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Applications Guide for BoTest™ A/E Botulinum Neurotoxin Detection Kit

Relevant products:

BoTest™ A/E Botulinum Neurotoxin Detection Kit	200 assays	Cat. A1004
	2000 assays	Cat. A1005

BoTest™ A/E Botulinum Neurotoxin Detection Kit- Detailed Description

BoTest™ A/E Botulinum Neurotoxin Detection Kit (BoTest™) will detect the proteolytic activity of botulinum neurotoxin (BoNT) sero-types A and E, both holotoxin and light-chain preparations. The degree of response and sensitivity of the assay will be dependent on the preparations of BoNT used, the incubation conditions, and the buffer conditions. The data shown below are provided to illustrate the utility of the assay and to help end-users optimize their experiments.

Assay description and performance

BoTest™ contains a reporter that consists of residues 141-206 of SNAP-25, the naturally occurring substrate of BoNT/A and E. The substrate is sandwiched between two fluorescent proteins, a cyan fluorescent protein (CFP) derivative, and a yellow fluorescent protein (YFP) derivative. The CFP and YFP moieties form a donor-acceptor Förster Resonance Energy Transfer (FRET) pair.

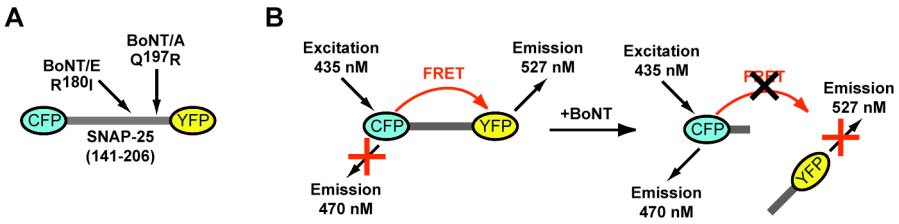


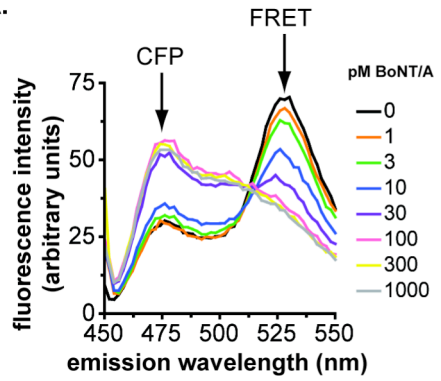
Figure 1. BoTest™ A/E Reporter. (**A**) BioSentinel's BoTest™ A/E Reporter. CFP and YFP are connected by SNAP-25 (amino acids 141-206). The cleavage sites for each BoNT sero-type are indicated. (**B**) A FRET assay for measuring BoNT protease activity. If CFP and YFP are in close proximity to each other, excitation of CFP leads to emission from YFP via FRET. Emission from CFP is attenuated. If the SNAP-25 linker between CFP and YFP is cleaved by BoNT, the CFP and YFP are no longer in close proximity. FRET is lost, corresponding to a decrease in YFP emission and an increase in CFP emission.

The use of a FRET donor-acceptor pair separated by the substrate for BoNT allows for the real-time detection of BoNT proteolytic activity (**Fig. 1**). In the absence of BoNT, the reporter is intact and the CFP and YFP moieties are in close proximity. Excitation of CFP results in energy transfer to YFP due to FRET. As a consequence, CFP emission is quenched while YFP emits fluorescence due to FRET. In the presence of BoNT, the reporter is cleaved by the proteolytic activity of BoNT. The CFP and YFP moieties are physically separated and FRET can no longer occur. CFP emission is restored and YFP emission is reduced.

Figure 2 illustrates how the BoTest™ reporter's fluorescence spectra respond to increasing concentrations of BoNT. In the absence of BoNT, the YFP emission is strong due to energy

transfer from CFP and CFP emission is reduced. Incubation of the reporter with varying concentrations of BoNT results in cleavage of the reporter and the physical separation of the CFP and YFP moieties. The emission of the YFP due to FRET falls and the CFP emission increases. Changes in fluorescence are dose-dependent, thus allowing for the quantification of BoNT preps when a suitable activity standard is used.

