throughput 96-well plate format[56]. Increased sensitivity can be obtained using larger sample volumes since the assay allows concentration of the toxin on the bead surface.

The BoTest Matrix assays for BoNTs A, B, E, and F were developed and tested with food, pharmaceutical, and environmental samples[56,60]. Here, we describe procedures for executing these assays for the detection of BoNT in low complexity (*e.g.* pharmaceutical, BoNT in buffer) and high complexity (*e.g.* food, environmental) samples. Specific processing methods for several sample types are addressed in this protocol and sample types not described here can usually be adapted using a combination of the presented methods. The protocol was developed and tested with BoNT/A but is adaptable to other BoNT serotypes using their respective assays as demonstrated elsewhere[56,60].

Protocol

1. Preparation of Assay Reagents

1. Thaw 200x dithiothreitol (DTT), 10x Matrix Binding buffer (10x Binding buffer hereafter), 10x Neutralization buffer (food or pH imbalanced samples only), and 10x BoTest Reaction buffer (10x Reaction buffer hereafter) at room temperature (RT) for 15 min or until completely thawed. See **Table 1** for a list of buffers and reagents used in this protocol. See **Table 2** for a list of materials and equipment required for this protocol.
2. Vortex the thawed buffers for 5 sec to mix. 10x Neutralization buffer, 200xDTT, and 10x Reaction buffer should appear clear while the 10x Binding buffer will have a cloudy appearance. Warm the 10x Reaction buffer for 5 min at 37 °C and repeat vortexing if it appears cloudy following thawing.
3. Generate 3.8 ml of 1x Reaction buffer.
    1. Label a 15 ml conical tube "1x Reaction buffer" and add 3.42 ml molecular biology grade water, 380 µl 10x Reaction buffer, and 19 µl 200x DTT. Mix buffer well by inversion.
    2. Cut a single EDTA-free protease inhibitor tablet in quarters using a clean razor blade and add one quarter of the tablet to the 1x Reaction buffer (the remaining tablet portion can be stored at 4 °C for later use). *N.B.* Protease inhibitors may not be necessary if using purified toxin and simple buffers (*e.g.* pharmaceutical samples).
    3. Vortex the 1x Reaction buffer until the protease tablet is fully dissolved.
4. Warm the Matrix A beads (IP-A beads hereafter) for 20 min at RT.
5. Thaw the BoTest A/E reporter (A/E reporter hereafter) at RT protected from light.
6. Label a 15 ml conical tube "0.5 µM A/E reporter" and add 45 µl of the 20 µM A/E reporter stock to 1.8 ml 1x Reaction buffer. Mix thoroughly by pipetting and store on ice protected from light.

2. Standard Curve Sample Generation

The standard curve described here spans 10-30,000 mLD_{50}/g food or per ml buffer in half-log dilutions (**Table 3**). The end-user is free to use alternate concentrations as applicable.

1. **Spiking and incubating the matrices with reference material.** Generate the standard curve using a diluent of the same matrix as the unknown, if possible, to reduce matrix effects. Use gelatin phosphate buffer (GPB) or phosphate-buffered saline (PBS) as a diluent if additional, BoNT-negative matrix is not available (*e.g.* field or BoNT outbreak sample testing). Generate the standard curve by spiking BoNT/A into the matrix of choice following the appropriate protocol below.
    1. **Low complexity samples (*e.g.* PBS, GPB, or pharmaceutical)**
        1. Add 10 ml of the appropriate buffer to a 15 ml conical tube and set aside. This sample will be used as diluent for the standard curve and unknown dilutions (Section 4).
        2. Add 1.2 ml buffer to a microcentrifuge tube.

3. **CAUTION:** This step uses BoNT and extreme caution must be used when handling and disposing of any reagents and materials that come in contact with the toxin. Use of personal protective equipment and proper disposal of all materials must be performed according the US Department of Labor OSHA guidelines for BoNT (http://www.osha.gov/SLTC/botulism/index.html). Add BoNT/A to the 1.2 ml sample such that the final concentration is 30,000 mLD_{50}/ml (36,000 mLD_{50} total).

2. **Liquid food samples (or other complex liquid samples)** Note: Initial testing is recommended to determine the volume of supernatant recovered from clarified samples as the particulate matter (*e.g.* pulp) in liquid matrices will vary. This protocol assumes at least 1.2 ml of clarified supernatant will be recovered from a 1.4 ml sample. Increase sample size if necessary.
    1. Add 10 ml liquid food to a 15 ml conical tube and set it aside. This sample will be used as diluent for the standard curve and unknown dilutions (Section 4).
    2. Weigh an empty 1.5 ml microcentrifuge tube and record its mass.
    3. Add 1.4 ml of the liquid food to the weighed microcentrifuge tube.
    4. Reweigh the microcentrifuge tube and calculate the mass of the added food sample by subtracting the mass of the empty tube.
    5. **CAUTION:** This step uses BoNT and extreme caution must be used when handling and disposing of any reagents and materials that come in contact with the toxin. Use of personal protective equipment and proper disposal of all materials must be performed according the US Department of Labor OSHA guidelines for BoNT (http://www.osha.gov/SLTC/botulism/index.html). Add BoNT/A to the 1.4 ml sample at a final concentration of 30,000 mLD_{50}/g food.
    6. Incubate the diluent and spiked samples at RT or 4 °C for 2 hr to give the BoNT time to interact with the food matrix, mimicking a natural contamination.

3. **Solid food samples (or other solid samples)** Note: Initial testing is recommended to determine the volume of supernatant recovered from homogenized and clarified samples following the addition of 1 ml GPB/g food. This protocol assumes that at least 1.2 ml clarified supernatant will be recovered from a 2 g sample. Increase sample size if necessary.
    1. Weigh out 10 g solid sample into a 50 ml conical tube and set aside. This will be used as diluent.
    2. Weigh out 2 g solid food sample into a second 50 ml conical tube.
    3. **CAUTION:** This step uses BoNT and extreme caution must be used when handling and disposing of any reagents and materials that come in contact with the toxin. Use of personal protective equipment and proper disposal of all materials must be performed according the US Department of Labor OSHA guidelines for BoNT (http://www.osha.gov/SLTC/botulism/index.html). Add BoNT/A to the surface of the 2 g sample to a final concentration of 30,000 mLD_{50}/g food (60,000 total mLD_{50}).
    4. Incubate the diluent and spiked samples at RT or 4 °C for 2 hr to give the BoNT time to interact with the food matrix, mimicking a natural contamination.

2. **Sample homogenization and buffer adjustment.** Process the spiked standard curve sample and diluent generated above according to sample type.
    1. **Low complexity samples**
        1. No further sample processing is necessary.

    2. **Liquid food samples (or other complex liquid samples)**
        1. No sample homogenization is necessary.
        2. Add 140 µl 10x Neutralization buffer to the 1.4 ml spiked sample and 1 ml 10x Neutralization buffer to the 10 ml diluent sample. Mix samples well by inversion.
        3. Partially clarify both samples by centrifuging for 10 min at 6,000 x g and 4 °C. Immediately remove the supernatants and transfer to new tubes.

