Nomination to ICCVAM: BoTest™ 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™ botulinum neurotoxin (BoNT) kits are biochemical assays for the detection of BoNT/A, B, D, E, F, and G proteolytic activity. The kits rely upon substrates or reporters that are identified and cleaved by the six detectable serotypes of BoNT. Currently BioSentinel offers two kits: BoTest™ A/E for the detection of BoNT/A and E and BoTest™ B/D/F/G for the detection of BoNT/B, D, F, and G. **BoTest™ kits' intended uses are drug discovery and the detection and quantification of purified BoNT preparations.**

The BoTest™ reporters were invented by Dr. Edwin Chapman's group at the University of Wisconsin and reported on 2004[1]. 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 Förster Resonance Energy Transfer (FRET) pair enabling the detection of reporter cleavage in real time and with high sensitivity (**Fig. 1**).

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™ A/E BoNT Detection Kit in May 2009 and the BoTest™ B/D/F/G kit in January 2010. The kits have since been used by BioSentinel, collaborators (including DoD laboratories), or clients for the quantification of drug formulations and drugs, high throughput drug screening campaigns for the discovery of BoNT inhibitors, and the development of novel methods for the detection of BoNT including BioSentinel's own BoTest™ Matrix assays.

The BoTest™ reporters were developed because no other commercially available assay detects BoNT activity with the sensitivity of the current standard, the mouse bioassay. The mouse bioassay is currently used by pharmaceutical manufacturers, government labs, and clinicians to detect and quantify BoNT in a wide range of samples. The BoTest™ kits offer mouse level or near mouse level sensitivity, depending of the BoNT serotype. In addition, the BoTest™ reporters

offer real-time detection capabilities, tunable sensitivity, and low training requirements.

1.1.2 **Peer review to date**. A pharmaceutical client has validated the BoTest™ assays for the quantification of drug formulations and drug products and is currently completing comparability studies between the BoTest™ assay and the mouse bioassay. In addition, BioSentinel has published a peer reviewed article on the development and performance of the BoTest™ assays[2].

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™ assays detect BoNT protease activity, the biological activity responsible for BoNT's toxicity. The assays were designed to provide an easy, high throughput means to assess BoNT activity. The assays currently detect six of seven serotypes of BoNT.

The assay uses a reporter that contains a FRET donor-acceptor pair separated by a segment of SNAP-25 or synaptobrevin sequence, the natural substrates of BoNT (**Fig. 1**). The BoTest™ reporters are recombinant proteins with cyan fluorescent protein (CFP) fused to the N-terminus and yellow fluorescent protein (YFP) fused to the C-terminus. In the intact reporter, CFP excitation leads to YFP excitation by FRET, thus quenching the fluorescence emission from CFP, while increasing the fluorescence emission from YFP. Treating the reporter with BoNT leads to cleavage of the linker, and thus decreasing YFP emission and increasing CFP emission. The reaction can be followed in real-time by measuring the ratio of YFP to CFP emission. Thus, the BoTest™ reporters allow for kinetic determinations of BoNT activity in a mix-and-read format allowing for quantitative determinations of enzymatic activity.

The BoTest™ A/E assay uses a SNAP-25 based reporter specific for BoNT/A and E while the BoTest™ B/D/F/G assay uses a synaptobrevin based reporter specific for BoNT/B, D, F, and G. These reporters differ from other commercially available assays, in that they use much larger segments of SNAP-25 or synaptobrevin sequence resulting in BoNT having a much higher affinity for the reporter than other reporter systems. This high affinity results in the ability to detect BoNT activity with sensitivity that matches or nearly matches that of the mouse bioassay (femtomolar to picomolar BoNT concentrations). For BoNT/A, the BoTest™ A/E assay is capable of detecting the amount of proteolytic activity corresponding to a single mouse median lethal dose (mLD_{50}), 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™ assays detect and quantify this endopeptidase activity using reporters that are modeled after the native

targets of BoNT. Thus, the BoTest reporters account for BoNT biological activity rather than simply toxin mass.

The BoTest™ 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.

1.2.3 **Range of testable substance.** The BoTest™ assays are to be used with purified BoNT preparations or with substances that do not have high concentrations of salts or proteins. The assays can also be used with samples containing high BoNT concentrations where any interfering substances can be diluted to below inhibitory thresholds. The BoTest™ assays have been shown to work with native and recombinant purified preparations of BoNT, BoNT-based drug products, and with bacterial supernatants. Applications include high throughput drug screening, quantification of BoNT-based drug products, and specific activity determinations of BoNT preparations.

The BoTest™ assays tolerate < 20 mM NaCl and < 1 % albumins without inhibiting BoNT proteolytic activity *in vitro*.

2.0 Test Method Protocol Components.

A DETAILED PROTOCOL IS CONTAINED IN ATTACHMENT 8.1.

2.1 Protocol overview. The BoTest™ assays rely on a simple mix-and-read format. BoNT-containing samples are dispensed into wells of a 96-well (or 384- or 1536-well) microtiter plate. Each sample is then mixed with BoTest reaction buffer supplement with a reducing agent such as dithiothreitol. 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.**

- BoTest™ A/E or B/D/F/G reporter (20 μM)
- 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 ^{0}C or 37 ^{0}C (optional)
- BoNT/A or E (BoTest™ A/E) or BoNT/B, D, F, or G (BoTest™ B/D/F/G)
- Nanopure H_2O
- Pipettes and tips for 1μl – 100 μl dispensing
- Microplate mixer

2.2.2 **Dose selection procedure.** Due to its real-time nature, assay sensitivity is time tunable 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.** The BoTest™ assays have a low tolerance to salts (<20 mM) and albumins (<1 %). The assays are to be used with samples with known buffering components or samples that can be extensively diluted.

