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BoLISA® BoNT Sandwich ELISA Protocol

BoLISA® A BoNT/A Sandwich ELISA Detection Kit	100 assays	Cat. A1029
BoLISA® B BoNT/B Sandwich ELISA Detection Kit	100 assays	Cat. A1045
BoLISA® B4 BoNT/B4 Sandwich ELISA Detection Kit	100 assays	Cat. A1048
BoLISA® C BoNT/C Sandwich ELISA Detection Kit	100 assays	Cat. A1042
BoLISA® CD BoNT/CD Sandwich ELISA Detection Kit	100 assays	Cat. A1050
BoLISA® E BoNT/E Sandwich ELISA Detection Kit	100 assays	Cat. A1034

1.0 INTRODUCTION

The BoLISA® Botulinum Neurotoxin (BoNT) Detection Kits are used to detect and quantify BoNT mass from liquid matrices using a traditional sandwich ELISA approach. The BoLISA BoNT Detection Kits capture and quantify BoNT complex or holotoxin contained in 50 to 200 µl samples in a 96-well format with limits of detection of 300 fM or less, depending on sample composition and BoNT serotype. Each BoLISA BoNT Detection Kit is highly serotype specific and it is important to choose the correct kit for the serotype of interest (see **Fig. 1**). The BoLISA BoNT Detection Kits provide enough antibody-based reagents for 100 wells or a complete 96-well plate.

BoLISA BoNT ELISA Detection Kits consist of a serotype-specific anti-BoNT capture and a biotinylated anti-BoNT detection antibody. The capture antibody is first absorbed onto a 96-well ELISA plate followed by blocking and the addition of a BoNT-containing sample. Following washing, the biotinylated detection antibody is bound to the captured BoNT, providing a bridge for downstream detection with a user-defined streptavidin-conjugated detection reagent system.

2.0 DESCRIPTION

2.1 Materials Supplied

A1029 BoLISA A BoNT/A Sandwich ELISA Detection Kit:

Description	Composition	A1029	
		Size	Part #
BoLISA A Capture Antibody	0.5 µg/µl IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1027
BoLISA A Detection Antibody	0.5 µg/µl biotinylated IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1028

A1045 BoLISA B BoNT/B Sandwich ELISA Detection Kit:

Description	Composition	A1045	
		Size	Part #
BoLISA B Capture Antibody	0.5 µg/µl IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1043
BoLISA B Detection Antibody	0.5 µg/µl biotinylated IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1044

A1048 BoLISA B4 BoNT/B4 Sandwich ELISA Detection Kit:

Description	Composition	A1048	
		Size	Part #
BoLISA B Capture Antibody	0.5 µg/µl IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1046
BoLISA B Detection Antibody	0.5 µg/µl biotinylated IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1047

A1042 BoLISA C BoNT/C Sandwich ELISA Detection Kit:

Description	Composition	A1042	
		Size	Part #
BoLISA C Capture Antibody	0.5 µg/µl IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1040
BoLISA C Detection Antibody	0.5 µg/µl biotinylated IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1041

A1050 BoLISA CD BoNT/CD Sandwich ELISA Detection Kit:

Description	Composition	A1042	
		Size	Part #
BoLISA C Capture Antibody	0.5 µg/µl IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1040
BoLISA D Detection Antibody	0.5 µg/µl biotinylated IgY in PBS and 0.01% (w/v) thimerosal	40 µl	A1049

A1034 BoLISA E BoNT/E Sandwich ELISA Detection Kit:

Description	Composition	A1034	
		Size	Part #
BoLISA E Capture Antibody	0.5 µg/µl IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1032
BoLISA E Detection Antibody	0.5 µg/µl biotinylated IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1033

2.2 Additional Required/Optional Materials

- Clear or opaque flat-bottom 96-well ELISA microtiter plates [NUNC Maxisorp plates, Thermo Scientific cat. # 442404 (clear, for TMB detection), 436110 (white, for QuantaRed detection), or 437111 (black, for QuantaRed detection), recommended]
- Microplate covers (Thermo Scientific cat. # AB0752) or seals (Thermo Scientific cat. # 15036)
- BoNT (optional, required for control, standardization, and quantification purposes)
- ELISA plate washer (optional)
- Plate reader capable of reading absorbance at 450 nm (TMB), excitation at 570 nm and emission at 585 nm (QuantaRed), or other wavelengths specific to the detection method used
- High-quality (i.e. nanopure) H_2O

