United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 207

Supplemental Assay Method for Potency Testing *Clostridium novyi* Type B Alpha Antigen

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

Approvals:

/s/Larry R. Ludemann Date: 09Jul14
Larry R. Ludemann, Section Leader
Bacteriology

/s/Byron E. Rippke Date: 10Jul14
Byron E. Rippke, Director
Policy, Evaluation, and Licensing
Center for Veterinary Biologics

/s/Rebecca L.W. Hyde Date: 10Jul14
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

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Supplemental Assay Method for Potency Testing *Clostridium novyi* Type B Alpha Antigen

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Supplemental Assay Method for Potency Testing *Clostridium novyi* Type B Alpha Antigen

1. Introduction

This Supplemental Assay Method (SAM) describes the method used to determine whether biological products containing *Clostridium novyi* type B alpha antigen can stimulate the production of satisfactory immunity as prescribed by the title 9, *Code of Federal Regulations* (9 CFR), section 113.108. 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°C and -70°C

2.1.5 Refrigerator, 2°- 7°C

2.1.6 Micropipettes, 100-µL and 1000-µL

2.2 Reagents/supplies

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

2.2.1 *C. novyi* type B alpha antitoxin, IRP 507 (04) (available from the Center for Veterinary Biologics (CVB))

2.2.2 *C. novyi* type B alpha toxin IRP 581 (available from the CVB)

2.2.3 Peptone diluent

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

2.2.5 Syringes, needle-locking, 1-cc, 10-cc, 20-cc, or 30-cc

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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 Polystyrene snap-top tubes, 17 x 100-mm

2.2.15 Polystyrene screw-cap conical tubes, 17 x 120-mm

2.2.16 Glass screw-cap tubes, 13 x 100-mm

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 (Five mice are required for each toxin-antitoxin mixture.)

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 Sterilize all glassware before use.

3.2.2 Use only sterile supplies (pipettes, syringes, needles, etc.).

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3.2.3 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 following manufacturer's recommendations. Cool flasks and tighten caps. Store at 2°- 7°C for up to 3 months.

3.3.2 Preparation of *C. novyi* type B alpha antitoxin

1. *C. novyi* type B alpha antitoxin, IRP 507 (04), contains 140 antitoxin units per mL (AU/mL) and has been standardized against the World Health Organization gas gangrene (*C. novyi*) International Antitoxin, equine origin. Each vial contains 4.5 mL of antitoxin.

2. Prepare a solution of *C. novyi* type B alpha antitoxin that contains 10 AU/mL by adding 1.0 mL of IRP 507 (04) to 13 mL of peptone diluent in a 17 x 100-mm snap-top tube. Dispense in 2.25 mL amounts in glass 13 x 100-mm screw-cap tubes and store at -70°± 10°C until used.

3.3.3 Preparation of *C. novyi* type B alpha toxin

Each vial of *C. novyi* type B standard toxin IRP 581 contains 1.3 mL of toxin. Store the toxin at -70°± 10°C 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. Use 10-, 20- or 30-cc syringes fitted with 20-gauge x 1-inch needles to vaccinate the rabbits.

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

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 5 times. Let the tubes of blood sit at 22°- 26°C (room temperature) for 30 to 60 minutes.

4.2.4 Centrifuge blood at approximately 1000 x g for 10 to 20 minutes at room temperature.

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 (provided that, if more than 7 rabbits are bled per vaccinated group, then equal volumes from each rabbit are 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 Use 0.2 mL pooled serum diluted with 0.8 mL peptone diluent to test for 0.5 AU/mL of antitoxin.

4.3.4 Use 0.1 mL pooled serum diluted with 0.9 mL peptone diluent to test for 1.0 AU/ml of antitoxin.

4.4 Toxin neutralization

4.4.1 Preparation of standard alpha toxin

1. Dilute the *C. novyi* type B alpha toxin to 1:10 by adding 1.0 mL of IRP 581 toxin to 9 mL of peptone diluent in a 17 x 100-mm snap-top tube. Further dilute the toxin to 1:35 by adding 1 mL of the 1:10 dilution to

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2.5 mL of peptone diluent in a 17 x 100-mm snap-top tube. For the purpose of this test, the 1:35 dilution of IRP 581 is referred to as the standard alpha toxin.

2. A volume of 0.5 mL of standard alpha toxin and 0.5 mL of peptone diluent represents 0.1 L_o dose. A volume of 0.8 mL of standard alpha toxin plus 0.2 mL of peptone diluent represents 0.1 L_+ dose.

3. For the purposes of this SAM, 0.1 L_o dose is defined as the greatest amount of toxin that, when mixed with 0.1 AU, results in 100% survival of all mice inoculated intravenously (IV) with 0.2 mL of this mixture. The 0.1 L_+ dose is defined as the least amount of toxin that, when mixed with 0.1 AU, results in the death of 80%-100% of all mice inoculated IV with 0.2 mL of this mixture.