2.2.5 **Nature of response assessed**. The BoTest 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. The negative control is essential for discriminating between BoNT proteolytic activity and non-specific proteolytic activity that may be introduced by the buffering system used.

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 = Bottom + (Top – 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_{50} 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.

2.4 Proprietary components and their integrity over time and production runs. BioSentinel manufacturers the BoTest™ 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 has tested product integrity in accelerated and long-term stability experiments and found the products to be robust and maintain stability upon long-term storage.

3.0 Product Performance, Stability, and Third Party Validations.

3.1 Product performance

3.1.1 **Figure 2** shows the time and temperature dependence of BoNT/A and E cleavage of the BoTest™ A/E reporter. At 37 ^{0}C BoNT/A is largely inactivated after one hour and assay sensitivity cannot be improved with increased incubation time. At 25 or 30 ^{0}C, BoNT/A remains stable and assay sensitivity is increased with further incubation time.

3.1.2 **Figure 3** shows the time dependence of BoNT/B, D, F, and G cleavage of the BoTest™ B/D/F/G reporter.

3.1.3 **Table 1** summarizes the limits of detection of the BoTest™ assays with six serotypes of BoNT. For reference, 1 pM BoNT/A in 100 μl is equivalent to ~4 mLD_{50} units. 1 pM BoNT/B in 100 μl if equivalent to ~1.5 mLD_{50} units.

3.1.4 **Figure 4** demonstrates how the BoTest™ assays can be used to determine the enzymatic specific activity of a lot of BoNT/A and the variability associated with such determinations.

3.2 Product stability

3.2.1 **Figure 5** shows the performance of the BoTest™ A/E and B/D/F/G reporters after 1 – 7 days storage at elevated temperatures. Neither reporter exhibited any loss of performance.

3.2.2 **Figure 6** shows the performance of the BoTest™ A/E reporter upon extended storage at -20 and -80 ^{0}C. Long-term stability studies are currently ongoing for the BoTest™ B/D/F/G reporter.

3.2.3 **Figure 7** demonstrates that the BoTest™ reporters are stable upon repeated freeze-thaw cycling.

3.2.4 **Figure 8** outlines BioSentinel's manufacturing process for the BoTest™ reporters and quality assurance testing. BioSentinel maintains complete raw products and procedures tracking.

3.3 Third party validation

3.3.1 The BoTest™ A/E BoNT Detection Kit was successfully validated by a pharmaceutical manufacturer of BoNT for the quantification of preformulated and formulated drug substances. **Data available upon request.**

3.3.2 The BoTest™ A/E kit is currently undergoing comparability testing to the mouse bioassay by a pharmaceutical manufacturer. Early results indicate that the BoTest™ assay is a suitable mouse bioassay replacement for the quantification of BoNT-based drug products. **Data available upon request.**

3.3.3 The above pharmaceutical client intends to file for regulatory approval to use the BoTest™ assay for in-process sample testing.

4.0 Other scientific reports or reviews.

4.1 BoTest™ 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[1;2].

4.2 Similar methods. Several other methods that detect the proteolytic activity of BoNT have been reported [3-8]. 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.

5.0 Animal Welfare Considerations

5.1 Refinement, Reduction, and Replacement. The BoTest™ 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. The BoTest™ 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™ assays can replace the mouse bioassay for many applications, particularly applications (drug discovery and botulinum drug manufacturing and testing) where the increased throughput of the BoTest™ assays would great facilitate outcomes.

6.0 Practical Considerations

6.1 Transferability. The BoTest™ 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. Other equipment and reagents required is typically found in most laboratories (e.g. pipettes, nanopure H_2O, 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™ assay can be transferred to a much larger segment of Federal and State testing laboratories.

6.2 **Training.** The BoTest™ 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™ assay would require one kit ($425 per kit, enough reagents for 200 wells, US government pricing) and an estimated 3 hours of labor at $150/ hour for a total cost of $875 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 takes 2 – 24 h to complete with incubation periods. In contrast, a typical mouse bioassay protocol takes 48 – 96 h to complete.

7.0 References.

1. Dong, M., Tepp, W. H., Johnson, E. A. & Chapman, E. R. (2004). Using fluorescent sensors to detect botulinum neurotoxin activity in vitro and in living cells. *Proc Natl Acad Sci U S A* **101**, 14701-6.
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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 endoproteinase activities of botulinum neurotoxins A-G by mass spectrometry. *Anal Chem* **77**, 3916-24.
4. Schmidt, J. J., Stafford, R. G. & Millard, C. B. (2001). High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. *Anal Biochem* **296**, 130-7.
5. Schmidt, J. J. & Stafford, R. G. (2003). Fluorigenic substrates for the protease activities of botulinum neurotoxins, serotypes A, B, and F. *Appl Environ Microbiol* **69**, 297-303.
6. Anne, C., Cornille, F., Lenoir, C. & Roques, B. P. (2001). High-throughput fluorogenic assay for determination of botulinum type B neurotoxin protease activity. *Anal Biochem* **291**, 253-61.
7. Kalb, S. R., Goodnough, M. C., Malizio, C. J., Pirkle, J. L. & Barr, J. R. (2005). Detection of botulinum neurotoxin A in a spiked milk sample with subtype identification through toxin proteomics. *Anal Chem* **77**, 6140-6.
8. Kalb, S. R., Moura, H., Boyer, A. E., McWilliams, L. G., Pirkle, J. L. & Barr, J. R. (2006). The use of Endopep-MS for the detection of botulinum toxins A, B, E, and F in serum and stool samples. *Anal Biochem* **351**, 84-92.