A.



B.

[BoNT]	RFU FRET	RFU CFP	emission ratio
0	70.0	26.5	2.64
1	66.1	27.1	2.44
3	63.0	28.5	2.21
10	53.5	31.1	1.72
30	43.4	46.6	0.93
100	36.9	50.4	0.73
300	35.0	49.1	0.71
1000	33.9	49.3	0.69

Figure 2. Fluorescence response of BoTest™ A/E to BoNT/A. The BoTest™ reporter was incubated with varying concentrations of BoNT/A holotoxin in 100 µl of 1X BoTest™ Reaction Buffer. After four hours, the reporter was excited at 434 nm and the emission spectrum was collected from 450 to 550 nm on a Thermo-Fisher Varioskan plate reader. **(A)** Fluorescence emission spectra of the reporter after a four-hour incubation period with the indicated concentrations of BoNT/A. The emission peaks for CFP (470 nm) and FRET (526 nm) are indicated. **(B)** Fluorescence intensity and emission ratio responses of the reporter to BoNT/A cleavage. The fluorescence intensity in relative fluorescence units (RFU) was measured for the FRET fluorescence (526 nm) and for the CFP fluorescence (470 nm) at the indicated concentrations of BoNT/A holotoxin. The emission ratio was calculated by dividing the RFU value of the FRET fluorescence by RFU value of the CFP fluorescence.

Determining the fluorescence spectra of the reporter is not required for the assay and is not recommended in most applications. Instead, the fluorescence emission of CFP (470 nm) and YFP (526 nm) are measured during excitation of CFP (434 nm). The data is then converted to an emission ratio by dividing the relative fluorescence at 526 nm by the relative fluorescence at 470 nm (**Fig. 2**). Typically, the emission ratio is greater than 2.0 in the absence of BoNT and less than 1.0 in the presence of saturating concentrations of BoNT although the actual ratio is dependent on the plate reader used and the buffer conditions.

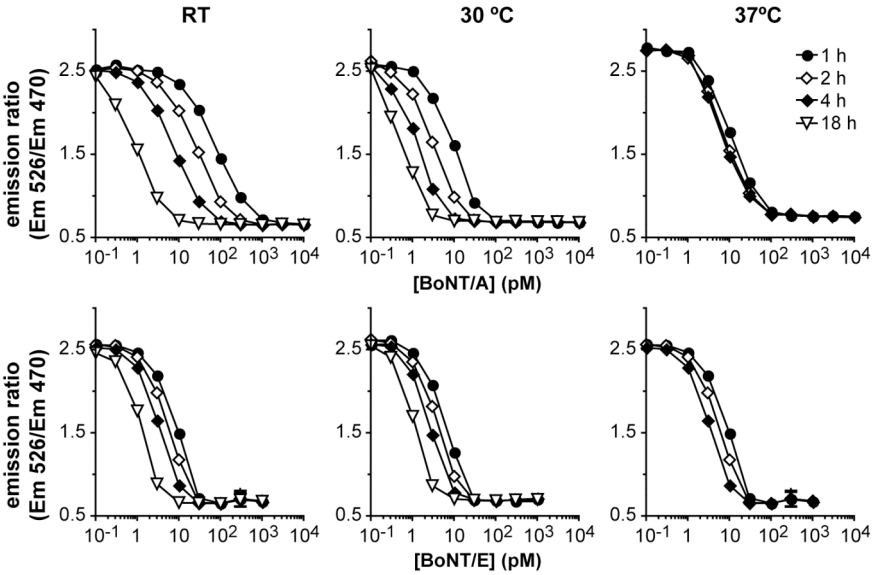
Assay sensitivity, incubation conditions, and BoNT preparations.

The sensitivity of the assay will depend on a number of factors including incubation time, incubation temperature, and the preparation of BoNT used. BoNT/A and BoNT/E activity has been reported to be maximum at 37 ^{0}C (ref. 1) but is more stable over long incubation temperatures at lower temperatures. Thus if maximum assay sensitivity is desired, incubation temperatures of 20-30 ^{0}C and incubation periods of greater than two hours are recommended. If kinetic information is required, shorter incubation periods are recommended (see Application Note 2.0).

