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BoTest® Matrix E Botulinum Neurotoxin Detection Kit Protocol

This protocol is intended for users who have purchased the *combination* BoTest Matrix E BoNT Detection Kit (product # A1019). Users who purchased either the Matrix E (product # A1018) or the BoTest A/E (product # A1004) Kits as standalone solutions should refer to protocols specific for those kits.

BoTest Matrix E BoNT Detection Kit 200 assays Cat. A1019

1.0 INTRODUCTION

The BoTest Matrix E Botulinum Neurotoxin (BoNT) Detection Kit is an *in vitro* assay for the detection and quantification of BoNT serotype E (BoNT/E) in complex matrices such as serum, blood, food, water, bacterial supernatant, and pharmaceutical samples. The BoTest Matrix E Kit consists of two primary reagents- the Matrix E Beads and the BoTest A/E Reporter. The Matrix E Beads capture, concentrate, and isolate BoNT/E complex or holotoxin out of complex matrices from samples ranging in size from 100 µl to 50 ml. The BoTest A/E Reporter then detects the amount of captured BoNT/E providing real-time assessments of BoNT/E activity and quantity. Depending on the matrix composition and sample size, picomolar sensitivities can be achieved in < 4 hours.

The Matrix E Beads consist of a proprietary anti-BoNT/E antibody covalently conjugated to magnetic beads. These beads are added to a sample containing BoNT/E and incubated to allow BoNT/E binding. Interfering compounds that might otherwise impede BoNT/E activity determinations are removed by washing the Matrix E Beads. The BoTest A/E Reporter is modeled after the naturally occurring substrates of proteolytic BoNT and detects the endopeptidase activity of BoNT/E. 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/E. The reporter has a high affinity for BoNT/E and, when incubated with BoNT/E bound to the Matrix E Beads, rapidly and sensitively detects BoNT/E proteolytic activity.

2.0 DESCRIPTION

The BoTest Matrix E Kit is supplied as a combination of the Matrix E (product # A1018) 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	A1009	
		Size	Part #
BoTest A/E Reporter	20 µM in 50 mM Hepes-NaOH, pH 7.1,10 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 $ZnCl_2$	2 x 1.25 ml	A1002

Matrix E BoNT/E Immunoprecipitation Kit (A1018)

Description	Composition	A1022	
		Size	Part #
Matrix E Beads	Magnetic beads covalently conjugated to a mononclonal anti-BoNT/E antibody in PBS, 0.1% Tween-20, 0.05% Sodium Azide, 0.25% Casein, and 50% Glycerol	4 ml	A1017
10x Matrix Binding Buffer	500 mM Hepes-NaOH, pH 7.1, 250 mM NaCl, 1% Tween-20, 5% Casein, 0.05% Sodium Azide	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 96-well microtiter plates with covers
- Dithiothreitol (DTT)
- Microtiter plate mixer
- Orbital shaker at room temperature or 37 °C (preferred)
- BoNT/E (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_2O

3.0 STORAGE

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

4.0 SAFETY PRECAUTIONS

Normal precautions exercised in handling laboratory reagents should be followed.

5.0 GENERAL ASSAY CONSIDERATIONS

5.1 Required instrumentation and equipment

The Matrix E Kit uses antibody-conjugated magnetic beads (the Matrix E Beads) and requires a 96-well magnetic separation plate compatible with the microtiter plates being used. 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). Several other designs are available from various manufacturers that may provide satisfactory results although testing will be required.

Washing the Matrix E 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 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 then maintaining the suspension during all incubation steps. A microplate mixer can be used to thoroughly resuspend the Matrix Beads after pelleting. Bead suspensions can then be maintained using an orbital shaker or an incubating microtiter plate shaker during incubation periods.

The BoTest Matrix E 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 E 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 heating elements.

5.2 Buffer considerations

The BoTest Matrix E Kit comes with a 10x Matrix Binding Buffer that is added to samples in order 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 although exclusion may result in poor assay performance.

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

5.3 Matrix E Bead use consideration

Assay performance is critically dependent on executing washing and supernatant removal steps without accidental removal or loss of the beads. 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 completion of mixing and incubation periods as noted in 7.0 BASIC ASSAY PROTOCOL. This prevents the beads from settling to the bottom of the wells before the separation step and ensures complete bead sequestering to the sides of the wells, limiting accidental aspiration of beads from the well bottom during supernatant removal. When removing supernatants manually, insert the pipette tip in the center of the well and slowly remove the liquid.

