

## Nomination to ICCVAM: **BoTest™ Matrix** Botulinum Neurotoxin Detection Kits.

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**Preamble:** BioSentinel is submitting nominations for three different product types- the **BoTest™** BoNT Detection Kits, the **BoTest™ Matrix** BoNT Detection Kits, and the **BoCell™** A Cell-based assay. These products differ in their BoNT sensitivity and tolerance to complex matrices. While an independent evaluation of each assay is required, these products should be considered a suite of solutions for detecting and quantifying BoNT. Thus, the products can be used in combination to meet criteria necessary for improved disease diagnosis, improved drug product production and development, and reduced animal testing.

### 1.0 Introduction and Rationale for the Proposed Test Method

#### 1.1 Introduction

1.1.1 **Background.** The BoTest™ Matrix A and E botulinum neurotoxin (BoNT) detection kits are biochemical assays for the detection of BoNT/A and E proteolytic activity contained in complex samples. The kits rely upon the use of immunoprecipitation technology (Matrix) and reporters that are identified and cleaved by BoNT/A and E (BoTest™). Currently BioSentinel offers two kits: BoTest™ Matrix A for the detection of BoNT/A and BoTest™ Matrix E for the detection of BoNT/E. **BoTest™ Matrix kits intended use is the detection and quantification of BoNT activity contained in complex matrices such blood, serum, water, pharmaceutical products, and food substances.**

The Matrix beads were developed by BioSentinel with the intent of adapting the BoTest™ BoNT Detection Kits for use with complex matrices. The Matrix beads consist of anti-BoNT/A or E specific antibodies covalently conjugated to a magnetic substrate. The beads are capable of binding and concentrating the BoNT contained in complex matrices (**Fig. 1**). Bound BoNT is then detected and quantifies using the BoTest™ reporters.

The BoTest™ reporter were invented by Dr. Edwin Chapman's group at the University of Wisconsin and reported on 2004<sup>1</sup>. Dr. Chapman's group described two reporters that utilize large sequences of SNAP-25 or synaptobrevin-2, the natural substrates of BoNT, fused to fluorescent proteins. The fluorescent proteins formed a Forster Resonance Energy Transfer (FRET) pair enabling the detection of reporter cleavage in real time and with high sensitivity (**Fig. 2**). The technology was subsequently licensed by BioSentinel who began operations in June, 2007 and initiated a R&D program with the goals of improving the reporters' performance, scaling up production, and developing drug discovery, biodefense testing, and human health testing.

BioSentinel commercialized the BoTest™ Matrix A BoNT Detection Kit in April of 2011 and is planning a commercial launch of the BoTest™ Matrix E BoNT Detection Kit in July of 2011. Pre-release kits have been used to quantify BoNT contained in pharmaceutical, blood, serum, and food samples. The BoTest™

Matrix E assay is currently being used by the National Wildlife Health Center (NHWCC) to quantify the amount of BoNT/E contained in avian blood samples. Kits for the other five serotypes are currently in development.

The BoTest™ Matrix assays were developed because there is currently no commercially available biochemical assay that can quantify the BoNT activity contained in complex samples. Salts, proteins, and other matrices interfere with BoNT activity *in vitro*; and high throughput, easy-to-perform methods that allow for the sequestering of BoNT and the removal of other matrix molecules are not described in the literature. The mouse bioassay is the current reference test method used by pharmaceutical manufacturers, government labs, and clinicians to detect and quantify BoNT in a wide range of samples. The BoTest™ Matrix assays offer mouse bioassay level sensitivity but with higher throughput than the mouse bioassay.

- 1.1.2 **Peer review to date.** BioSentinel has published a peer reviewed article on the development and performance of the BoTest™ assays<sup>2</sup>. Papers on the BoTest™ Matrix assays are in the preparation stage.

**The BoTest™ assays have not undergone any formal government panel peer review.**

- 1.1.3 **Confidential information.** No Information contained in these documents is considered confidential.

## 1.2 Scientific basis for the proposed test method

- 1.2.1 **Purpose and mechanistic basis** The BoTest™ Matrix assays detect BoNT protease activity, the biological activity responsible for BoNT's toxicity. The assays were designed to provide an easy, medium to high throughput means to assess the BoNT activity contained in samples such as blood, serum, food samples, and pharmaceutical samples. The assays currently detect two of seven serotypes of BoNT. Assays against the other five serotypes are in development.

The BoTest™ Matrix assay is a two-part assay ([Fig. 1](#)). First, samples containing BoNT are incubated with the Matrix beads. The beads contain a BoNT-specific antibody that binds and concentrates BoNT to the bead surface. After binding BoNT, the beads are then washed to remove any molecules that might otherwise interfere with the assay. In the second part of the assay, the Matrix beads (with bound BoNT) are incubated with the BoTest™ reporter. This reporter contains a FRET donor-acceptor pair flanking a segment of SNAP-25 or synaptobrevin sequence, the natural substrates of BoNT (see [Fig. 2](#) for explanation of FRET). The amount of BoNT bound to the Matrix beads is quantified by the bound toxin's ability to cleave the reporter. The reaction can be followed in real-time by measuring the ratio of YFP to CFP emission.

The BoTest™ Matrix A assay uses antibody-conjugated beads that are specific for the BoNT/A heavy chain, while the BoTest™ Matrix E assay uses beads that are specific for the BoNT/E heavy chain. BoNT is composed of heavy and light chains with the light chain responsible for BoNT's proteolytic activity. Thus, the BoTest™ Matrix assays provide identification of both the heavy chain (by

antibody binding) and the light chain (by cleavage of the BoTest™ reporter). The antibodies and reporters used in the assays were engineered for high affinity to BoNT. This high affinity allows the BoTest™ Matrix assays to detect BoNT activity contained in complex samples with femtomolar to picomolar sensitivity. For BoNT/A, the BoTest™ Matrix A assay is capable of detecting the amount of proteolytic activity corresponding to a single mouse median lethal dose (mLD<sub>50</sub>), the unit of activity currently used by the reference test method, the mouse bioassay.

- 1.2.2 **Similarities & differences between test method and mode of action.** BoNTs paralyze neurons and cause botulism by cleaving proteins that are essential for regulated neurotransmitter release. The BoTest™ Matrix assays detect and quantify this endopeptidase activity using reporters that are modeled after the native targets of BoNT. Thus, the BoTest™ Matrix assays account for BoNT biological activity rather than simply toxin mass.

The BoTest™ Matrix assays do not account for the ability of BoNT to bind to and enter neurons. Capturing these biological activities requires a cell-based assay such as BioSentinel's BoCell™ cell-based assays. The BoTest™ Matrix assays do, however, provide identification of both the heavy (antibody binding) and light (protease activity) chains of BoNT.

- 1.2.3 **Range of testable substance.** The BoTest™ Matrix assays are to be used with samples containing complex mixtures of molecules. BioSentinel has demonstrated BoNT detection and quantification using blood, serum, food, pharmaceutical, and environmental samples spiked with BoNT. The BoTest™ Matrix assay can also concentrate and quantify the BoNT contained in large volume (5 – 50 ml), low concentration (< 1 pM BoNT/A) samples. Potential applications include environmental testing (animal, plant, and water samples), clinical BoNT diagnostics, pharmaceutical quality control, and biodefense testing.