Figure 3 illustrates the relationship between assay sensitivity, incubation time, and incubation temperature. At 37 ^{0}C, the reaction is largely complete after one hour with a minimal increase in sensitivity for either BoNT/A or E with increased incubation times. This lack of increased

sensitivity likely reflects the low thermal stability of BoNT/A and BoNT/E at 37 ^{0}C under the buffer conditions used. Lowering the temperature to 30 ^{0}C or room-temperature (RT, ~23 ^{0}C), results in no increase in assay sensitivity after one hour. The increased thermal stability of the toxins at 30 ^{0}C or RT, however, allows the assay to be run longer, increasing the sensitivity over incubation periods longer than one hour. Even after incubation periods of 18 hours, BoNT/A and E remain active and femtomolar amounts of BoNT can be detected.

A.



B.

temp.	time (h)	EC_{50} (pM)		LOD (pM)	
		BoNT/A	BoNT/E	BoNT/A	BoNT/E
RT	1	76	8.2	<10	<3
	2	25	5.1	<3	<1
	4	7.4	3.2	<1	<1
	18	0.88	1.2	<0.3	<1
30 ^{0}C	1	9.0	5.6	<3	<1
	2	2.9	3.6	<0.3	<1
	4	1.7	2.4	<0.3	<1
	18	0.4	1.1	<0.3	<0.3
37 ^{0}C	1	9.9	4.6	<3	<1
	2	7.1	3.1	<3	<1
	4	6.3	2.3	<3	<1
	18	8.5	2.2	ND	ND

Figure 3. Incubation time and temperature dependency of the BoTest™ A/E BoNT Detection assay. The BoTest™ reporter was incubated at the indicated temperature with varying concentrations of BoNT/A holotoxin or trypsin-treated BoNT/E 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). (**A**) Sensitivity of the BoTest™ Assay to incubation time and temperature. The emission ratio was plotted as a function of BoNT/A (upper panels) or BoNT/E (lower) 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). (**B**) Half maximal effective concentration (EC_{50}) and limits of detection (LOD) 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 panel 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. For the LOD values, the minimum concentration of BoNT that is three standard deviations from control values in the absence of BoNT (n=6) is listed.

The sensitivity of the BoTest™ A/E Botulinum Neurotoxin assay will be influenced by the quality and source of BoNT preparation used. **Figure 4** shows two different recombinant light-chain preparations from different manufacturers. The data shows that one preparation was nearly ten-times as active as another preparation. These data also illustrated the need for

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suitable standards when quantifying the activity of preps of BoNT. Standards should ideally be prepared under identical buffer conditions as the unknown.

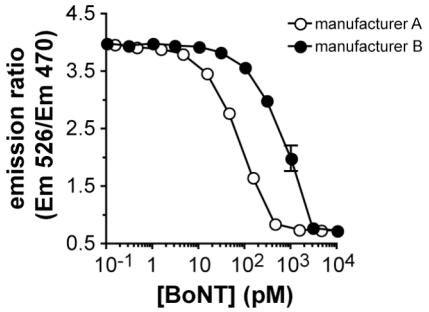

Figure 4. Sensitivity of BoTest™ A/E BoNT Detection assay to different preparations of BoNT/A light-chain. The BoTest™ reporter was incubated at 37 ^{0}C with varying concentrations of recombinant BoNT/A light-chain from two different manufacturers in 100 μl of 1X BoTest™ Reaction Buffer. After four 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 light-chain 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).

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Application Note 1.0: Converting changes in emission ratio to substrate concentrations.

BoTest™ can be used to determine the amount of substrate (BoTest™ reporter) remaining in a reaction. This information may be helpful for determining turnover rates, product inhibition/stimulation studies, or determining the specific activity of BoNT preparations.