- Non-fat dry milk or other blocking reagent
- 10x phosphate-buffered saline (PBS), pH 7.4
- Tween 20
- Streptavidin poly-horseradish peroxidase (streptavidin poly-HRP; Thermo Scientific cat. # 21140, or similar, see **General Assay Considerations**)
- Poly-HRP dilution buffer (Thermo Scientific cat. # N500, or similar, see **General Assay Considerations**)
- Color development (see **General Assay Considerations**):
    - Option 1:
        - TMB (Thermo Scientific cat. # N301)
        - Sulfuric Acid, 95 – 99% (Fisher cat. # A300S or other high-quality reagent, diluted 1:100 in high-quality H_2O for use)
    - Option 2:
        - QuantaRed™ Enhanced Chemifluorescent HRP Substrate Kit (Thermo Scientific cat. # 15159
- 1 M HEPES, pH 7.2 – 8 (optional, see **General Assay Considerations**)
- NaCl (optional, see **General Assay Considerations**)

3.0 STORAGE

Description	Storage Temp.	Notes
Antibody	-80 ºC	Avoid repeated freeze/thaw, aliquots are stable for up to a week at 4 °C

4.0 SAFETY PRECAUTIONS

Normal precautions exercised in handling laboratory reagents and BoNT should be followed.

5.0 GENERAL ASSAY CONSIDERATIONS

5.1 Optional equipment

ELISA plate washing can be performed by hand using single- or multi-channel pipettes. However, reduced assay times and increased throughput can be achieved by using an automatic plate washer or vacuum apparatus. Refer to the instrument's user's manual for proper settings and washing protocols.

5.2. Buffer and sample considerations

The BoNT ELISA Detection Kits are not supplied with a sample binding or dilution buffer; however, assay performance is dependent on sample pH and salt concentration. The protocol below may need to be modified to account for sample pH, viscosity, non-specific binding, or other factors, depending on the nature of the BoNT-containing sample. Of critical importance is sample pH. The pH of the assayed samples must be between 6.5 and 8 for effective binding between the capture antibody and the core BoNT holotoxin. Sample pH can be adjusted either by diluting the sample into an appropriate buffer, e.g., PBS or HBS, or by adding 1/10 to 1/5 volume of 1 M HEPES at pH 7.2 – 8.0. It is also recommended that the sample NaCl concentration be at least 150 mM. Addition of 0.1% final concentration Tween 20 and 1% bovine serum albumin (BSA) or other blocking reagent to the samples is recommended to reduce non-specific binding to the plate and antibody. Centrifugation of samples containing particulate matter is also recommended to pellet insoluble material from the samples before addition to the plate. Additional sample treatments or washing steps may be required to optimize assay performance.

5.3 Detection chemistry and reagents

Several different streptavidin-conjugated detection reagents (e.g. HRP and alkaline phosphatase) and substrate chemistries (colorimetric, fluorescent, chemiluminescent) are available for use with biotinylated detection antibodies. The protocol below describes BioSentinel's optimized HRP/TMB system. BioSentinel has tested and demonstrated increased sensitivity in the BoLISA A BoNT/A Sandwich ELISA

using the QuantaRed Enhanced Chemifluorescence HRP Substrate Kit (see **Fig. 2**). The QuantaRed Kit uses fluorescence to detect HRP and can be read over time until the desired sensitivity is obtained with minimal risk of saturation, unlike TMB detection. Independent optimization to minimize background and maximize specific assay signal may be required if an alternate detection method is chosen. Refer to the instructions provided by the detection reagent supplier for details on how to use other detection methods.

6.0 ADDITIONAL INFORMATION

Additional information can be found at www.biosentinelpharma.com.

7.0 BASIC ASSAY PROTOCOL

The protocol below provides basic, optimized parameters for the assay. As described in 5.0 **GENERAL ASSAY CONSIDERATIONS**, complex samples may require additional treatments to account for particulate matter, non-neutral pH, or low ionic strength. These sample treatments should be incorporated and tested on a case-by-case basis. In addition, the protocol does not specify the composition of standards for quantitative assays.

Part 1. Coating the ELISA plate with capture antibody.

1. Calculate the number of wells required.
    a. At least one well is required for each sample (unknown, control, and standard) to be assayed.
    b. It is recommended that each sample (unknown, control, and standard) be assayed in duplicate or triplicate.
    c. Include one or more negative control samples containing no BoNT, ideally using the same matrix or buffer as the unknown samples.
    d. For quantitative assays, it is recommended that a standard dilution series of known BoNT quantities be prepared. A starting range of 300 pM to 100 fM BoNT is recommended.
    e. Add a 3 – 5% overage to the total well count to account for reagent loss during pipetting.
    f. For high precision applications, only the inner 60 wells of the ELISA plate should be used to avoid edge effects. Fill unused wells with 200 µl of room temperature water during all steps of the assay**.**
2. Prepare 10 ml of 1x PBS.
    a. Add 9 ml of high-quality water to a 15 ml conical tube.
    b. Add 1 ml 10x PBS.
    c. Mix solution well by inversion.
3. Dilute capture antibody to 4 µg/ml in 1x PBS.
    a. Calculate the total volume required by multiplying the number of sample wells (with overage) by 50 µl, e.g., 100 wells x 50 µl = 5,000 µl or 5 ml.
    b. Dilute the stock capture antibody 1:125 in the calculated volume of PBS, e.g., dilute 40 µl of stock capture antibody in 5 ml of PBS.
4. Coat 96-well plate wells with capture antibody.
    a. Add 50 µl of the diluted capture antibody to each sample well.
    b. Seal and incubate the plate at 4 °C overnight.