## **2.0 Test Method Protocol Components.**

### **A DETAILED PROTOCOL IS CONTAINED IN ATTACHMENT 8.1.**

**2.1 Protocol overview.** The BoTest™ Matrix assay requires a two-step protocol. BoNT-containing samples are dispensed into wells of a 96-well microtiter plate before the addition of a 10x Matrix Binding buffer to provide buffering and reduction of non-specific binding. Matrix beads are then dispensed into each well and incubated with shaking. The beads are then washed three times before resuspension into the BoTest™ Reaction Buffer supplemented with dithiothreitol (DTT). The assay is initialized by the addition of the BoTest™ reporter. Depending on the application and desired sensitivity, the assay is read after an incubation period of a few minutes up to 24 hours. No secondary reagents are required, so repetitive data reads can be carried out until the desired sensitivity is reached. Data is captured using a fluorescence microplate reader capable of detecting emissions at two wavelengths. The fluorescence emission are converted into a ratiometric value and plotted as a function of BoNT concentration or time. Unknowns can be quantified using an appropriate BoNT standard and basic curve fitting software.

## **2.2 Protocol details**

- 2.2.1 **Materials, equipment, and supplies needed.**

- Matrix beads
- 10x Matrix Binding Buffer
- 10x Matrix Wash Buffer
- BoTest™ A/E reporter
- BoTest™ Knock-out reporter (optional)
- 10x BoTest™ Reaction Buffer
- Fluorescence microplate reader with 434 nm excitation, 470 nm emission, and 526 emission filters
- Black, flat-bottom microtiter plates with covers
- Incubator set to 30 °C or 37 °C (optional)
- Microplate washer (optional)
- BoNT/A or E
- Nanopure H<sub>2</sub>O
- Dithiothreitol (DTT)
- Pipettes and tips for 1 µl – 100 µl dispensing
- Microplate mixer

- 2.2.2 **Dose selection procedure.** Due to its real-time nature, assay sensitivity is time tuneable with an expected range of quantifiable BoNT concentrations between <300 fM and 10 nM when including an appropriate standard curve containing 0.5 log BoNT dilutions.
- 2.2.3 **Endpoint measured.** For each data capture event, emissions are collected at 470 nm and 526 nm with 434 nm excitation. The relative fluorescence unit (RFU) value at 526 nm is divided by the RFU value of 470, yielding a ratiometric value. The ratiometric value is then compared to the ratiometric values obtained using a standard BoNT curve.
- 2.2.4 **Known limits of use.** BioSentinel has only tested a limited set of matrices; some matrices may interfere with assays. For samples containing bacterial contaminants, non-specific protease activity should be controlled for using a knock-out reporter lacking BoNT binding and cleavage sites (BioSentinel provided).
- 2.2.5 **Nature of response assessed.** The BoTest™ Matrix assays detect and quantify BoNT proteolytic activity.
- 2.2.6 **Appropriate positive and negative controls.** Each assay should contain a standard curve composed of known BoNT concentrations. A negative control will contain no BoNT in a buffer ideally identical to the unknowns. To identify non-specific protease activity and false positives, a knock-out reporter that lacks BoNT binding and cleavage sites should be included.
- 2.2.7 **Acceptable range of positive and negative controls.** The standard curve should contain concentrations of BoNT that give a dose-response curve that clearly demonstrates assay saturation (complete reporter cleavage) and no activity (reporter intact). Clear evidence of both is required for accurate curve fitting of the standard curve and solving unknowns.
- 2.2.8 **Methods to analyze the resulting data.** Ratiometric values obtained from the standard curve are plotted versus the known BoNT concentration contained in

each standard sample. The data is then fitted with equation  $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{[(\text{LogEC}_{50} - X) * \text{Hill slope}]})$ , where X is the logarithm of BoNT concentration and Y is assay response, yielding an EC<sub>50</sub> value. The fit is then used to solve for unknown concentrations.

**2.3 Basis for test method system.** The test system was selected because it is the only commercially available BoNT detection method with femtomolar to picomolar BoNT sensitivity that detects and quantifies a BoNT biological activity. In addition, the method does not require the use of animals and is compatible (and adaptable to) with a wide range of complex matrices.

**2.4 Proprietary components and their integrity over time and production runs.** BioSentinel manufactures the BoTest™ Matrix BoNT Detection Kits in their entirety. BioSentinel maintains manufacturing records that document all raw materials (manufacturer, part and lot numbers), each step in the manufacturing process, and quality control steps taken to ensure expected performance. In addition, BioSentinel's quality assurance program securely maintains all manufacturing records, standard operation procedures, and employee training. Finally, BioSentinel tests product integrity with accelerated and long-term stability experiments.

### 3.0 Product Performance and Stability.

#### 3.1 Product performance

- 3.1.1 **Figure 3** shows an example data resulting from a BoTest™ Matrix A assay used to quantify BoNT spiked into 100% serum and the time dependency of assay sensitivity.
- 3.1.2 **Figure 4** demonstrates that the BoTest™ Matrix A assay is capable of detecting femtomolar concentrations of BoNT/A in large volumes. A LOD of < 10 fM after 6 hours was achieved using higher volume samples.
- 3.1.3 **Figure 5** shows that the BoTest™ Matrix A assay is capable of detecting BoNT/A spiked into carrot juice, whole milk, whole blood, 100% human serum, and municipal water. In all cases, the LOD was ≤ 1 pM BoNT/A.
- 3.1.4 **Figure 6** demonstrates that the BoTest™ Matrix A assay can detect and quantify the BoNT/A contained in drug products. Detection is dependent on sequestering the BoNT with the Matrix beads before assaying for BoNT proteolytic activity.
- 3.1.5 **Figure 7** shows that the BoTest™ Matrix E assay is capable of quantifying the BoNT/E contained in archived avian blood samples. Note that these samples were field collected four or more years ago and tested by NWHC with the mouse bioassay for the presence of BoNT.

#### 3.2 Product stability

- 3.2.1 **Figure 8** demonstrates that lot-to-lot variability of the anti-BoNT/A antibody conjugated to the Matrix beads is low. The assay performs as expected when antibodies are isolated from completely different animals or from the same animals but on different days.
- 3.2.2 **Figure 9** shows that the BoTest™ Matrix assay is stable at 4 °C for at least 21 days with loss of assay performance. (Additional stability studies are in process.)

3.2.3 **Figure 10** outlines BioSentinel's manufacturing process for the BoTest™ reporters and quality assurance testing. A similar process is in place for the Matrix beads. BioSentinel maintains complete raw products and procedures tracking.

#### 4.0 Other scientific reports or reviews.

**4.1 BoTest™ reporter development and use.** The first report on the BoTest™ reporter can be found in the literature, as can a more recent report on the final development of the reporter<sup>1;2</sup>.

**4.2 Similar methods.** Several other methods that detect the proteolytic activity of BoNT have been reported<sup>3-8</sup>. These methods are less desirable than the BoTest™ assays due to their lack of throughput, sensitivity, or ease-of-use. Further, most of these methods were never commercialized and are unavailable to general scientific and clinical community. Finally, only mass spectrometry-based methods have demonstrate abilities to detect BoNT in complex matrices. Mass spectrometry-based methods are not easily transferable to most clinical, industrial, and research laboratories.

#### 5.0 Animal Welfare Considerations

**5.1 Refinement, Reduction, and Replacement.** The BoTest™ Matrix assays do not require the use of animals and represent the only commercially available assays with the sensitivity of the mouse bioassay, the current standard for BoNT testing, and the ability to measure BoNT activity in complex matrices. The BoTest™ Matrix assays measure and quantify the proteolytic activity of BoNT preparations, the activity most directly responsible for its toxicity. Thus, if properly validated with comparability studies, the BoTest™ Matrix assays can replace the mouse bioassay for many applications, particularly applications where testing of complex samples is required (diagnostic, environmental, and biodefense) and where the increased throughput of the BoTest™ Matrix assays would great facilitate outcomes.

#### 6.0 Practical Considerations

**6.1 Transferability.** The BoTest™ Matrix assay can be carried out in most any clinical or research laboratory. The only specialized equipment requirement is a fluorescence microplate reader with the ability to measure emissions at two wavelengths. A microtiter plate washer with magnetic capabilities will greatly improve throughput but is not required. Other equipment and reagents required is typically found in most laboratories (e.g. pipettes, nanopure H<sub>2</sub>O, etc).