    3. **Solid food samples (or other solid samples)**
        1. Add 2 ml GPB (1 ml GPB/g food) to the 2 g BoNT/A-spiked solid food sample and 10 ml GPB to the 10 g diluent sample.
        2. Homogenize the samples using a pestle until thoroughly blended. Depending on the nature of the sample, there may be small chunks of material that cannot be homogenized, which is acceptable. Alternately, mechanical homogenization methods may be used. **Use of a blender is not recommended as it may inactivate as well as aerosolize the toxin.**
        3. Add 1/10th volume of 10x Neutralization buffer to the diluent sample based on the approximate total volume (*e.g.* 2 ml if the volume is 20 ml). Extrapolate the total volume of the BoNT/A-spiked sample and add 1/10th volume of 10x Neutralization buffer. Mix samples well by inversion.
        4. Partially clarify both samples by centrifuging for 10 min at 6,000 x g and 4 °C. Immediately remove the supernatants and transfer to new tubes.

3. **Prepare standard curve serial dilutions for testing**
    1. Using the BoNT/A-spiked standard curve sample as D1 and the nonspiked sample as diluent, generate the remaining standard curve samples in 1.5 ml microcentrifuge tubes according to **Table 3**.

3. Prepare Unknown Samples

This section can be completed in parallel to section 2.

1. **Determine the number and dilutions of unknowns.** Test unknowns in triplicate if possible.
    1. For qualitative assays, run the unknowns without any further dilution than required to process the sample as described below.

2. For quantitative assays, prepare at least two 1:10 dilution samples, as described below, to ensure that one or more samples fall within the linear range of the assay response. Generate dilutions using a diluent of the same matrix as the unknown, if possible, to reduce matrix effects as described in Section 3. Otherwise, use PBS or GPB as the diluent.

2. **Generating and diluting unknown samples according to sample type.**
    1. **Low complexity unknowns (*e.g.* PBS, GPB, or pharmaceutical)**
        1. Add at least 750 µl unknown to a microcentrifuge tube to generate Unknown dilution 1.
        2. Add 675 µl diluent to two microcentrifuge tubes labeled Unknown dilution 2 and 3. The diluent will be the same material used for standard curve generation.
        3. Serially dilute dilution 1 by transferring 75 µl of dilution 1 into the dilution 2 tube and mixing.
        4. Serially dilute dilution 2 by transferring 75 µl of dilution 2 into the dilution 3 tube and mixing.

    2. **Liquid food unknowns (or other complex liquid samples)** Note: Initial testing is recommended to determine the volume of supernatant recovered from clarified samples as discussed in Section 3. Increase sample size if necessary.
        1. Add ≥ 875 µl of the liquid unknown to a microcentrifuge tube.
        2. Add 1/10th volume of 10x Neutralization buffer to the sample (*e.g.* 87.5 µl for a 875 µl sample).
        3. Partially clarify the sample by centrifuging for 10 min at 6,000 x g and 4 °C. Immediately transfer the supernatant to a new tube. This is Unknown dilution 1.
        4. Add 675 µl diluent to two tubes labeled Unknown dilution 2 and 3. The diluent will be the same ***processed*** material used for standard curve generation.
        5. Serially dilute dilution 1 by transferring 75 µl of dilution 1 into the dilution 2 tube and mixing.
        6. Serially dilute dilution 2 by transferring 75 µl of dilution 2 into the dilution 3 tube and mixing.

    3. **Solid food unknowns (or other solid samples)** Note: Initial testing is recommended to determine the volume of supernatant recovered from clarified samples as discussed in Section 3. Increase sample size if necessary.
        1. Weigh out 2 g solid unknown sample into a 50 ml conical tube.
        2. Add 2 ml GPB (1 ml GPB/g food) to the 2 g solid sample.
        3. Homogenize the samples as described in **Section 3.2.3.2.**
        4. Add 1/10th volume of 10x Neutralization buffer to the sample based on the approximate total volume (*e.g.* 0.4 ml if the volume is 4 ml). Mix samples well by inversion.
        5. Partially clarify the sample by centrifuging for 10 min at 6,000 x g and 4 °C. Immediately transfer ≥750 µl supernatant to a microcentrifuge tube. This is Unknown dilution 1.
        6. Add 675 ml diluent to two tubes labeled Unknown dilution 2 and 3. The diluent will be the same ***processed*** material used for standard curve generation.
        7. Serially dilute dilution 1 by transferring 75 µl of dilution 1 into the dilution 2 tube and mixing.
        8. Serially dilute dilution 2 by transferring 75 µl of dilution 2 into the dilution 3 tube and mixing.

4. Final Sample Clarification

If testing liquid or solid food samples, centrifuge all samples for 5 min at ≥14,000 x g in a microcentrifuge to fully clarify the samples. Immediately remove the supernatants and transfer to new tubes.

5. Plate Setup and BoNT/A Pull Down

1. The specific plate layout is application dependent; however, do not use the outside wells so as to avoid edge effects. Each unknown sample and standard curve sample D1-D8 requires 3 wells while sample D9 requires 6 wells. A suggested plate layout is shown in **Figure 1**.
2. Add 20 µl 10x Binding buffer to each well to be used.
3. Add 200 µl of each clarified dilution and unknown to each of three wells (six wells for D9) for triplicate testing. Mix the plate for 10 sec on a microplate mixer.
4. Add the IP-A beads.
    1. Vortex the IP-A beads for 10 sec at the highest speed. Continue vortexing if beads are not fully resuspended and homogeneous.
    2. Pipette 20 µl IP-A beads to each sample well.
    3. Mix the plate for 30 sec on a microplate mixer.

5. Incubate the plate using a rotating plate incubator for 2 hr at 750 rpm, 25 °C or RT. Assay performance is highly dependent on generating and maintaining the IP-A bead suspension during all incubation steps. Always resuspend beads with a microplate mixer after pelleting and maintain the suspension using an orbital microtiter plate shaker during all incubation steps.

6. Plate Washing and Bead Resuspension

1. Wash the plates either by hand or by using a **magnetic bead-compatible** automated plate washer. An automated plate washer configured for magnetic beads greatly increases assay throughput.
    1. **Manual Washing**
        1. Label a 50 ml conical tube "1x Wash buffer" and add 45 ml molecular biology grade water and 5 ml 10x Matrix Wash Buffer. Mix buffer well by inversion.
        2. Remove the plate from the rotating plate incubator.
        3. Immediately place the plate on a 96-well magnetic bead separation plate for 5 min.

4. While keeping the plate on the 96-well magnetic bead separation plate, gently remove and discard the supernatants from the sample wells using a single- or multi-channel pipette. Do not aspirate beads- visually monitor the aspirated buffer in the pipette tip for accidental bead removal. If removal is witnessed, gently add the tip contents back to the well, reseparate, and repeat removal.
5. Add 300 µl 1x Wash buffer to each sample well.
6. Fully resuspend the beads by mixing the plate for 30 sec on a microplate mixer.
7. Incubate the plate on the 96-well magnetic bead separation plate for 2 min.
8. Remove and discard the supernatants from the sample wells as before.
9. Repeat steps 6.1.1.5-6.1.1.8 three more times for a total of 4 washes.
10. With the plate on the 96-well magnetic bead separation plate, visually inspect the wells to confirm even supernatant removal; use a pipette to remove excess residual buffer as necessary. Some buffer will remain in the wells and care must be taken to avoid bead removal or bead drying.
11. Add 50 µl 1x Reaction buffer to each sample well and mix the plate for 30 sec on a microplate mixer to fully resuspend the beads. If needed, use a pipette to fully resuspend the beads.