Procedure:

1. Run BoTest™ as described in the "**BoTest™ A/E Botulinum Neurotoxin Detection Kit Customer Protocol."**
    a. Include samples that contain no BoNT.
    b. Include samples that contain saturating amounts of BoNT resulting in complete cleavage of the BoTest™ reporter. [The BoTest™ reporter is completely cleaved with 1 nM of BoNT/A holotoxin from Metabiologics (Madison, WI) after two hours incubation at room temperature.]
2. Determine the average maximum emission ratio value from the samples containing no BoNT.
3. Determine the average minimum emission ratio value from the samples containing saturating amounts of BoNT.
4. Normalize data by setting the average maximum emission ratio value to 1.0 and the average minimum emission ratio value to 0.0.
    a. This can often be done automatically using graphing software.
5. Multiply the normalized values by the starting substrate concentration. (i.e. 250 nM if using standard, recommended BoTest™ reaction conditions.)
6. A step-by-step example of how to determine substrate concentrations for a given experiment is shown in **Application Note 2.0**.

The ability to correlate changes in emission ratio to the amount of substrate left in solution has been confirmed experimentally (**Fig. 5**). The BoTest™ reporter substrate was incubated with varying concentrations of BoNT/A holotoxin before analyzing samples for both fluorescence emission and the presence of intact BoTest™ reporter (substrate) by electrophoresis. These data show that changes in emission ratio is tightly correlated with a loss of intact BoTest™ reporter. Thus, the concentration of substrate can be directly determined from the emission ratio as described in the above procedure. (Note that electrophoretic analysis of the BoTest™ assay is NOT required for determining remaining substrate concentration. These data are shown for confirmative and illustrative purposes.)

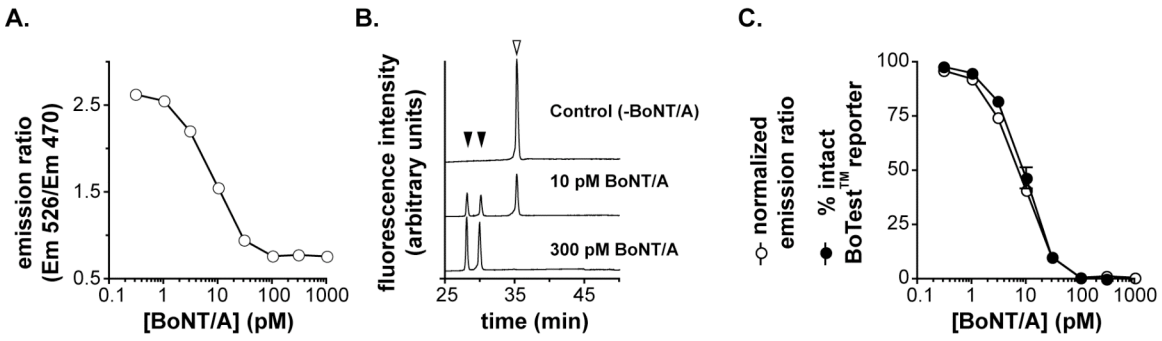

Figure 5. Changes in emission ratio correlate with the disappearance of intact BoTest™ reporter. The BoTest™ reporter was incubated at the room temperature with varying concentrations of BoNT/A holotoxin in 100 μl of 1X BoTest™ Reaction Buffer. After two hours, the reporter was excited at 434 nm and the emission was collected at 470 and 526 nm on a Varioskan plate reader (Thermo-Fisher). Samples were then immediately stopped in electrophoresis buffer and analyzed using an Experion automated electrophoresis station (Bio-Rad). (**A**) Sensitivity of the BoTest™ Assay to BoNT/A holotoxin. 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). (**B**) Electropherogram of the BoTest™ reporter at varying BoNT/A concentrations. Shown are samples treated with no, 10, or 300 pM BoNT/A. Intact reporter is indicated by the open arrowhead and cleavage products are indicated by the closed arrowheads. (**C**) Loss of the intact BoTest™ reporter is directly proportional to the change in normalized emission ratios. The data in (A) were normalized by setting the emission ratio of the reporter in the absence of BoNT to 100 and the emission ratio of the reporter in the presence of saturating amounts of BoNT (1 nM) to 0. The percent of intact BoTest™ reporter was determined by electrophoresis as shown in (B). Both the normalized emission ratio data and the electrophoretic data were plotted as a function of BoNT/A concentration.

