

Purification of *Clostridium botulinum* Type A Neurotoxin

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1. Introduction

Botulinum neurotoxins produced by strains of the spore-bearing bacterium *Clostridium botulinum* have long been known to cause a distinctive paralytic disease in humans and animals (1). In recent years, injection of crystalline botulinum toxin type A has been demonstrated to provide relief from certain involuntary muscle disorders, dystonic conditions, pain syndromes, and headaches (2,3,4). The use of botulinum toxin for treatment of human disease and its usefulness in cell biology (5) has stimulated interest in the study of the toxin complexes and the neurotoxin component within the complexes. The use of botulinum toxin complex in medicine will benefit from the development of more potent, less antigenic, and longer-lasting toxin preparations. In this chapter we describe methods for production of high-quality botulinum type A toxin complex and neurotoxin. The procedures described in this chapter are not those used for preparation of botulinum toxin for medical use (3).

C. botulinum produces seven serotypes of neurotoxin designated A–G. These neurotoxins exist in nature as toxin complexes, in which the neurotoxin is noncovalently bound to various nontoxic protein components and to ribonucleic acid (1,3). The neurotoxins are considered the most potent poisons known for humans and certain animals (3,6). The toxicity of type A botulinum neurotoxin has been estimated to be 0.2 ng/kg of body weight (3,6), and as little as 0.1–1 µg may be lethal to humans (3). Consequently, considerable care and safety precautions are necessary in working with botulinum neurotoxins.

Because the consequences of an accidental intoxication with botulinum neurotoxins are severe, safety must be a primary concern of scientists interested in the study of these toxins. The Centers for Disease Control and Prevention

(CDC) recommends Biosafety Level 3 primary containment and personnel precautions for facilities producing large (milligram) quantities of the toxins (7,8). All personnel who work in the laboratory should be immunized with pentavalent (A–E) toxoid available from the CDC, and antibody titers of immunized personnel should be confirmed.

In 1997, *C. botulinum* cultures and toxins were included in a group of select agents whose transfer is controlled by the CDC. To transfer these agents both the person sending and the person receiving them must be registered with the CDC (7). To ensure safety of personnel, a biosafety manual should be placed in the laboratory containing the proper emergency phone numbers and procedures for emergency response, spill control, and decontamination. All personnel should be trained in these procedures as well as in safe laboratory practices. When performing steps where aerosols may be created (such as in centrifugation) special precautions need to be taken. Toxins should be handled in sealed, unbreakable containers and manipulated in a Class II or III biological safety cabinet, and/or respiratory protection should be employed. The use of syringes and needles to perform bioassays using mice or for inoculation of rubber septum sealed tubes also requires caution and proper training.

The methods outlined here were developed or modified from previously described production and purification methods to limit the introduction of antigenic or contaminating material (3,9). Any steps that could be simplified, omitted, or improved from these earlier methods with equivalent or improved results were modified as appropriate.

Type A neurotoxin is produced in cultures as part of a protein complex (1,3,10). Under the growth conditions described, the neurotoxin molecule is noncovalently associated with 6–7 nontoxic proteins (Fig. 1). Some of these nontoxic proteins have hemagglutinating activity (3,10) and stabilize the neurotoxin molecule (3,9,10). The fully active neurotoxin is an approx 150-kDa dichain protein (10). The heavy (ca. 100 kDa) and light (ca. 50 kDa) chains are covalently bound by a disulfide bridge. *C. botulinum* Hall A strain produces proteolytic enzymes during culture. These proteases cleave the single-chain neurotoxin into the dichain form. The chains can be separated by reducing the sulfide bond as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1).