The current reference test method, the mouse bioassay, requires specialize mouse handling equipment often housed in a space with purpose-designed air handling infrastructure. For this reason, the mouse bioassay is currently only carried out at a handful of state, federal, and commercial labs. The BoTest™ Matrix assay can be transferred to a much large segment of Federal and State testing laboratories.

**6.2 Training.** The BoTest™ Matrix assays require minimal training to perform. With a background in clinical or research laboratory science, an individual can be trained within a few hours. The training level is no more extensive than that required to run a typical ELISA or BCA assay. In contrast, the mouse bioassay requires training and certification is animal handling and extensive experience with animal injections, observing animals for the clinical symptoms of botulism, and transforming data into a useful predictor of toxicity.

**6.3 Cost Considerations.** Costs are difficult to estimate without a specific endpoint and application to consider. However, BioSentinel can estimate the costs for quantification of a single BoNT preparation using a commercial laboratory. (BioSentinel does not have enough data to estimate costs at a government laboratory.) Assuming a single sample to be tested, the BoTest™ Matrix assay would require one kit (\$1180 per kit, enough reagents for 200 wells, US government pricing) and an estimated 5 hours of labor at \$150/ hour for a total cost of \$1930 at a commercial laboratory. Since more than one sample can be quantified with a single kit, the costing would be drastically reduced on a per sample basis as long as the samples were run at the same time.

Quantifying the same sample using the mouse bioassay would be much more expensive. For regulatory approved, drug product-grade mouse bioassays, a single quantification assay can require as many as 200 – 300 mice at an estimated cost of \$6,000 if completed at a commercial laboratory operating under good laboratory practices. Cost savings are minimal when multiple samples require quantification as each sample would require the same number of injections to maintain assay accuracy and precision measures.

**6.4 Time considerations.** Depending on the number of samples and the desired sensitivity, the BoTest™ assay take 2 – 24 h to complete. In contrast, a typical mouse bioassay protocol takes 48 – 96 h to complete.

## **7.0 References.**

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3. Boyer, A. E., Moura, H., Woolfitt, A. R., Kalb, S. R., McWilliams, L. G., Pavlopoulos, A., Schmidt, J. G., Ashley, D. L. & Barr, J. R. (2005). From the mouse to the mass spectrometer: detection and differentiation of the endoprotease activities of botulinum neurotoxins A-G by mass spectrometry. *Anal Chem* **77**, 3916-24.
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## **8.0 Supporting Materials (Appendices).**

### **8.1 BoTest™ Matrix A BoNT Detection Kit Protocol.**

### **8.2 Figures, legends, and tables.**



## Attachment 8.1: BoTest™ Matrix A Botulinum Neurotoxin Detection Kit Protocol

This protocol is intended for users who have purchased the **combination BoTest™ Matrix A BoNT Detection Kit**. Users who purchased either the **Matrix A (product # A1014)** or the **BoTest™ A/E (product # A1004)** kits as standalone solutions should refer to protocols specific for those kits.

BoTest™ Matrix A BoNT Detection Kit

200 assays Cat. A1015

### 1.0 INTRODUCTION

The BoTest™ Matrix A Botulinum Neurotoxin (BoNT) Detection Kit is an *in vitro* assay for the detection and quantification of BoNT serotype A (BoNT/A) in complex matrices such as serum, blood, food, water, and pharmaceutical samples. The BoTest™ Matrix A kit consists of two primary reagents- the Matrix A beads and the BoTest™ A/E reporter. The Matrix A beads capture, concentrate, and isolate BoNT/A complex or holotoxin out of complex matrices from a variety of samples sizes (100 µl – 50 ml). The BoTest™ A/E reporter then detects the amount of captured BoNT/A providing real-time assessments of BoNT/A activity and quantity. Depending on the matrix composition and sample size, picomolar sensitivities can be achieved in < 3 hours and femtomolar sensitivities in 24 hours.

The Matrix A beads consist of a proprietary anti-BoNT/A antibody covalently conjugated to magnetic beads. These beads are added to a sample containing BoNT/A and incubated to allow BoNT/A binding. Interfering compounds that might otherwise interfere with BoNT/A activity determinations are removed by washing the Matrix A beads. The BoTest™ A/E reporter is modeled after the naturally occurring substrates of proteolytic BoNT and detects the endopeptidase activity of BoNT/A. The BoTest™ A/E reporter contains amino acids 141-206 of SNAP-25, encompassing both the exosite binding sites and the cleavage site of BoNT/A. The reporter has a high affinity for BoNT/A and, when incubated with BoNT/A bound to the Matrix A beads, rapidly and sensitively detects BoNT/A proteolytic activity.

### 2.0 DESCRIPTION

The BoTest™ Matrix A kit is supplied as a combination of the Matrix A (product # A1014) AND the BoTest™ A/E (product # A1004) kits. **COMPLETION OF THIS PROTOCOL REQUIRES BOTH OF THESE PRODUCTS.**

#### 2.1 Materials Supplied

BoTest™ A/E Botulinum Neurotoxin Detection Kit (A1004)

Description	Composition	A1004	
		Size	Part #
BoTest™ A/E Reporter	20 µM in 50 mM Hepes-NaOH, 10 mM NaCl, 15% Glycerol	250 µl	A1001
10x BoTest™ Reaction Buffer	500 mM Hepes-NaOH, pH 7.1, 50 mM NaCl, 1% Tween-20, 100 µM ZnCl <sub>2</sub>	2 x 1.25 ml	A1002



## Matrix A™ BoNT/A Pull-down Kit (A1014)

Description	Composition	A1004	
		Size	Part #
Matrix A Beads	Magnetic beads covalently conjugated to anti-BoNT/A antibody in PBS, 0.1% Tween-20, 0.05% Sodium Azide, 0.25% Casein, and 50% Glycerol.	4 ml	A1012
10x Matrix Binding Buffer	500 mM Hepes-NaOH, pH 7.1, 250 mM NaCl, 1% Tween-20, 5% Casein	5 ml	A1016
10x Matrix Wash Buffer	119 mM Phosphates, pH 7.4, 1370 mM NaCl, 27 mM KCl, 1% Tween-20	25 ml	A1013

### 2.2 Additional Required Materials

- Fluorescence microplate reader with 434 nm excitation, 470 nm emission, and 526 emission filters
- Black, flat-bottom microtiter plates w/covers
- Dithiothreitol (DTT)
- Microtiter plate mixer
- Orbital shaker at room temperature or at 25 °C if temperature control is available
- BoNT/A (optional, required for standardization and quantification purposes)
- 96-well magnetic separation plate compatible with chosen microtiter plates
- Plate washer or vacuum apparatus set up for washing magnetic beads (optional)
- Microcentrifuge (optional)
- High-quality (i.e. nanopure) H<sub>2</sub>O

### 3.0 STORAGE

Description	Storage Temp.	Notes
BoTest™ Reporters	-80 °C	Upon thawing, aliquot into single use amounts to avoid repeated freeze-thaw cycles. Stable for a minimum of five days at 4 °C upon thawing.
10x BoTest™ Reaction Buffer	-20 or -80 °C	Stable for a minimum of five days at 4 °C upon thawing.
Matrix A Beads	-20 °C	Stable for a minimum of five days at 4 °C upon removal from -20 °C. DO NOT FREEZE AT -80 °C.
10x Matrix Binding Buffer	-20 or -80 °C	Stable for a minimum of five days at 4 °C upon thawing.
10x Matrix Wash Buffer	-20 or -80 °C	Stable for a minimum of five days at 4 °C upon thawing.

