Detection of Botulinum Neurotoxin Serotype A, B, and F Proteolytic Activity in Complex Matrices with Picomolar to Femtomolar Sensitivity

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Rapid, high-throughput assays that detect and quantify botulinum neurotoxin (BoNT) activity in diverse matrices are required for environmental, clinical, pharmaceutical, and food testing. The current standard, the mouse bioassay, is sensitive but is low in throughput and precision. In this study, we present three biochemical assays for the detection and quantification of BoNT serotype A, B, and F proteolytic activities in complex matrices that offer picomolar to femtomolar sensitivity with small assay volumes and total assay times of less than 24 h. These assays consist of magnetic beads conjugated with BoNT serotype-specific antibodies that are used to purify BoNT from complex matrices before the quantification of bound BoNT proteolytic activity using the previously described BoTest reporter substrates. The matrices tested include human serum, whole milk, carrot juice, and baby food, as well as buffers containing common pharmaceutical excipients. The limits of detection were below 1 pM for BoNT/A and BoNT/F and below 10 pM for BoNT/B in most tested matrices using 200- μ l samples and as low as 10 fM for BoNT/A with an increased sample volume. Together, these data describe rapid, robust, and high-throughput assays for BoNT detection that are compatible with a wide range of matrices.

Botulinum neurotoxins (BoNTs) are zinc-dependent endopeptidases produced by members of the bacterial genus *Clostridium* (17, 36, 64). The seven BoNT serotypes, designated A to G, are structurally similar, each comprising a heavy chain that governs neuron-specific cell binding, cell uptake, and translocation into the cytosol and a light chain that contains endopeptidase activity (35, 43, 45). While cell surface receptor and endopeptidase substrate specificity differ between serotypes, all BoNTs act by specifically cleaving one or more soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, inhibiting neurotransmitter release at the neuromuscular junction (11, 12, 44, 49, 52–54, 67). This block in neurotransmitter release leads to the flaccid paralysis characteristic of the disease botulism and can cause death by respiratory failure resulting from paralysis of the diaphragm and intercostal muscles. BoNTs are extremely toxic, with estimated human lethal doses of BoNT serotype A of 1 to 3 ng/kg by the intravenous route, 10 to 13 ng/kg by inhalation, and 1 μ g/kg orally, making BoNTs the most lethal substances known (4, 27).

Its extreme toxicity, neuronal specificity, and ease of dissemination make BoNT a human health and biodefense concern. Human botulism is most commonly associated with BoNT/A, BoNT/B, BoNT/E, and BoNT/F contracted through ingestion and wounds (37). In the United States, infant botulism, commonly contracted by the ingestion of honey or dust containing *Clostridium* spores (2, 3, 15), was responsible for ~66% of the reported botulism intoxications from 2001 to 2009 (<http://www.cdc.gov/national-surveillance/botulism-surveillance.html>). BoNTs are also considered a biodefense threat of the utmost concern, warranting a class A designation from the CDC and the Department of Health and Human Services (4). Illicit manufacturing of BoNT for terrorism purposes is documented (63, 69). In all botulism cases, whether naturally occurring or intentional, early diagnosis

of intoxication is critical for effective treatment and identification of the source of the toxin (60).

BoNT's specificity for neurons and the neuromuscular junction and long biological half-life (>4 months for BoNT/A) (59) are extensively and increasingly used for cosmetic and therapeutic applications. The Food and Drug Administration approved BoNT-based drug products for treatment of a variety of conditions, including glabellar lines, strabismus, cervical dystonia, and chronic migraines. Dozens of "off-label" applications are also documented (9, 21, 58), and new applications are being pursued through modification of the toxin (26, 48). However, the extreme toxicity of BoNT requires accurate toxin quantification for correct dosing. Underdosing may result in a lack of effective treatment, while overdosing puts patients at risk of dangerous and potentially deadly side effects.

The mouse lethality bioassay is the accepted standard method of detecting and quantifying BoNT activity in clinical, environmental, and pharmaceutical samples (1, 37, 51); however, there is no single mandated protocol and different testing facilities may follow unique protocols. The mouse bioassay is applicable to all BoNT serotypes and is extremely sensitive, with a limit of detection (LOD) of 5 to 10 pg of BoNT/A (25). The mouse bioassay also reports a physiological response, death, that requires a fully functional toxin capable of binding and entering neurons before cleaving its SNARE substrate. The assay is performed by injecting mice

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intraperitoneally with an ~0.5- to 1-ml sample per mouse and recording the time(s) and number of deaths over 2 to 7 days, depending on the protocol being followed. As expected, the assay is low throughput and requires highly trained personnel operating in specialized animal facilities. In addition, ethical concerns associated with animal use has led to public calls to replace the mouse bioassay in clinical and pharmaceutical testing (6, 7, 10, 33). The lack of standardized assay protocols among testing labs also leads to variable quantification results with the same sample (14, 56). Indeed, while both Botox (onabotulinumtoxinA) and Dysport (abobotulinumtoxinA), two BoNT/A therapeutics, share the same unit definition (1 mouse 50% lethal dose [mLD₅₀]/U), one study found that 1 U of Botox was equivalent to 2 to 11 U of Dysport, depending on the testing lab (39), although a more recent study concluded a Botox:Dysport equivalency of 1:2 to 4 U (66). A similar dose inequality between the BoNT/A therapeutic Xeomin (incobotulinumtoxinA) and Botox was reported (31). These dose inequalities pose a patient safety risk. Alternative, animal-free, standardizable assays for the detection of BoNT activity with mouse bioassay sensitivity are a critical need for diagnostic, environmental, biodefense, and pharmaceutical testing.

