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**United States Department of Agriculture
Center for Veterinary Biologics**

Testing Protocol

**Potency Test for *Clostridium perfringens* Type D epsilon Antitoxin Using a
Cell Assay**

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1. Introduction

This Testing Protocol (PRO) describes an *in vitro* method to test anti-*Clostridium perfringens* Type D epsilon levels in serum as a final step in the potency assay for *Clostridium perfringens* Type D toxoids. It is a cell assay using Madin-Darby Canine Kidney (MDCK) epithelial cells. Relative potency is determined by comparing cell death patterns between a standard antitoxin and an unknown serum.

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 (405 and 590 nm)
- 2.1.2 Micropipettors, to cover the range of 10.0-μL to 1000-μL
- 2.1.3 8- or 12-channel micropipettor
- 2.1.4 Orbital shaker
- 2.1.5 A computer software program capable of reading optical densities (OD)
- 2.1.6 Incubator, CO₂ (5-7%)

2.2 Reagents/supplies

- 2.2.1 Small glass or snap top plastic tubes (3 mL or larger)
- 2.2.2 Non-binding dilution plate, Falcon or equivalent
- 2.2.3 MDCK cells plated at 1.0×10^5 – 2.0×10^5 cells/mL concentration on a cell culture plate
- 2.2.4 Minimal Essential Medium (MEM) with Earle's F-15 with 0.5% Lactalbumin Enzymatic Hydrolysate (LAH)
- 2.2.5 Fetal Bovine Serum (FBS)
- 2.2.6 L-Glutamine (200 mM)

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2.2.7 Penicillin/streptomycin solution

2.2.8 Crystal violet solution

2.2.9 Sterile water

2.2.10 Isopropyl alcohol

2.2.11 *Clostridium perfringens* Type D (epsilon) standard toxin, current Center for Veterinary Biologics (CVB) lot

2.2.12 *Clostridium perfringens* Type D (epsilon) standard antitoxin, current CVB lot

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.

3.3 Preparation of reagents/control procedures

3.3.1 Crystal violet, 0.25% (National Centers for Animal Health (NCAH) Media #30216)

Crystal violet	2.5 gm
QH ₂ O	1000 mL

Mix the above ingredients until in solution.

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3.3.2 MEM with Earle's F-15 with 0.5% LAH supplemented with 5% (v/v) FBS, 1% L-glutamine, and 0.1% Penicillin/Streptomycin

Ingredients

A. MEM with Earle's F-15 with 0.5% LAH (NCAH Media #20030)

MEM with Earle's	9.61 gm
LAH	5.0 gm
Sodium Bicarbonate	1.1 gm
Sterile Super Q Water (QS to)	1000 mL

Heat to dissolve. Filter sterilize and store at 2°- 7°C for up to 6 months.

B. Penicillin/Streptomycin (50/50) solution (NCAH Media #20049)

Penicillin G Potassium Salt (1586 units/mg)	15.77 gm
Streptomycin Sulfate (747 units/mg)	100.0 gm
Super Q H ₂ O (QS to)	1000 mL

C. FBS (sterile)

D. L-glutamine (200 mM) Sigma-Aldrich Catalog #G6392 or equivalent

Preparation of cell culture media

At time of use, add 1.0 mL of Ingredient B, 50 mL of Ingredient C, and 10 mL of Ingredient D to Ingredient A. Mix and store at 2°- 7°C. Solution can be stored up to 14 days. After the 14-day period, 1% of L-glutamine can be added to extend the expiration of media an additional 14 days. This can be done repeatedly.

3.4 Preparation of samples

3.4.1 Standard antitoxin preparation

The standard antitoxin preparation is diluted in cell culture media according to the current lot's reagent data sheet. The test is conducted at 0.1 AU/mL.

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3.4.2 Standard toxin preparation

The standard toxin preparation is diluted in cell culture media according to the current lot's reagent data sheet.

3.4.3 Unknown serum sample preparation

To test for 2.0 AU/mL of antitoxin, dilute pooled serum 1:2 with cell culture media. Perform an additional 1:10 dilution immediately prior to adding the serum sample to the dilution plate.

4. Performance of the Test

4.1 Use 5 small glass or snap top tubes that hold at least 3 mL each. Pipette 2 mL of cell culture media into each tube.

4.2 Dilute the standard toxin according to the reagent data sheet. Further dilute the toxin by adding the specified volumes of toxin to each of the 5 tubes to obtain the graduated toxin levels.