### 4.0 SAFETY PRECAUTIONS

Sodium azide is toxic if ingested. Normal precautions exercised in handling laboratory reagents should be followed.

## **5.0 GENERAL ASSAY CONSIDERATIONS**

### **5.1 Required instrumentation and equipment**

The BoTest™ Matrix A kit uses antibody-conjugated magnetic beads and requires a 96-well magnetic separation plate compatible with the microtiter plates being used. Several different designs are available that may provide satisfactory results although testing will be required. We recommend the 96-well magnetic plate from V&P Scientific (cat. #VP771H) used with black, flat-bottomed F96 MicroWell™ microtiter plates from NUNC (cat. #237105).

Washing the Matrix A beads can be performed by hand with a single- or multi-channel pipette. However, assay times can be reduced and throughput increased by using an automatic plate washer or vacuum apparatus configured for washing magnetic beads. The washer or vacuum should be tested and adjusted to minimize bead loss during washing. Refer to the instrument's manual for proper settings and testing protocols.

Assay performance is highly dependent on thorough resuspension of the Matrix beads where specified in the protocol and maintaining the suspension during all incubation steps. A microplate mixer can be used to thoroughly resuspend the Matrix beads after pelleting. Beads suspensions can then be maintained using an orbital shaker or an incubating microtiter plate shaker during incubation periods.

The BoTest™ Matrix A assay requires the use of a fluorescent plate reader that allows for sequential detection at two emission wavelengths. For monochromator-based readers, the excitation wavelength should be set to 434 nm and the two emission wavelengths should be set to 470 and 526 nm. An excitation cut-off of 5 nm above the excitation wavelength is recommended if instrumentation allows. For filter-based readers, a 430-435 nm excitation filter and emission filters at 465-475 and 520-530 nm should be used.

The BoTest™ Matrix A assay should be performed with microplates designed for fluorescence-based assays such as black 96-well plates. Transparent plates should not be used. White-well plates can be used but are not recommended for assays where high accuracy is required. Covered plates or plate sealers are required for long incubation periods. Sample evaporation during incubation periods can be minimized by using a microtiter plate incubator with top and bottom heating elements.

### **5.2. Buffer considerations**

The BoTest™ Matrix A kit comes with a 10x Matrix Binding Buffer that is spiked into the samples to adjust buffer conditions and reduce non-specific binding to the beads. In cases where maximum sample volume is critical, this buffer may be excluded; however, exclusion may result in poor assay performance.

The protocol below may need to be modified to account for sample viscosity, non-specific binding, or other factors, depending on the BoNT/A-containing Matrix. Additional sample treatments or bead washing steps may be required to optimize assay performance (see 6.0 ADDITIONAL INFORMATION).

### **5.3 Matrix A bead use consideration**

Assay performance is critically dependent on executing washing and supernatant removal steps without accidental removal of the beads (bead loss). The recommended magnetic separation times should not be reduced without thorough testing to minimize bead loss. In addition, separation times may need to be increased if magnets other than the recommended magnets are used (see 5.1).

The microtiter plate should be placed on the magnetic 96-well separator as soon as possible after removing the plate from mixing and incubation periods as noted in 7.0 BASIC ASSAY PROTOCOL. This prevents beads from settling to the bottom of the wells before separation and ensures complete bead sequestering to the sides of the wells, limiting the possibility of accidentally aspirating the beads from the bottom of the well during supernatant removal. When removing supernatants, insert the pipette tip in the center of the well and slowly remove the liquid.

Finally, the beads must remain in solution during the pull-down and during the incubation with BoTest™ A/E reporter to ensure optimal pull-down efficiency and BoTest™ A/E reporter cleavage as noted in 7.0 BASIC ASSAY PROTOCOL.

## 6.0 ADDITIONAL INFORMATION

An applications guide along with sample data for the BoTest™ Matrix A BoNT Detection Assay can be found at [www.biosentinelpharma.com](http://www.biosentinelpharma.com).

## 7.0 BASIC ASSAY PROTOCOL

### Part 1. Reagent preparation.

1. Thaw the 10x Matrix Wash, 10x Matrix Binding, and 10x BoTest™ Reaction Buffers at room temperature.
  - a. Allow the buffers to warm completely to room temperature.
  - b. Vortex buffers for 5 seconds to mix.
  - c. If the 10x BoTest™ Reaction buffer appears cloudy, warm to 37 °C for 5 minutes.
2. Thaw the BoTest™ A/E Reporter at room temperature.
  - a. Mix gently upon thawing.
3. Calculate the number of wells required.
  - a. Include two or more control wells containing no BoNT/A, ideally using samples containing the same matrix or buffers as the unknown samples.
  - b. For quantitative assays, it is recommended that a dilution series of known BoNT/A quantities be prepared. A range of 0.1 pM to 1 nM BoNT is recommended.
  - c. The calculated number of wells will be used to calculate the buffers volumes to be prepared in the following steps. **It is recommended that the calculated number of wells be increased 10 – 25% to account for pipetting errors and loss during dispensing.**
4. Prepare 1 ml 1x Matrix Wash buffer per well.
  - a. Prepare 1x Matrix Wash buffer by diluting 1 part 10x BoTest™ Matrix A Wash Buffer with 9 parts H<sub>2</sub>O and mixing well by inversion.
  - b. Keep 1x buffer at room temperature until used.
5. Prepare 100 µl 1x BoTest™ Reaction Buffer with DTT per well.
  - a. Prepare 1X BoTest™ Reaction Buffer prepared by diluting 1 part 10x BoTest™ Reaction Buffer with 9 parts high-quality H<sub>2</sub>O.
  - b. Supplement 1x BoTest Reaction buffer with 5mM DTT (final concentration). DTT is required to fully activate BoNT/A activity during detection.
6. For each well to be run, prepare a BoTest™ A/E **Master Stock** containing 1.25 µl BoTest™ A/E Reporter and 3.75µl 1x BoTest™ Reaction Buffer with DTT per well.

### Part 2. BoNT/A binding and isolation.

7. Warm the Matrix A beads for 15 minutes at room temperature.
8. Prepare samples.
  - a. Sample volumes of 100 – 225 µl/well can be tested using the microtiter plate format. For smaller volumes, increase the sample volume to at least 100 µl using a buffer compatible with your samples (e.g. PBS or the matrix being tested). It is recommended that all samples be of the same volume.
  - b. (Optional) For samples containing particulate matter, a pre-clearing step is recommended to remove solid material from the sample. Spin samples in a microcentrifuge for >10 minutes at >12,000 x g and carefully collect the supernatants for testing.
  - c. For sample volumes > 225 µl, please consult the application guide (see 6.0 ADDITIONAL INFORMATION).
9. Dispense samples including unknowns, standards, and controls into wells.
10. Dispense a 1/10-sample volume of 10x Matrix Binding Buffer to each well containing samples.
  - a. Example: If the sample volume is 100 µl/ well, dispense 10 µl/well of 10x Matrix Binding Buffer.
11. Thoroughly resuspend the Matrix A beads by rigorously vortexing for 5 seconds.