8.0 Supporting Materials (Appendices).

8.1 BoTest™ A/E and B/D/F/G BoNT Detection Kit Protocol.

8.2 Figures, legends, and tables.

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BioSentinel Part No: L1002, Release Date: 12/17/2009

Attachment 8.1: BoTest™ Botulinum Neurotoxin Detection Kit Protocol

BoTest™ A/E Botulinum Neurotoxin Detection Kit	200 assays	Cat. A1004
	2000 assays	Cat. A1005
BoTest™ B/D/F/G Botulinum Neurotoxin Detection Kit	200 assays	Cat. A1009
	2000 assays	Cat. A1010

1.0 INTRODUCTION

BoTest™ is an *in vitro* assay for the detection of botulinum neurotoxins (BoNT). BoTest™ can be used to assess the quality of BoNT preparations, carry out biochemical studies on BoNT function, screen for antagonists to BoNT, and quantify BoNT preparations. BoNT detection can be monitored in real-time using a standard fluorescent microplate reader with appropriate excitation and emission filters. Alternatively, BoTest™ can be used as an end-point assay for maximum sensitivity. BoTest™ can detect picomolar concentrations of BoNT within a few minutes to a few hours depending on the BoNT sero-type tested.

The BoTest™ reporters are modeled after the naturally occurring substrates of proteolytic BoNT and detect the endopeptidase activity of BoNT sero-types A and E (BoTest™ A/E) or sero-types B, D, F, and G (BoTest™ B/D/F/G). The BoTest™ reporters contain amino acids 141-206 of SNAP-25 (BoTest™ A/E) or 33-94 of synaptobrevin (BoTest™ B/D/F/G), encompassing both the exosite binding sites and the cleavage site of BoNT. These reporters have a high affinity for BoNTs and closely resemble the natural substrates of BoNT, resulting in a sensitive, quick assay for BoNT detection.

2.0 DESCRIPTION

2.1 Materials Supplied

BoTest™ A/E Botulinum Neurotoxin Detection Kit

Description	Composition	A1004		A1005	
		Size	Part #	Size	Part #
BoTest™ A/E Reporter	20 μM in 50 mM Hepes-NaOH, 10 mM NaCl, 15% Glycerol	250 μl	A1001	10 x 250 μl	A1001
10x BoTest™ Reaction Buffer	500 mM Hepes-NaOH, pH 7.1, 50 mM NaCl, 1% Tween-20, 100 μM $ZnCl_2$	2 x 1.25 ml	A1002	25 ml	A1003

BoTest™ B/D/F/G Botulinum Neurotoxin Detection Kit

Description	Composition	A1009		A1010	
		Size	Part #	Size	Part #
BoTest™ B/D/F/G Reporter	20 μM in 50 mM Hepes-NaOH, 10 mM NaCl, 15% Glycerol	250 μl	A1008	10 x 250 μl	A1008
10x BoTest™ Reaction Buffer	500 mM Hepes-NaOH, pH 7.1, 50 mM NaCl, 1% Tween-20, 100 μM $ZnCl_2$	2 x 1.25 ml	A1002	25 ml	A1003

2.2 Additional Required Materials

- 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 ^{0}C or 37 ^{0}C (optional)
- BoNT/A or E (BoTest™ A/E) or BoNT/B, D, F, or G (BoTest™ B/D/F/G)

3.0 STORAGE

Description	Storage Temp.	Notes
BoTest™ Reporters	-80 ^{0}C	Upon thawing, aliquot into single use amounts to avoid repeated freeze-thaw cycles. Stable for a minimum of five days at 4 ^{0}C upon thawing.
10x BoTest™ Reaction Buffer	-20 or -80 ^{0}C	Stable for a minimum of five days at 4 ^{0}C upon thawing.

4.0 SAFETY PRECAUTIONS

All reagents in this kit are considered non-hazardous according to 29 CFR 1910.1200. Normal precautions exercised in handling laboratory reagents should be followed.

5.0 GENERAL ASSAY CONSIDERATIONS

5.1 Required instrumentation and equipment

The 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 assay should be performed with microplates designed for fluorescence-based assays such as 96-well black-well plates. Transparent plates should not be used. White-well plates can be used but are not recommended for assays were high accuracy is required. Covered plates are required for long incubation periods.

Although room-temperature incubations are adequate for many applications, an incubator set to 30 ^{0}C or 37 ^{0}C may be required for long incubation periods or for plate readers that do not have on-board heating. For long incubation periods (>4 hours), it is recommended that the plate be covered. Evaporation of sample is minimized by using a microplate incubator with top and bottom heating elements.

5.2. Buffer considerations

BoNTs are metalloproteases that require the presence of Zn^{2+} for activity. Sample buffers that contain metal chelators such as EDTA or EGTA should be avoided. In addition, BoNT can be sensitive to moderate to high salt concentrations. It is recommended that the final salt concentration not exceed 20 mM. BoNT/A endopeptidase activity has been reported to be sensitive to sodium phosphate buffers and high concentrations (>50 mM) of other common buffers such Hepes and Tris[1].

Addition of fresh 5 mM dithiothreitol (DTT) to all buffers is recommended for use of the assay with holotoxin preparations of BoNT. Reduction of holotoxin with DTT is required for maximum proteolytic activity. For kinetic experiments, holotoxin preparations should be reduced prior to initiating the assay.

Use of 0.1% (w/v) BSA can stabilize some BoNT preparations and is recommend for use with light-chain preparations of BoNT/A. Other BoNT preparations should be tested on a case-by-case basis.

Concentrations of albumins above >1% (w/v) inhibit BoNT/A activity (BioSentinel Pharma LLC, unpublished results) and should be avoided.

Solvents such as ethanol, methanol, and DMSO affect BoNT activity and will cause changes in the fluorescence properties of the reporter. When using these solvents, a constant solvent concentration should be maintained in all samples including controls. It is recommended that the concentration of these solvents not exceed 10% (v/v).

