United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Potency Testing *Clostridium septicum* Antigen

Date: **June 24, 2011**

Number: BBPRO1301.03

Supersedes: BBPRO1301.02, July 31, 2008

Contact: Janet M. Wilson, (515) 337-7245

Approvals:

/s/Geetha B. Srinivas Date: 12Jul11
Geetha B. Srinivas, Section Leader
Bacteriology

/s/James D. McCord for Date: 13Jul11
Rebecca L.W. Hyde, Section Leader
Quality Management
Center for Veterinary Biologics

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

INTERNAL USE ONLY

Potency Testing *Clostridium septicum* Antigen

Table of Contents

Potency Testing *Clostridium septicum* Antigen

1. Introduction

This Testing Protocol (PRO) describes the method used to determine whether biological products containing *Clostridium septicum* 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. septicum* alpha antitoxin, IRP 228

2.2.2 *C. septicum* alpha toxin, IRP 455

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 lb (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. Dispense and 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. septicum* alpha antitoxin

1. *C. septicum* alpha antitoxin, IRP 228, contains 960 antitoxin units per vial (AU/vial).

2. A dilution of *C. septicum* alpha antitoxin containing 1 AU/mL is used in the toxin-neutralization test. Rehydrate the antitoxin by adding 5.0 mL of peptone diluent to a vial of IRP 228 and placing it at 2°- 7°C for 16 to 24 hours. Transfer the reconstituted antitoxin to a tube. Wash the vial with 15.0 mL of peptone diluent using 5.0 mL per wash. Add an additional 28.0 mL of peptone diluent to the tube and thoroughly mix the contents. This antitoxin mixture, 48.0 mL total, (containing 20 AU/mL) may be dispensed into individual tubes and stored at -70°C or lower.

3.3.3 Preparation of *C. septicum* alpha toxin

Each vial of *C. septicum* standard alpha toxin IRP 455 contains 1.3 mL of toxin. Store the toxin at -70°C or lower until used.

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 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 12.5-mL serum separation tube fitted with a 20-gauge x 1 1/2-inch Vacutainer® needle to collect blood from the heart. Collect approximately 12.5 mL of blood from each rabbit. Gently invert tubes 4 to 6 times. 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 the 1 AU/mL level.

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.

Potency Testing *Clostridium septicum* Antigen

Antitoxin Levels	Serum Dilutions (**Section 4.3.4**)
1.0 AU/mL	No dilution
1.5 AU/mL	Dilute 1:1.5 (2.0 mL serum + 1.0 mL diluent)
2.0 AU/mL	Dilute 1:2 (1.5 mL serum + 1.5 mL diluent)
2.5 AU/mL	Dilute 1:2.5 (1.0 mL serum + 1.5 mL diluent)
3.0 AU/mL	Dilute 1:3 (1.0 mL serum + 2.0 mL diluent)

Table 1

4.4 Toxin neutralization

4.4.1 Preparation of standard alpha toxin

1. Dilute the *C. septicum* alpha toxin 1:40 by adding 1.0 mL of well mixed IRP 455 to 39.0 mL of sterile peptone diluent. For the purpose of this test, the 1:40 dilution of IRP 455 is referred to as the standard toxin.

2. A volume of 1.0 mL of standard alpha toxin and 2.0 mL of peptone diluent represents 1 L_o dose. A volume of 1.6 mL of standard toxin plus 1.4 mL of peptone diluent represents 1 L_+ dose.

3. 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 Preparation of standard alpha antitoxin

Further dilute the 20 AU/mL *C. septicum* alpha antitoxin (see **Section 3.3.2**) by adding 1.0 mL to 19.0 mL of peptone diluent. This solution contains 1 AU/mL and is referred to as the standard antitoxin.

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 alpha 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 72 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 antitoxin level in **Table 1** that protects all 5 mice.

5.2.3 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.

7. References

7.1 History of reagents: *C. septicum* alpha antitoxin (IRP 228) was produced in ponies at the National Veterinary Services Laboratories in Ames, Iowa, in June 1976.

7.2 *C. septicum* alpha toxin (IRP 455) was produced at the National Veterinary Services Laboratories, Ames Iowa, in October 2000. The toxin was made from *C. septicum* strain CN3204. The culture was obtained from Wellcome Research Laboratories, Beckenham, England, on July 29, 1977. The number of passages is unknown.

8. Summary of Revisions

Version .03

- The Contact information has been updated.
- **3.3:** This section has been updated to reflect current procedures.

Version .02

- One of the signatories has changed due to personnel reassignment.