We previously reported the development of the BoTest A/E and B/D/F/G assays (50). These assays use fluorogenic protein reporters consisting of an N-terminal cyan fluorescent protein (CFP) moiety and a C-terminal yellow fluorescent protein derivative Venus moiety linked by a BoNT substrate, residues 141 to 206 of SNAP25 in BoTest A/E and residues 33 to 94 of synaptobrevin in BoTest B/D/F/G. In the absence of cleavage, excitation of CFP results in Förster resonance energy transfer (FRET) to Venus, quenching CFP emission and exciting Venus emission. Upon linker cleavage by BoNT, CFP and Venus become separated, preventing FRET; CFP emission increases, and Venus emission decreases. The ratio of these two emissions gives a quantitative measure of BoNT activity. Furthermore, as long as the BoNT remains active, the BoTest reporters will continue to be cut; thus, the assay can be read until the desired sensitivity is achieved. Femtomolar (BoNT/A) and picomolar (BoNT/B and BoNT/F) LODs are possible with the BoTest assays using purified toxins.

In this study, we report the development of *in vitro* assays for the quantification of BoNT/A, BoNT/B, and BoNT/F activities in complex matrices. These assays use the BoTest A/E and B/D/F/G substrates coupled to immunological methods in a medium- to high-throughput format and are capable of detecting BoNT with picomolar to femtomolar sensitivity. Recently, we used such an approach to develop an assay for the detection of BoNT/E in avian blood samples (47). Together, these assays present an ideal alternative for routine and rapid testing and quantification of BoNT in a wide variety of applications.

MATERIALS AND METHODS

Protein expression. Protein expression plasmids containing heavy chain receptors (HcRs) A and F were kindly provided by J. T. Barbieri (Medical College of Wisconsin—Milwaukee) and expressed as previously described (47). The BoTest A/E and B/D/F/G reporters were purified as previously described (50).

Antibodies and preparation of immunoprecipitation (IP) beads. Purified anti-HcR/A and anti-HcR/F IgY was generated by GeneTel Laboratories (Madison, WI) using HcR/A and HcR/F, respectively, for immunization and antigen-specific purification. Anti-BoNT/B mouse monoclonal antibody mcs92-32-1-10 was kindly provided by L. Stanker (USDA, ARS). Tosyl-activated BcMag magnetic beads (Bioclone, San Di-

ego, CA) were coated with anti-HcR/A (BoTest Matrix A), mcs92-32-1-10 (BoTest Matrix B), or anti-HcR/F (BoTest Matrix F) antibody (5 µg antibody/mg beads) by following the manufacturer's protocol, blocked for 1.5 h at 37°C with Blocker Casein (Pierce, Rockford, IL), washed, and resuspended to 6 mg/liter with phosphate-buffered saline–0.1% Tween 20 (PBS-t).

BoNT activity assays. All reactions were run in black, flat-bottom, 96-well plates (Nunc, Rochester, NY). For assays that did not include IP, triplicate dilutions of the indicated BoNT/A, BoNT/B, or BoNT/F holotoxin concentrations (Metabio, Madison, WI) were made in 100 µl BoTest reaction buffer (50 mM HEPES [pH 7.1], 5 mM NaCl, 0.1% Tween 20, 10 µM ZnCl₂, 5 mM dithiothreitol) supplemented, when indicated, with various concentrations of human serum albumin (HSA), NaCl, or other test material. Assays were initiated by adding a 0.25 µM final concentration of BoTest A/E or B/D/F/G reporter and incubating the mixture at 25°C (BoTest A/E) or 37°C (BoTest B/D/F/G). After the indicated period of time, the reporter was excited at 434 nm and emissions were collected at 470 and 526 nm using a Varioskan microplate reader (Thermo-Fisher, Waltham, MA). The fluorescence emission ratio was calculated by dividing the emission at 526 nm by the emission at 470 nm and plotted as a function of the BoNT concentration.

For IP experiments including the BoTest Matrix assays, unless otherwise indicated, a 20-µl volume of antibody-conjugated magnetic beads (BoTest Matrix beads) was mixed with 20 µl of BoTest Matrix binding buffer (250 mM NaCl, 1% Tween 20, 5% casein, 0.05% sodium azide, 500 mM HEPES-NaOH, pH 7.1). A 200-µl sample was then added, and the mixture was incubated for 2 h at 25°C. The beads were then washed three times with 200 µl of PBS-t using a BioTek Elx405 magnetic-bead-capable plate washer and resuspended in 50 µl BoTest reaction buffer. Proteolytic activity determinations were then initiated by the addition of 0.5 µM BoTest A/E or B/D/F/G reporter in 50 µl BoTest reaction buffer. Where indicated, control BoNT dilutions representing 100% pull-down efficiency were made in 50 µl BoTest reaction buffer and assayed along with the pull-down samples. Samples were incubated at 25°C (BoNT/A) or 37°C (BoNT/B and BoNT/F) in a temperature-controlled microplate mixer at 750 rpm (Discovery Scientific, Kelowna, British Columbia, Canada). At the desired time points, the beads were separated on a 96-well magnetic separator (V&P Scientific, San Diego, CA). Following separation, the reporter was excited at 434 nm, emission values at 470 and 526 nm were collected, and the emission ratio (526/470) was calculated. For further incubations, where applicable, the beads were resuspended and placed back into incubation.

In all experiments, negative-control samples were run in either triplicate or nonuplicate (when a LOD determination was made) to monitor for non-specific protease contamination. While these data are not plotted, no background protease activity was observed in any of the data presented.

