505 S. Rosa Road, Suite 105
Madison, WI 53719
1-608-441-8174
Email: info@biosentinelpharma.com

BioSentinel Part No: L1002, Release Date: May 8, 2014

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 where 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. Gently mix and place the BoTest Reporter on ice.
    a. After thawing and mixing, material recovery can be maximized by briefly (~3 seconds) centrifuging the BoTest Reporter vial in a microcentrifuge set to the highest speed.

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 **Reporter 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 Reporter Master Stock can be increased 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

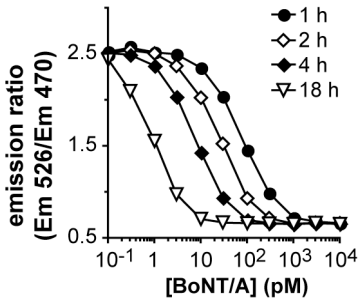

Figure 1. Sensitivity of BoTest® A/E to BoNT/A holotoxin at varying incubation times. BoTest **A/E** reporter was incubated at room temperature with varying concentrations of BoNT/A holotoxin in 100 µl of 1X BoTest Reaction Buffer. After one, two, four and 18 hours, the reporter was excited at 434 nm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