Part 2. Performing the ELISA Assay.

5. Make 50 ml of PBS-t (PBS with 0.1% Tween 20).
    a. Add 45 ml of high-quality water to a 50 ml conical tube.
    b. Add 5 ml of 10x PBS.
    c. Add 50 µl of Tween 20.
    d. Mix the solution well by inversion.
6. Make 30 ml of Blocking Buffer (5% nonfat dry milk in 1x PBS-t).
    a. Add 1.5 g of nonfat dry milk to a 50 ml conical tube.
    b. Bring the volume to 30 ml with PBS-t.

c. Mix the solution by inversion until the dried milk is completely dissolved.

7. Wash and block the capture antibody-coated wells.
    a. Wash the coated wells six times with 300 µl of PBS-t.
    b. Following removal of the last volume of wash buffer, gently tap the plate, inverted, three times onto a paper towel to remove residual liquid.
    c. Add 300 µl of Blocking Buffer to each coated well.
    d. Incubate the plate for 1 to 2 h at room temperature.

8. Add samples to the ELISA plate.
    a. Wash the coated wells 6 x 300 µl with PBS-t.
    b. Following removal of the last volume of wash buffer, gently tap the plate, inverted, three times onto a paper towel to remove residual liquid.
    c. Add 50 – 200 µl of sample to each test well.
        i. See 5.0 **General Assay Conditions** for pretreatment of samples prior to addition to the ELISA plate.
    d. Incubate the plate for 1 to 2 h at room temperature.

9. Add the biotinylated detection antibody to the ELISA plate.
    a. Make a 2 µg/ml dilution of biotinylated detection antibody in Blocking Buffer.
        i. Calculate the total volume required by multiplying the number of sample wells (with overage) by 100 µl, e.g., 100 wells x 100 µl = 10,000 µl or 10 ml.
        ii. Dilute the stock detection antibody 1:250 in the calculated volume of Blocking Buffer, e.g., dilute 40 µl of stock capture antibody in 10 ml of Blocking Buffer.
    b. Wash the sample wells 6 x 300 µl with PBS-t.
    c. Following removal of the last volume of wash buffer, gently tap the plate, inverted, three times onto a paper towel to remove residual liquid.
    d. Add 100 µl of the 2 µg/ml biotinylated detection antibody to each test well.
    e. Incubate the plate for 1 to 2 h at room temperature.

10. Add streptavidin poly-HRP reagent to the ELISA plate.
    a. Make a 1:5,000 dilution of streptavidin poly-HRP in poly-HRP dilution buffer.
        i. Calculate the total volume required by multiplying the number of sample wells (with overage) by 100 µl, e.g., 100 wells x 100 µl = 10,000 µl or 10 ml.
        ii. Dilute the stock streptavidin poly-HRP 1:5,000 in the calculated volume of poly-HRP dilution buffer, e.g., dilute 2 µl of stock streptavidin poly-HRP in 10 ml of poly-HRP dilution buffer.
    b. Wash the sample wells 6 x 300 µl with PBS-t.
    c. Following removal of the last volume of wash buffer, gently tap the plate, inverted, three times onto a paper towel to remove residual liquid.
    d. Add 100 µl of the 1:5,000 dilution of streptavidin poly-HRP to each test well.
    e. Incubate the plate for 1 to 2 h at room temperature.
    f. During incubation, aliquot the required detection volume of TMB and bring to room temperature.
        i. Calculate the total volume required by multiplying the number of sample wells (with overage) by 100 µl, e.g., 100 wells x 100 µl = 10,000 µl or 10 ml.
        ii. Aliquot the required volume into a sterile tube and store at room temperature protected from light.

11. Develop and read the ELISA plate
    a. Wash the sample wells 12 x 300 µl with PBS-t
    b. Following removal of the last volume of wash buffer, gently tap the plate, inverted, three times onto a paper towel to remove residual liquid.
    c. Add 100 µl of room temperature TMB to each test well.
    d. Incubate the plate for 5 – 30 min at room temperature. The required incubation time will depend on the amount of BoNT in the test samples and desired assay sensitivity. Increased incubation time will increase signal but may also lead to higher background and/or signal saturation.
    e. Add 100 µl of H_2SO_4 diluted 1:100 (100 mM) in high-quality H_2O.
    f. Immediately measure the A_{450nm}.

