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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 B4 Capture Antibody	0.5 µg/µl IgG in PBS and 0.01% (w/v) thimerosal	40 µl	A1046
BoLISA B4 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** and **Appendix A**)
- 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 8.5 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 Food sample testing

Food samples require special processing before application to the BoLISA assay. The special processing steps are dependent on the type of foodstuff being tested and the type of detection chemistry being performed. See **Appendix A**.

5.4 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 activity and can be read over time until the desired sensitivity is obtained with minimal risk of saturation, unlike TMB detection. 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 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 water or PBS 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 100% 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

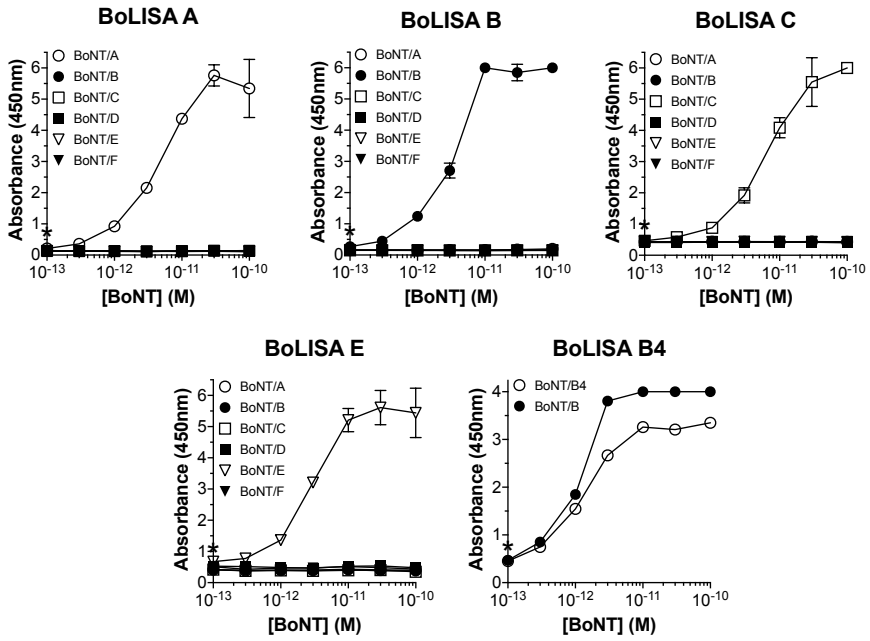

Figure 1. Sensitivity and specificity of the BoLISA BoNT Sandwich ELISA Kits to BoNT serotypes A - F. Dilutions of the indicated BoNT holotoxin (serotypes A – F) were made from 100 pM to 100 fM in PBS containing 5% non-fat dry milk and 0.1% Tween 20. The BoLISA B4 BoNT Sandwich ELISA Kit was only tested 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 was 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).

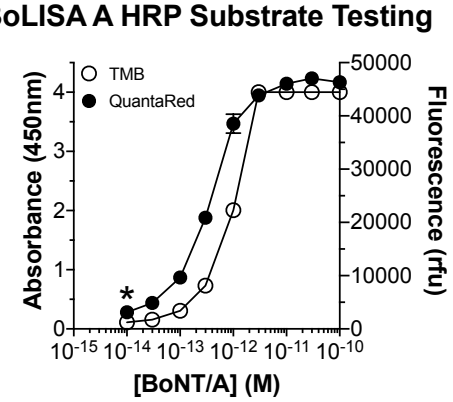

Figure 2. Sensitivity of the BoLISA A BoNT/A Sandwich ELISA to BoNT/A using two different HRP substrate kits. Dilutions of BoNT/A holotoxin were made from 100 pM to 10 fM in gelatin phosphate buffer (GPB) containing 5% non-fat dry milk and 0.1% Tween20. Two replicate dilution series of 100 µl of each 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).

APPENDIX A: Food testing sample preparation

A.1 Food sample testing considerations

- **Sample types:** Depending on user needs, test samples may consist of the following:
  - Field-collected food samples – samples with unknown BoNT content
  - Spiked/challenge samples – samples spiked with BoNT before (pre-spike) or after (post-spike) sample clarification steps
    - Spiking timepoints are denoted in the protocol below. Decisions regarding the spiking of samples is dependent on end-user needs.
  - Liquid foods – foods that require clarification before testing
  - Solid foods – foods that require the addition of buffer and homogenization before clarification

- **Assay types:** Food sample testing can be run in several formats including:
  - Non-quantitative – samples are tested and compared against positive and negative controls
  - Quantitative – samples are tested and interpolated onto a standard curve
  - Performance testing – spiked samples are diluted and tested to determine the assay performance (e.g., limit of detection) across a range of BoNT concentrations

- **Diluent**: An appropriate dilution buffer is required when testing food samples. If matrix effects are anticipated, the dilution buffer can be made from clarified control material (*i.e.*, an identical food sample lacking BoNT) prepared alongside the test samples. Gelatin phosphate buffer (GPB) may be used when control material is not available; however, uncontrolled matrix effects may result in false positives or inaccurate quantification of test samples. When included as part of the assay, 3.75 ml of diluent is required to generate each test sample or standard curve described below.

- **Sample size/required volumes**: This protocol requires 350 µl of clarified sample for testing of a single dilution in triplicate or 500 µl for a half-log serial dilution of the test sample in triplicate (100 µl/well). The ratio of solid to liquid material will vary between matrices. Initial testing is recommended to determine the volume of liquid recovered from food samples clarified as described below. Volumes up to 200 µl/well can also be used for increased sensitivity with scaling of the procedure below.