Toxin production is carried out in an undefined complex medium (3,11). After 4 d of incubation, acid precipitation is used to concentrate the toxin complex from the culture fluid. The precipitated toxin complex (“mud”) is stable at these lower pHs (ca. 3.5) and can be stored in this crude form for several months to years. Toxin complex is solubilized from the mud in sodium phosphate buffer and precipitated with ammonium sulfate. The toxin complex is purified

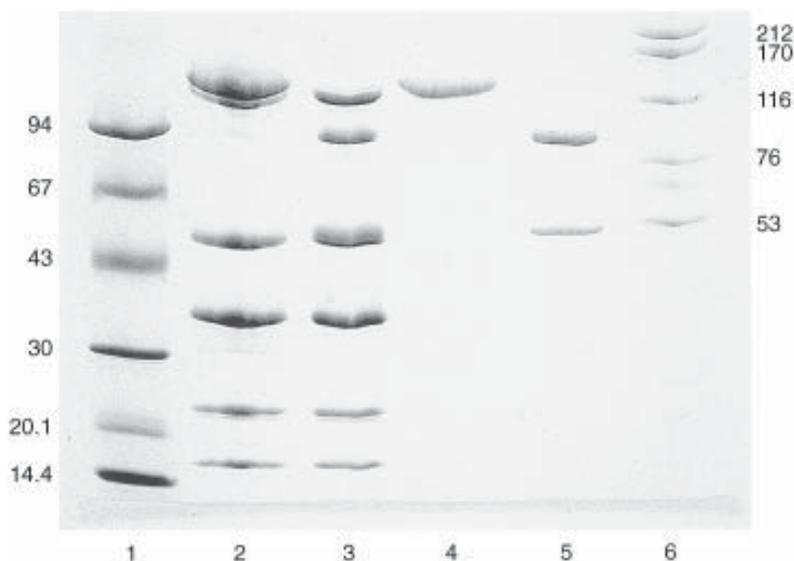


Fig. 1. Analytical SDS-polyacrylamide (10–15% gradient gel) of botulinum toxin complex and neurotoxin. Lanes: 1, Low molecular weight markers; 2, Crystalline complex; 3, Crystalline complex reduced; 4, Neurotoxin eluted from DEAE column; 5, Neurotoxin reduced; 6, Molecular weight markers (top to bottom): myosin, $M_r = 212,000$; α_2 -macroglobulin, $M_r = 170,000$; β -galactosidase, $M_r = 116,000$; transferrin, $M_r = 76,000$; glutamic dehydrogenase, $M_r = 53,000$.

and the nucleic acids are removed by anion ion-exchange chromatography at an acidic pH. The toxin complex is then crystallized in 0.9 *M* ammonium sulfate (3,12–14). These crystals are not true crystals, but consist of needle-shaped paracrystals that contain the neurotoxin and associated proteins (3).

The neurotoxin is separated from the nontoxic complex proteins by chromatography at alkaline pH on an anionic exchange gel (15–17). The use of a shallow sodium chloride gradient (0–0.3 *M*) increases the efficiency of this procedure. If present, trace contaminants can be removed by treatment with immobilized *p*-aminophenyl- β -D-thiogalactopyranoside (18) and/or chromatography on a cation-exchange column (17).

The biological activity of botulinum toxin preparations can be assayed by the intravenous (IV) time to death method (19,20) or the intraperitoneal (IP) end point dilution method (21). The IV method has a variance of ~15% when performed properly (21) and requires three to five mice per sample. The IP method results in a variance of as low as 5% if correct dilutions and sufficient animals are used (22).

The best storage method for the neurotoxin is dependent on the end-use of the material. Neurotoxin standards with stabilizing protein added have been kept for years at 20–22°C in low-pH buffers with no appreciable loss of activity (21). Ammonium sulfate precipitated neurotoxin can be stored at 4°C for several weeks. However, within a month, SDS-PAGE will show fragmentation and breakdown products of the neurotoxin stored in this manner. The neurotoxin can also be stored at –20°C with 50% glycerol as a cryoprotectant for years. Because crystals of the complex are very stable, purifying the neurotoxin from the complex as required could be the best option.