Data analysis. All data analysis was performed using Prism 4.0 (GraphPad Software, La Jolla, CA). Data were fitted to the four-parameter logistic equation $Y = \text{bottom} + [(\text{top} - \text{bottom}) / (1 + 10^{(\log EC_{50} - x)}) \times \text{Hill slope}]$, where Y is the response that starts at a top (no activity) and goes to a bottom (assay saturation), x is the logarithm of the concentration, and EC_{50} is the half-maximal (50%) effective concentration. For LOD determinations, serial dilutions of BoNT-spiked samples were compared to non-spiked control samples ($n = 9$). The LOD was defined as having an emission ratio value less than 3 standard deviations (SDs) below that of control samples. Data interpolations to standard curves were performed using Prism. Percent coefficients of variation (CVs) were calculated by dividing the SD of intra- or interassay runs by the average of the run or runs, respectively.

Complex-matrix testing. Complex-matrix testing was performed as described above, with the following modifications. BoNT dilutions were made by first spiking the tested matrix with BoNT holotoxin. Following BoNT addition, the baby food sample (Beech-Nut Classic Garden Vegetables) was diluted 1:3 with PBS to reduce sample viscosity. Serial dilutions of the toxin were then made in the tested matrix, except for baby food, where 1:3-diluted matrix was used as the diluent. Following dilu-

FIG 1 NaCl inhibits BoNT/A and BoNT/B activities, while HSA significantly affects only BoNT/A activity. The indicated concentrations of BoNT/A (A, B) or BoNT/B (C, D, E) holotoxin were incubated with the BoTest A/E (BoNT/A) or BoTest B/D/F/G reporter (BoNT/B) in the presence of the indicated concentration of HSA (A, C), NaCl (B, D), or Na succinate, pH 5.6 (E). Control samples (open circles) were not supplemented with HSA, NaCl, or Na succinate. Emission (Em) ratios (526/470 nm) were collected after 4 h of incubation.

tion, baby food and carrot juice samples were spun for 10 min at $14,000 \times g$ to pellet particulate matter. Samples were then assayed with BoTest Matrix assays as described above, except that the post-IP beads were washed four times with 200 μ l PBS-t.

Protein blotting. Ten-microliter samples of input, nonbound, BoTest assay, and bead fractions collected during the pulldown assay and containing 10 nM (anti-BoNT/A blot) or 10 pM (anti-HSA blot) BoNT/A were separated by SDS-PAGE and transferred to Immobilon P polyvinylidene difluoride membrane (Millipore, Billerica, MA). Blots were probed with anti-BoNT/A (Metabio) or anti-HSA (15C7; Abcam, Cambridge, MA) antibody, followed by an appropriate horseradish peroxidase-conjugated secondary antibody. A signal was generated using SuperSignal West Pico substrate (Pierce) and imaged on a ChemiDoc gel system (Bio-Rad). Densitometry was performed using QuantityOne software (Bio-Rad).

RESULTS

Sample composition influences *in vitro* BoNT activity determinations. Detection of BoNT in environmental, food, and pharmaceutical samples is complicated by the presence of non-BoNT matrix molecules that affect BoNT activity. For example, BoNT-based drug products such as Botox, Dysport, Myobloc (rimabotulinumtoxinB), and Xeomin all contain various HSA levels along with other stabilizers or excipients such as NaCl, sucrose, and lactose. We tested the effects of common pharmaceutical excipients on BoNT/A and BoNT/B activities using the BoTest A/E and B/D/F/G reporters. The concentrations tested ranged from 0.1 to 10 times the concentration of each excipient commonly found in saline resuspensions of BoNT-based drug products (see Table S1 in the supplemental material). Both HSA and NaCl dramatically affected BoNT/A activity determinations (Fig. 1). The BoNT/A EC_{50} increased from 1.4 pM in the absence of HSA to 5.9 and 36.9 pM in the presence of 0.05 and 0.5% HSA, respectively (Fig. 1A). The EC_{50} increased from 1.6 pM in the absence of NaCl to 19 pM in the presence of 154 mM NaCl, while the assay's dynamic range (the difference between the emission ratio of the uncleaved reporter and that of the fully cleaved reporter) decreased from 2.0 to

1.1 (arbitrary units; Fig. 1B). Sucrose and lactose had no significant impact on the assay at up to 4.7 and 2.5%, respectively (data not shown).

HSA had a modest impact on BoNT/B activity determinations using the BoTest B/D/F/G reporter (Fig. 1C). The addition of 0.5% HSA increased the BoNT/B EC_{50} from 236 pM to 312 pM, and the dynamic range decreased from 3.4 to 2.9. In contrast, NaCl impacted both BoNT/B activity determinations. Addition of 100 mM NaCl increased the BoNT/B EC_{50} from 261 pM to 3.4 nM and decreased the reporter dynamic range from 3.3 to 1.7 (Fig. 1D). Addition of Na succinate, pH 5.6, also greatly impacted the BoNT/B EC_{50} and the BoTest B/D/F/G dynamic range at concentrations above 10 mM (Fig. 1E). These data demonstrate the large impact even well-defined matrix molecules can have on BoNT activity determinations, highlighting the need for methods that allow the removal of matrix molecules before BoNT activity quantification.

Development of IP-based BoNT enrichment methods. Several strategies can be used to reduce the effects of interfering compounds, including immunological enrichment of desired molecules, immunological depletion of undesired molecules, and simple dilution to reduce matrix effects. We chose to use immunological enrichment through IP, as this would allow for (i) adaptation of the assay to multiple sample types (e.g., food, pharmaceutical, diagnostic), (ii) buffer exchanges, and (iii) concentration of BoNT from dilute solutions. In the assay envisioned, a BoNT serotype-specific antibody is conjugated to a substrate such as a magnetic bead. The antibody-conjugated beads are added to a sample, binding and sequestering the available toxin before removal of unwanted contaminants by washing of the beads. Bead-captured BoNT is resuspended in an optimized reaction buffer and quantified with a BoTest reporter (Fig. 2A). While mechanistically straightforward, this method requires an antibody with high specificity and affinity to match the femtomolar-to-picomolar LOD found with current animal-based assays.