2. **Automated Plate Washing**
    1. Setup and program the washer according to **Table 4**. Clean and flush the washer with high-quality (*e.g.* nanopure) water.
    2. Prime the washer by running the program "Prime".
    3. Remove the plate from the rotating plate incubator.
    4. Immediately place the plate on the 96-well magnetic bead separation plate on the plate washer.
    5. Run the link program "Master Wash". The first program step is a 5 min incubation step where the washer will be stationary.
    6. Following program completion, remove the plate from the washer, add 50 µl 1x Reaction buffer to each sample well, and mix the plate for 30 sec on a microplate mixer to fully resuspend the beads. If needed, use a pipette to fully resuspend the beads.

7. Assay Initiation and Incubation

1. Add 50 µl 0.5 µM A/E reporter (see Section 1) to each sample well and mix for 30 sec on a microplate mixer to fully resuspend the beads.
2. Add 100 µl water to each unused well on the plate to prevent edge effects.
3. Seal the plate with plate sealing tape and incubate the plate using a rotating plate incubator at 750 rpm, 25 °C or RT. Protect the plate from light during incubation.

8. Data Collection and Analysis

Note: This assay is a real-time assay that can be measured multiple times until the desired sensitivity is obtained, there are no stop reagents required. Recommended initial read times are 2, 4, and 24 hr incubation time with assay sensitivity increasing with incubation time.

1. Data collection
    1. At each read time, remove the plate from the rotating plate incubator, remove the sealing tape, and immediately place the plate on the 96-well magnetic bead separation plate. Allow the beads to separate for 2 min.
    2. Place the plate in the microplate reader and measure the emissions at ~470 and ~526 nm under excitation at ~434 nm.
    3. If additional read times are desired, resuspend the beads for 30 sec on the microplate mixer, reseal the plate, and return the plate to the rotating plate incubator.

2. Data analysis
    1. Calculate the emission ratio for each sample by dividing the relative fluorescence unit (RFU) value at 526 nm by the RFU value at 470 nm.
    2. Plot the emission ratio versus the log[BoNT/A] for the standard curve data points. Depending on the BoNT/A potency range tested, a sigmoidal dose-response curve will be obtained (see Representative Results).
    3. Fit the standard curve data with the variable slope dose-response curve Y=Bottom+(Top-Bottom)/(1+10^((logEC_{50}-X)*Hillslope)) where X is the logarithm of concentration, Y is the response, and Y starts at Bottom and goes to Top with a sigmoidal shape.
    4. Determine the limits of detection (LOD), limits of quantification (LOQ), and half-maximal effective concentration (EC_{50}). Limits of detection are defined as a sample having an emission ratio less than 3 standard deviations (SDs) below the blank controls (n = 6). Limits of quantification are defined as a sample having an emission ratio less than 10 SDs below the blank controls (n = 6). EC_{50} is determined from the sigmoidal dose-response curve fit.
    5. Interpolate the potency of any unknown samples against the sigmoidal dose-response standard curve.
        1. For quantitative results, the unknown sample should ideally fall within the linear portion of the standard curve. Approximate the linear portion of the standard curve by calculating the 20-80% total assay response window (*e.g.* if the emission ratio of the standard curve ranges from 0.5-2.5, the linear range would be the portion of the standard curve that ranges from 0.9-2.1).
        2. For qualitative results, compare the unknown sample against the LOD and LOQ of the standard curve.
        3. Do not extrapolate unknown samples beyond the limits of the standard curve.

Representative Results

A diagram summarizing the steps in the described protocol is shown in **Figure 2**. The assay requires between 4-26 hr to complete depending on sample type and desired assay sensitivity, but only ~2 hr of hands-on time. The assay is performed in 96-well plates and, depending on the type of testing being performed, allows triplicate testing of up to 20 samples including standards per plate.

Figure 3 shows representative assay results using BoNT/A holotoxin spiked into PBS and tested with the described protocol following 2, 4, and 24 hr incubation with the A/E reporter. Purified BoNT/A holotoxin was chosen for this experiment because its defined molecular weight of ~150 kDa, compared to the more broadly defined 700-900 kDa for the toxin complex, and purity allow very accurate spiking at precise concentrations. The emission ratio, the ratio of emission at 526 nm to that at 470 nm following excitation at 434 nm, at each time point is plotted versus BoNT/A concentration (the mLD_{50} value is based on the BoNT/A manufacturer's potency testing). Cleavage of the reporter by BoNT is measured as a reduction in the emission ratio, which with our plate reader ranges between about 2.7 for the intact reporter to about 0.7 for the fully cleaved reporter. It is important to note that, since each plate reader measures fluorescence in RFUs, the actual value of the emission ratio will be dependent on the plate reader used. Prolonged incubation time with the reporter results in increased reporter cleavage as seen by the leftward shift in the curve. The data points, tested in triplicate, display low SD of the mean and follow the expected trend of increased emission ratio with decreased toxin load. Failure of the assay to follow this expected trend may indicate an error during dilution generation or data plotting. The emission ratios of the controls containing no BoNT/A also remain steady during the incubation (**Figure 3**, inset), indicating the lack of nonspecific protease activity.

An example of using this protocol to quantify pharmaceutical BoNT/A samples is shown in **Figure 4**. A standard curve was generated in PBS with purified BoNT/A holotoxin and processed in parallel with dilutions of drug product generated from a single 100 U vial of lyophilized BOTOX rehydrated in 0.9% saline. (Ideally, the standard curve would be composed of reference lots of BOTOX but such material was not readily available.) Samples were tested using the described protocol with the exception that 50 µl samples were tested in duplicate without the use of 10x Binding buffer. The standard curve was plotted as a function of BoNT/A concentration following 24 hr incubation with the A/E reporter. The emission ratio of each unknown sample was then visualized by plotting its intersection across the standard curve. In this assay, the linear portion of the standard curve (the 20-80% assay response window) falls between an emission ratio of 2.11-1.05 and is indicated by the dashed box in the figure. The concentrations of the three unknowns that fall within this linear range were then interpolated from the standard curve (**Figure 4**, inset). This example demonstrates the general methodology that would be used to detect or quantify any unknown sample against a standard curve.

Fresh tomatoes and 2% milk were chosen to demonstrate the protocol's performance and sensitivity using both a solid (tomato) and liquid food (2% milk) matrix. BoNT/A complex comprised of the core holotoxin and neurotoxin-associated proteins (NAPs) was selected for these experiments because this preparation resembles the toxin produced during a natural *Clostridium* contamination. As shown in **Figure 5A**, recovery of BoNT/A, measured as cleavage of the A/E reporter, is observed with both matrices. Increased incubation time with the reporter increases assay sensitivity, but does not result in decreased emission ratios for the no toxin controls (**Figure 5A**, insets), indicating the observed cleavage results from BoNT/A and not nonspecific protease carry over from the food in the assay. As with **Figure 3**, the data displays low variability and follows the expected trend of decreased reporter cleavage with decreased toxin concentration.