2. Materials

2.1. Toxin Production

1. Hall A strain: *Clostridium botulinum* (see ref. [23] for genetic characterization).
2. Inoculum medium: 500 mL of dH₂O, 2.0% casein hydrolysate, 1.0% yeast extract, 0.5% glucose (w/v), pH 7.2. Autoclave for 30 min at 121°C.
3. 50 g of glucose solution in 500 mL of dH₂O. Autoclaved for 30 min.
4. 10 L of toxin production medium in 13 L of carboy: 9.5 L of dH₂O, 2.0% casein hydrolysate, 1.0% yeast extract (w/v), pH 7.2. Autoclave for 90 min at 121°C.

2.2. Precipitation and Extraction

1. 3 N Sulfuric acid.
2. Sterile water.
3. 0.2 M Sodium phosphate buffer, pH 6.0.
4. 1.0 N Sodium hydroxide.
5. Ultrapure ammonium sulfate.

2.3. Purification of Type A Complex

1. 0.05 M Sodium citrate buffer, pH 5.5.
2. DEAE-Sephadex A-50 gel (80 g dry) swelled to a volume of 1–1.5 L and degassed.
3. Chromatography column (5 cm × 50 cm).
4. Dialysis tubing.
5. Fraction collector.
6. UV-spectrophotometer.
7. Ultrapure ammonium sulfate.

2.4. Crystallization of Type A Complex

1. 0.05 M Sodium phosphate buffer, pH 6.8.
2. Dialysis tubing.
3. UV-spectrophotometer.
4. 4 M Sterile ammonium sulfate solution.
5. 0.9 M Sterile ammonium sulfate solution.

2.5. Purification of Type A Neurotoxin

1. 0.02 M sodium phosphate buffer, pH 7.9.
2. Dialysis tubing.
3. Chromatography column (2.5 cm × 20 cm).
4. DEAE-Sephadex A-50 (19 g dry) swelled and degassed.
5. Gradient maker apparatus.
6. Fraction collector.
7. UV-spectrophotometer.

2.6. Additional Purification Steps

2.6.1. Treatment with Carbohydrate-Binding Affinity Gel

1. Washed and equilibrated *p*-aminophenyl- β -D-thiogalactopyranoside (pAPTG, Sigma).
2. 10-mL Chromatography column.
3. Fraction collector.
4. UV-spectrophotometer.

2.6.2. Chromatography on SP-Sephadex C-50

1. 25-mL Chromatography column.
2. Dialysis tubing.
3. 0.02 M Sodium phosphate buffer, pH 7.0.
4. SP-Sephadex C-50 gel swelled and degassed.
5. Gradient maker.
6. Fraction collector.
7. UV-spectrophotometer.

2.7. Bioassays

2.7.1. IV Assay

1. Female ICR mice, 18–22 g.
2. Mouse restraint (“mouse trap”).
3. 30 mM Sodium phosphate buffer, pH 6.3, + 0.2% (w/v) gelatin.
4. IV Standard curve for crystalline type A toxin.
5. 1-mL Syringe with 25–26-gage needle.

2.7.2. IP Assay

1. Female ICR mice, 18–22 g.
2. Sterile 30 mM sodium phosphate buffer, pH 6.3, + 0.2% (w/v) gelatin.
3. Sterile serial dilution blanks.
4. 1-mL Syringe with 25–26-gage needle.

2.8. Precipitation and Storage of Toxin Preparations

2.8.1. Precipitation

Ultrapure ammonium sulfate.

2.8.2. Storage of Standard Toxin Solutions

Sterile 0.05 M sodium acetate buffer + 3% bovine serum albumin + 2% gelatin (w/v), pH 4.2.

2.8.3. -20° or -80° C Storage

1. Sterile glycerol.
2. Sterile vials.

3. Methods

3.1. Toxin Production

1. Inoculate 500 mL of inoculum medium with frozen stock culture of *C. botulinum* strain Hall A and incubate without shaking at 37°C until turbid (12–24 h).
2. Add 500 mL of cooled glucose solution to carboy.
3. Inoculate carboy with 500 mL of a 12–24-h inoculum culture.
4. Incubate production carboy for 4 d at 37°C (*see Note 1*). A schematic of toxin production is presented in **Fig. 2**.