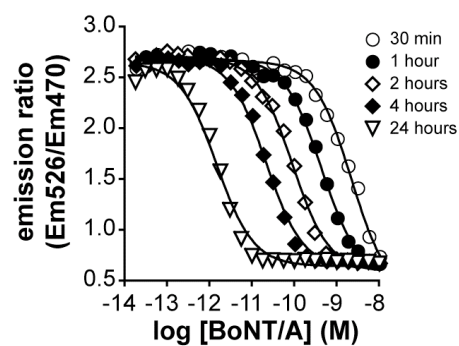
- a. The beads will settle to the bottom of the stock tube over time and must be thoroughly resuspended before removal. Verify that the beads are in a homogenous suspension, if needed, repeat vortexing.
12. **Immediately** dispense 20 µl Matrix A Beads into each well containing sample.
  - a. Maintain a homogenous bead stock suspension during dispensing. If needed, vortex the bead stock again for 5 seconds.
13. Cover plate and incubate at 25 °C or room temperature with shaking for 2 hours.
  - a. The beads must be maintained in suspension for optimal assay performance. Add Matrix A beads to wells and mix plate for 10 seconds using a microtiter plate mixer before transferring to an orbital shaker.
14. Wash the Matrix beads with 1x Matrix Wash Buffer.
  - a. Remove the plate from the orbital shaker and **immediately** separate beads for ≥ 2 minutes using a 96-well magnetic separation plate.
  - b. Using a pipette tip, gently remove the supernatants being careful not to remove any beads.
  - c. Remove the plate from the magnetic separator and add 250 µl 1x BoTest™ Matrix A **Wash Buffer**.
  - d. Mix plate for 10 seconds using a 96-well plate mixer. After mixing, **immediately** separate beads for 2 minutes using a 96-well magnetic separation plate.
  - e. Repeat Steps (b) through (d) twice for a total of 3 washes.

### Part 3. Detection of BoNT/A activity with BoTest™.

15. Remove 1x Matrix Wash Buffer supernatant.
  - a. Mix plate for 10 seconds using a microtiter plate mixer. After mixing, **immediately** separate beads for 2 minutes using a 96-well magnetic separation plate.
  - b. Using a pipette tip, gently remove the supernatants being careful not to remove any beads.
16. Add 95 µl 1x BoTest™ Reaction Buffer with DTT to each well.
17. Add 5 µl BoTest™ **Master Stock** to each well and mix plate for 10 seconds using a 96-well plate mixer set to the settings determined earlier.
18. Cover plate(s) and **immediately** incubate on orbital shaker at room temperature or 25 °C for 30 min – 24 hours.
19. At the desired time point, measure the fluorescence at ~470 and ~526 nm using 434 nm excitation.
  - a. Remove the plate from the orbital shaker and **immediately** separate beads for > 2 minutes using a 96-well magnetic separation plate.
  - b. Gently remove the plate from the magnetic separator and place in fluorescent plate reader making sure not to disturb the pelleted beads.
  - c. Measure the fluorescence at ~470 and ~526 nm using ~434 nm excitation.
  - d. If additional incubation time is desired, mix plate for 10 seconds using a 96-well plate mixer.
  - e. Cover plate and immediately transfer to an orbital shaker at room temperature or 25 °C until the next time point.

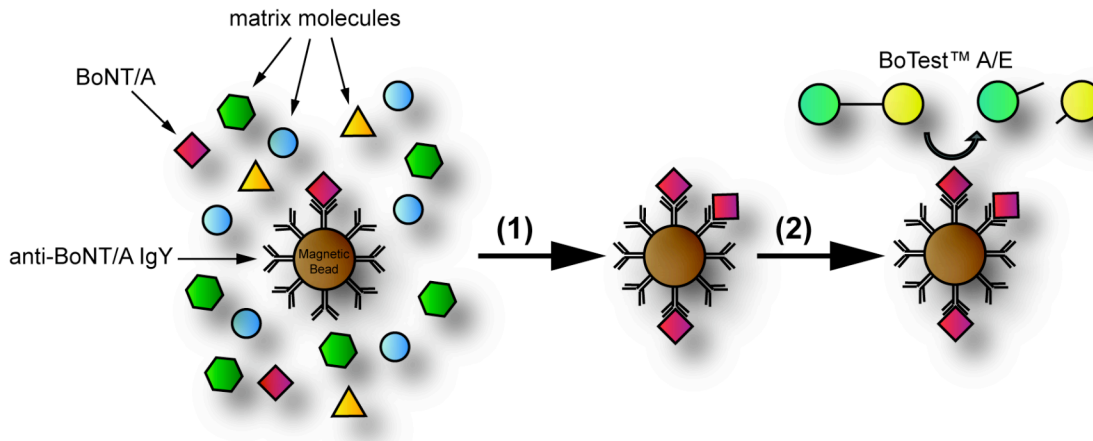
(Note: Some plate readers will disturb the pelleted beads during reading, resulting in increased data scatter. In these cases, supernatants (50 – 100 µl) from the BoTest™ reaction can be transferred to another plate for readings. The volume transferred should be consistent among all wells.)
20. For each well, obtain an emission ratio by dividing the relative fluorescence unit (RFU) value at 526 nm by the RFU value at 470 nm. BoNT activity is detected by a decrease in the emission ratio compared to control wells (See **Figure 1**).

## 8.0 EXAMPLE DATA

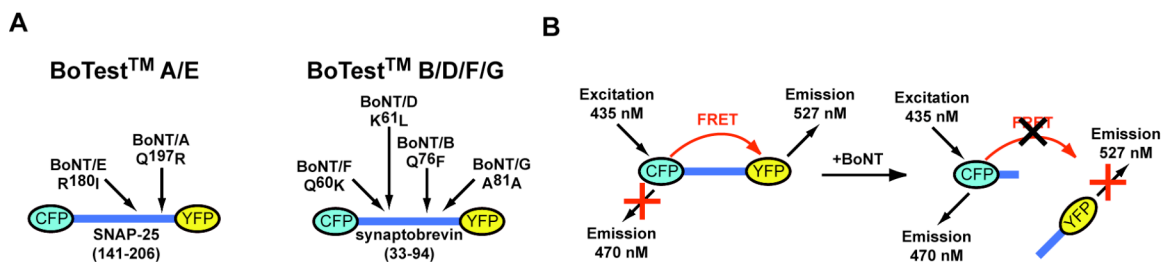


**Figure 1. Femtomolar to picomolar BoNT/A detection with the BoTest™ Matrix A BoNT Detection Kit.** Samples containing the indicated BoNT/A concentration were immunoprecipitated with the Matrix A beads according to protocol. BoTest™ reporter was then added to the beads and incubated at 22 °C. After 30 min – 24 hours, the reporter was excited at 434 nm and the emission was collected at 470 and 526 nm on a Varioskan plate reader (Thermo-Fisher). The emission ratio was plotted as a function of BoNT/A concentration where the emission ratio is the relative fluorescence units (RFU) value of the FRET peak (526 nm) divided by the RFU value of the donor/CFP peak (470 nm).

## Attachment 8.2: BoTest™ Matrix BoNT Detection Kit data

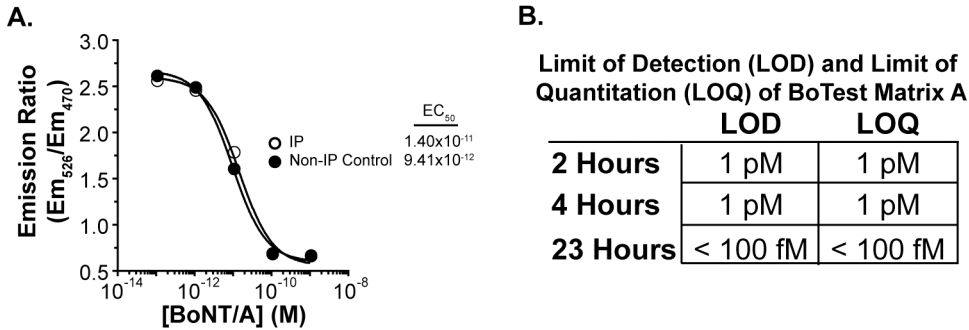


**Figure 1. The BoTest™ Matrix A BoNT Detection Assay.** Magnetic beads conjugated to anti-BoNT/A IgY Ab (the Matrix beads) are incubated with samples containing complex mixtures of matrix molecules (1) The beads are washed removing the matrix molecules while retaining BoNT/A on the bead surface via binding of the anti-BoNT/A IgY to BoNT/A. (2) The beads and bound BoNT/A are resuspended in reaction buffer along with the BoTest™ A/E reporter. The reporter is cleaved by BoNT/A resulting in a change in reporter fluorescence emission at two wavelengths (470 and 526 nm). Bound BoNT/A's proteolytic activity is quantified by the change in reporter emission.