4.3 Dilute the standard antitoxin according to the reagent data sheet.

4.4 Add 100 µL of the standard antitoxin diluted to 0.1 AU/mL to Rows B through G, Columns 2-6 on a dilution plate.

4.5 Add 100 µL of the diluted unknown serum to Rows B through G, Columns 8-11 on a dilution plate.

4.6 Add 100 µL of each graduated standard toxin level to the dilution plate in order of graduated level. In other words, add the most dilute level to Column 2, then the next level to Column 3 and 8, then the third highest level to Column 4 and 9, and so forth. See the current CVB reagent data sheet for dilutions and plate template.

4.7 Add 200 µL of cell culture media to Column 7, Rows B-D, for the live cell control.

4.8 Add 200 µL of toxin at the standard toxin use dilution level to Column 7, Rows E-G, for the killed cell control.

4.9 Rotate the dilution plate on an orbital shaker (80-120 RPM) at 20°- 25°C for 60 ± 5 minutes.

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4.10 Remove a cell culture plate from the CO₂ incubator and confirm cell growth is between 90 to 100% confluent in all inside wells. **NOTE: Once the wells are deemed 90-100% confluent, plates must be used within 3 days.**

4.11 Dump the plate. Gently tap the plate on absorbent paper to remove any excess liquid.

4.12 Transfer 100 µL of each column of the dilution plate to the corresponding wells on the cell culture plate. Change tips between each column.

4.13 Incubate the cell culture plate in a CO₂ incubator at 35°- 37°C with a plate cover on overnight (16-24 hours).

4.14 Remove the cell culture plate from the CO₂ incubator and dump the plate. Gently tap the plate on absorbent paper to remove any excess liquid.

4.15 Add 75 µL of crystal violet to each well and rotate the plate on an orbital shaker (80-120 RPM) at 20°- 25°C for 5 ± 1 minutes.

4.16 Wash the plate three times by running sterile water onto Rows 1-3 and allowing the water to run into all the wells.

4.17 Tap the plate to remove excess water. Add 100 µL isopropyl alcohol to resuspend the live cell stain.

4.18 Rotate the stained plate on an orbital shaker (80-120 RPM) at 20°- 25°C for 5 ± 1 minutes, then read the OD of the plate at 590 nm and 405 nm. Set the reader to reduce the data (590-405).

5. Interpretation of the Test Results

5.1 Average live cell control

Calculate the average OD of the live cell control wells (Column 7, B-D). Divide the average by 2 to establish the 50% cutoff value.

5.2 Average of standard antitoxin and unknown serum sample

Calculate the average OD of each column (graduated toxin level) of standard antitoxin (Columns 2-6) and the average OD of each column of unknown serum sample (Columns 8-11). The columns with the average ODs greater than the live cell control 50% cutoff value are considered “live.” The columns with the average ODs less than the 50% cutoff are considered “dead.”

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5.3 Requirements for a valid test

5.3.1 The average OD reading from Column 2, Row B-G, must be above the 50% cutoff value and classified as “live.”

5.3.2 The average OD reading from Column 6, Rows B-G, must be below the 50% cutoff value and classified as “dead.”

5.3.3 The toxin control wells (Column 7, E-G) must have an average OD reading less than the average OD reading from Column 6, Rows B-G.

5.3.4 The live cell control wells (Column 7, B-D) must have an average OD reading of greater than 2.0.

5.4 Requirements for a satisfactory serial

In a valid test, if the unknown serum sample has “live” cells at a toxin concentration higher (less dilute column) than the standard antitoxin, it is considered satisfactory.

If the last “live” column of the unknown is the same last “live” column of the standard, then additional evaluation is required. The unknown serum will be considered satisfactory if the average of the last live column in the unknown serum is higher than the corresponding column OD average of the standard wells.

5.5 Unsatisfactory serial

In a valid test, if the last “live” column of the unknown serum sample contains a toxin concentration lower (more dilute) than the standard, the serial is unsatisfactory.

If the last “live” column of the unknown is the same last “live” column of the standard, then additional evaluation is required. The unknown serum will be considered unsatisfactory if the average of the last live column in the unknown serum is lower than the corresponding column OD average of the standard wells.

5.6 Retest Criteria

Unsatisfactory tests may be repeated a maximum of 2 times to rule out technical errors. If both retests are satisfactory in valid tests, the test may be reported as satisfactory.

6. Report of the Test Results

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