

United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

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Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

Table of Contents

- 1. Introduction**
 - 2. Materials**
 - 2.1 Equipment/instrumentation**
 - 2.2 Reagents/supplies**
 - 3. Preparation for the Test**
 - 3.1 Personnel qualifications/training**
 - 3.2 Preparation of equipment/instrumentation**
 - 3.3 Preparation of reagents/control procedures**
 - 3.4 Preparation of the sample**
 - 4. Performance of the Test**
 - 5. Interpretation of the Test Results**
 - 6. Reporting of the Test Results**
 - 7. Summary of Revisions**
- Appendix**

Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

1. Introduction

This Testing Protocol (PRO) describes an *in vitro* technique for potency testing inactivated *Clostridium chauvoei* bacterins. It is a capture enzyme-linked immunosorbent assay (ELISA) to measure the flagellar protein of *C. chauvoei*. Relative potency is determined by comparing the flagellar protein content of the test serial to the flagellar protein content present in a non-expired, suitably qualified reference bacterin.

2. Materials

2.1 Equipment/instrumentation

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

- 2.1.1 Microplate reader with dual wavelengths (450 and 650 nm)
- 2.1.2 Micropipettors, to cover the range of 10.0- μ L to 1000- μ L
- 2.1.3 8- or 12-channel micropipettor
- 2.1.4 Orbital shaker
- 2.1.5 Balance, validated for 150 mg to 15 g
- 2.1.6 A computer software program capable of implementing the calculations described in **CVBSOP0102**, *Using Software to Estimate Relative Potency*.
- 2.1.7 Ultrasonic processor with 50 Watt output

2.2 Reagents/supplies

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

- 2.2.1 Carbonate coating buffer
- 2.2.2 Phosphate buffered saline
- 2.2.3 Blocking solution/reagent diluent
- 2.2.4 Wash solution
- 2.2.5 Stop solution

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Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

- 2.2.6** Phosphate buffer for antigen elution (optional)
- 2.2.7** 0.5% sodium desoxycholate buffer for antigen elution (optional)
- 2.2.8** Sodium citrate, reagent grade, for antigen elution (optional)
- 2.2.9** 3,3',5,5'-tetramethylbenzine (TMB) substrate (Kirkegaard-Perry Laboratories, Inc.)
- 2.2.10** 96-well flat bottom microtitration plates (Immulon 2; Dynex Laboratories, Inc.)
- 2.2.11** 96-well microtitration plates (transfer plates)
- 2.2.12** Plate sealers or parafilm
- 2.2.13** Capture antibody: Monoclonal antibody against flagellar protein of *C. chauvoei*, (Current Lot), provided by the Center for Veterinary Biologics (CVB)
- 2.2.14** Detection antibody: Rabbit origin monospecific polyclonal antiserum against flagellar protein of *C. chauvoei* (Current Lot), provided by the CVB
- 2.2.15** Horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc.)
- 2.2.16** Qualified, non-expired reference bacterin containing *C. chauvoei*
- 2.2.17** Water, distilled or deionized, or water of equivalent purity

3. Preparation for the Test

3.1 Personnel qualifications/training

Technical personnel should have working knowledge of the use of general laboratory chemicals, equipment, and glassware and knowledge and experience in the operation of an automated microplate reader and data recording and evaluation software.

3.2 Preparation of equipment/instrumentation

- 3.2.1** Operate and maintain all equipment according to manufacturers' recommendations.
- 3.2.2** Calibrate the ELISA microplate reader.

Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

3.3 Preparation of reagents/control procedures

Caution: Concentrated solutions of acids and bases are used to prepare some of the following reagents. Both are hazardous and should be handled properly. Consult Material Safety Data Sheets (MSDS) (current version) for proper safety procedures.

3.3.1 Carbonate coating buffer (pH 9.6)

Na ₂ CO ₃	1.59 g
NaHCO ₃	2.93 g
Water	q.s. 1 L

Store at 2°- 7°C for up to 1 week.

3.3.2 Phosphate Buffered Saline (PBS)

NaCl	8.50 g
NaH ₂ PO ₄ ·H ₂ O	0.25 g
Na ₂ HPO ₄	1.19 g
Water	q.s. 1 L

Adjust pH to 7.2 with 5 M NaOH or 0.1 M HCl. Store at 2°- 7°C for up to 6 months.