6.0 ADDITIONAL INFORMATION

An applications guide for the BoTest™ BoNT Detection Kits can be found at www.biosentinelpharma.com.

7.0 BASIC ASSAY PROTOCOL

1. Thaw the BoTest™ Reporter and the 10x BoTest™ Reaction Buffer at room temperature.
    a. Allow the 10X BoTest™ Reaction Buffer to warm completely to room temperature.
    b. If the reaction buffer appears cloudy, warm to 37 ^{0}C for 5 minutes.

2. Place the BoTest™ A/E Reporter on ice.

3. Calculate the number of wells required.
    a. Include two control wells that do not include BoNT.
    b. For quantitative assays, it is recommended that a dilution series of known quantities of BoNT be prepared. A range of 0.1 pM to 1 nM BoNT is recommended.

4. Based on the number of wells to be run, prepare a **Master Stock** containing 1.25 µl BoTest™ reporter and 3.75µl **1X** BoTest™ Reaction Buffer per well.
    a. 1X BoTest™ Reaction Buffer can be prepared by diluting the 10X BoTest™ Reaction Buffer in high-quality (i.e. nanopure) H_2O. Add 1 part 10x BoTest™ Reaction Buffer to 9 parts H_2O.
    b. The total volume of the Master Stock can be increase 10-25% to account for pipetting error and loss during dispensing.

5. Dispense 85.5 µl of unknowns, known dilution-series, or control samples into each well (see **Table 1**).
    a. For accurate comparison of unknown, knowns, or controls, all samples should be in the same starting buffer or H_2O.

6. Add 9.5 µl of 10x BoTest™ Reaction buffer to each well.

7. Add 5 µl of BoTest™ Reporter Master Stock to each well and begin timing the reaction.

8. Incubate plate(s) for 0 - 24 hours at desired temperature.
    a. Incubation conditions will depend on the desired application. (See **6.0 Additional Information.)**

9. Measure the florescence at ~470 and ~526 nm using 434 nm excitation.

10. 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**).

Table 1: Distribution of sample and reagents.

sample name	volume of sample or control	volume of protease-free H_20	10x BoTest™ Reaction Buffer	BoTest™ Reporter Master Stock	Total Volume
Negative Control	----	85.5 µl	9.5 µl	5 µl	100 µl
Unknown or standard	0-85.5 µl	To 85.5 µl	9.5 µl	5 µl	100 µl

8.0 EXAMPLE DATA

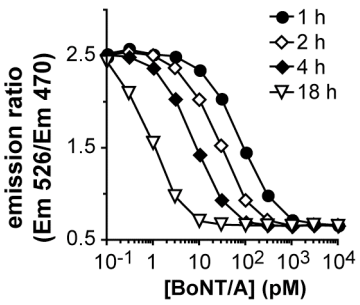


Figure 1. Sensitivity of BoTest™ A/E to BoNT/A holotoxin at varying incubation times. BoTest™ reporter was incubated at room temperature with varying concentrations of BoNT/A holotoxin in 100 µl of 1X BoTest™ Reaction Buffer. After one, two, four and 18 hours, the reporter was excited at 434 nm and the 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).

Reference:

1. Ekong et al (1997) *Microbiology*, **143:** 3337-47.

Attachment 8.2. Figures, Legends, and Tables.

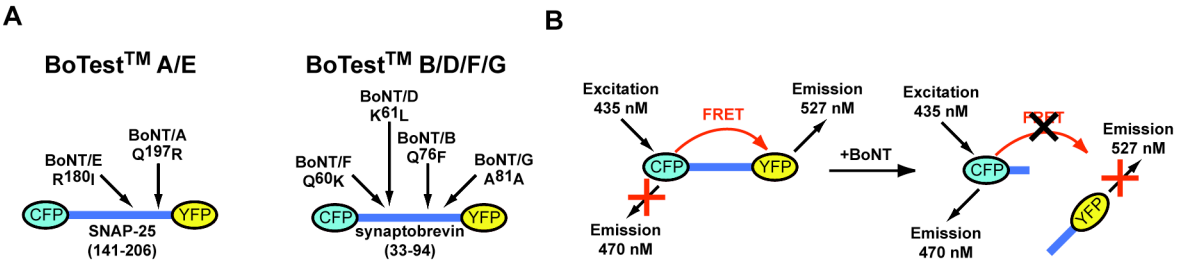


Figure 1. BioSentinel's FRET-based BoTest™ reporters. (**A**) BioSentinel's *in vitro* reporters. CFP and YFP are connected by SNAP-25 (amino acids 141–206, BoTest™ A/E) or by synaptobrevin (amino acids 33–94, BoTest™ B/D/F/G). The cleavage sites for each BoNT serotype are indicated. (**B**). A FRET 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.