FIG 2 Development of BoTest Matrix assays for the detection of BoNT/A, BoNT/B, and BoNT/F in complex matrices. (A) Overview of the BoTest Matrix assays. Anti-BoNT antibodies are conjugated to magnetic beads. Upon incubation with BoNT-containing samples, the beads bind BoNT from solution, fractionating the toxin from unwanted matrix molecules that can then be removed by washing. The proteolytic activity of the BoNT bound to the beads is then assayed with a BoTest reporter. (B) Identification of anti-BoNT/A, BoNT/B, and BoNT/F antibodies that do not interfere with BoNT proteolytic activity. The antibodies indicated were added at a 10-fold molar excess to 100 pM BoNT/A or 1 nM BoNT/B or BoNT/F and assayed with a BoTest A/E (BoNT/A) or BoTest B/D/F/G (BoNT/B and BoNT/F) reporter. Fluorescence emission (Em) ratios were collected every minute and plotted as a function of time. (C) Efficient IP and recovery of BoNT/A, BoNT/B, and BoNT/F activities using the BoTest Matrix A, B, and F assays. Magnetic beads coated with the appropriate anti-BoNT antibody (open circles) were used to immunoprecipitate BoNT/A (left panel), B (middle), or F (right) from PBS; this was followed by washing and assaying with the appropriate BoTest reporter. Non-IP controls (closed circles) were composed of identical concentrations of BoNT diluted in BoTest reaction buffer and incubated with the BoTest reporter without IP.

We screened >60 mono- and polyclonal antibody preparations from in-house, commercial, and academic sources. Most of the antibodies tested were not appropriate for an IP-based activity assay because of antibody-specific inhibition of BoNT proteolytic activity or insufficient affinity to achieve the desired assay LOD (data not shown). We did, however, identify three preparations with sufficient affinity and specificity to meet assay development needs: For BoNT/A and BoNT/F, we developed IgY polyclonal preparations obtained from chickens immunized with the HcR subdomains of BoNT/A and BoNT/F, respectively. HcR immunization has the distinct advantage of directing the antibodies to the heavy chain rather than the proteolytically active light chain. For BoNT/B, we identified a mouse monoclonal antibody previously developed at the USDA. None of these antibodies altered BoNT endopeptidase substrate cleavage kinetics using the BoTest re-

porters (Fig. 2B). When magnetic beads were coated with anti-BoNT/A, BoNT/B, or BoNT/F antibodies and used to immunoprecipitate BoNT from human serum, nearly 100% BoNT activity recovery was observed compared to a control curve representing 100% assay efficiency (Fig. 2C).

Assay performance was optimized for bead volume, antibody concentration, incubation temperatures, incubation time, and use in a 96-well format to maximize BoNT sensitivity and minimize assay times (see Fig. S1 in the supplemental material). The final optimized assays, which combine IP using magnetic beads coated with anti-BoNT antibodies (BoTest Matrix beads) and activity determination using BoTest reporters, were named the BoTest Matrix A, B, and F assays, according to the serotype specificity of each assay.

Serotype specificity of the BoTest Matrix assays. BoNT sero-

FIG 3 Serotype specificity of BoTest Matrix assays. (A) The BoTest Matrix assay indicated was used to detect the concentration of assay-specific or nonspecific BoNT indicated. Fluorescence emission (Em) values were collected after 4 h for the BoTest Matrix A assay (24-h data in inset) or 24 h for the BoTest Matrix B and F assays. Emission ratios were plotted as a function of the BoNT concentration. (B) Ability of the BoTest Matrix assays to quantify assay-specific BoNT in a mixed background. Test samples containing a known concentration of the assay-specific BoNT serotype (10 pM for the BoTest Matrix A assay, 100 pM for BoTest Matrix B and F assays) in the absence (closed circles) or presence of 100 pM each of the remaining BoNT/A through BoNT/F serotypes (open diamonds) were assayed with the respective BoTest Matrix assays and quantified against a standard curve run in parallel. The fluorescence emission ratios of the test samples were interpolated against the ratio obtained from the standard curve. The BoTest reporters were incubated with beads for 4 h (BoTest Matrix A assay) or 24 h (BoTest Matrix B and F assays).

types exhibit significant homology; thus, serotype specificity cannot be assumed when developing BoNT detection assays using antibodies (35). To test assay serotype specificity, the BoTest Matrix A assay was titrated with BoNT/A, BoNT/C, and BoNT/E and the BoTest Matrix B and F assays were titrated with BoNT/B, BoNT/D, and BoNT/F (Fig. 3). These experiments evaluated the specificity of the bead-conjugated antibodies, since the nonspecific BoNTs tested can cleave the reporters used in their respective assays. The BoTest Matrix A assay detected BoNT/A with EC_{50} s of 6.2 and 1.2 pM after 4 and 24 h, respectively (Fig. 3A). Cross-reactivity between the BoTest Matrix A assay and BoNT/E was seen, although the assay's BoNT/E EC_{50} at 24 h was ~ 438 pM, more than 2 orders of magnitude higher than that obtained with BoNT/A (~ 1.2 pM). The BoTest Matrix A assay BoNT/E LOD was ~ 1 nM after 4 h and ~ 100 pM at 24 h, while the BoNT/A LOD was 1 pM after 4 h and 100 fM at 24 h. Thus, the data indicate that the BoTest Matrix A is serotype selective but not specific for BoNT/A. Confirmation of BoNT/A activity, however, could be made by the introduction of a serotype-specific reporter, as previously reported for BoNT/E (47). The BoTest Matrix B assay demonstrated no significant cross-reactivity with BoNT/D or BoNT/F, and the BoTest Matrix F assay exhibited no significant cross-reactivity with BoNT/B or BoNT/D at up to 1 nM toxin (Fig. 3A).