Finally, the beads must remain in solution during the immunoprecipitation and BoTest A/E Reporter incubation steps to ensure optimal assay performance as noted in 7.0 BASIC ASSAY PROTOCOL.

6.0 ADDITIONAL INFORMATION

Additional information can be found at www.biosentinelpharma.com or by contacting info@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. 10x Matrix **Binding** Buffer will have a cloudy appearance.
    c. Vortex buffers for 5 seconds to mix.
    d. If the 10x BoTest **Reaction** Buffer appears cloudy, warm to 37 ºC for 5 minutes.
2. Thaw the BoTest A/E Reporter at room temperature.
    a. Mix gently upon thawing and store protected from light.
    b. Material recovery from the vial 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. At least one well is required for each unknown, control, and standard to be assayed.
    b. It is recommended that each sample (unknown, control, and standard) be assayed in duplicate or triplicate, increasing the number of wells used to 2 or 3 wells per sample.
    c. Include one or more control wells containing no BoNT/E, ideally using samples containing the same matrix or buffers as the unknown samples.
    d. For quantitative assays, it is recommended that a standard dilution series of known BoNT/E quantities be prepared. A range of 0.1 pM to 1 nM BoNT is recommended.
    e. The calculated number of wells will determine the volumes of buffers 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 of 1x Matrix Wash Buffer for every test well.
    a. Prepare 1x Matrix Wash Buffer by diluting 1 part 10x BoTest Matrix Wash Buffer with 9 parts H_2O and mixing well by inversion.
    b. Keep 1x Matrix Wash Buffer at room temperature until used.
5. Prepare 100 µl 1x BoTest Reaction Buffer with DTT per well.
    a. Prepare 1X BoTest Reaction Buffer by diluting 1 part 10x BoTest® Reaction Buffer with 9 parts high-quality H_2O.
    b. Supplement 1x BoTest Reaction Buffer with DTT to a final concentration of 5 mM. DTT is required to fully activate BoNT/E activity during detection.
6. Prepare 5 µl of BoTest A/E **Master Stock** per well
    a. Prepare BoTest A/E **Master Stock** by diluting 1.25 µl BoTest A/E Reporter with 3.75 µl 1x BoTest Reaction Buffer with DTT per well.
    b. Mix gently and store on ice protected from light.

Part 2. BoNT/E binding and isolation.

7. Warm the Matrix E Beads for 15 minutes at room temperature.
8. Prepare samples including unknowns, controls, and standards.
    a. Sample volumes of 100 to 225 µl per 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 have 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 see 6.0 ADDITIONAL INFORMATION.

9. Dispense the appropriate volume of unknowns, controls, and standards into wells.
10. Dispense a volume of 10x Matrix Binding Buffer to each well equal to one tenth ($1/10^{th}$) of the sample volume.
    a. Example: If 100 µl of the sample was dispensed into a well, add 10 µl of 10x Matrix Binding Buffer to the sample in the well.
11. Thoroughly resuspend the Matrix E Beads by vigorously 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. Visually verify that the beads are in a homogenous suspension, if needed, repeat vortexing.
12. **Immediately** dispense 20 µl Matrix E 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 the plate and incubate at 25 ºC or room temperature with shaking for 2 hours.
    a. The beads must be maintained in suspension for optimal assay performance. 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 200 µl 1x BoTest Matrix **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. Additional wash steps may be required for high viscosity samples or samples containing particulate matter.

Part 3. Detection of BoNT/E 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 37 ºC (preferred, for maximum assay sensitivity) for 2 – 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 37 ºC 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

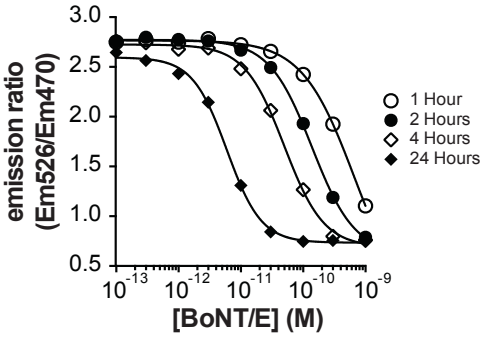

Figure 1. Picomolar BoNT/E detection with the BoTest® Matrix E BoNT Detection Kit. Samples containing the indicated BoNT/E concentration in PBS were immunoprecipitated with the Matrix E Beads according to protocol. BoTest A/E reporter was then added to the beads and incubated at 37 ºC. After 1 – 24 hours, 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/E 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).