The BoNT/A LOD, LOQ, and EC_{50} for both foods at each time point shown are summarized in **Table 5**. The LOD and LOQ are defined as the lowest concentration sample with an emission ratio lower than three and ten SDs below background (samples containing no BoNT/A), respectively. These limits are restricted to the data points tested, although interpolation to the sigmoidal dose-response curve can be used to calculate lower theoretical limits. Some matrix-to-matrix variability in LOD and LOQ is expected, as matrix effects may influence the binding of the toxin to the beads and the recovery of beads during washing. While it would appear that there was more toxin recovery from 2% milk than tomatoes from the data in **Figure 5A**, much of this difference results from the additional dilution required to homogenize tomato samples in GPB.

In addition to being a solid food matrix, tomatoes are a noteworthy sample type where pH and ionic strength adjustment, achieved by the addition of 10x Neutralization buffer, is critical to assay success. **Figure 5B** illustrates assay responses when testing tomatoes with or without inclusion of the 10x Neutralization buffer. Failure to add the 10x Neutralization buffer results in poor recovery of BoNT/A from samples and is demonstrated by a constant emission ratio across all tested BoNT/A concentrations. Addition of the 10x Neutralization buffer, though, results in sensitive detection of the toxin.

Nonspecific proteases contained in complex matrices can lead to false positives if not addressed since proteases other than BoNT may cleave the A/E reporter. Nonspecific proteases may be endogenous to the food sample or introduced when using nonpurified BoNT preparations such as *Clostridium* culture supernatants. Thorough bead washing will remove most nonspecific proteases; however, the addition of protease inhibitors to the reaction buffer is critical. **Figure 6** demonstrates nonspecific protease activity found in *Clostridium* BoNT/A culture supernatants using a modified A/E reporter, BoTest KO (KO reporter). The KO reporter is the same as the A/E reporter with the exception that the BoNT/A cleavage site has been mutated such that it is no longer cleaved by BoNT/A. Therefore, any observed reporter cleavage results from nonspecific protease activity. The high levels of KO reporter cleavage indicates the culture supernatant contains a high level of protease activity, but that activity is effectively negated by the addition of protease inhibitors.

The sample data demonstrate the utility of the protocol for high throughput BoNT/A detection in simple buffers and food matrices. Certain foods may require small assay adjustments, but the described protocol should yield good results with the majority of food types.

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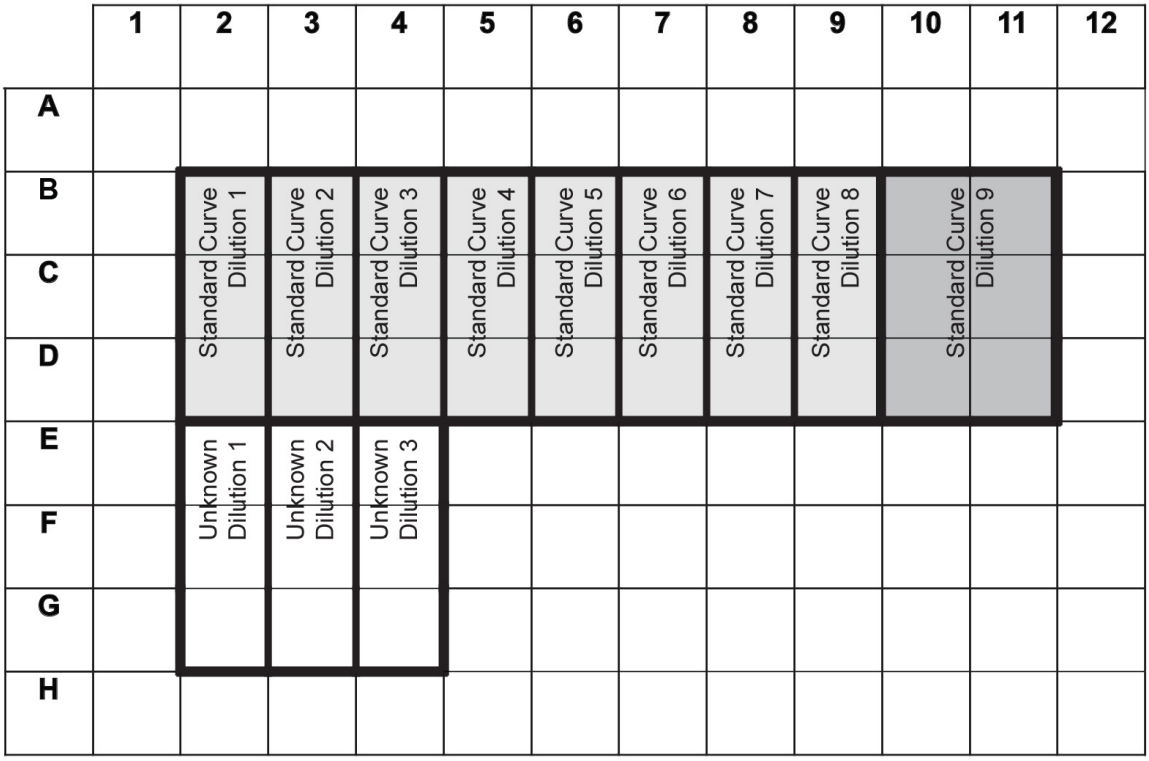

Figure 1. Suggested plate layout. Samples are restricted to the inner 60 wells of the plate to avoid possible edge effects. Unknown or standard curve samples can be added as desired. All unused wells should be filled with 100 µl water during reporter incubation. Click here to view larger image.