4.4.2 Preparation of standard alpha antitoxin

Thaw the 10 AU/mL *C. novyi* type B alpha antitoxin previously described (see **Section 3.3.2**). Further dilute the antitoxin to 1 AU/mL by adding 1 mL of the well mixed 10 AU/mL antitoxin to 9 mL of diluent in a 17 x 100-mm snap-top tube. Finally dilute the antitoxin to 0.1 AU/mL by adding 1 mL of the well mixed 1 AU/mL antitoxin to 9 mL of diluent in a 17 x 100-mm snap-top tube. For the purpose of this test, this 0.1 AU/mL dilution of antitoxin is referred to as the standard alpha antitoxin.

4.4.3 Product and standard alpha toxin

1. Mix a sufficient volume of standard alpha toxin and peptone diluent (0.5 mL of standard alpha toxin and 0.5 mL peptone diluent [0.1 L_o dose]) for each serum pool and the L_o control in a 17 x 120-mm screw-cap tube. Add 1 mL of each of the serum dilutions to 1 mL of this alpha toxin-peptone diluent L_o mixture in 17 x 100-mm snap-top tubes. Mix each tube with a vortex-type mixer.

2. Let the mixtures sit at 22°- 26°C (room temperature) for 1 hour.

3. Place tubes in ice.

4.4.4 Standard alpha toxin and antitoxin controls

1. Add 1.0 mL of standard alpha antitoxin (0.1 AU/mL) to 1 mL of the alpha toxin-peptone diluent (0.1 L_o dose) mixture (see **Section 4.4.3**) in a 17 x 100-mm snap-top tube**.** Mix well with a vortex-type mixer.

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2. Add 1.0 mL of standard alpha antitoxin (0.1 AU/mL) to a 17 x 100-mm snap-top tube containing 0.1 mL peptone diluent and 0.9 mL of standard alpha toxin (0.1 L_+ dose). Mix well with a vortex-type mixer.

3. Let the mixtures sit at 22°- 26°C (room temperature) for 1 hour.

4. Place tubes in ice.

4.5 Inoculation of mice

4.5.1 Inject 0.2 mL of each standard test toxin-product antitoxin mixture into each of 5 mice.

4.5.2 Inject 0.2 mL of each standard test toxin-standard antitoxin mixture into each of 5 mice.

4.5.3 Inoculate all mice intravenously into 1 of the lateral tail veins. Use 1-cc 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 Mouse inoculations need to be completed within 1 hour of placing the toxin-antitoxin mixtures in ice.

4.5.6 The test is concluded 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 0.1 L_o/0.1 AU control mixture must survive.

5.1.2 At least 4 of the 5 mice inoculated with the standard 0.1 L_+/0.1 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.

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5.2 Interpretation of test results

5.2.1 If 5 of the 5 mice inoculated with the 1:5 diluted serum-toxin mixture (see **Section 4.3.3**) survive, the serum contains at least 0.5 AU/mL of *C. novyi* type B alpha antitoxin and the product is satisfactory.

5.2.2 If 5 of the 5 mice inoculated with the 1:10 diluted serum-toxin mixture (see **Section 4.3.4**) survive, the serum contains at least 1.0 AU/mL of *C. novyi* type B alpha antitoxin.

5.2.3 The product is considered unsatisfactory if the serum pool from at least 7 rabbits contains less than 0.5 AU/mL of *C. novyi* type B alpha antitoxin.

6. Report of Test Results

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

7. References

7.1 Title 9, *Code of Federal Regulations*, Part 113.108, U.S. Government Printing Office, Washington, DC.

7.2 History of reagents. *C. novyi* type B alpha antitoxin IRP 507 (04) was produced in goats at the National Veterinary Services Laboratories/Center for Veterinary Biologics in Ames, Iowa.

7.3 *C. novyi* type B alpha toxin (IRP 581) was produced at the Center for Veterinary Biologics, Ames, Iowa, in April 2009. The toxin was made from *C. novyi* type B alpha strain CN234.3. The culture was obtained from Wellcome Research Laboratories, Beckenham, England, on July 16, 1965. The number of passages is unknown.

8. Summary of Revisions

<u>Version .07</u>

- The Bacteriology Section Leader was updated.
- Minor word changes for clarification of procedures.

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Version .06

- *C. novyi* type B alpha toxin IRP 581 has replaced IRP 425 throughout the document.

Version .05

- The Contact information has been updated.

Version .04

- The standard antitoxin IRP 298 has been changed to IRP 507(4) throughout the document.

- The 0.1 L_o and 0.1 L_+ doses have been updated throughout the document.

Version .03

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **4.4.3** The wording was changed to add clarity.

- **4.4.4** The wording was changed to add clarity.

- Humane endpoint language was added.

- Dilution/holding vessel sizes were added for clarification.

- The contact person was changed to Janet M. Wilson.

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