United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Potency Testing *Clostridium perfringens* Type A Antigen

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Potency Testing *Clostridium perfringens* Type A Antigen

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Potency Testing *Clostridium perfringens* Type A Antigen

1. Introduction

This Testing Protocol (PRO) describes the method used to determine whether biological products containing *Clostridium perfringens* Type A antigen can stimulate the production of satisfactory immunity. For products that require 2 vaccinations, rabbits are vaccinated twice 20 to 23 days apart and bled 14 to 17 days following the second vaccination. For products that require a single vaccination, rabbits are vaccinated and bled 34 to 40 days later. The serum is titrated by a toxin-antitoxin neutralization test, using mice as an indicator.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Mixer, vortex-type

2.1.2 Centrifuge

2.1.3 Autoclave

2.1.4 Freezers, -20° and -70°C or lower

2.1.5 Refrigerator, 2°- 7°C

2.1.6 Micropipettes, 100-µL to 1000-µL

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 *C. perfringens* Type A antitoxin, current Center for Veterinary Biologics (CVB) lot

2.2.2 *C. perfringens* Type A alpha toxin, current CVB lot

2.2.3 Peptone diluent

2.2.4 Screw-top Erlenmeyer flask, 500-mL, with cap

2.2.5 Syringes, Luer-lok, 1-cc, 10-cc, 20-cc, or 30-cc

2.2.6 Needles, 25- to 27-gauge x 7/8- to 1 1/4-inch, 20-gauge x 1-inch

2.2.7 Vacutainer® needles, 20-gauge x 1 1/2-inch and needle holder

2.2.8 Serum separation tubes, 12.5-mL

2.2.9 Pipettes, 2-mL, 5-mL, 10-mL, 25-mL

2.2.10 Tips for micropipettes

2.2.11 Ketamine hydrochloride, 100 mg/mL solution

2.2.12 Xylazine, 20 mg/mL solution

2.2.13 Water, distilled or deionized, or water of equivalent purity

2.2.14 Tubes (with caps), various sizes

2.3 Test animals

2.3.1 New Zealand White rabbits, nonpregnant females, 4-8 pound (Eight rabbits are required per serial to be tested.)

2.3.2 White Swiss nonpregnant female mice, 16-20 g, 5 mice for each toxin-antitoxin mixture. Refer to the manufacturer's Outline of Production for variations.

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel need a working knowledge of the use of general laboratory chemicals, equipment, and glassware; and must have specific training and experience in the safe handling of clostridial toxins. Personnel must have specific training in the care and handling of laboratory rabbits and mice.

3.2 Preparation of equipment and supplies

3.2.1 Use only sterile supplies.

3.2.2 Operate all equipment according to the manufacturers' instructions.

3.3 Preparation of reagents

3.3.1 Peptone diluent

Peptone (Difco)	8 g
NaCl, reagent grade	2 g
Water, q.s. to	800 mL

Dissolve peptone and sodium chloride in water. Adjust pH to 7.2 with 1N sodium hydroxide. Fill a 500-mL Erlenmeyer flask no more than 3/4 full with diluent. Autoclave with caps loosened at ≥ 121°C for 25 to 30 minutes. Cool flasks and tighten caps. Store at 2°- 7°C for up to 3 months.

3.3.2 Preparation of *C. perfringens* Type A antitoxin

Prepare according to the current Reagent Data sheet.

IRP 538 (06) contains 6.0 International units of alpha antitoxin per mL. A dilution of standard antitoxin containing 1.0 antitoxin unit per mL (AU/mL) is used in the mouse assay. The dilution is prepared by adding 1.0 mL of IRP 538 (06) to 5.0 mL of peptone diluent (1.0% peptone, 0.25% sodium chloride, pH 7.2).

3.3.3 Preparation of *C. perfringens* Type A toxin

Prepare according to the current Reagent Data sheet.

IRP 560-07 is diluted 1:80. The toxin dilution is prepared by adding 1.0 mL of well mixed IRP 560-07 to 9.0 mL of peptone diluent (1.0% peptone, 0.25% sodium chloride, pH 7.2). The toxin is further diluted to 1:80 by adding 2.0 mL of the 1:10 dilution to 14.0 mL of diluent.

4. Performance of the Test

4.1 Vaccination of rabbits

4.1.1 Thoroughly shake each bottle of product and wipe the top with alcohol before filling the syringe.

4.1.2 Vaccinate each rabbit subcutaneously in the shoulder region with not more than half of the largest recommended dose for any species indicated on the product label or manufacturer's Outline of Production. Use 5-, 10-, 20- or 30-cc syringes fitted with 20-gauge x 1-inch needles to vaccinate the rabbits.

4.1.3 For products that require 2 vaccinations, give the second vaccination 20 to 23 days after the first vaccination.

4.2 Collection and preparation of rabbit serum

4.2.1 Collect blood from the test rabbits 34 to 40 days after vaccination, or 14 to 17 days after the second vaccination for products that require 2 vaccinations.

4.2.2 Anesthetize rabbits for bleeding with a mixture of 1.32 mg/kg of xylazine and 8.8 mg/kg of ketamine hydrochloride. Give the anesthetic mixture by intramuscular injection.