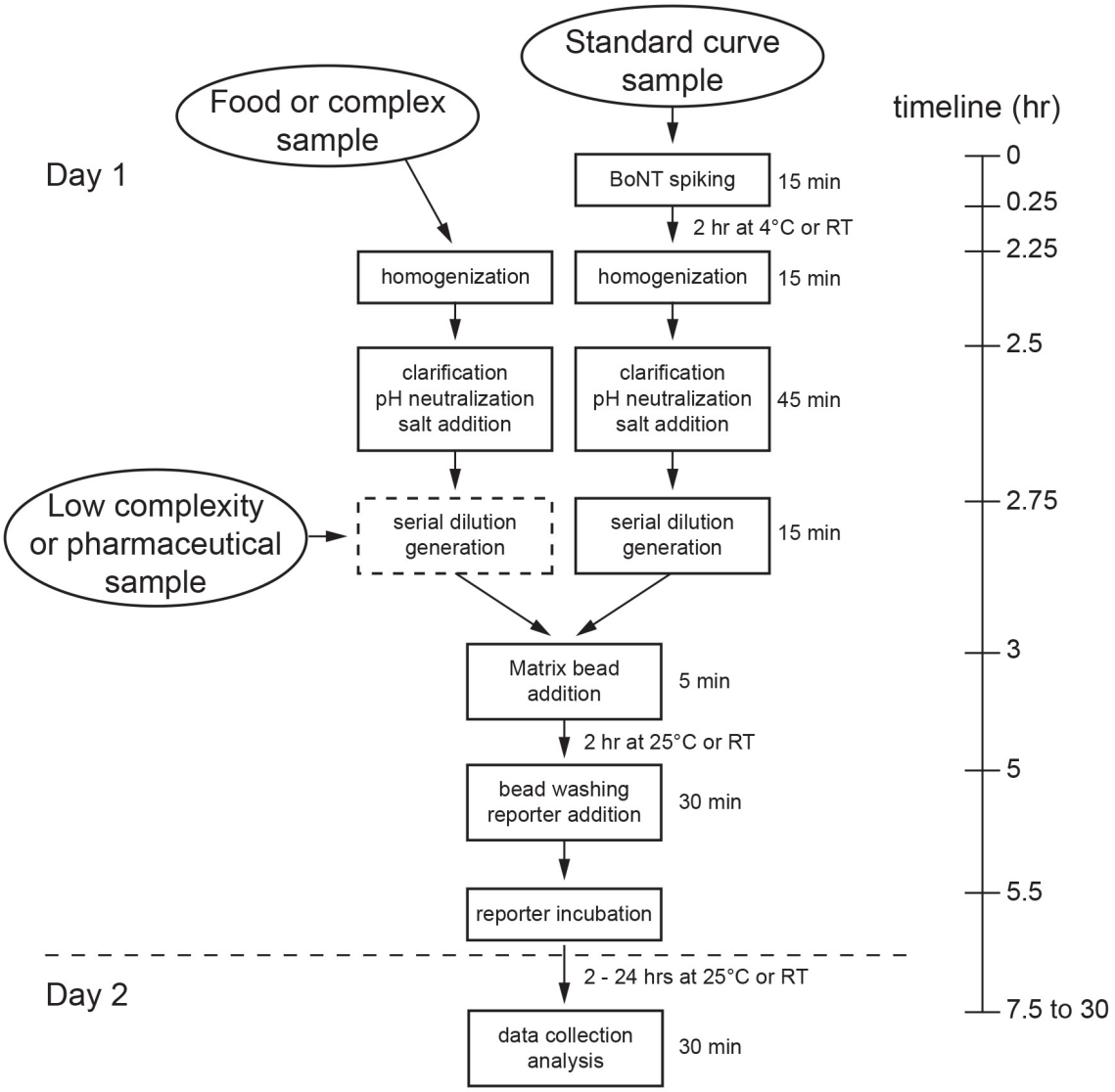

Figure 2. Schematic overview of the described protocol. Each major step in the protocol is indicated by a labeled box and aligned next to an estimated assay timeline (not to scale). Nonfood samples do not require sample processing and so enter the protocol downstream of food samples. The dashed box around serial dilution generation for the unknowns indicates this is an optional, but recommended, step. Click here to view larger image.

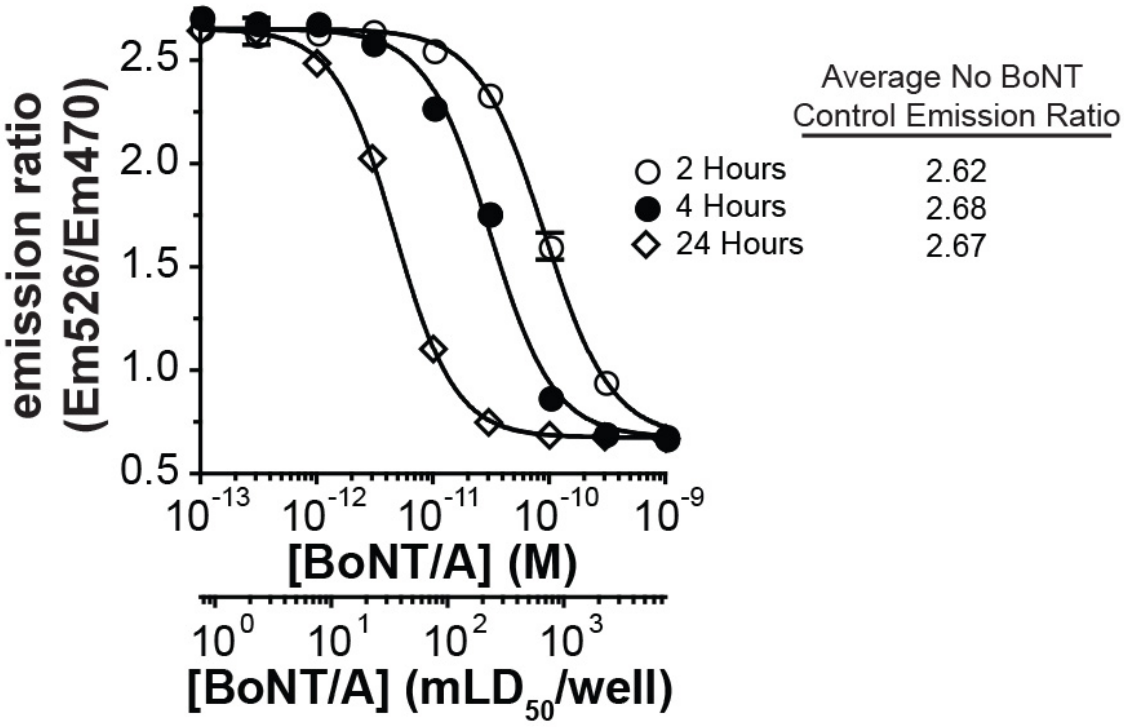

Figure 3. Detection of BoNT/A holotoxin in PBS using the described protocol. Purified BoNT/A holotoxin was spiked into PBS and tested using the described protocol with an upper BoNT/A concentration of 1 nM. All samples were tested in triplicate and the error bars represent the standard deviation of the mean. Reported potency is based on the manufacturer's mouse bioassay testing of the toxin. The emission ratio (the ratio of emission at 526 nm to 470 nm upon excitation at 434 nm) of the standard curve was measured following 2, 4, and 24 hr incubation with A/E reporter and plotted as a function of BoNT/A concentration. Click here to view larger image.