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Application Note 2.0: Initial velocity and specific activity.

BoTest™ can be used to determine the initial velocity of a reaction. This information is essential for determining the specific activity of BoNT preparations, for determining overall enzyme kinetics, or for drug discovery.

Figure 6 illustrates the procedure for determining the specific activity of a preparation of BoNT/A holotoxin. BoTest™ reporter was incubated with varying concentrations of BoNT/A and the fluorescence emission was collected in kinetic mode over a 150-minute period. The data were normalized and converted to substrate concentrations as described in Application Note 1.0. Data between two time points were fitted by linear regression to determine the initial velocity at each BoNT concentration. Finally, initial velocity was plotted as a function of BoNT concentration. Specific activity is determined by fitting the data that shows a linear relationship between initial velocity and BoNT concentration (**Fig. 6, panel E, inset**). For the example shown here, the specific activity of this BoNT/A prep is 0.031 +/- 0.002 μmol substrate cleaved per min per mg BoNT/A.

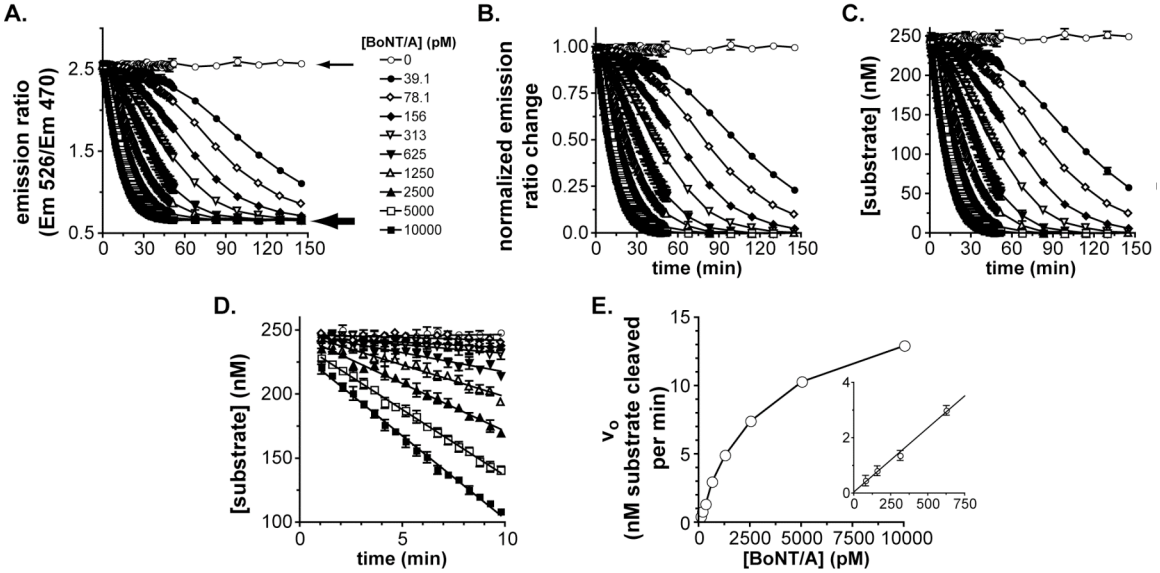

Figure 6. Assessment of enzymatic activity using BoTest™ A/E. 250 nM BoTest™ reporter was incubated at the room temperature with varying concentrations of BoNT/A holotoxin in 100 μl of 1X BoTest™ Reaction Buffer. At 30-sec intervals for 50 min and at 15 min intervals thereafter, the reporter was excited at 434 nm and the emission was collected at 470 and 526 nm on a Varioskan plate reader. (**A**) Kinetics of BoTest™ A/E Reporter cleavage by BoNT/A. The emission ratio was plotted as a function of time where the emission ratio is the RFU value of the FRET peak (526 nm) divided by the RFU value of the donor/CFP peak (470 nm). (**B**) Normalized kinetics of the BoTest™ A/E Reporter cleavage by BoNT/A. The data shown in panel (A) were normalized by determining the maximum emission ratio value in the absence of BoNT [small arrow, panel (A)] and setting that value to 1.0. The minimum emission ratio value in the presence of a saturating concentration of BoNT [large arrow, panel (A)] was set to a value of 0. (**C**) Kinetics of substrate disappearance. Intact BoTest™ A/E Reporter (substrate) concentration was plotted as a function of time. The data in panel (B) was transformed by multiplying the values in the y-axis by 250 nM (the initial concentration of BoTest™ reporter). (**D**) Early kinetics of substrate disappearance. Disappearance of the BoTest™ Reporter at 1-10 min was plotted as a function of time and fitted by linear regression. (**E**) Initial velocity as a function of BoNT/A concentration. The negative of the slopes from the linear regression fits in panel (D) were plotted as a function of BoNT/A concentration. The linear range of BoNT/A concentrations are shown in the inset, fitted by linear regression. The slope of the fit was 4.63 pM substrate cleaved min^{-1} pM^{-1} BoNT/A. Given a 100 μl reaction volume and a molecular weight of 150,000 Daltons for BoNT/A holotoxin, this gives a specific activity of 0.0309 μmol substrate cleaved min^{-1} mg^{-1} BoNT/A.