3.2. Precipitation and Extraction

1. Adjust pH of the production medium to 3.4 by addition of 3 N sulfuric acid. Allow the precipitate to settle for 1–3 d (*see Note 2*). Remove the supernatant by siphoning and centrifugation (12,000g for 10 min at 20°C). Discard the supernatant to waste and decontaminate by autoclaving.
2. Wash the precipitated toxin with 1 L of sterile water and collect the washed precipitate by centrifugation (12,000g for 10 min at 20°C).
3. Resuspend pelleted toxin in 600 mL of 0.2 M sodium phosphate buffer, pH 6.0.
4. Adjust the pH of dissolved toxin to 6.0 with 1 N sodium hydroxide and gently stir for 1 h at 20°C.
5. Centrifuge (12,000g for 10 min at 20°C) the extracted toxin to clarify.
6. Save supernatant and repeat **steps 3–6** with the pellets using 400 mL of the buffer.
7. Pool clarified extracts and precipitate by bringing to 60% saturation (39 g/100 mL) with ammonium sulfate (*see Note 3*).

3.3. Purification of Type A Complex

1. Collect precipitated toxin by centrifugation (12,000g for 10 min at 8°C) and dissolve in 25 mL of 0.05 M sodium citrate buffer, pH 5.5.
2. Dialyze sample for 18 h at 4°C against 3× changes of 500 mL of the same buffer.
3. Pack and wash DEAE-Sephadex A-50 in the column with 1–2 column volumes of the citrate buffer.

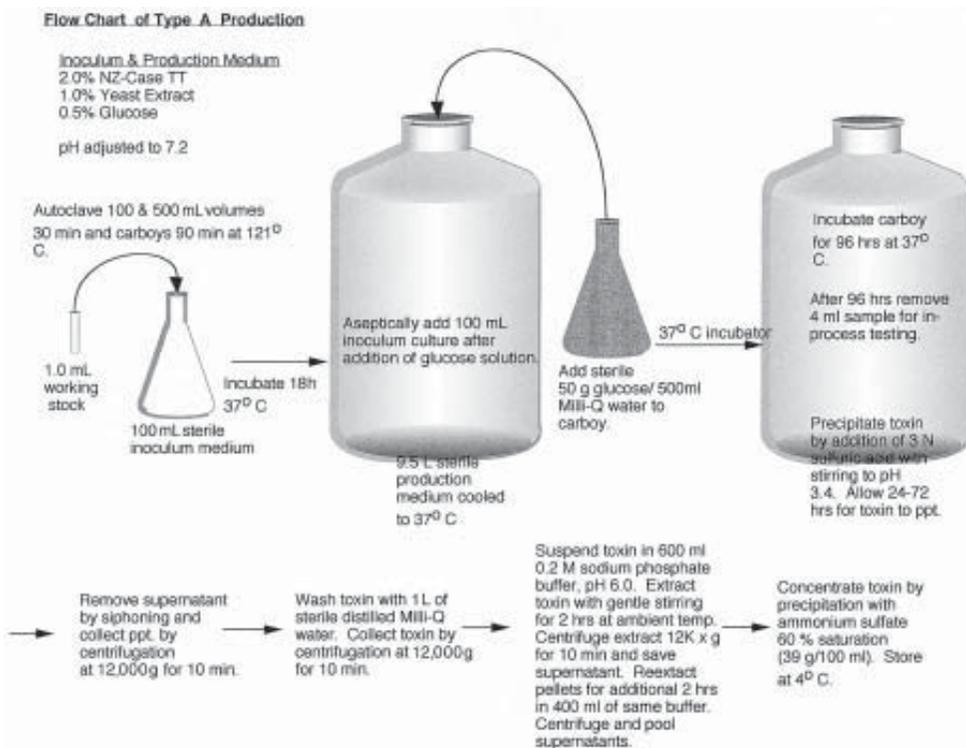


Fig. 2. Schematic for toxin production.