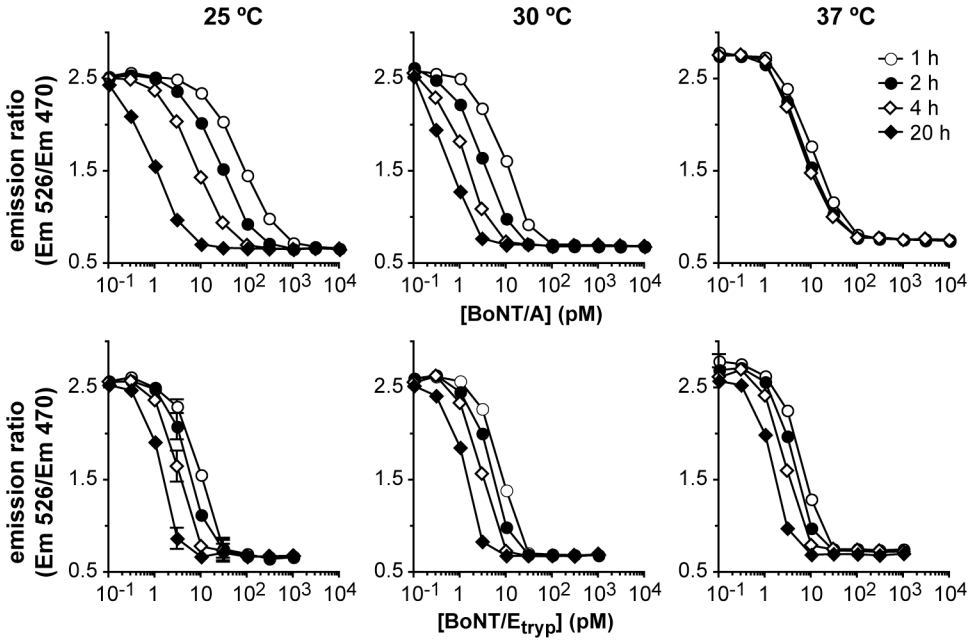


Figure 2. Incubation time and temperature dependency of the BoTest™ A/E BoNT detection assay. The BoTest™ A/E reporter was incubated at the indicated temperature with varying concentrations of BoNT/A holotoxin or trypsin-treated BoNT/E (BoNT/E_{tryp}) holotoxin. After 1, 2, 4 and 20 h, the reporter was excited at 434 nm, and the emission was collected at 470 and 526 nm. The emission ratio (526/470) was plotted as a function of BoNT/A (upper panels) or BoNT/E_{tryp} (lower panels) concentration.

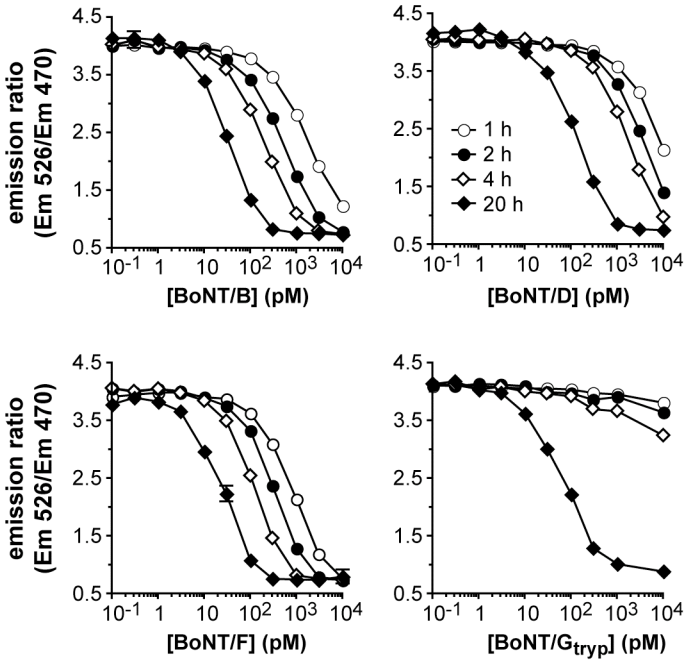
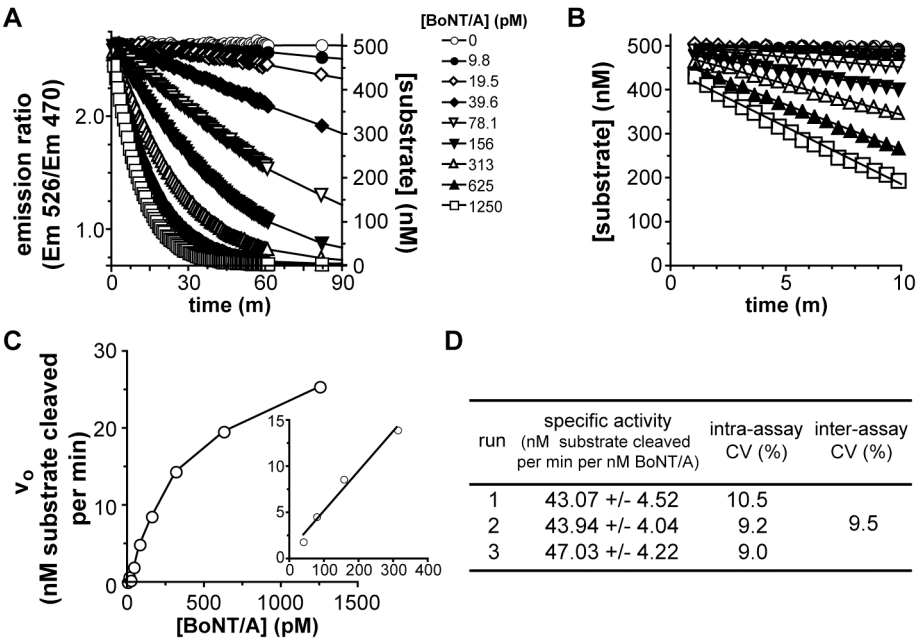
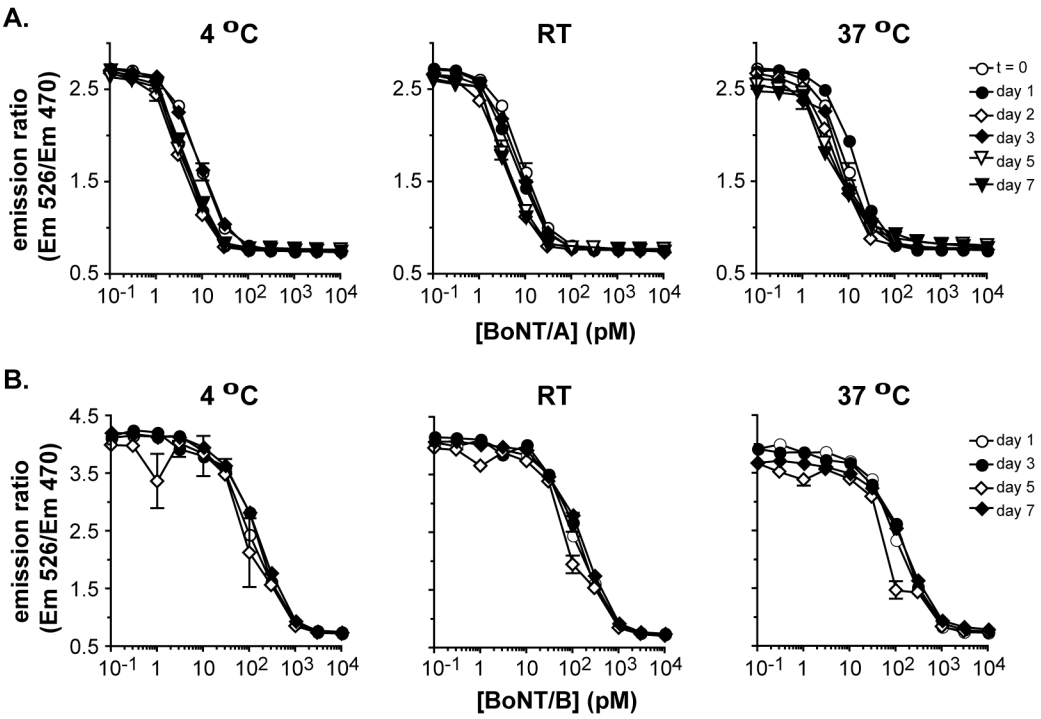