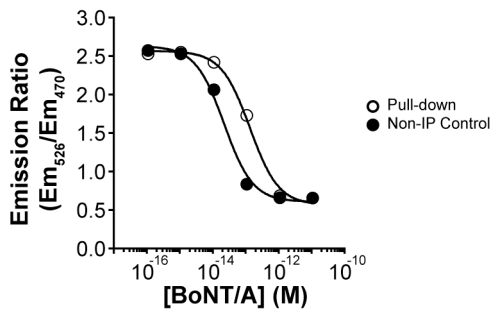


**Figure 2. BioSentinel's FRET-based BoTest™ reporters.** (A) BioSentinel's *in vitro* reporters. CFP and YFP are connected by SNAP-25 (amino acids 141–206, BoTest™ A/E) or by synaptobrevin (amino acids 33–94, BoTest™ B/D/F/G). The cleavage sites for each BoNT serotype are indicated. (B). A FRET assay for measuring BoNT protease activity. If CFP and YFP are in close enough proximity to each other, excitation of CFP leads to emission from YFP via FRET and concomitant attenuated emission from CFP. If the SNAP-25 or synaptobrevin linker between CFP and YFP is cleaved by BoNT, CFP

and YFP are no longer in close proximity. In this case, FRET is lost, corresponding to decreased YFP emission and increased CFP emission.



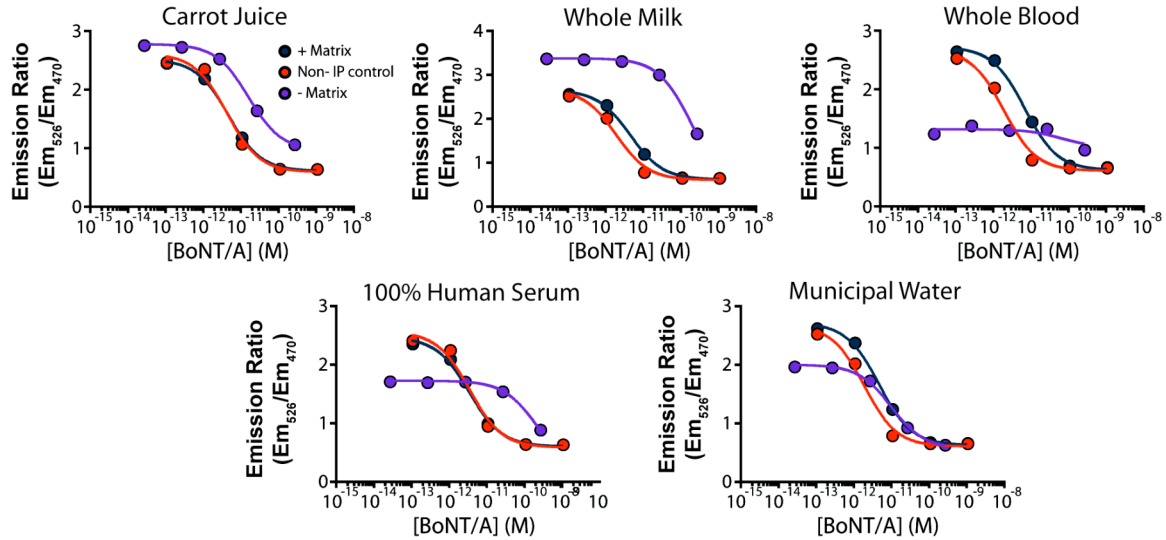
**Figure 3. Performance of the BoTest™ Matrix A assay in quantifying BoNT/A in human serum.** (A) Ability of the optimized assay to detect BoNT/A. Matrix beads were used to pull down varying BoNT/A concentrations spiked into 200  $\mu$ L 100% human serum. After an 2 h incubation at room-temperature, beads were washed and assayed with the BoTest™ reporter for 2 h before collecting fluorescence emissions at 526 and 470 nm with 434 nm excitation. The emission ratios (526/470) were then plotted as a function of BoNT/A concentration. The non-IP control sample data is the result of incubating BoNT/A diluted directly into BoTest™ reaction buffer with the BoTest™ reporter. The non-IP control curve theoretically represents 100% BoTest™ Matrix assay BoNT/A pull-down efficiency. (B) Limits of detection (LOD) and quantification (LOQ) for the BoTest™ Matrix A assay at varying incubation times. The BoTest™ Matrix A assay was run with BoNT/A-spiked human serum samples as described in (A) except that the incubation of the BoNT/A-bound Matrix beads with the BoTest™ reporter was varied from 2 – 23 h. LOD and LOQ were calculated as the concentration for which the emission ratio was less than 3 (LOD) or 10 (LOQ) standard deviations below control samples without BoNT/A holotoxin.



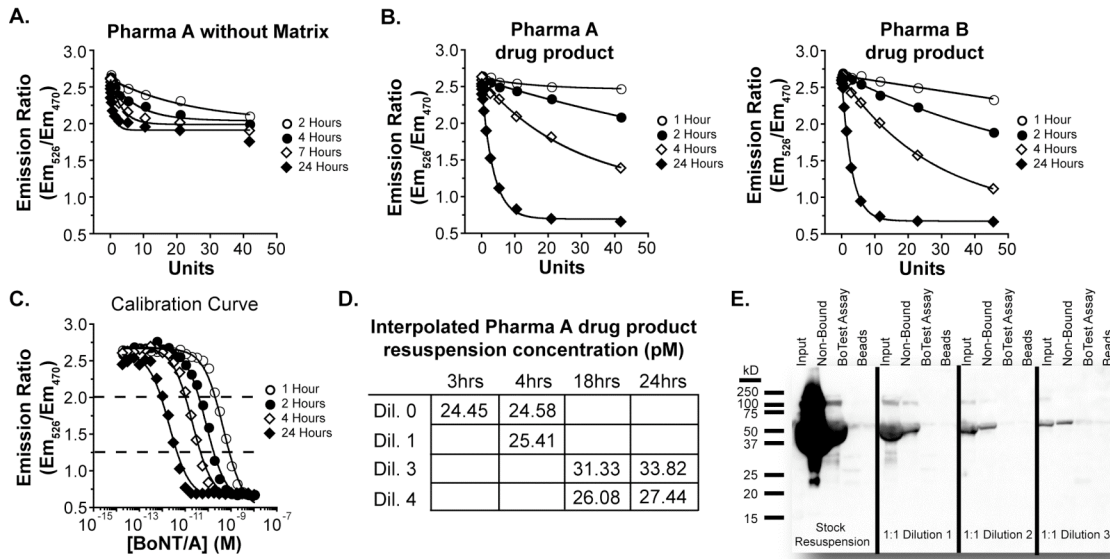
**Figure 4. The Matrix A beads can concentrate highly dilute samples.** Matrix beads were used to pull-down the indicated concentration of BoNT/A diluted in 15 mL PBS-t. The beads were then washed and incubated with the BoTest™ reporter as described in Figure 3. Non-IP controls were also run that represented the total BoNT/A in the 15 mL