The assays were also tested for the ability to quantify BoNT in samples containing a mixture of BoNT serotypes using two different test samples (Fig. 3B). One sample (alone) contained a known concentration of the serotype for which the assay is specific, while the other sample (mixed) contained a known concentration of the assay-specific serotype in a background of high concentrations of nonspecific serotypes. Each sample was then run alongside a stan-

dard curve generated by spiking with known concentrations of the assay-specific serotype. All samples and standards were diluted with 100% human serum. For the BoTest Matrix A assay, 10 pM BoNT/A was quantifiable with $96\% \pm 0.4\%$ and $91\% \pm 0.7\%$ recovery in the absence and presence of nonspecific serotypes, respectively. A 100 pM BoNT/B concentration was quantified by using the BoTest Matrix B assay with $93\% \pm 6.2\%$ and $97\% \pm 4.1\%$ recoveries in the absence and presence of nonspecific serotypes, respectively. Finally, 100 pM BoNT/F was quantified with the BoTest Matrix F assay with $95\% \pm 6.2\%$ and $100\% \pm 0.4\%$ recoveries in the absence and presence of nonspecific serotypes, respectively (Fig. 3B). The data indicate that BoTest Matrix assays are selective for their respective serotypes and suitable for serotype-specific activity determinations.

BoTest Matrix A assay precision. The precision of the BoTest Matrix A assay was assessed by determining the inter- and intra-assay percent CVs. Mock test samples in human serum were quantified against a BoNT/A standard curve run in parallel using interpolation (Fig. 4). The percent CV was calculated either for 10 test sample duplicates run at the same time on the same day (intra-assay; Fig. 4A) or from test samples assayed on 3 separate days (interassay; Fig. 4B). The calculated inter- and intra-assay CVs were 5.8 and 7.3% with interpolated mean concentrations of 3.0 ± 0.2 and 2.7 ± 0.2 pM, respectively.

Quantification of BoNT in pharmaceutical excipient mixes. We assessed the abilities of the BoTest and BoTest Matrix assays to quantify BoNT activity in the presence of pharmaceutical excipient mixtures. The compositions of the BoNT/A-based drug products Botox, Dysport, and Xeomin and the BoNT/B-based drug product Myobloc are readily available from the manufacturers

FIG 4 Inter- and intra-assay variabilities of the BoTest Matrix A assay. (A) Intra-assay percent CV determined by quantifying 10 duplicate 3 pM BoNT/A test samples against a standard curve run in parallel. (B) Interassay percent CV determined by quantifying a single duplicate 3 pM BoNT/A test sample against a standard curve run in parallel on 3 separate days. Fluorescence emission values were collected after 4 h, and emission (Em) ratios were plotted as a function of the BoNT/A concentration. Insets show data-fitting enforcements.

(see Table S1 in the supplemental material). We prepared mock excipient mixtures by using laboratory reagents with 0.1 to 10 times the excipient concentrations typically found in drug product resuspensions. The mixtures were spiked with various BoNT/A or BoNT/B concentrations, and BoNT activity was detected by using the non-IP BoTest A/E (mock Botox, Dysport, and Xeomin) and BoTest B/D/F/G (mock Myobloc) assays. The pharmaceutical excipient mixtures impacted BoNT activity and BoTest reporter performance to various degrees (Fig. 5A). The mock Botox mixture displayed the largest impact on BoNT/A activity determinations, shifting the BoNT/A EC_{50} from 1.3 pM in the absence of excipients to 5.1 and 57 pM in the presence of 1 and 10 times the excipient concentrations, respectively. Furthermore, the 10 $\times$ mock Botox excipient buffer strongly decreased the BoTest A/E reporter dynamic range. The mock Xeomin buffer also strongly inhibited BoNT/A activity, shifting the BoNT/A EC_{50} from 1.1 pM in the absence of excipients to 5.1 and 29 pM in the presence of 1 $\times$ to 10 $\times$ excipient concentrations, respectively. The mock Dysport buffer only significantly impacted the BoTest A/E assay at a 10 $\times$ excipient concentration, shifting the BoNT/A EC_{50} from 1.1 pM with no excipients to 7.5 pM with a 10 $\times$ excipient concentration. Mock Myobloc excipients dramatically impacted BoNT/B activity and BoTest B/D/F/G reporter performance. The BoNT/B EC_{50} shifted from 163 pM in the absence of excipients to 3.4 nM in the presence of a 1 $\times$ excipient concentration, and the assay dynamic range decreased from 3.5 to 1.8, respectively. Further increases in excipient the concentration to 5 $\times$ and 10 $\times$ resulted in complete failure of the assay, presumably as a result of high NaCl concentrations (>500 mM) and low pH. Clearly, accurate BoNT activity determination requires the removal of excipients.

We next tested whether the BoTest Matrix assays are capable of fractionating BoNT from excipients and quantifying BoNT activity regardless of the excipient load. Two master excipient buffers were generated that at 1 $\times$ contain the highest concentration of each excipient found among the three BoNT/A pharmaceuticals (BoTox, Dysport, and Xeomin) or the single BoNT/B pharmaceutical (Myobloc; see Table S1 in the supplemental material). Various BoNT concentrations were then used to spike 0.1 $\times$ to 10 $\times$ master excipient mixtures, and BoNT activity was assayed using

the BoTest Matrix A or B assay. The BoTest Matrix A assay efficiently detected BoNT/A endopeptidase regardless of the excipient concentration (Fig. 5B). BoTest Matrix A EC_{50} s ranged from 7.4 to 9.5 pM, with no correlation between the EC_{50} and the excipient concentration. The BoTest Matrix B assay also effectively quantified the BoNT/B activity contained in all of the pharmaceutical excipient mixtures. BoTest Matrix B EC_{50} s ranged from 48 to 74 pM with no correlation between assay EC_{50} s and excipient concentrations (Fig. 5C).