4.2.3 Use a serum separation tube fitted with a 20-gauge x 1 1/2-inch Vacutainer® needle to collect blood from the heart. Collect approximately 8-12 mL of blood from each rabbit. Let the tubes of blood sit at 20°- 25°C for 30 to 60 minutes.

4.2.4 Centrifuge the blood at 1000 x g for 10 to 20 minutes at 20°- 25°C.

4.3 Preparation of serum pools

4.3.1 Prepare a pooled sample using an equal volume of serum from at least 7 rabbits per vaccinated group. If more than 7 rabbits are bled per vaccinated group, an equal volume of serum from each rabbit is used for the serum pool. If less than 7 rabbits are bled, the test is invalid and must be repeated.

4.3.2 The pooled sample may be held at 2°- 7°C for up to 7 days if the test will be conducted within that time. If testing will not be completed within 7 days, store the pooled sample at -20°C or lower.

4.3.3 This test evaluates undiluted serum at several AU/mL levels. Refer to the Outline of Production for AU/mL level required for a satisfactory test.

4.3.4 Test for both the level of antitoxin required in the Outline of Production and the next higher level of antitoxin. See **Table 1** for antitoxin level and serum dilution examples.

Antitoxin Levels	Serum Dilutions (**Section 4.3.4**)
2 AU/mL	Dilute 1:2 (1.5 mL serum + 1.5 mL diluent)
3 AU/mL	Dilute 1:3 (1.0 mL serum + 2.0 mL diluent)
12AU/mL	Dilute 1:12 1.0 mL serum + 11 mL diluent
15AU/mL	Dilute 1:15 (1.0 mL serum + 14 mL diluent)
17AU/mL	Dilute 1:17 (1.0 mL serum + 16 mL diluent)

Table 1

4.4 Toxin neutralization

4.4.1 Prepare standard toxin according to the current Reagent Data sheet.

For the purposes of this PRO, 1 L_o dose is defined as the greatest amount of toxin that, when mixed with 1 AU, results in 100% survival of all mice inoculated intravenously (IV) with 0.5 mL of this mixture. The 1 L_+ dose is defined as the least amount of toxin that, when mixed with 1 AU, results in the death of at least 80% of the mice inoculated IV with 0.5 mL of this mixture.

4.4.2 Prepare standard antitoxin according to the current Reagent Data sheet

4.4.3 Product serum and standard alpha toxin

1. One L_o dose toxin contains 1.0 mL of standard toxin (**Section 4.4.1**) and 2.0 mL peptone diluent. Mix a sufficient volume of this L_o dose toxin for each serum pool and the L_o control.

2. Dispense 3.0 mL-aliquots of L_o dose toxin mixture into empty tubes.

3. Add 2.0 mL of diluted serum (**Section 4.3.4**) to the corresponding tube containing the L_o dose toxin mixture. Mix each tube with a vortex-type mixer.

4. Let the mixtures sit at 20°- 25°C for 1 hour ± 5 minutes.

5. Place tubes in ice.

4.4.4 Standard toxin and antitoxin controls

1. Add 2.0 mL of standard antitoxin (1 AU/mL, **Section 4.4.2**) to a tube containing 3.0 mL of L_o dose toxin mixture (**Section 4.4.3[2]).** Mix well with a vortex-type mixer.

2. Add 2.0 mL of standard alpha antitoxin (1 AU/mL, **Section 4.4.2**) to a tube containing 1.6 mL of standard alpha toxin and 1.4 mL peptone diluent (1 L_+ dose). Mix well with a vortex-type mixer.

3. Let the mixtures sit at 20°- 25°C for 1 hour ± 5 minutes.

4. Place tubes in ice.

4.5 Inoculation of mice

4.5.1 Inject each mouse in the group with 0.5 mL of each standard test toxin-product antitoxin mixture.

4.5.2 Inject each mouse in the group with 0.5 mL of each standard test toxin-standard antitoxin mixture.

4.5.3 Inoculate all mice intravenously into 1 of the lateral tail veins. Use 1-cc Luer-lok syringes fitted with 25- or 27-gauge x 7/8- to 1 1/4-inch needles.

4.5.4 Always inoculate the mice receiving the standard test toxin-standard antitoxin mixtures (controls) **last**.

4.5.5 Complete all mouse inoculations within 1 hour of placing the toxin-antitoxin mixtures in ice.

4.5.6 Conclude the test 48 hours after the mice are inoculated.

5. Interpretation of Test Results

5.1 Criteria for a valid test

5.1.1 All 5 mice inoculated with the standard 1 L_o/1 AU control mixture must survive.

5.1.2 At least 80% of the mice inoculated with the standard 1 L_+/1 AU control mixture must die.

Note: Moribund animals exhibiting clinical signs consistent with the expected disease pathogenesis that are unable to rise or move under their own power may be humanely euthanized and considered as deaths as outlined in 9 CFR 117.4.

5.2 Interpretation of test results

5.2.1 The antitoxin value of the test serum is determined as the highest level of antitoxin that protects all 5 mice.

5.2.2 The product is considered unsatisfactory if the serum pool from at least 7 rabbits does not protect all 5 mice at the dilution required in the manufacturer's Outline of Production.

6. Report of Test Results

Report results of the test(s) as described by standard operating procedures.