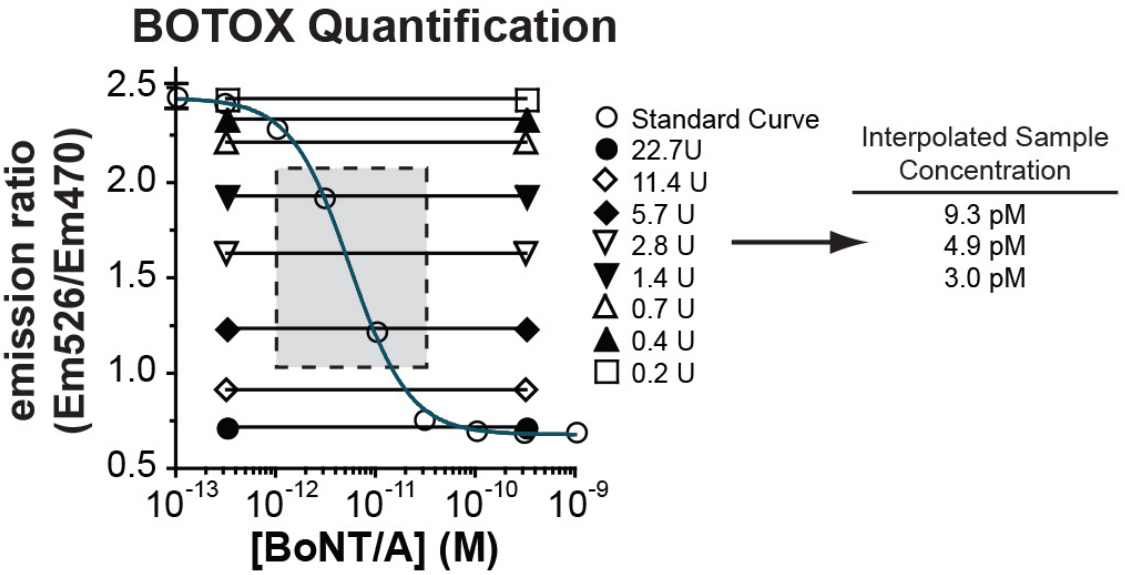

Figure 4. Quantification of BOTOX drug product using the described protocol. A single 100 U vial of lyophilized BOTOX drug product was resuspended in 220 µl 0.9% saline, serially diluted, and tested as unknowns against a standard curve made in PBS using purified BoNT/A holotoxin. Samples were tested according to the described protocol except that 50 µl samples were tested without 10x Binding buffer in duplicate. The standard curve was calculated by fitting the emission ratio of the standard curve samples to the variable slope sigmoidal dose-response equation and is plotted as a function of BoNT/A concentration. Each unknown sample is shown where it intersects with the standard curve following a 24 hr incubation with the A/E reporter. The concentrations of the unknown samples falling within the linear range (dashed shaded box) were interpolated from the standard curve. The label for each BOTOX unknown is the number of units present in the sample based on labeled potency. Error bars represent the standard deviation of the mean. Click here to view larger image.

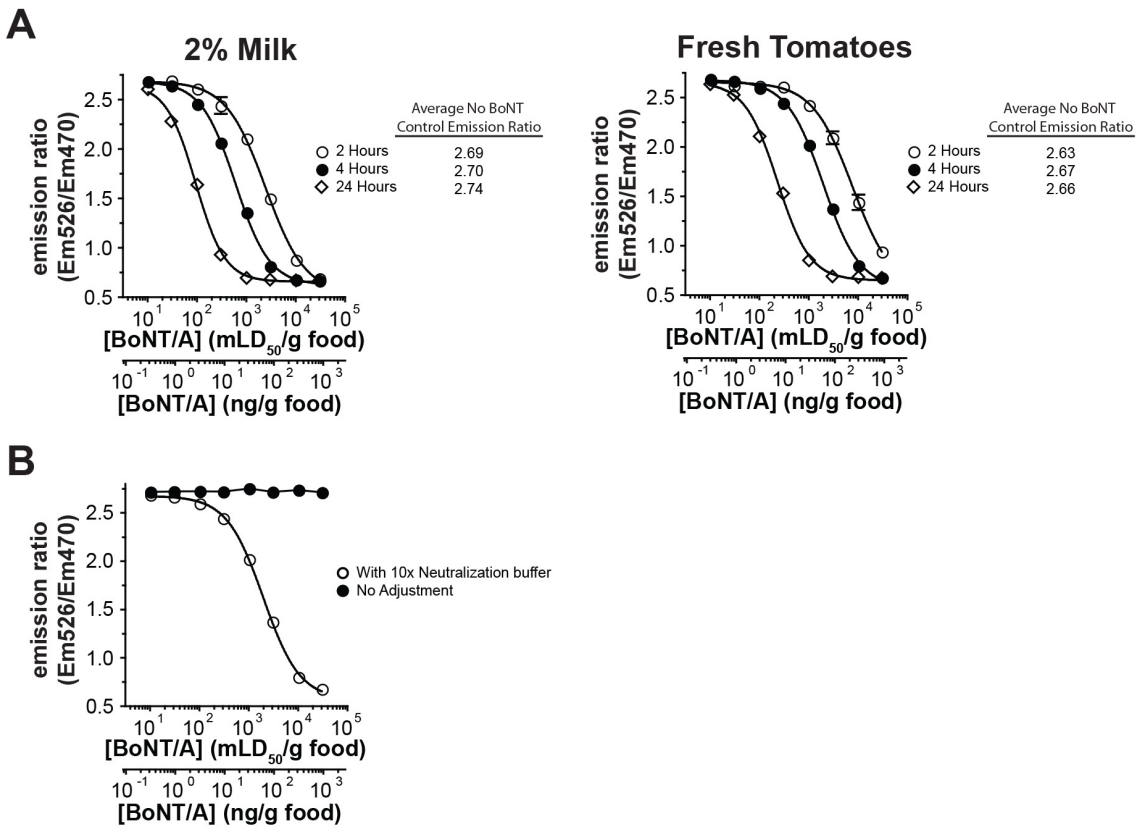

Figure 5. Recovery of BoNT/A-spiked 2% milk and fresh tomatoes testing using the described protocol. Samples of 2% milk and fresh tomatoes were spiked with purified BoNT/A complex and tested using the described protocol. All samples were tested in triplicate and the error bars represent the standard deviation of the mean. **(A)** The emission ratio of the 2% milk and fresh tomatoes standard curves were measured following 2, 4, and 24 hr incubation with A/E reporter and plotted as a function of BoNT/A potency. **(B)** Failure to include the 10x Neutralization buffer during tomato testing results in assay failure. Samples of fresh tomato were tested according to the described protocol either with or without addition of 10x Neutralization buffer. Data shown is for the 4 hr time point. Click here to view larger image.

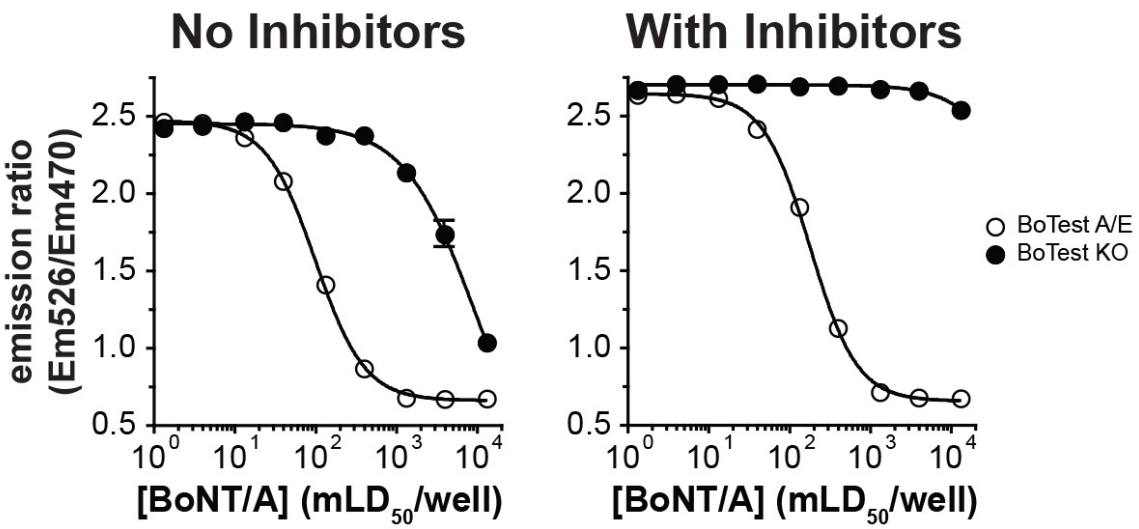

Figure 6. Protease inhibitors are required when testing complex matrices. *Clostridium* BoNT/A (strain Hall A) culture supernatant was serially diluted in PBS and tested using the described protocol either with or without protease inhibitors added to the reaction buffer and with both the A/E and KO reporters. The emission ratio was measured following 24 hr incubation with both the A/E or KO reporters and plotted as a function of BoNT/A potency. Significant cleavage of the KO reporter was seen without addition of protease inhibitors but was negated by their inclusion. All samples were tested in triplicate and the error bars represent the standard deviation of the mean. Click here to view larger image.