Figure 3. Incubation time dependency of the BoTest™ B/D/F/G BoNT detection assay. The BoTest™ B/D/F/G reporter was incubated with varying concentrations of BoNT/B, trypsin-treated BoNT/G (BoNT/G_{tryp}), BoNT/D, or BoNT/F holotoxin. After 1, 2, 4, and 20 h, the reporter was excited at 434 nm, and the emission was collected at 470 and 526 nm. The emission ratio (526/470) was plotted as a function of BoNT concentration.



run	specific activity (nM substrate cleaved per min per nM BoNT/A)	intra-assay CV (%)	inter-assay CV (%)
1	43.07 +/- 4.52	10.5	
2	43.94 +/- 4.04	9.2	9.5
3	47.03 +/- 4.22	9.0	

Figure 4. Assessment of assay precision and enzymatic activity using BoTest™ A/E. 500 nM BoTest™ A/E reporter was incubated at 25 ^{o}C with varying concentrations of BoNT/A holotoxin. At 30-s intervals for 60 min, and at 15-min intervals thereafter, the reporter was excited at 434 nm, and the emission was collected at 470 and 526 nm. (**A**) Kinetics of the BoTest™ A/E Reporter cleavage by BoNT/A. (Left y-axis) The emission ratios (526/470) for a given BoNT/A concentration were plotted as a function of time. (Right y-axis) The emission ratios were normalized and then converted to substrate concentration by multiplying the normalized values by 500 nM (the initial concentration of BoTest™ reporter). (**B**) Early kinetics of substrate disappearance. Disappearance of the BoTest™ A/E reporter at 1-10 min was plotted as a function of time and fitted by linear regression. (**C**) Initial velocity as a function of BoNT/A concentration. The negative of the slopes from the linear regression fits in panel (B) were plotted as a function of BoNT/A concentration. The linear velocity range of BoNT/A concentrations is shown in the inset and was fitted by linear regression. The slope of the fit gives specific activity of the BoNT/A preparation in nM substrate cleaved per m per pM BoNT/A. (**D**) Assay precision of the BoTest™ A/E assay. 3 independent determinations (runs) were made of a single preparation of BoNT/A. The intra-assay coefficients of variation (CV) were calculated by dividing the standard deviation of a single run by the average of the run. The inter-assay CV were calculated by dividing the standard deviation of the 3 runs by the average of the 3 runs, respectively.

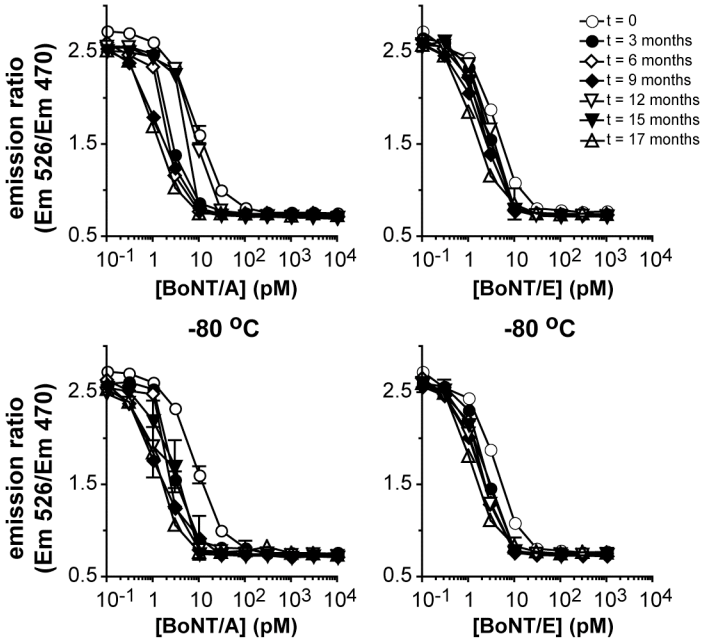


Day	EC_{50} (pM)		
	4 °C	RT	37 °C
0		8.0	
1	4.1	5.6	13.2
2	3.5	3.6	5.4
3	8.1	7.0	7.2
5	4.1	4.9	3.9
7	4.5	3.6	4.8

Day	EC_{50} (pM)		
	4 °C	RT	37 °C
1	109	116	108
3	140	125	132
5	103	87	63
7	148	150	146