3.3.3 Blocking solution/Reagent diluent (2% w/v nonfat dry milk in PBS)

Nonfat dry milk	10 g
PBS (see 3.3.2)	500 mL

Store at 2°- 7°C for up to 7 days.

3.3.4 Wash solution

NaCl	8.5 g
NaH ₂ PO ₄ ·H ₂ O	0.25 g
Na ₂ HPO ₄	1.19 g
Tween	0.5 mL
Water	q.s. 1 L

Adjust pH to 7.2 with 5 M NaOH or 0.1 M HCl. Store at room temperature (20°- 25°C) for up to 6 months. If long term storage (up to 1 year) is desired, autoclave to sterilize before storage.

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Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

3.3.5 Stop solution

H ₂ SO ₄ (2.5 M)	13.6 mL
Water	q.s. to 100 mL

Store at room temperature (20°- 25°C) for up to 1 year.

3.3.6 Bacterins containing *C. chauvoei* antigen

Reference bacterin (supplied by Firm)
Test serial

CRITICAL CONTROL POINT: The reference and test serials should be adjuvanted with the same adjuvant. Ideally, the reference and test serials should be produced by the same Outline of Production.

3.4 Preparation of the sample

Some bacterins do not require antigen-elution treatment prior to being tested in this assay. Representative serials should be tested with and without antigen-elution treatment to determine if the treatment specifically enhances the capture of flagellar protein. If no enhancement of antigen capture can be demonstrated, the bacterins should be tested without antigen-elution treatment. The reference bacterin and the test serials for each product must be treated by the same procedure. See the **Appendix**, which discusses some of the more common antigen elution treatments. Alternate elution procedures may be more appropriate for some bacterins. Antigen-elution treatments should be optimized for each manufacturer's bacterin(s).

Some bacterins may not require sonication treatment prior to being tested in this assay. Representative serials should be tested with and without sonication treatment to determine if the treatment specifically enhances the capture of flagellar protein. If no enhancement of antigen capture can be demonstrated, the bacterins should be tested without sonication treatment. The reference bacterin and the test serials for each product must be treated by the same procedure. Alternate preparation procedures may be more appropriate for some bacterins. Sample preparation treatments should be optimized for each manufacturer's bacterin(s).

4. Performance of the Test

4.1 Dilute capture MAb, current lot, to the current use dilution in cold carbonate coating buffer. Place 100 µL diluted MAb into each well of a 96-well flat-bottomed microtitration plate. Seal plate with adhesive plate sealers or parafilm. Incubate overnight at room temperature (20°- 25°C) for at least 16 hours. All incubations are conducted with agitation on an orbital shaker revolving at 80-120 cycles per minute.

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Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

- 4.2** Wash the plate three times (300 μL /well) with wash solution. Blot plate on absorbent toweling.
- 4.3** Place 200 μL blocking solution/reagent diluent into each well of test plate. Seal plate and incubate for 75 ± 5 minutes at $35^\circ - 37^\circ\text{C}$. After blocking, remove excess solution by emptying and blotting plates on absorbent toweling. Blocked plates may be sealed and held at $2^\circ - 7^\circ\text{C}$ up to 1 week.
- 4.4** Wash the plate as described in **Section 4.2**.
- 4.5** Make serial twofold dilutions of reference and test bacterins in a transfer plate. Place 125 μL blocking solution/reagent diluent into each well of a 96-well U-bottom microtitration plate. Add 125 μL of bacterin to the first well of each of a minimum of 2 rows per bacterin. Use a multichannel pipetting device to make serial twofold dilutions of each bacterin across the plate. At least 2 wells must be reserved without bacterin for use as a blank. The use of at least 7 serial twofold dilutions is recommended. The selected bacterin dilutions should delineate the sigmoid curve from antigen saturation to antigen extinction for each bacterin. The dilutions used for the reference and bacterin may differ. Alternatively, dilutions may be made in tubes and transferred to the microtitration plate.
- 4.6** Transfer 100 μL diluted bacterin from each well of the transfer plate to the corresponding well on the test plate. Seal plate and incubate for 75 ± 5 minutes at $35^\circ - 37^\circ\text{C}$.
- 4.7** Wash the plate as described in **Section 4.2**.
- 4.8** Dilute detection antibody, current lot, (monospecific rabbit antiserum) to current use dilution in blocking solution/reagent diluent. Place 100 μL diluted detection antibody in each well of the test plate. Seal plate and incubate for 75 ± 5 minutes at $35^\circ - 37^\circ\text{C}$.
- 4.9** Wash the plate as described in **Section 4.2**.
- 4.10** Dilute anti-rabbit horseradish peroxidase conjugate to appropriate use dilution as specified by manufacturer or determined by checkerboard titration for that lot. Place 100 μL diluted conjugate in each well of the test plate. Seal plate and incubate for 75 ± 5 minutes at $35^\circ - 37^\circ\text{C}$.
- 4.11** Allow the bottles of TMB substrate to come to room temperature (store in the dark) during the conjugate incubation step.
- 4.12** Wash the plate as described in **Section 4.2**.

Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

4.13 Measure the necessary volume (10 mL/plate) of TMB substrate. Place 100 µL of TMB substrate dilution into each well of the test plate. Incubate at room temperature (20°-25°C) for 10 - 30 minutes, or until adequate color change has occurred.

Warning: TMB is a potential carcinogen. Use caution and wear gloves when handling TMB.

4.14 Place 100 µL stop solution into each well of test plate.

4.15 Read the ELISA plate(s) at 450/650 nm. Calculate the mean absorbance for the blank wells. Subtract the mean absorbance of the blank wells from each bacterin test well absorbance value prior to data analysis.

5. Interpretation of the Test Results

5.1 Relative potency calculation method

Refer to the current version of **CVBSOP0102** for estimating the relative potency.

5.2 Requirements for a valid test

5.2.1 An assay must meet the validity requirements of **CVBSOP0102** (current version) to be considered valid.

5.2.2 Lines determined by first-order linear regression must have a correlation coefficient (r) of ≥ 0.95 .

5.2.3 The reference dose-response line and the test serial dose-response line must show parallelism.

5.2.4 Assays that are not valid may be repeated up to a maximum of 3 times. If a valid assay cannot be achieved, report the serial as unsatisfactory.

5.3 Requirements for a satisfactory serial

To be considered satisfactory, a test serial must have an RP value of ≥ 1.0 unless otherwise specified in the Outline of Production. Serials with RP values < 1.0 on a valid assay may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. If the reported RP values of both of the retests are ≥ 1.0 , the serial is satisfactory. If the reported RP values of both of the retests are ≤ 1.0 , please refer to Veterinary Services Memorandum No. 800.104 for the second stage testing option.

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Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

6. Reporting of the Test Results

Results of the test(s) are reported as described by standard operating procedures.

7. Summary of Revisions

Version .03

- **3.3.4:** Corrected wash solution (Tween 0.5 mL).

Version .02

- Entire protocol updated to reflect current procedures.

Potency Testing of *Clostridium chauvoei* Bacterins using an ELISA Procedure

Appendix

Antigen elution treatment examples

1. Aluminum-adjuvanted bacterins

Bacterins adjuvanted with aluminum hydroxide may be treated with either sodium citrate or phosphate buffer prior to making serial twofold dilutions in reagent diluent.

a) Sodium citrate elution

Add 1.0 g sodium citrate to 10 mL bacterin (10% w/v). Place on an orbital shaker (approximately 120 rpm) overnight (16 to 24 hours) at 35°- 37°C. Treated bacterin is considered undiluted.

b) Phosphate buffer elution

Add 1.0 mL of phosphate elution buffer to 1.0 mL of bacterin. Bacterin may be sonicated if desired. Place on an orbital shaker (approx. 80-120 rpm) overnight (16 to 24 hours) at 35°- 37°C. Eluted bacterin may be sonicated again prior to use. Treated bacterin is considered 1:2.

2. Oil-adjuvanted bacterins

Mix 1.0 mL 0.5% sodium desoxycholate with 1.0 mL bacterin. Place on an orbital shaker (approx. 120 rpm) overnight (16 to 24 hours) at 35°- 37°C. Treated bacterin is considered diluted 1:2.

Phosphate buffer for antigen elution

KH ₂ PO ₄	8.2 g
Deionized H ₂ O	q.s. 100 mL

Adjust pH to 6.5 with 5 M NaOH.

0.5% sodium desoxycholate for antigen elution

NaCl	0.85 g
NaH ₂ PO ₄ ·H ₂ O	0.02 g
Na ₂ HPO ₄	0.15 g
Sodium desoxycholate (Difco #02248-13, Sigma D6750, or equivalent)	0.50 g
Deionized H ₂ O	q.s. 100 mL

Store at 2°- 7°C for up to 30 days. Warm to room temperature (20°- 25°C) prior to using.

Warning: This solution gels at refrigerator temperatures.

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