4. Centrifuge (12,000g for 10 min at 20°C) dialyzed toxin solution to clarify.
5. Load sample onto the column at a flow rate at 35–45 mL/h (*see Note 4*). The toxin complex is eluted in the void volume in citrate buffer.
6. Start collecting fractions after 200 mL have passed through the column (*see Note 5*).
7. Measure absorbance of each fraction at 260 and 280 nm (A_{260} and A_{280}) with the UV-spectrophotometer.
8. Pool fractions from the first peak off the column with a A_{260}/A_{280} ratio of 0.6 or less (*see Note 6*).
9. Precipitate toxin pool by making 60% saturated with ammonium sulfate (*see Note 7*).

3.4. Crystallization of Type A Complex

1. Dissolve precipitated toxin at a concentration of ~10 mg/mL in 0.05 M sodium phosphate buffer, pH 6.8.
2. Dialyze sample thoroughly against 3× changes of 500 mL of the same buffer (*see Note 8*).

3. Centrifuge (12,000*g* for 10 min at 10°C) sample and dilute to a concentration of 6–8 mg/mL with 0.05 *M* sodium phosphate buffer, pH 6.8.
4. Slowly, with gentle stirring, add 4 *M* ammonium sulfate solution to a final concentration of 0.9 *M* (see **Note 9**).
5. Allow crystals to form for 1–3 wk at 4°C (see **Note 10**).

3.5. Purification of Botulinum Type A Neurotoxin

Recovery of neurotoxin from the complex is typically 10–13%. Because crystalline toxin is one of the more stable forms, only the required amount of neurotoxin (which is more labile than the crystals) is usually purified as needed.

1. Dissolve the required amount of crystals in 0.02 *M* sodium phosphate buffer, pH 7.9, for 1–2 h and dialyze for 18 h (10× with three buffer changes) to remove ammonium sulfate.
2. Pack and wash DEAE-Sephadex A-50 in a column sufficient in size to bind all the complex (see **Note 11**).
3. Centrifuge (12,000*g* for 10 min at 10°C) sample and load onto column. Wash column with at least 50 mL of starting buffer or until A_{280} is less than 0.01.
4. Set flow rate at 30 mL/h and begin collecting fractions.
5. Neurotoxin is eluted with a linear gradient of sodium chloride made of running buffer plus running buffer containing 0.3 *M* sodium chloride. Toxin elutes at ca. 0.15 *M* chloride ion. The volume of eluant is dependent on column size but is typically 4–5× the volume of the gel volume.
6. Read A_{280} of fractions and pool first peak eluted in the sodium chloride gradient (see **Note 12**).

3.6. Additional Purification Steps

The following methods can be used to eliminate any trace contaminants that may be present in the toxin pool that could interfere with sensitive protocols. Specific toxicity generally will not be improved, but trace contaminants are removed.

3.6.1. Treatment with Carbohydrate-Binding Affinity Gel

1. Mix pooled toxin with 1 mL of washed pAPTG gel and gently mix for 15 min at 20–22°C.
2. Load toxin–pAPTG slurry into the column and collect the toxin as it elutes using A_{280} to pool fractions. Neurotoxin does not bind to the gel matrix but nontoxic proteins including those with hemagglutinating activity do bind under these conditions.

3.6.2. Chromatography on SP-Sephadex C-50

1. Dialyze toxin against 0.02 *M* sodium phosphate, pH 7.0, at 4°C against several changes of buffer.

2. Pack and wash SP-Sephadex C-50 column with sodium phosphate buffer. Set flow rate at 30 mL/h.
3. Centrifuge toxin (12,000g for 10 min at 10°C) and load clarified solution onto column. Wash column with 2–3 column volumes of the running buffer. Elute neurotoxin with a 0–0.5 M sodium chloride gradient that is 4–5× the column volume.
4. Read A₂₈₀ of fractions eluted with the sodium chloride gradient and pool fractions containing neurotoxin.

3.7. Bioassays

Extreme caution should be taken when performing these procedures. The IV method will give toxicity data in 30–70 min with concentrated toxin solutions. The IP method is completed in 3–4 d, but requires less technical expertise to perform accurately.