Note that the specific activity of a preparation of BoNT is specific for the reaction conditions used. Changes in substrate concentration, incubation temperature, or buffer conditions will result in different specific activities for a given BoNT preparation. In addition, multiple concentrations of BoNT must be tested to determine the range of BoNT concentrations where the relationship between BoNT concentration and initial velocity is linear.

Depletion of the substrate or product inhibition/stimulation can affect the linearity of the reaction. When determining initial velocity, use time points where minimal changes in substrate concentration have occurred. This may not be possible for all data points; however, care should be taken to ensure that sufficient data points are collected to ensure linearity. If data significantly deviates from linearity, earlier time points should be taken and/or data points should be taken more often.

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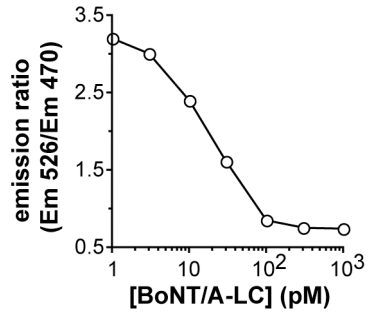
Application Note 3.0: Drug screening with BoTest™

The BoTest™ A/E BoNT detection assay can be used for high-throughput, primary screening of small molecule libraries for BoNT inhibitors. The assay has been tested in 96- and 384-well formats without a loss in assay sensitivity or assay robustness. BoTest™ is sensitive to picomolar amounts of BoNT, minimizing the per plate cost of BoNT and the overall biohazardous waste stream.

Initiation of small molecule inhibitor screens requires that assay robustness be tested using the buffer conditions, incubation conditions, and BoNT preparation to be used in the screen. **Figure 7** illustrates the use of Z'-factors to determine the suitability of the conditions for high-throughput applications and to determine the required amount of BoNT to maximize assay robustness. [Please see Zhang et al for a detailed explanation of Z-factor and how it relates to assay robustness (ref. 2).] As the data show, a minimum of 10 pM BoNT/A light-chain is required to obtain a Z'-factor of at least 0.5, the accepted lower Z' limit (ref. 2). Increasing the BoNT concentration above 10 pM will improve assay robustness & the Z'-factor value although the cost per well of BoNT will increase.

A.



B.

[BoNT]	Z'
0	NA
1	-0.45
3	0.40
10	**0.70**
30	0.85
100	0.93
300	0.95
1000	0.95

Figure 7. Z'-factor testing of BoTest™ A/E BoNT Detection Assay and BoNT/A light-chain. (**A**) The BoTest™ A/E reporter was incubated with varying concentration of BoNT/A light-chain (BoNT/A-LC) in 100 µl along with 1X BoTest™ Reaction Buffer supplemented with 0.1% BSA in a 96-well plate. After two hours at 37 ^{0}C, 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 light-chain 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). (**B**) Z'-factor determinations at varying BoNT/A light-chain concentrations. For each BoNT/A light-chain concentration, a Z' factor was calculated using the equation $Z' = 1 - [3x (\sigma_p + \sigma_n)] / |\mu_p - \mu_n|$, where σ is the standard deviation, μ is the mean, p is the positive control (emission ratio in the presence of the indicated BoNT/A light-chain concentration), and n is the negative control (emission ratio in the absence of BoNT/A light-chain).