We verified separation of BoNT/A from the excipient HSA during the course of a BoTest Matrix A assay. Four fractions were collected during the testing of BoNT/A samples in 1 $\times$ excipients—the preassay input, a post-IP nonbound supernatant, a BoTest A/E reaction supernatant, and the final postassay Matrix beads. Each fraction was analyzed by Western blotting for the presence of HSA and BoNT/A (Fig. 5D). As expected, BoNT/A was found in the input and postassay Matrix bead fractions, indicating that BoNT/A was effectively sequestered to the BoTest Matrix bead surface during the assay. HSA was largely present only in the input and post-IP nonbound fractions. Densitometry analysis indicated that 89% HSA was left in the post-IP supernatant, while <6% nonspecifically bound the BoTest Matrix A beads. These data indicate that the BoTest Matrix assay can effectively fractionate BoNT/A from unwanted matrix molecules.

As a final test of the assay's ability to detect the activity contained in reconstituted drug products, we used the BoTest Matrix A assay to detect the BoNT/A activity contained in 100-U vials of clinical-grade Botox. These experiments directly addressed whether the BoTest Matrix A assay can detect the activity contained in a lyophilized and then reconstituted drug product. A single Botox vial was reconstituted in 0.9% saline and serially diluted in PBS-t from 454.5 to 0.2 U/ml. Each sample was then subjected to the BoTest Matrix A assay with reaction and read times between 30 min and 24 h (Fig. 5E). Less than 10 U of activity could be detected in 2 h, and <1 U could be detected in 24 h, demonstrating the BoTest Matrix assays' sensitivity and immunity to pharmaceutical excipients.

BoTest Matrix assays are compatible with a variety of complex matrices. We assessed whether the BoTest Matrix assays are

FIG 5 Detection of BoNT activity in pharmaceutical excipient mixtures using the BoTest and BoTest Matrix assays. (A) Excipient effects on BoNT activity determinations using BoTest. Non-IP BoTest A/E and B/D/F/G reactions were carried out using the indicated BoNT/A or BoNT/B concentration in PBS buffer without excipients (open circles) or supplemented with 0.1 to 10 times the concentration of excipients found in manufacturer-recommended resuspensions of the indicated BoNT-based pharmaceuticals. The 1× excipient solutions contain, in addition to PBS, mock Botox (0.05% HSA, 15.4 mM NaCl), mock Dysport (0.0125% HSA, 0.25% lactose), mock Xeomin (0.1% HSA, 0.47% sucrose), and mock Myobloc (0.05% HSA, 100 mM NaCl, 10 mM Na succinate, pH 5.6). Fluorescence emission (Em) values were collected after 4 h (BoTest Matrix A) or 24 h (BoTest Matrix B), and emission ratios were plotted as a function of the BoNT concentration. (B) BoTest Matrix A was used to immunoprecipitate the indicated BoNT/A concentrations in either PBS (open circles) or PBS spiked with 0.1 to 10 times the combined highest concentration of each excipient present in the BoNT/A pharmaceutical formulations tested in panel A. The 1× solution contains, in addition to PBS, 0.1% HSA, 15.4 mM NaCl, 0.25% lactose, and 0.47% sucrose. (C) BoTest Matrix B was used to immunoprecipitate the indicated BoNT/B concentration from either PBS (open circles) or PBS spiked with 0.1 to 10 times the concentration of each excipient found in Myobloc. In panels B and C, the fluorescence emission values were collected at 4 h (BoTest Matrix A) or 24 h (BoTest Matrix B) and emission ratios were plotted as a function of the BoNT concentration. (D) A protein blot analysis was run on the 10 nM and 10 pM BoNT/A fractions generated in panel B and probed with anti-BoNT/A (10 nM samples) or anti-HSA (10 pM samples) antibody. Fractions include the untreated input, the post-IP supernatant (nonbound), the post-BoTest assay supernatant (BoTest assay), and the post-IP beads. The positions of the BoNT heavy chain (HC) and light chain (LC) and HSA are indicated. (E) Detection of BoNT/A activity contained in Botox using the BoTest Matrix A assay. A single vial of Botox drug product was resuspended, serially diluted, and assayed with BoTest Matrix A. Fluorescence emission values were collected at the indicated times, and the emission ratios were plotted as a function of vial label-defined units.

compatible with highly complex matrices using a panel of sample types consisting of milk, human serum, baby food, and carrot juice. Unlike the well-defined mixture of excipients presented by pharmaceutical samples, environmental and food samples are highly varied, often with unpredictable compositions. Nonneutral pH, fat content, salt content, and viscosity, to name a few variables, may not only present sample-processing issues but directly interfere with BoNT activity determinations. To account for these potential issues, additional sample-processing steps were introduced into the BoTest Matrix assay protocol. A binding buffer containing HEPES and casein was used to spike all samples before IP to neutralize the pH and to reduce nonspecific binding to the Matrix beads. Baby food samples were diluted to reduce viscosity, and matrices containing particulate matter were centrifuged fol-

lowing dilution to clarify the supernatant before the performance of the assay (see Materials and Methods; Fig. 6).