3.7.1. IV Mouse Assay

1. Dilute complex to 15 µg/mL and the neurotoxin to 4 µg/mL in 30 mM sodium phosphate buffer, pH 6.3, + 0.2% (w/v) gelatin (gel-phosphate buffer).
2. Inject 0.1 mL of the diluted sample into the lateral tail veins of three to five animals. Record the time the animals were injected and mark the animals (*see Note 13*).
3. Record the time to death and determine the average time to death (ATTD) for the mice (*see Note 14*).
4. Convert ATTD to IP LD₅₀/mL using the previously prepared standard curve (**Fig. 3**) (*see Note 15*).

3.7.2. IP Assay

1. Dilute toxin to ~10 LD₅₀/mL using gel-phosphate buffer (*see Note 16*).
2. Make several (approx five or six) serial twofold dilutions.
3. Inject 0.5 mL of diluted toxin IP into groups of 5–10 mice per dilution.
4. Record the number of deaths within 96 h (*see Note 17*).
5. The dilution that kills 50% of the animals is taken as the LD₅₀/0.5 mL. Multiply dilution ×2 to obtain LD₅₀/mL.

3.8. Storage of *Botulinum Toxin Preparations*

Purified neurotoxin is not as stable as the toxin complex. Precipitated neurotoxin will show degradation within a month when analyzed by SDS-PAGE. Diluted toxin solutions can be stored for years at 20°C by the addition of stabilizing protein excipients and adjustment of pH and ionic strength. Solutions of toxin in 50% glycerol stored at –20 to –70°C are stable for years. The preferred method of storage will depend on the project requirements.

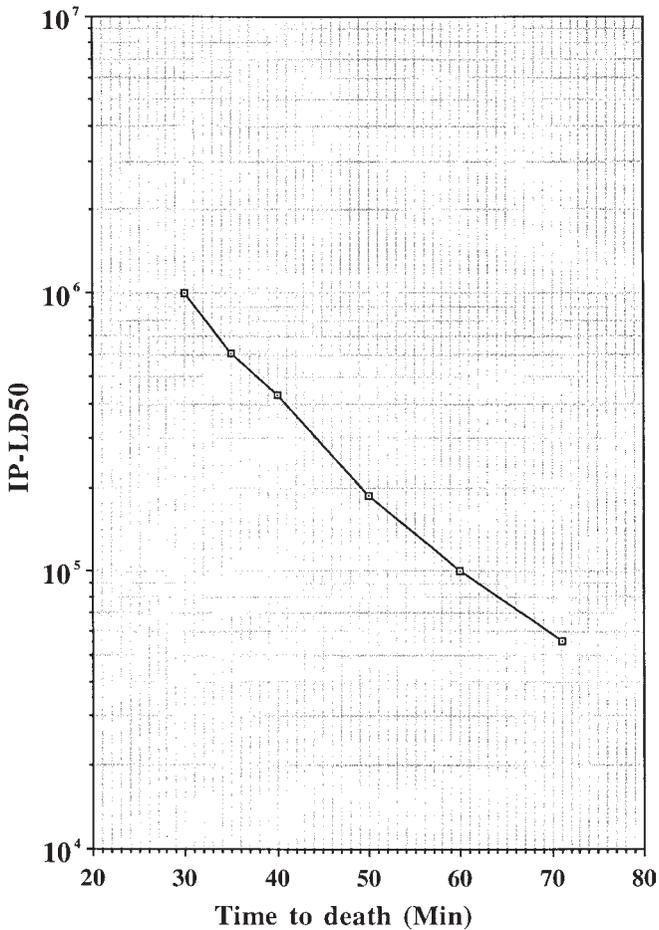


Fig. 3. IV time-to-death standard curve for botulinum type A crystalline toxin complex.

3.8.1. Precipitation

Make the toxin solution to 60% saturation with ammonium sulfate and store at 4°C.

3.8.2. Storage of Standard Toxin Solutions

1. Dilute filter-sterilized toxin (0.2 μ m filter) to desired concentration in sterile 0.05 M sodium acetate buffer + 3% bovine serum albumin + 2% gelatin, pH 4.2.
2. Aliquot toxin solution to desired volumes and store at 4–25°C. Do not freeze these samples to avoid toxin inactivation.