8.0 EXAMPLE DATA

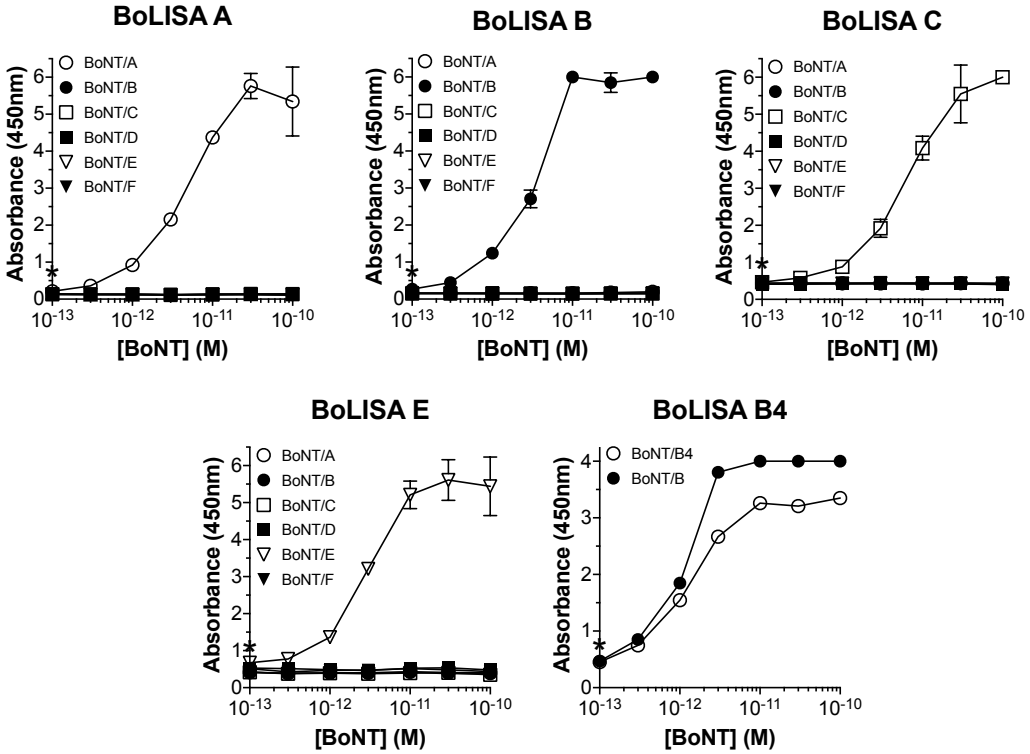

Figure 1. Sensitivity and specificity of the BoLISA BoNT Sandwich ELISA Kits to BoNT serotypes A - F. Dilutions of the indicated BoNT holotoxin (serotypes A – F) were made from 100 pM to 100 fM in PBS containing 5% non-fat dry milk and 0.1% Tween 20. The BoLISA B4 BoNT Sandwich ELISA Kit was only tested with BoNT/B and BoNT/B4 holotoxin. 100 µl of each dilution was then assayed in triplicate using the indicated BoLISA BoNT Sandwich ELISA Kit. Horseradish peroxidase/TMB were used for assay readout and the absorbance at 450 nm was plotted as a function of BoNT concentration. Error bars represent the standard deviation of the mean. The asterisk indicates the limit of detection determined by taking the first data point that is 3 standard deviations above background (n = 6).

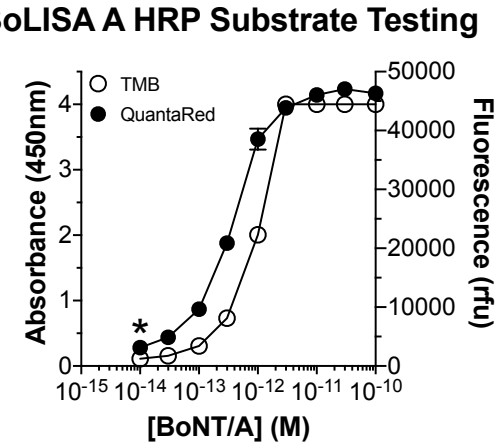

Figure 2. Sensitivity of the BoLISA A BoNT/A Sandwich ELISA to BoNT/A using two different HRP substrate kits. Dilutions of BoNT/A holotoxin were made from 100 pM to 10 fM in GPB containing 5% non-fat dry milk and 0.1% Tween20. Two replicate dilution series of 100 µl of each dilution was then assayed in triplicate using the BoLISA A BoNT/A Sandwich ELISA. Each dilution series was then developed with either TMB or QuantaRed and the response (rfu, relative fluorescence units) was plotted as a function of BoNT concentration (TMB on the left y-axis, QuantaRed on the right). Error bars represent the standard deviation of the mean. The asterisk indicates the limit of detection determined by taking the first data point that is 3 standard deviations above background (n = 6).