For these experiments, as with the other experiments presented in this study, we used the purified 150-kDa holotoxin form of BoNT/A, BoNT/B, and BoNT/F (38). *Clostridium* naturally produces the toxin as a complex of variable molecular weight consisting of the core neurotoxin and neurotoxin-associated proteins (NAPs) (32, 62). These toxin complexes are produced during food contamination; however, the stability of BoNT complexes is highly pH dependent. For example, the BoNT/A complex disassembles into holotoxin and NAPs above a pH of ~6.25 (22, 29). The inclusion of a binding buffer during food sample IP results in pH neutralization, and thus, the toxin will be expected to exist in holotoxin form regardless of whether the test samples were gen-

FIG 6 Quantification of BoNT/A, BoNT/B, and BoNT/F activities in complex food and human-derived matrices using the BoTest Matrix assays. (A) Sample-dependent loss of BoNT activity and assay performance using the non-IP BoTest assay. Each matrix indicated was spiked with the concentration of BoNT/A, BoNT/B, or BoNT/F indicated and assayed with the BoTest A/E (BoNT/A) or B/D/F/G (BoNT/B) reporter. Following 24 h of incubation with the reporter, fluorescence emission (Em) values were collected and emission ratios were plotted as a function of the BoNT concentration. (B) Detection of BoNT activity in various sample types with the BoTest Matrix assays. BoTest Matrix A was used to immunoprecipitate and quantify the proteolytic activity of the same BoNT/A-spiked samples described in panel A at 2, 4, and 24 h. (C) BoTest Matrix B and F assays were used to immunoprecipitate and quantify the BoNT/B (open circles) and F (closed circles) proteolytic activities, respectively, from the same BoNT/B- and BoNT/F-spiked samples described in panel A. The fluorescence emission values were collected at 24 h, and emission ratios were plotted as a function of the BoNT concentration.

erated with a BoNT holotoxin or complex. Holotoxin was used in these experiments because of its high purity and precise molecular weight, allowing accurate spiking of food samples with known BoNT concentrations. In addition, we performed experiments comparing the BoNT/A holotoxin and complex used to spike human serum at equal mLD₅₀ dosages and found very similar results regardless of the toxin form (see Fig. S2 in the supplemental material).

Sample compatibility issues were first identified by spiking samples with various BoNT/A, BoNT/B, or BoNT/F concentra-

tions and attempting to measure BoNT activity directly with the BoTest assays with no IP treatment. The ability to detect BoNT activity was highly matrix dependent (Fig. 6A and Table 1). BoNT/A was detectable in all samples, but assay sensitivity and dynamic range were highly matrix dependent. BoNT/B and BoNT/F were even less detectable in the different matrices, with BoNT/B and BoNT/F proteolytic activity completely undetectable in human serum. These results re-emphasize the need for separation methods to reliably detect BoNT activity in complex matrices.

TABLE 1 LODs for BoTest Matrix assays using complex matrices

Diluent	LOD (pM BoNT holotoxin) for:											
	Matrix A (BoNT/A)				Matrix B (BoNT/B)				Matrix F (BoNT/F)			
	2 h	4 h	24 h	24 h non-IP	2 h	4 h	24 h	24 h non-IP	2 h	4 h	24 h	24 h non-IP
Carrot juice	3	1	0.1	1	300	100	10	100	30	30	10	300
Baby food	3.3	3.3	0.3	1	100	33.3	3.3	100	100	10	1	NA ^a
Human serum	3	1	0.1	30	300	100	10	NA	30	30	10	NA
Whole milk	3	1	0.1	10	300	30	10	30	30	10	0.1	NA

^a NA, not applicable (could not be determined).

FIG 7 Concentration and detection of BoNT/A in high-dilution samples. Equal masses of BoNT/A were used to spike 200- μ L (open circles) and 15-ml (closed circles) volumes of PBS-t and serially diluted in their respective volumes. BoTest Matrix A was then used to immunoprecipitate and quantify the BoNT/A proteolytic activity contained in each sample following a 4-h reporter incubation. The fluorescence emission (Em) values were collected, and emission ratios were plotted as a function of the BoNT/A concentration in each dilution.

The BoTest Matrix assays, however, readily detected BoNT/A, BoNT/B, or BoNT/F activity in all of the matrices (Fig. 6B and C). Assay sensitivity was time dependent in all of the matrices tested, as demonstrated with BoNT/A-spiked samples and the BoTest Matrix A assay (Fig. 6B). Furthermore, BoNT/B and BoNT/F activities were readily detected regardless of the sample type, with picomolar sensitivities possible within 4 h (Fig. 6C). Table 1 summarizes the BoTest Matrix assay LODs as a function of the BoNT serotype, sample type, and read time. LODs varied with the BoNT serotype and the matrix tested and were only calculated on the basis of half-log dilutions (for a given sample type, the LOD was the first data point that fell 3 SDs below control values). BoNT/A sensitivity was the least dependent on matrix effects, with LODs below 3.33 pM after 4 h and 0.333 pM by 24 h with all of the matrices. BoNT/B and BoNT/F sensitivities were more variable across the matrices tested. BoNT/B detection ranged from 30 to 100 pM after 4 h to 3.3 to 10 pM after 24 h. Sensitivity to BoNT/F ranged from 10 to 30 pM after 4 h to 1 to 10 pM after 24 h. These data are presented as BoNT holotoxin concentrations because of the unit's highly defined nature. While toxin picogram-to-mLD₅₀ conversion values are dependent on the testing lab and mouse bioassay protocol being run, 1 mLD₅₀ can be approximated as 10 pg of BoNT/A, 30 pg of BoNT/B, and 100 pg of BoNT/F (~0.3 pM, 1 pM, and 3.3 pM BoNT when in 200 μ L, respectively) (20, 68) (Metabiologics, personal communication). These results indicate that the BoTest Matrix A, B, and F assays are powerful tools for quantifying BoNT in highly complex matrix environments, including both serum and foodstuffs.