Similar results were found when using 384-well plates using BoNT/A light-chain or BoNT/A holotoxin.

Figure 8 shows an example of a high-throughout, small molecule screen for BoNT inhibitors using the BoTest™ A/E BoNT Detection Assay, a 1536-well microtiter plate, and automated liquid handling. The position of "hits" are denoted by an *. The data show that BoTest™ A/E BoNT Detection Assay is adaptable to high-throughput, automated drug screening.

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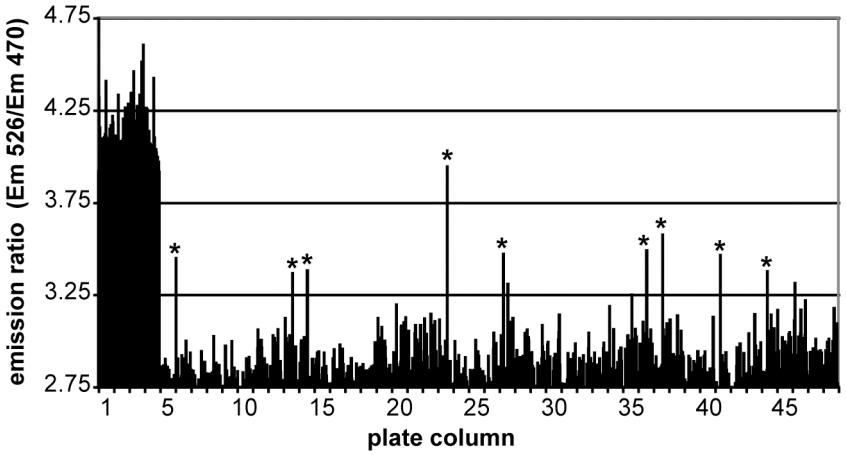

Figure 8. A high-throughput screen for BoNT/A antagonists using BoTest™ A/E BoNT Detection Assay. The BoTest™ A/E reporter was incubated in 10 μl in the presence or absence of 10 pM BoNT/A light-chain and in the presence or absence of 50 μM test compound from a commercially available small molecule library (10% final DMSO concentration). Each well also contained 1X BoTest™ Reaction Buffer supplemented with 0.1% BSA. Distribution and mixing of reagents was carried out using a Beckman-Coulter (Fullerton, CA) BioMek Laboratory Automation Workstation. After two hours incubation at 37 ^{0}C, each well was excited at 434 nm and fluorescence emission was collected at 470 nm and 526 nm using Tecan (Switzerland) Safire^{2} plate reader. The emission ratio was calculated by dividing the RFU value of the FRET peak (526 nm) by the RFU value of the donor/CFP peak (470 nm). Emission ratios were plotted a function of well position with wells grouped by column. Columns 1-4 contain the BoTest™ reporter but no BoNT/A light-chain or test compounds (negative control). Columns 45-48 contain the BoTest™ reporter and 10 pM BoNT/A light-chain but no test compounds (positive control). Columns 5-44 contain reporter, BoNT/A light-chain, and test compounds where each well contains a unique compound. Well with apparent BoNT/A inhibitors are marked by (*).

