Tetracore, Inc.
Immunochemical Services Division

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PROCEDURE TITLE: ELISA Antigen Capture to Detect Botulinum Toxin A Complex

For products: TC-4045-001 & TC-4047-001

ASSAY OBJECTIVE

To detect Botulinum toxin A Complex in unknown samples using an indirect capture ELISA.

SAFETY

Botulinum toxin is highly toxic and should be handled accordingly.

EQUIPMENT/MATERIALS

(NOT SUPPLIED):
-Automated ELISA plate washer (optional)
-Automatic ELISA plate reader (optional)
-12 or 8 channel pipettor
-Pipettes in common laboratory sizes (i.e. Gilson Pipetman P20, P200, P1000)
-Appropriate pipette tips
-De-ionized or filtered water
-37^{o}C incubator
- Botulinum toxin A Complex (toxoid may be used as an alternative), or other positive control antigen
-Magnetic stir bar
-Stir plate
-Vacuum pump (optional)
-0.22 μm vacuum filter unit (Corning brand or equivalent) (optional)
-PBS (Phosphate Buffered Saline) packet, yielding 1 liter 0.01 M phosphate buffered saline, pH 7.4 (Sigma, St. Louis, cat. no. P3813 or equivalent)
-Tween-20 (Sigma, St. Louis, cat. no. P1379)
-Aluminum foil

(SUPPLIED) Storage and Handling:
In a Sealed Foil Pouch (Storage at 4° C):
-One 96 well flat bottom ELISA plate

Other reagents included:
-Positive Capture Antibody (rabbit anti-bot tox), 2.5 mg/mL, Lot T041007-01 *
-Detector Antibody (Mab anti-Bot Toxin A), 3.3 mg/mL, Lot T040213-01
-Conjugate Antibody (Goat anti-Mouse IgG - HRP), 0.4 mg/mL
- Dry skim milk; *Storage at 4° C*
- ABTS peroxidase Substrate (one-component); *Storage at 4° C*

PROCEDURE

- **IMPORTANT:** Users of the non-coated (+ only) botulinum toxin A complex BioThreat Alert ELISA kit (TC-4045-001) should follow the instructions below. Users of the pre-coated (+ only) botulinum toxin A complex BioThreat Alert ELISA kit (TC-4047-001) will omit steps 2 and 3 below, and their kits will not contain the Positive Capture Antibody listed above.

- **NOTE:** It is recommended to give tubes a quick low-speed centrifuge spin prior to using to ensure the full volume is in the bottom of the tube and to prevent loss.

1. Preparation of buffers:

    a. PBS Coating Buffer: To one liter of de-ionized or filtered water, add one packet of PBS. Mix solution using a magnetic stir bar on a stir plate until solution is dissolved. **Optional**: Sterile filter using a 0.22 μm filter unit with vacuum pump. Store at 4°C for up to one month.

    b. PBST (Phosphate Buffered Saline with Tween-20): To 800 mL of PBS Coating Buffer add 0.8 mL Tween-20 and allow solution to mix until well dissolved. Store at 4°C for up to one month.

    c. ELISA Dilution/Blocking Buffer: For each plate being used, add 5 grams of dry skim milk to 90mL PBST. Allow mixture to go into solution using a stir bar and a stir plate. Adjust pH to 7.4 using sodium hydroxide or equivalent. Add PBST to bring total volume to 100 mL. Store at 4°C for up to 3 days, or in aliquots at –20°C for up to one month.

    Note: For long term storage, 0.001% thimerosal (Sigma T5125) can be added to the

buffers. Typically, a 1% solution of thimerosal in de-ionized or filtered water is prepared, then 1 mL of this mixture is added per 1 liter of buffer, creating a 0.001% solution.

2. Add Positive Capture Antibody.
   Coat all rows of the ELISA plates with 100 µL per well of Positive Capture Antibody at a concentration of 10µg/mL in PBS Coating Buffer. To obtain this dilution, add 44 µL of Positive Capture Antibody to 11 mL of PBS Coating Buffer. This dilution provides enough material for the entire plate. Wrap coated plates in aluminum foil and store at 4°C overnight.

3. Wash plates 4 times with PBST.

4. Block plates by adding 150 µL ELISA Dilution/Blocking Buffer per well. Wrap plates with aluminum foil and incubate 1 hour at 37°C.

   Note: Wrap plates in foil when incubating in subsequent steps.