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Buffer	Composition	Storage Temperature	Stability	Notes
10x Matrix Binding Buffer	500 mM HEPES-NaOH, pH 7.1, 250 mM NaCl, 1% Tween-20, 5% Casein, 0.05% NaN_3	-20 or -80 °C	Stable for a minimum of five days at 4 °C upon thawing	Supplied with BoTest Matrix A Botulinum Detection Kit
10x Matrix Wash Buffer	119 mM Phosphates, pH 7.4, 1,370 mM NaCl, 27 mM KCl, 1% Tween-20	-20 or -80 °C	Stable for a minimum of five days at 4 °C upon thawing	Supplied with BoTest Matrix A Botulinum Detection Kit
10x Neutralization Buffer	1 M HEPES-NaOH, pH 8.0, 1 M NaCl	4 °C	Stable for up to six months at 4 °C	
10x BoTest Reaction Buffer	500 mM HEPES-NaOH, pH 7.1, 50 mM NaCl, 1% Tween-20, 100 µM $ZnCl_2$	-20 or -80 °C	Stable for a minimum of five days at 4 °C upon thawing	Supplied with BoTest Matrix A Botulinum Detection Kit
BoTest A/E Reporter	20 µM in 50 mM HEPES-NaOH, 10 mM NaCl, 15% Glycerol	-80 °C	Store in small aliquots. Stable for a minimum of five days at 4 °C upon thawing.	Supplied with BoTest Matrix A Botulinum Detection Kit
Gelatin Phosphate Buffer (GPB)	33.3 mM NaH_2PO_4, pH 6.2, 2 g/L gelatin	4 °C	Stable for up to 1 month at 4 °C	
200x dithiothreitol (DTT)	1 M DTT	-20 °C	Stable for up to 6 months at -20 °C	Make and store small (100 µl) aliquots
1x PBS-t	11.9 mM Phosphates, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1 % Tween-20	4 °C	Stable for up to 1 month at 4 °C	Can be made using 10x PBS from Fisher (BP399-1)
Matrix A Beads (IP-A Beads)	Magnetic beads covalently conjugated to chicken anti-BoNT/A antibody in PBS. 0.1% Tween-20, 0.05% Sodium Azide, 0.25% Casein, and 50% Glycerol	-20 °C	Stable for a minimum of five days at 4 °C upon removal from -20 °C. DO NOT FREEZE AT -80 °C	

Table 1. Buffers required for the described protocol. The 10x Neutralization Buffer is only for highly complex (*e.g.* food) samples and may not be required depending on the nature of the samples being assayed.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
BoTest Matrix A Botulinum Neurotoxin Detection Kit	BioSentinel	A1015	Detection kits for BoNT/B and F are also available.
Varioskan Flash fluorescence microplate reader	Thermo-Fisher Scientific	5250040	Most monochromator- or filter-based units with 434 nm excitation and 470 nm and 526 nm emission capability can be used.
96-well Magnetic Bead Separation Plate	V&P Scientific	VP771H	Other magnetic plates may be used, but the plate should be designed to separate the beads to the side of the well.
Magnetic Bead-Compatible Plate Washer	BioTek	ELx405 VSRM	Optional, only required for automated plate washing. Other magnetic bead-compatible plate

			washers may also be used, but should be tested before use.
Microcentrifuge	Various	N/A	Optional, only required for samples needing centrifugation.
MixMate plate mixer	Eppendorf	22674200	
Orbital Shaker	Various	N/A	Used at RT or at 25 °C If temperature control is available
EDTA-free Protease Inhibitor Tablets	Roche	4693132001	Only required for food or environmental testing. Protease inhibitors must be EDTA-free.
BoNT/A	Metabiologics	N/A	Optional, only required for standardization and quantification purposes
Black, Flat-bottomed 96-well Plates	NUNC	237105	Plates should not be treated
96-well Plate Sealing Tape	Thermo Scientific	15036	

Table 2. Materials and equipment required for the described protocol. Some materials and equipment are optional or can be substituted depending on the equipment available. Additional suitability testing and optimization may be required if using alternate equipment.

Sample Name	Volume Toxin	Volume Diluent	[BoNT/A] (mLD_{50}/ml) or (mLD_{50}/g food)	log[BoNT/A] (mLD_{50}/ml) or (mLD_{50}/g food)
D1	1,200 µl Stock	N/A	30,000	4.48
D2	300 µl D1	600 µl	10,000	4
D3	90 µl D1	810 µl	3,000	3.48
D4	90 µl D2	810 µl	1,000	3
D5	90 µl D3	810 µl	300	2.48
D6	90 µl D4	810 µl	100	2
D7	90 µl D5	810 µl	30	1.48
D8	90 µl D6	810 µl	10	1
D9	N/A	1,400 µl	N/A	N/A

Table 3: Dilution table for standard curve samples. This table generates a standard curve in half-log dilutions over 3.5 orders of magnitude. Enough No BoNT blank sample (D9) is generated to run an n = 6. Additional dilutions can be added if desired.

Program Name	Variable	Value	Comments
Prime	Reagent Bottle	A	
Prime Volume	400		
Prime Flow Rate	7		
Soak After Prime?	N		
Matrix Wash	Reagent Bottle	A	The number of washes can be increased if residual protease activity is observed.
Method			
Number of Cycles	4		
Soak/Shake	Y		
Soak Duration	180		
Shake Before Soak?	N		
Prime After Soak?	N		
Disp			
Dispense Volume	300		
Dispense Flow Rate	5		
Dispense Height	130		
Hor. Dispense Pos.	0		

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Disable Aspirate?	Y		
Bottom Wash First?	N		
Prime Before Start?	N		
Aspir			
Aspiration Height	40		
Hor. Aspirate Pos.	0		
Aspiration Rate	5		
Aspiration Delay	0		
Crosswise Aspirate?	N		
Final Aspiration?	Y		
Final Aspiration Delay	0		
Matrix Soak	Soak Duration	300	Increased soak duration may increase bead recovery from more viscous foods.
Shake Before Soak?	N		
Master Wash	Matrix Soak		This is a 'Link' program to run the Matrix Soak and Matrix Wash programs together.
Matrix Wash			

Table 4. Magnetic bead-compatible automated plate washer program settings. The following programs assume that the plate washer is equipped with a magnet that pulls beads to the side of the wells and that the buffer switching module is set up such that 1x Wash Buffer (PBS-t) is attached to valve A. These programs are specific to the BioTek ELx405. Refer to the instrument manual for programming instructions. Other magnetic bead-compatible automatic plate washers or vacuum manifolds can be used; however, testing will be required to define settings that maximize washing efficiency and minimize bead loss during washing. Initial testing of bead recovery following washing is recommended regardless of the specific plate washer used.