Figure 5. Accelerated long-term stability study of the BoTest™ reporters. Aliquots of the BoTest™ reporters were stored at the indicated temperatures. After 1 - 7 days, reporter aliquots from each storage temperature were incubated at 37 °C with varying concentrations of BoNT/A (BoTest™ A/E) or BoNT/B (BoTest™ B/D/F/G) holotoxin. After 4 h, the reporter was excited at 434 nm, and the emission was collected at 470 and 526 nm. (**A**) Sensitivity of the BoTest™ A/E reporter to storage temperature. The emission ratio (526/470) was plotted as a function of BoNT/A concentration. The reporter storage temperature is indicated above each panel, and the days in storage before testing are indicated by symbol legend. Half maximal effective concentration (EC_{50}) values for cleavage of the BoTest™ reporters by BoNT/A at the indicated time and temperature are indicated in the table. For EC_{50} value calculations, the plotted data were fitted with the variable slope dose response equation $Y=Bottom + (Top-Bottom)/(1+10^{((LogEC50-x)*HillSlope)})$, where X is the logarithm of concentration and Y is the response. (**B**) Sensitivity of the BoTest™ B/D/F/G reporter to storage temperature. Data were plotted as a function BoNT/B concentration and EC_{50} values calculated as described in (A).

A.

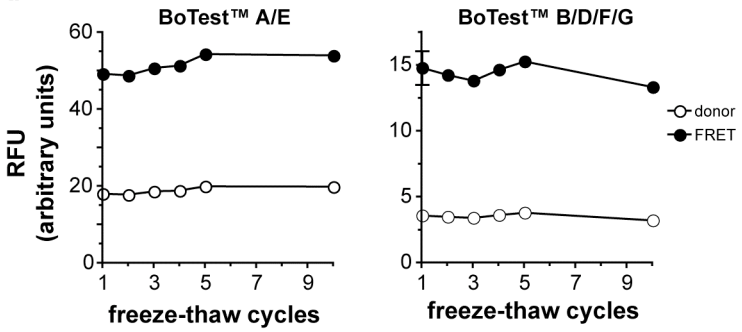


B.

storage time (months)	EC_{50} (pM), **-20 °C**		EC_{50} (pM), **-80 °C**	
	BoNT/A	BoNT/E	BoNT/A	BoNT/E
0	8.1	3.7	8.1	3.7
1	2.3	2.0	2.6	2.2
2	4.0	2.3	4.0	2.3
3	2.4	2.5	2.7	2.4
4	2.1	1.9	3.6	1.8
5	2.5	2.6	2.6	3.1
6	2.0	2.1	2.3	1.9
7	1.9	2.2	2.5	1.8
8	1.8	2.5	0.6	2.1
9	1.3	1.9	1.1	1.7
10	13.6	4.3	13.8	3.8
11	1.4	1.9	1.4	1.7
12	2.1	2.1	2.5	1.7
13	1.7	1.5	1.8	1.2
14	1.8	1.7	1.6	1.8
15	4.6	1.8	3.0	1.8
16	1.4	1.2	1.2	1.7
17	1.1	1.3	1.3	1.2

Figure 6. Long-term stability study of the BoTest™ A/E Reporter. Aliquots of the BoTest™ A/E reporter were stored at -20 and -80 ^{0}C. The storage containers and the concentration of reporter used were set according to manufacturing specification. After 1-17 months, samples were thawed at room temperature and assayed with varying concentrations of BoNT/A holotoxin or trypsin-treated BoNT/E holotoxin in 100 μl of 1X BoTest™ Reaction Buffer. 10X BoTest™ Reaction Buffer previously stored under normal storage specifications was used for the assay. After 4 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). (**A**) Sensitivity of the BoTest™ A/E Reporter to storage time and temperature. The emission ratio was plotted as a function of BoNT/A (left panels) or BoNT/E (right) 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). The reporter storage temperature is indicated above each panel and the months in storage before testing are indicated by symbol legend. (**B**) Half maximal effective concentration (EC_{50}) values for cleavage of the BoTest™ reporters by BoNT/A and E at varying time and temperature. For the EC_{50} values, the data in (A) were fitted with the variable slope dose response equation Y=Bottom + (Top-Bottom)/(1+10$^{((LogEC50-x)*Hill\ Slope)}$), where X is the logarithm of concentration and Y is the response.

A.



B.

freeze-thaw cycles	BoTest™ A/E EC_{50} (pM)		BoTest™ B/D/F/G EC_{50} (pM)	
	BoNT/A	BoNT/E_{tryp}	BoNT/B	BoNT/F
1	7.6	7.4	119	90.0
2	7.3	8.2	140	96.5
3	7.3	7.8	157	95.6
4	9.2	8.8	130	108
5	7.2	6.0	135	106
10	7.1	6.0	136	100

Figure 7. Resistance of the BoTest™ reporters to freeze-thaw cycling. For each freeze-thaw cycle, tubes of BoTest™ reporter (20 μM, 105 μl per tube) were thawed at RT for 10-15 min, mixed gently, and placed at -80 °C for 20 min. Complete thawing and freezing was confirmed by visual inspection of tubes. (**A**) Effect of freeze-thaw cycling on donor and FRET emission. BoTest™ A/E (left) or B/D/F/G (right) reporter samples were subjected to the indicated number of freeze-thaw cycles. Samples were then diluted to 1 μM in 100 μl of 50 mM HEPES (pH 7.1), 10 μM $ZnCl_2$, 5 mM NaCl, 5 mM DTT, and 0.1% Tween-20 in a well of a 96-well plate. Each well was excited at 434 nm, and the emission was collected at 470 (donor) and 526 (FRET) nm. Relative fluorescent units (RFU) were plotted as a function of freeze-thaw cycles. (**B**) Effect of freeze-thaw cycling on BoTest™ reporter sensitivity to BoNT. BoTest™ reporter samples were subjected to freeze-thaw cycling and diluted to 1 μM as described in panel A except dilutions were made in the presence of the indicated concentration of each BoNT serotype. After 4 h incubation at 37 °C, donor and FRET fluorescence emission was collected as described in panel A. The emission ratio (526/470) and the EC_{50} values were, for each of the indicated toxins, as described in Figure 6.