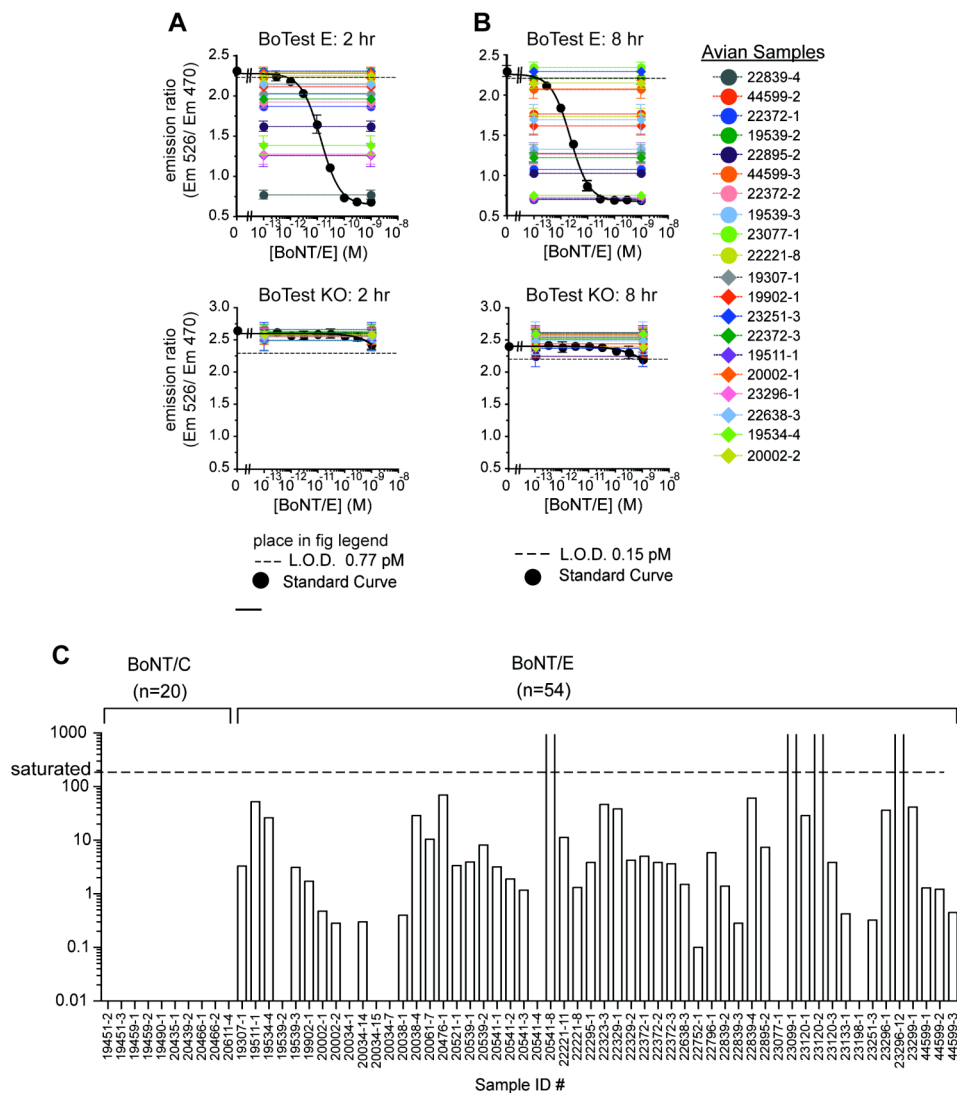
sample (theoretical 100% pull-down efficiency). A LOD of < 10 fM after 6 hours was achieved using higher volume samples.



**Figure 5. The addition of the Matrix A beads allows quantification of BoNT/A activity in highly complex matrices using BoTest™ A/E reporter.** The indicated BoNT/A concentrations were spiked into carrot juice, whole milk, whole blood, human serum, and municipal water and assayed in duplicate with BoTest™ A/E either with (+Matrix) or without (-Matrix) pull down steps before incubating with the BoTest™ reporter as described in Figure 3. Non-IP control reactions were made by directly diluting BoNT/A into BoTest™ reaction buffer and incubating with the BoTest™ reporter without use of the Matrix beads (theoretical 100% pull-down efficiency). Reactions were incubated at 25°C and emission ratio data was collected after 4 hours.

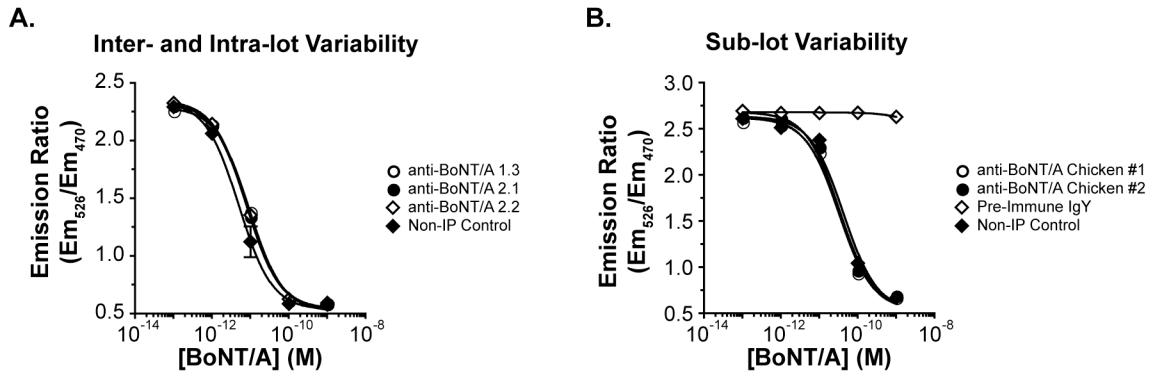


**Figure 6. Capture, detection and quantification of BoNT/A in drug products using the BoTest™ Matrix A Assay.** Two drug products were renamed Pharma A and B. **(A)** Detection of BoNT/A activity without first using the Matrix beads to sequester BoNT/A. A single vial (100U) of Pharma A drug product was resuspended to 0.83 U/μl in 0.9% saline and serially diluted 1:1 in saline 12 times. 50 μl of each dilution was then mixed with 50 μl BoTest™ Reaction Buffer containing 0.5 μM BoTest™ A/E reporter and incubated at 25°C. Emission ratio data was collected at 2, 4, 7, and 24 h and plotted as a function of drug product units. **(B)** Detection of BoNT/A activity in two drug products using the BoTest™ Matrix A assay. A 100 U vial of each drug product was resuspended in saline to 0.83 or 0.91 U/μl, respectively, and serially diluted as described in (A). Each dilution was then incubated with Matrix A beads for 2 h at RT. Beads were washed and assayed as described in Fig. 3 with 1, 2, 4, and 24 h BoTest™ reporter incubation times. **(C)** Calibration curves for quantifying BoNT/A activity in Pharma A drug product. An internal BoNT/A standard was serially dilute 1:1 in PBS-t with 33.3 mg/ml HSA (to mimic drug product excipients) and subjected to the BoTest™ Matrix assay as described in (B). The dotted line indicates the linear portion of assay response. **(D)** Quantification of BoNT/A contained in Pharma A drug product. The BoNT/A concentration of a Pharma A drug product resuspension can be interpolated from the BoNT/A calibration curves in (C) using the emission ratio values of the drug product dilutions that fell within the linear range of the calibration curve. An empty box in the table indicates that the emission ratio value for that particular dilution fell out of the linear range of the corresponding calibration curve. **(E)** Capture of BoNT/A and removal of HSA. Samples from (B) were subjected to SDS-PAGE and western blotting using an anti-HSA Ab.