Food Matrix	Metric	2 hr		4 hr		24 hr	
		mLD_{50}/g food	mLD_{50}/well	mLD_{50}/g food	mLD_{50}/well	mLD_{50}/g food	mLD_{50}/well
Milk	LOD	300	58	100	19	30	6
	LOQ	1,000	193	300	58	100	19
	EC_{50}	2,404	464	590	114	92	18
Fresh Tomatoes	LOD	1,000	91	300	27	30	3
	LOQ	1,000	91	1,000	91	100	9
	EC_{50}	7,561	687	1,932	176	229	21

Table 5. Limits of detection (LOD), limits of quantification (LOQ), and half-maximal effective concentration (EC_{50}) for the 2% milk and fresh tomato testing shown in Figure 2A. The EC_{50} is derived from the sigmoidal dose-response curve fit while the LOD and LOQ are defined as being the lowest concentration data point that falls either 3 or 10 standard deviations below the no BoNT controls (n = 6), respectively. The data are presented in both mLD_{50}/g food and total mLD_{50}s tested per well in the 200 µl sample volume.

Discussion

This protocol describes procedures for quantifying BoNT/A complex, holotoxin, or *Clostridium* culture supernatant in complex matrices. The protocol is the same, however, when testing other BoNT serotypes (*e.g.* BoNT/B, E, and F) with their respective Matrix assays[56,60], although assay sensitivity will vary across serotypes and assays. This protocol does not account for every type of sample possible and some modifications may be required depending on the specific sample composition and desired application. Matrices can include food samples (*e.g.* food challenge testing) or pharmaceutical excipient buffers (*e.g.* BoNT-based drug product testing). Complex nonfood (*e.g.* animal tissue or environmental) liquid and solid samples should be treated as food samples. This protocol uses 200 µl/well sample volumes but as little as 50 µl/well can be tested with a corresponding adjustment to the volume of 10x Binding buffer. Samples smaller than 50 µl should be diluted with GPB or PBS to ≥50 µl before testing.

The highly variable nature of foodstuffs represents a particular challenge for BoNT detection and quantification in food. Sample pH, viscosity, ionic strength, and the presence of particulate matter can all potentially affect the activity of the toxin within the food matrix and necessitate that the toxin be isolated from the food for accurate, *in vitro* quantification. Many foods or toxin preparations also contain endogenous proteases that must be removed or inactivated when using protein-based reporters to ensure that only BoNT-specific protease activity is measured. Even simple, well-defined buffer matrices, such as pharmaceutical excipient buffers, can contain compounds that interfere with BoNT activity that must

be removed before quantification[35,56-59,61]. Immunoprecipitation offers a fast, highly serotype-specific BoNT purification method but requires high affinity antibodies to isolate the low toxin concentrations found in "real-world" food, environmental, and pharmaceutical samples.

The described protocol purifies the toxin using paramagnetic beads coated with anti-BoNT/A antibodies directed towards the toxin heavy chain receptor domain of the BoNT/A core holotoxin[56]. *Clostridium* species, however, naturally produce toxin complexes consisting of the core holotoxin in association with NAPs[62-64]. The NAPs protect the toxin from protease attack in the gastro-intestinal tract and are believed to facilitate toxin transport across the intestine epithelium[63,65]. The NAPs inhibit binding of the IP-A bead anti-BoNT/A antibodies to the core holotoxin (data not shown). This inhibition is relieved by increasing the sample pH to above ~6.25, causing the BoNT/A complex to dissociate into holotoxin and NAPs and allowing effective toxin binding to the IP-A beads[66]. For this reason, adjusting the sample pH to above 6.5 is critical to the success of the assay (**Figure 5B**). The 10x Binding buffer used in the protocol contains a buffering agent to help raise the pH to standard assay conditions. However, many acidic foods can overwhelm the buffering capacity of the Binding buffer. The additional 10x Neutralization buffer greatly increases the sample buffering capacity and should neutralize the majority of food matrices.

Another important parameter for the assay is sample ionic strength. Clarification of the samples by centrifugation is required for effective bead washing and recovery following immunoprecipitation. Testing with fresh tomatoes revealed that no BoNT/A recovery was seen when the samples were simply processed and centrifuged. We hypothesized that BoNT/A may associate with the tomato flesh causing it to pellet during centrifugation and become absent from the tested supernatant. We found that increasing the ionic strength or, more significantly, neutralizing the pH limited interactions between BoNT and the matrix resulting in improved toxin recovery (**Figure 5B**). While not required for BoNT recovery, it is expected, although not demonstrated, that higher salt concentrations will also increase the stringency of the immunoprecipitation and result in less nonspecific protein recovery, especially in low salt foods.

Sample pH and ionic strength adjustment in the protocol is performed by the addition of 10x Neutralization buffer and should be compatible with a wide range of foods. While the 10x Neutralization buffer has a high buffering capacity, some highly acidic foods may require additional pH adjustment. Testing of sample pH following buffer addition is recommended if poor assay performance is observed and sample volume allows. Additional pH adjustment can be achieved through the addition of small volumes of 1 M HEPES pH 8, if required. The additional NaCl introduced by the 10x Neutralization buffer should be acceptable for all but the saltiest foods; however, 10x Neutralization buffer can be replaced with 1 M HEPES pH 8 if poor results are seen with a given foodstuff. No significant differences have been observed when testing the described protocol at NaCl concentrations up to 1.2 M in PBS.

While this protocol is applicable to the majority of samples, foods at the extremes of pH, ionic strength, and/or protease content may not yield good results with the assay. Some foods may also remain too viscous following the addition of GPB, resulting in poor bead recovery. Predilution of samples with a simple buffer such as PBS may improve results. Increasing the number of washes or wash stringency (by increasing NaCl concentration) and increasing the concentration of EDTA-free protease inhibitors may also improve results if nonspecific protease contamination is observed. Instrument cleanliness is also very important when using automatic plate washing. Contamination of the plumbing and injection nozzles with proteases (*e.g.* trypsin) from other laboratory assays can lead to the unintentional introduction of proteases during the assay. Thorough cleaning of the plate washer according to the manufacturer's user's manual is recommended before running the assay.

The BoTest Matrix assays are the first commercialized, activity-based assays for BoNT detection and quantification in complex matrices. Compared to the standard mouse bioassay, the assay is fast, less expensive, and allows high-throughput testing without the need for specialized facilities or ethical concerns regarding animal use[56]. Other *in vitro* BoNT detection methods have been described but have not been shown to be compatible with complex sample matrices, require specialized equipment, or are not commercially available[35,42-44,46-55]. The assays are also activity-based assays that measure toxin endoprotease activity, whereas traditional enzyme-linked immunosorbant assay (ELISA) techniques only report toxin mass. Activity-based ELISA assays were previously described but these assays were not demonstrated to work with food matrices and do not encompass purification of the toxin from the sample matrix[41]. Significant underestimation of the toxicity of complex samples may occur if the toxin is not purified from the sample since matrix compounds often interfere with the BoNT activity[35,56-59].

Once the protocol is mastered, modifications can be made to increase sample volumes and increase sample sensitivity. For example, binding of the toxin to the beads can be performed in larger, bulk samples (*e.g.* 10 ml or greater) before collection by centrifugation. These beads can then be added to a 96-well plate and assayed following the described protocol. Increases in sensitivity greater than one log have been observed with increased sample size[56]. Thus, the described protocol can be used with larger volume samples to detect even trace amounts of BoNT contained in samples that may go undetected by the mouse bioassay.

Disclosures

F. M. Dunning, T. M. Piazza, F. Zeytin, and W. C. Tucker are employees or owners of BioSentinel Inc. BioSentinel currently manufactures and has commercialized some of the reagents presented in this report.

Acknowledgements

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