3.8.3. -20 to -80°C Storage

1. Dilute toxin to twice the desired concentration in 0.05 M sodium phosphate buffer, pH 6.8.
2. Add an equal volume of sterile glycerol.
3. Aliquot toxin into convenient volumes and store at -20 to -80°C .

4. Notes

1. After 4 d, production culture fluid should have a pH of 5.6–5.9, a toxicity greater than $10^6\text{LD}_{50}/\text{mL}$ and 1:1000 dilution should be neutralized by 1 IU of type A antitoxin (CDC).
2. NZ Amine A, NZ Amine B, and NZ Case TT all have lot-to-lot variation and need to be pretested to achieve adequate toxin production and precipitation qualities. In testing, toxin production should be $>10^6\text{LD}_{50}/\text{mL}$. Culture supernatant should contain less than 10% of the toxicity 24–72 h after precipitation with acid.
3. To process larger culture volumes (up to 40 L) using the same volume of DEAE-Sephadex, treat extracts with RNase before dialysis. Dissolve toxin in 0.05 M sodium phosphate, pH 6.0, and add 0.05 mg/mL of RNase A (Sigma) and treat for 3 h at 37°C . Precipitate by bringing to 60% saturation with ammonium sulfate.
4. The volume of toxin solution loaded onto the column must be $<10\%$ of column volume.
5. Elution of protein from the column can be monitored at A_{280} with a UV detector. Collect fractions when protein (A_{280}) begins to elute.
6. The approximate toxin concentration in the sample eluting off the first DEAE column can be determined using an extinction coefficient of 1.65 for type A complex. The yield from 20 L of culture should be approx 350 mg of complex.
7. The crystallization procedure can be omitted and neurotoxin purification accomplished using toxin complex off of the first DEAE-Sephadex A-50 column if desired. Additional purification (*see Subheading 3.6.*) may be required.
8. Toxin precipitates if the concentration of ammonium sulfate is too high, so all residual ammonium sulfate must be removed prior to crystallization.
9. The last few milliliters of ammonium sulfate should be added very slowly. When the toxin solution becomes opalescent, the concentration of ammonium sulfate is usually sufficient to initiate crystallization of toxin complex.
10. Recovery of crystalline toxin is $\sim 80\%$ after 10 d. High quality crystalline toxin complex has a specific toxicity of approx $3.5 \times 10^7\text{LD}_{50}/\text{mg}$. Crystals are stable in 0.9 M ammonium sulfate for several years.
11. DEAE-Sephadex A-50 will bind approx 0.9 mg/mL of the complex under these running conditions.
12. Protein concentration can be determined using an extinction coefficient of 1.63 for type A neurotoxin. Analysis of pure neurotoxin off of the second DEAE-Sephadex column by SDS-PAGE should show one band ($M_r = 150\text{ kDa}$) when unreduced (**Fig. 1**). Toxin should be $>98\%$ homogeneous and have a specific toxicity of $>10^8\text{LD}_{50}/\text{mg}$.

13. If the needle is in the vein, the plunger of the syringe will slide easily. A drop of blood will appear immediately upon retraction of the needle. If the needle is not in the vein, the plunger will be difficult to depress. Repeat any injections where the vein was missed, using a new animal.
14. Animals injected with same sample should die within 5 min of each other if titration is valid.
15. IP LD₅₀/mL can be taken from the standard curve prepared with type A complex (**Fig. 2**), or calculated from the equation $IP\ LD_{50}/mL = 6.9 \times 10^6 - 3.4 \times 10^5(ATTD) + 5.7 \times 10^3(ATTD)^2 - 31.9(ATTD)^3$ where ATTD = the average time to death.
16. The toxin complex is diluted to 4×10^{-6} /mg and neurotoxin 10^{-7} /mg.
17. Mice must die with clinical symptoms of botulism toxicity: contraction of abdominal muscles resulting in a wasp shape of the animal's waist, reduced mobility, labored breathing, convulsions, and eventual death.

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