Detection of low femtomolar BoNT/A concentrations in large sample volumes. One advantage of an IP strategy is that the antibody-coated matrix beads can concentrate BoNT from highly dilute samples. We therefore tested the ability of the BoTest Matrix A assay to detect highly diluted BoNT/A activity in a large sample volume. Parallel assays were run on various BoNT/A concentrations diluted in either 200 μ L or 15 ml PBS-t. After a 4-h BoTest A/E reaction time, the EC₅₀s for the 200- μ L and 15-ml samples were 5.3 pM and 250 fM and the LODs were 1 pM and 10 fM, respectively (Fig. 7). The results demonstrate that the BoTest Matrix A assay can recover highly diluted BoNT/A from large sample volumes and that low femtomolar assay LODs are possible with increased sample volumes.

DISCUSSION

The development of rapid, medium- to high-throughput assays to measure BoNT activity in environmental, food, and pharmaceutical samples is of the utmost importance for patient, biodefense, and food safety, as well as reducing government and industry dependence on animal methods. Several *in vitro* BoNT detection assays were reported in recent years that detect BoNT endopeptidase activity using peptide substrates with fluorescence-, chromatography-, or mass spectrometry-based outputs, some with sufficient sensitivity to be useful for diagnostic and food-testing purposes (5, 18, 28, 30, 40, 55). Notably, several publications have detailed the coupling of mass spectrometry methods to immunological techniques, resulting in assays that detect BoNT in a wide range of substances with a sensitivity equal to that of the mouse bioassay (8, 13, 34, 46, 65). Indeed, these are the first assays described that can detect multiple BoNT serotypes with demonstrated applicability to diagnostic samples, including fecal samples. The use of these methods, however, is limited to a few qualified laboratories because of the instrument and training requirements associated with mass spectrometry-based readouts. Other reported assays lack the throughput required for routine BoNT testing, are not applicable to multiple BoNT serotypes, or have not been demonstrated to be applicable to complex-sample testing.

The BoTest Matrix assays offer several distinct advantages over existing BoNT *in vitro* detection platforms (50). The BoTest Matrix assays can be executed with instrumentation and techniques commonly found in government and industrial laboratories. The only specific instrument requirement for the assays is a microplate reader with the ability to detect emission at two wavelengths, although a magnetic-bead-compatible plate washer greatly increases assay throughput. The assays detect picomolar-to-subpicomolar concentrations of BoNT in a wide variety of matrices, including pharmaceutical, human-derived, and food samples (Fig. 5 and 6). The assays are largely immune to sample composition, with only minor protocol modifications required to account for sample-specific characteristics (e.g., pH and particulate matter). Finally, along with the recently reported BoTest Matrix E assay (47), the BoTest Matrix assays offer coverage of the four BoNT serotypes most relevant to human health, BoNT/A, BoNT/B, BoNT/E, and BoNT/F (41). Thus, the BoTest Matrix assays offer a single solution for human-related BoNT testing.

The BoTest Matrix assays also address many of the shortcomings of the mouse bioassay, including high variability, long assay times, and the general use of animals. The interassay and interlaboratory CVs of the mouse bioassay were reported to be 20 to 40% (42, 56). Pharmaceutical testing laboratories overcome these precision issues by using as many as 200 mice per sample, which, combined with a need to test products at many points during production, leads to very high costs and animal usage. The BoTest Matrix assays, however, have inter- and intra-assay CVs of <10% (Fig. 4) using 96-well plate formatting with assay times under 24 h. Thus, these assays can dramatically increase sample throughput while reducing overall assay times. Finally, as an animal-free alternative for BoNT testing, the BoTest Matrix assays can aid testing laboratories in conforming with recent U.S. and European government efforts to reduce animal use in manufacturing and research, including the European Union's ban on animal testing in cosmetics and the U.S. Animal Welfare Act's three R's—reduc-

tion, refinement, and replacement (24). Replacement of the mouse bioassay in drug product manufacturing alone would result in a large reduction in animal use, as it is estimated that 600,000 mice are used during BoNT production each year (10).

The BoTest Matrix assays will not be applicable to all testing procedures; applications that require assessments of BoNT's ability to enter neurons and impair synaptic activity will continue to rely on animal testing until a suitable cell-based assay is developed. However, there are many situations where animal testing is not required. Examples include in-process BoNT-based drug substance manufacturing (the mouse bioassay is required for the final drug product) (61), bioterrorism or health emergency events where BoNT detection needs to be completed in hours at laboratories not qualified to run animal assays (16, 23), environmental sampling where the cost of the mouse bioassay prevents the acquisition and analysis of comprehensive data sets (47), and food challenge testing where scientists need to determine the susceptibility of toxin production in food (57). The assays presented in this study offer low-cost, high-throughput, standardized methods that, for many applications, are adequate as a testing endpoint and can potentially replace most pharmaceutical, biodefense, and diagnostic procedures where a quantitative determination of the presence of BoNT is required.

The samples tested in this study represent a large cross-section of matrix properties, from high fat (whole milk) and foodstuff (baby food) to clinical (human serum) and a vector for a documented outbreak (carrot juice) (19). No clear trending was observed toward one tested matrix being more difficult than another, and assay performance is expected to vary slightly from sample to sample. While it is impossible to test all possible sample matrices, we were able to detect BoNT/A, BoNT/B, and BoNT/F at sensitivities equal to or nearly equal to that of the mouse assay in this broad matrix sampling with small sample sizes (200 μ l) (Fig. 6). As demonstrated with PBS, increasing the sample size should further increase assay sensitivity (Fig. 7).

The BoTest Matrix assays will ultimately require interlab validation and studies using "real-world" samples to be accepted for many applications. For food testing, as an example, food challenge studies are required to verify the ability of the assay to detect BoNT produced by *Clostridium*, rather than more artificial introductions, and ensure that assay sensitivity is sufficient. Such studies, if successful, would empirically demonstrate the BoTest Matrix assays' utility and eventually lead to a viable, long-term replacement of current animal-based methods.

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