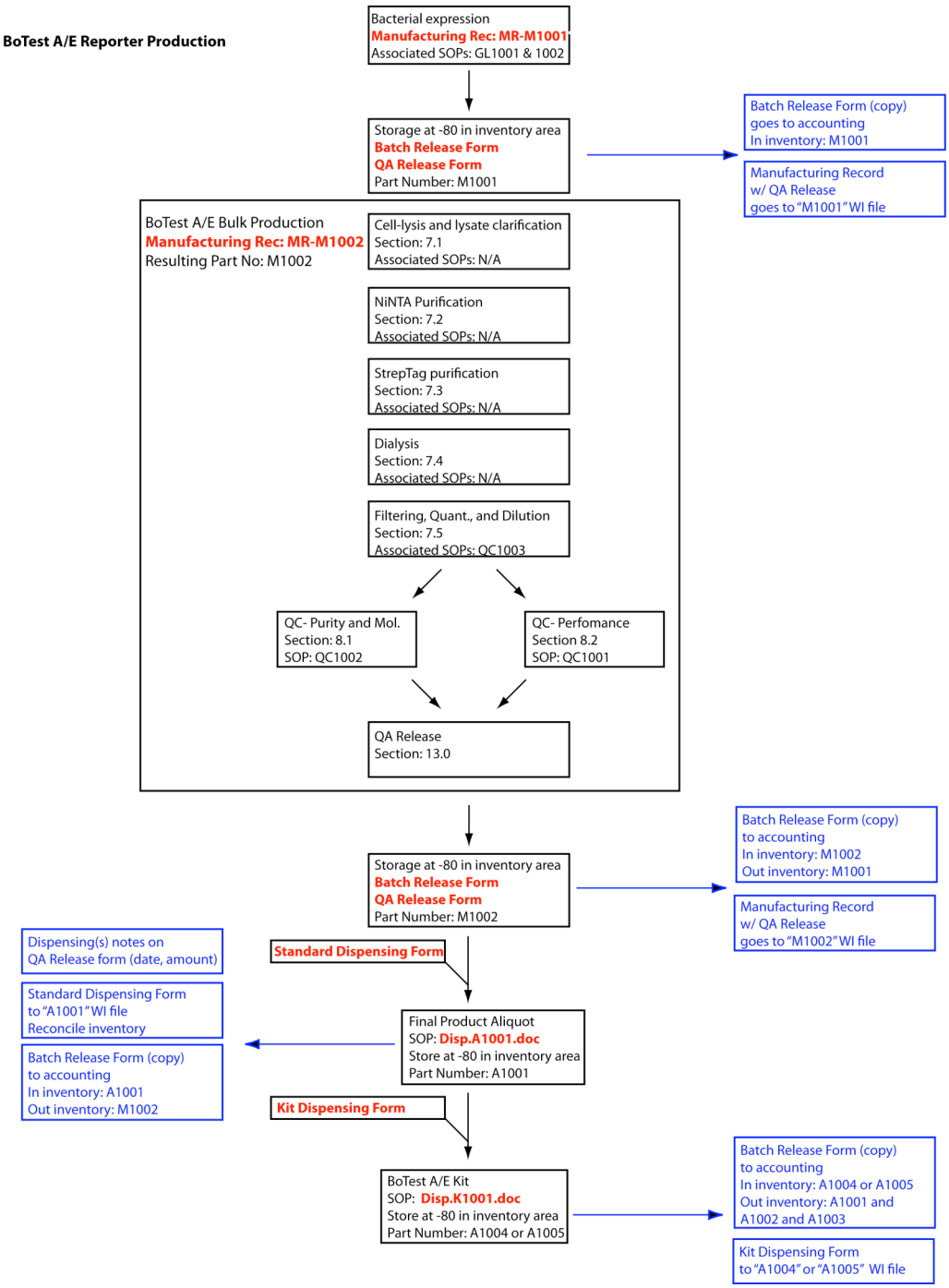


Figure 8. Manufacturing process flow for the BoTest™ A/E reporter. The key step or resulting product is indicated at the top of each box followed by the manufacturing record, associated SOPs, quality assurance release forms, and/or resulting product part number. The documents highlighted in red indicate 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.

Table 1. Limits of detection for the BoTest™ reporters. (Values in pM BoNT, ND = not determined)

		BoTest™ A/E			**BoTest™ B/D/F/G**				
temp.	time (h)	BoNT/A lot 1	BoNT/A lot 2	BoNT/E_{tryp}	BoNT/B	BoNT/B_{tryp}	BoNT/D	BoNT/F	BoNT/G_{tryp}
25 ^{O}C	1	10	30	3	1000	100	1000	100	>10000
	2	3	10	3	100	100	1000	100	10000
	4	3	3	1	100	30	300	30	1000
	20	0.3	0.3	1	10	3	100	3	30
30 ^{O}C	1	3	10	3	100	100	1000	100	>10000
	2	1	10	3	30	10	300	100	300
	4	0.3	3	1	30	10	300	30	300
	20	0.3	0.3	0.3	10	3	100	3	30
37 ^{O}C	1	3	10	3	100	30	1000	100	>10000
	2	3	10	3	30	10	300	30	300
	4	3	10	1	30	10	300	30	300
	20	ND	3	1	10	3	30	10	30