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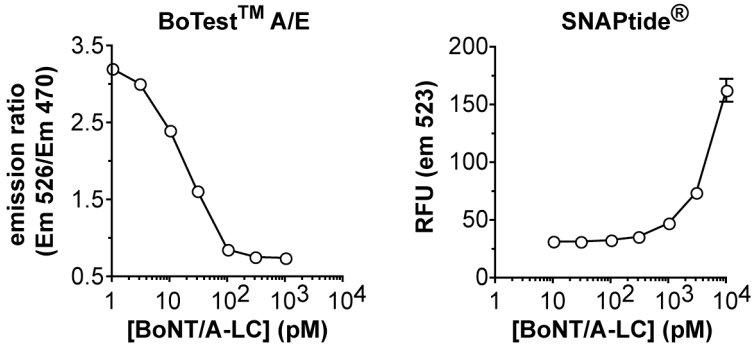
Application Note 4.0: Comparison of assay sensitivities- BoTest™ versus SNAPtide

An alternative to the BoTest™ A/E BoNT Detection Assay is SNAPtide® sold by List Biological Laboratories (Campbell, CA). The SNAPtide® reporter, like the BoTest™ reporter, is FRET-based reporter that has a donor-acceptor FRET pair separated by protein sequences that serves as the substrate for BoNT/A. However, the substrate contained in SNAPtide® is much shorter than that found in BoTest™. SNAPtide® is thus a lower affinity substrate for BoNT and requires higher concentrations of BoNT to be cleaved than does BoTest™.

The sensitivities of SNAPtide® and BoTest™ A/E have been compared side-by-side under identical conditions (**Fig. 9A**). Under the conditions tested, BoTest™ A/E responded to as little as 3 pM BoNT/A light-chain while SNAPtide® required at least 1 nM toxin to respond. Z' values where calculated at each BoNT/A light-chain concentration for both BoTest™ and SNAPtide® using assay responses in the absence of BoNT/A light-chain as the negative control (**Fig. 9B**). Z' values of >0.5 were found at 10 pM or greater toxin for BoTest™ A/E while toxin concentrations of 3 nM or greater were required for SNAPtide®. These data indicate that 300 times more BoNT is required for the SNAPtide assay than for BoTest™ A/E assay. The higher sensitivity of BoTest™ results in an assay that can be run with lower concentrations of BoNT, reducing overall assay costs.

A.



B.

[BoNT] (pM)	Z' BoTest™	Z' SNAPtide®	BoNT cost per plate ($)
1	-0.45	ND	0.01
3	0.40	ND	0.03
10	**0.70**	-22	**0.08**
30	0.85	-20	0.25
100	0.93	-4.6	0.84
300	0.95	-0.60	2.52
1000	0.95	0.48	8.40
3000	ND	**0.69**	**25.20**
10000	ND	0.75	84.00

Figure 9. Comparison of BoTest™ A/E BoNT Detection Kit and SNAPtide®. (**A**) The BoTest™ A/E or SNAPtide® reporter was incubated with varying concentration of BoNT/A light-chain (BoNT/A-LC) in 100 μl along with 1X BoTest™ Reaction Buffer supplemented with 0.1% BSA in a 96-well plate. After two hours at 37 ^{0}C, the BoTest™ reporter was excited at 434 nm and the emission was collected at 470 and 526 nm on a Varioskan plate reader. The emission ratio was plotted as a function of BoNT/A light-chain concentration where the emission ratio is the RFU value of the FRET peak (526 nm) divided by the RFU value of the donor/CFP peak (470 nm). The SNAPtide® reporter was excited at 490 nm and the fluorescence emission was collected at 523 nm. The RFU value was plotted as a function of BoNT/A-LC concentration. Averages and standard deviation are shown for 12 replicates. (**B**) Z'-factor determinations at varying BoNT/A light-chain concentrations. For each BoNT/A light-chain concentration, a Z' factor was calculated using the equation $Z' = 1 - [3x (\sigma_p + \sigma_n)] / |\mu_p - \mu_n|$, where σ is the standard deviation, μ is the mean, p is the positive control (emission ratio in the presence of the indicated BoNT/A light-chain concentration), and n is the negative control (emission ratio in the absence of BoNT/A light chain). BoNT cost per plate was based on a 96-well microtiter plate at 100 μl per well and the retail cost of a commercially available preparation of BoNT/A light-chain.

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References:

1. Washbourne, P.et al (1997) *FEBS Lett* **418**: 1.
2. Zhang et al (1999) *Journal of Biomolecular Screening*, **4:** 67.