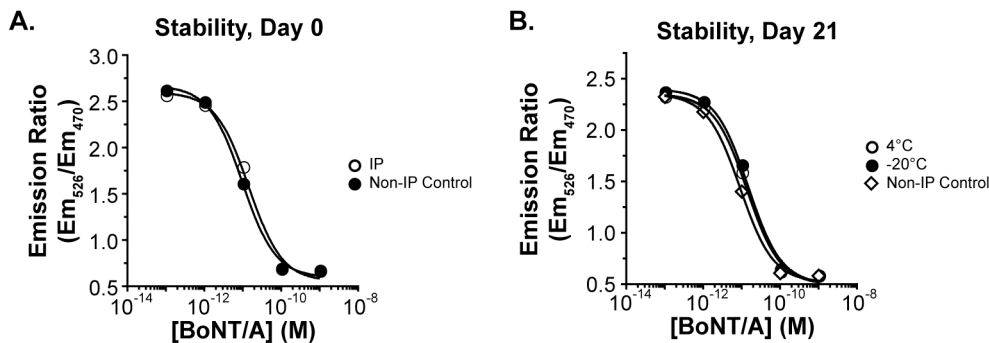


**Figure 7. BoNT/E detection in avian blood: diagnostics for BoNT/E associated die-offs.** Field collected avian blood samples from botulism-related die-off events were obtained from the NWHC. Each sample was confirmed to be positive for BoNT/C or E by the mouse bioassay using NWHC protocols. **(A)** and **(B)** Each sample (listed to the right) was assayed for the presence of BoNT/E using the BoTest™ Matrix E assay. Activities obtained were compared to a standard curve (closed black symbols) generated using the BoTest™ Matrix E assay and a standard preparation of BoNT/E. Emission spectra were measured as described in Figure 3 at 2 (panel A) and 8 (panel B) hours. Plotted is the dose-response curve of the standard with the individual emission ratio responses of the samples overlaid to show where the signals fall on the standard curve. LOD were calculated at each time-point by determining the minimal standard BoNT/E concentration producing a signal >3 standard deviations below samples containing no BoNT (---, n=6). LOD 4hr = 770 fM, LOD 8hr = 150 fM. Samples with emission ratios below calculated LOD values are considered positive for BoNT/E. Top panels show the responses of samples using the BoTest™ E reporter in the assay. Bottom panels show the response of samples using a knock out reporter that is insensitive to BoNT/E. **(C)** Quantified avian samples plot. Mouse bioassay-confirmed BoNT/C (n=20) and BoNT/E

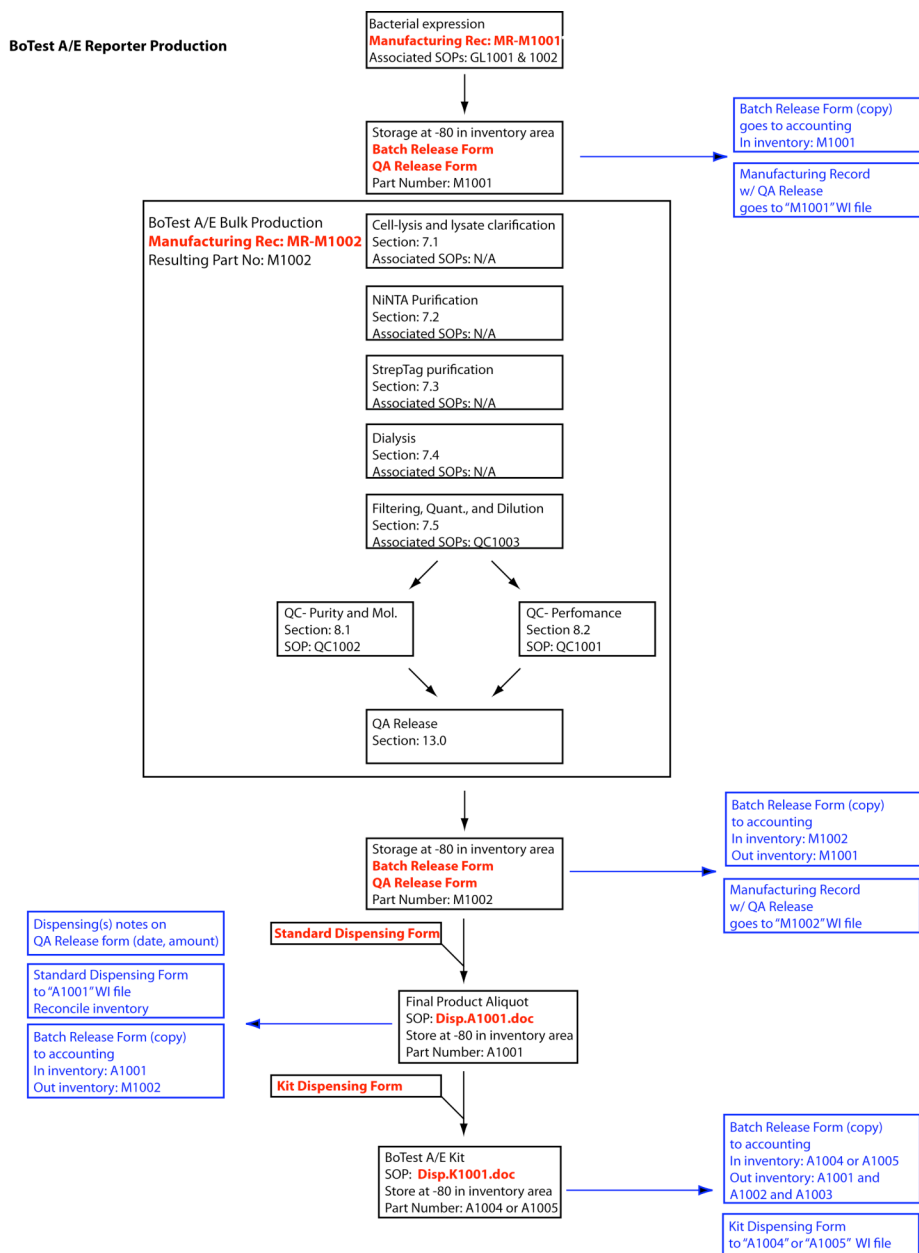
(n=54) samples were tested using the BoTest™ Matrix E assay. Calculated BoNT/E concentrations (pM) were plotted against sample identification number. Note that some samples completely cleaved the reporter under the conditions tests and are listed as saturated.



**Figure 8. Little lot-to-lot variability is seen with the anti-BoNT/A IgY. (A)** There is no inter-lot (different hens, compare lots 1.3 to 2.1) or intra-lot variability (same hens, different IgY collection times, compare lots 2.1 and 2.2) seen with anti-BoNT/A IgY lots. Matrix A beads were manufactured with each antibody lot and used to assay samples containing the indicated concentration of BoNT/A diluted into 100% human serum. BoNT/A activity was assayed as in Figure 3. **(B)** Sub-lot variability, the variability between two chickens from the same immunization clutch, had no effect on assay performance. Assays were carried out as described in (A).



**Figure 9. Matrix A beads are stable for at least 21 days at 4°C and -20°C. (A)** BoNT/A diluted into 100% human serum were subjected to the BoTest™ Matrix assay as described in Figure 3. **(B)** Matrix beads from the same lot as those used in panel (A) were stored at either 4°C or -20°C and assayed after 21 days storage. No significant loss in bead performance was seen following storage at either temperature.



**Figure 10. Manufacturing process flow for the BoTest™ A/E reporter.** The key step or resulting product is indicated at the top of each box followed by the manufacturing record, associated SOPs, quality assurance release forms, and/or resulting product part number. The documents highlighted in red indicate the primary documents used for tracking of a particular lot of crude or final product. These documents are filed by part number. Steps highlighted in blue indicate release forms and accounting steps. Key intermediates and final products in the manufacturing scheme are M1001, the bacterial cell paste containing over-expressed reporter; M1002, the purified, bulk preparation of the BoTest™ A/E reporter; A1001, the final, dispensed BoTest™ A/E reporter; and A1004, the final kit sold to customers which includes the BoTest™ A/E reporter, the 10X BoTest™ reaction buffer, a protocol, and a certificate of analysis.