- **Standard curve and controls**: At the minimum, positive (+ BoNT) and negative (-BoNT) controls are required for assay performance verification. A standard curve is required for quantification of BoNT in the test samples. The standard curve can be generated by diluting BoNT into GPB for relative quantification between samples, but the standard curve should be made in the same, BoNT-free clarified material as the test sample for accurate quantification that is excipient controlled.

- **Sample pH**: The antibodies used in the BoLISA kits bind to BoNT holotoxin and require a neutral sample pH to dissociate any complexing proteins and enable effective antibody binding. This protocol calls for the addition of a one-tenth volume of 1 M HEPES pH 8.0 to neutralize the sample before testing. When possible, the user should verify that this addition effectively neutralizes the sample (pH 6.5 – 8.5) and increase the added volume, if necessary.

A.2 Food sample preparation protocol by food type:

1. **Liquid foods**
   a. Determine the volume of test sample(s) and control materials needed for testing (see above).
   b. Add the test sample(s) and control material to appropriately sized centrifuge tubes able to withstand centrifugation at >6,000 x *g*.
   c. Spike samples with BoNT, if applicable (**pre-spike sample,** see above).
   d. Add one-tenth volume of 1 M HEPES pH 8 to the sample.
   e. Mix the sample well by pipetting or inversion.
   f. Perform an initial clarification by centrifugation for 10 min at 6,000 x *g*, 4°C.
   g. Transfer the clarified supernatant to a new tube.

h. Spike samples with BoNT, if applicable (**post-spike sample,** see above).
i. Liquid sample preparation is now complete.

2. Solid foods

a. Determine the volume of test sample(s) and control materials needed for testing (see above).
b. Add the test sample(s) and control material to appropriately sized centrifuge tubes able to withstand centrifugation at >6,000 x *g* or a plastic bag.
    i. Solid foods can either be processed manually with a pestle in a 50 ml conical tube or mechanically (*i.e.,* with a Stomacher) in a plastic bag.
c. Spike samples with BoNT, if applicable (**pre-spike sample,** see above).
d. Add 1 ml GPB/g of food to the sample.
e. Add one-tenth volume of 1 M HEPES pH 8 to the sample.
f. Homogenize the sample either manually with a pestle or mechanically.
    i. If using mechanical means, transfer the processed material to a 50 ml conical tube after homogenization.
g. Perform an initial clarification by centrifugation for 10 min at 6,000 x *g*, 4°C.
h. Transfer the clarified supernatant to a new tube.
i. Spike samples with BoNT, if applicable (**post-spike sample,** see above).
j. Solid sample preparation is now complete.

A.3 Test, standard, and control sample dilutions, as applicable:

3. Serial dilution of reference and/or test samples

a. Using the processed control material or GPB as diluent, generate the standard curve and test sample dilution series in 1.5 ml tubes following the tables below. For the standard curve, choose a toxin concentration range appropriate for your testing- in this example a top concentration of 100 pM is used.

Standard/reference curve serial dilution:

Sample	Volume of Toxin	Volume of Diluent	[BoNT] (pM)
SC1	500 µl spiked to 100 pM	N/A	100
SC2	120 µl of SC1	280 µl	30
SC3	40 µl of SC1	360 µl	10
SC4	40 µl of SC2	360 µl	3
SC5	40 µl of SC3	360 µl	1
SC6	40 µl of SC4	360 µl	0.3
SC7	40 µl of SC5	360 µl	0.1
SC8	40 µl of SC6	360 µl	0.03
SC9	40 µl of SC7	360 µl	0.01
SC10	N/A	360 µl	N/A

Test sample serial dilution:

Sample	Volume of Toxin	Volume of Diluent
TS1	500 µl of clarified sample	N/A
TS2	120 µl of SC1	280 µl
TS3	40 µl of SC1	360 µl
TS4	40 µl of SC2	360 µl
TS5	40 µl of SC3	360 µl
TS6	40 µl of SC4	360 µl
TS7	40 µl of SC5	360 µl
TS8	40 µl of SC6	360 µl
TS9	40 µl of SC7	360 µl
TS10	N/A	360 µl

4. **Single concentration dilution of positive and negative controls as well as test samples**
    a. Positive and negative controls
        i. Label two 1.5 ml tubes ‘PosCtrl’ and ‘NegCtrl’.
        ii. Add 400 µl of processed control material to each tube.
        iii. Spike BoNT into the ‘PosCtrl’ at a concentration appropriate for your testing.
    b. Test samples
        i. Multiple dilutions of a test sample are recommended if sample volume allows; otherwise, generate a single sample if sample is limiting.
        ii. Label two 1.5 ml tubes ‘1:2’ and ‘1:10’
        iii. Add 200 µl and 360 µl of processed control material or GPB to the ‘1:2’ and ‘1:10’ tubes, respectively
        iv. Add 200 µl and 40 µl of processed test sample to the ‘1:2’ and ‘1:10’ tubes, respectively
        v. Mix samples well by pipetting

5. **Final preparation (all samples)**
    a. Perform a final clarification
        i. If necessary, transfer >400 µl of all assay samples (*i.e.,* standard curve, test, and control samples) to 1.5 ml tubes.
        ii. Spin all samples at >14,000 x *g* for 5 min.
        iii. Immediately transfer the supernatants to new, labeled tubes.
    b. Samples are now ready for testing using the BoLISA BoNT Sandwich ELISA as described in Section 7.0.