5. Wash plate 4 times with PBST.

6. Add positive controls (Botulinum Toxin A Complex) and negative controls (ELISA Dilution/Blocking Buffer).
   Prepare controls using either of the two following methods:

   Method 1 (see page 6): Performed by diluting antigen at high, medium, and low concentrations (500ng/mL, 50ng/mL, and 10ng/mL).
   Add 100 µL of each positive control antigen dilution to a separate well of the plate (example: wells A1, A2, and A3). Also add 100 µL of ELISA Dilution/Blocking Buffer to three wells as a negative control. This series of controls can be run in multiples, if desired.

   Method 2 (see page 7): Performed by serially diluting antigen from 1µg/mL to 1ng/mL in ELISA Dilution/Blocking Buffer.
   Add 100 µL of ELISA Dilution/Blocking Buffer to each well of one row of the plate. Add 100 µL of positive control antigen dilution at 1µg/mL to one well (example: well A1), and mix well- this results in 200 µL of antigen at 500ng/mL in this well. Remove 100 µL of this antigen mixture from first well and transfer it to adjacent well, mixing well (example: from A1 to A2). Continue this serial dilution process across the row to column 11. Discard the excess 100 µL of serially diluted antigen from the well in column 11. Each well of the control row will contain 100 µL of antigen dilution or negative control once this process is completed. If room on the plate is allowed, it is recommended to include more than one negative control (buffer blank).

7. Add unknown samples to plate.
   For each unknown sample, add 100 μL of material to be tested to a well. Where space on the plate permits, it is recommended to test each sample both undiluted, and diluted 1:2 in ELISA Dilution/Blocking Buffer. Samples may be tested in multiples, if desired. Incubate plates for 1 hour at 37°C.

8. Wash plate 4 times with PBST.

9. Add Detector Antibody.
   For one full plate being tested, add 17 μL of Detector Antibody at 3.3 mg/mL to 11 mL of ELISA Dilution/Blocking Buffer for a concentration of 5μg/mL. Add 100 μL of this Detector Antibody dilution to each well. Incubate plate for 1 hour at 37°C.

10. Wash plate 4 times with PBST.

11. Add Conjugate Antibody.
    Perform a quick centrifuge spin on conjugate prior to making dilution to insure all liquid is at the bottom of the tube.

    Dilute Conjugate Antibody 1:5,000 using either of the two following methods:

    Method 1: For a full plate being tested, dilute the Conjugate Antibody to a 1:5,000 dilution by adding 2.2 μL of conjugate to 11 mL of ELISA Dilution/Blocking Buffer.

    Method 2: Serially dilute the antibody in two steps:
    I. Add 5 μL of Conjugate Antibody to 245 μL of ELISA Dilution/Blocking Buffer (a 1:50 dilution).
    II. For each ELISA plate, add 110 μL of the 1:50 dilution to 11 mL ELISA Dilution/Blocking Buffer (a 1:100 dilution). Steps I and II together are a 1:5,000 dilution.

    Add 100 μL of diluted Conjugate Antibody to each well. Incubate plate for 1 hour at 37°C.

12. Wash plate 4 times with PBST.

13. Add ABTS peroxidase Substrate.
    Add 100 μL of substrate to each well. Incubate plate for 30 minutes at 37°C.

14. If using an Automatic ELISA plate reader, read ODs between 405 and 410 nm. A suggested positive cutoff is determined by adding three times the standard deviation to the mean of the three negative control wells containing no antigen, then adding 0.150 to this value.

Note: ELISA plates can also be read by eye. A blue/green color represents a positive reaction. A well with color of greater intensity than that of the negative controls is considered a positive result.

Botulinum Toxin A Complex Capture ELISA - Method #1

ASSAY: _____ **DATE & INITIALS:** _____

POSITIVE COATING: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

ANTIGEN: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

DETECTOR: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

CONJUGATE: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

SUBSTRATE: _____ **LOT#** _____

DILUTION: _____

		1	2	3	4	5	6	7	8	9	10	11	12
Standard Curve	A	500ng/mL	50ng/mL	10ng/mL	**BLANK**								
Unknowns	B												
	C												
	D												
	E												
	F												
	G												
	H												

COMMENTS:

Botulinum Toxin A ComplexCapture ELISA - Method #2

ASSAY: _____ **DATE & INITIALS:** _____

POSITIVE COATING: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

ANTIGEN: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

DETECTOR: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

CONJUGATE: _____ **LOT#** _____

CONCENTRATION: _____ DILUTION: _____

SUBSTRATE: _____ **LOT#** _____

DILUTION: _____

		1	2	3	4	5	6	7	8	9	10	11	12
Standard Curve [ng/mL]	A	500	250	125	62.5	31.3	15.6	7.8	3.9	2.0	1.0	0.5	Blank
Unknowns	B												
	C												
	D												
	E												
	F												
	G												
	H												

COMMENTS: