United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 411

Supplemental Assay Method for the Titration of Newcastle Disease Vaccine, Infectious Bronchitis Vaccine, and Combination Newcastle Disease-Infectious Bronchitis Vaccine in Chicken Embryos

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Contact: Sandra K. Conrad, (515) 337-7200
Debra R. Narwold

Approvals: /s/Alethea M. Fry for _____ Date: 13Feb18
Geetha B. Srinivas, Section Leader
Virology

/s/Paul J. Hauer _____ Date: 26Feb18
Paul J. Hauer, Director
Policy, Evaluation, and Licensing
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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by: /s/Linda S. Snavely 28Feb18
Linda S. Snavely Date
Quality Management Program Assistant

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Supplemental Assay Method for the Titration of Newcastle Disease Vaccine, Infectious Bronchitis Vaccine, and Combination Newcastle Disease-Infectious Bronchitis Vaccine in Chicken Embryos

1. Introduction

This Supplemental Assay Method (SAM) describes a procedure for titrating Newcastle disease virus (NDV) vaccine, infectious bronchitis virus (IBV) vaccine, and combination NDV-IBV vaccine. The vaccines are reconstituted and inoculated into embryonated chicken eggs in tenfold dilutions such that the 50% egg infective doses (EID_{50}) can be calculated directly on a per field dose basis by the Reed-Muench method.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Centrifuge (Beckman J6-MI, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model 252)

2.1.3 Vortex mixer (Thermolyne Maxi Mix II, Model No. M37615)

2.1.4 Pipette (Rainin Pipetman, P1000)

2.1.5 Cool-lite tester (Val-A)

2.1.6 Egg candling light on stand (Speed King)

2.1.7 Etcher electric engraver (Vibro-graver Acme Burgess Inc.)

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cotton swabs/cotton balls

2.2.2 Serological pipettes (Falcon)

2.2.3 Specific-pathogen-free (SPF) chick embryos, 9- to 11-day-old

2.2.4 Pipette tips (Rainin Clean-Pak disposable microliter pipette tips RT-200)

2.2.5 Syringe, 1-cc tuberculin, single use (Becton, Dickinson and Company)

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2.2.6 Hypodermic needle, 18-gauge x 1 1/2-inch (Becton, Dickinson and Company)

2.2.7 Hypodermic needle, 25-gauge x 5/8-inch (Becton, Dickinson and Company)

2.2.8 Glass test tubes, 16 x 125-mm with Morten closures

2.2.9 Glass test tubes, 13 x 100-mm with Morten closures

2.2.10 Duco cement

2.2.11 Pipette tips (Rainin 0-100, 0-200, 100-1000 or equivalent)

2.2.12 Solutions

1. Tryptose phosphate broth (TPB)

TPB	29.5 g
q.s. with distilled or deionized water (DW)	1000.0 mL

Sterilize by autoclaving.

2. Penicillin/Streptomycin (pen/strep)

penicillin g	500 units/mL
streptomycin	2 mg/mL
q.s. with DW	1000 mL

3. Sterile DW

Sterilize by autoclaving.

4. 70% alcohol

ethyl alcohol	70.0 mL
q.s. with DW	30.0 mL

5. Iodine 2% in alcohol

iodine	2.0 g
ethyl alcohol (70%)	100.0 mL

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies and training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturers' instructions and monitor in compliance with current corresponding standard operating procedures or equivalent.

3.3 Preparation of reagents/control procedures

Prepare reference viruses in the same manner as sample preparation.

3.4 Preparation of the sample

3.4.1 NDV vaccine

1. Reconstitute NDV vaccine, frozen or lyophilized, in 4°C tryptose phosphate broth (TPB) to a total volume as listed in **Table 1**. Vigorously mix for a minimum of 30 seconds, and hold in an ice bath for 15 minutes to allow for virus disaggregation. Mix again and further dilute the reconstituted vaccine in TPB to obtain the final concentration (1 dose per 0.1 mL) as shown in **Table 1**.

2. Make tenfold dilutions of the final virus concentration, 10^{-1} through 10^{-8}, by serially mixing 0.5 mL of the virus with 4.5 mL of TPB plus antibiotics (500-u penicillin and 2.0-mg streptomycin per mL TPB). Use a separate pipette or pipette tip for each transfer. Mix each dilution well before proceeding to the next dilution, and keep all dilutions on ice.

3.4.2 IBV vaccine

Prepare the same as for the NDV vaccine (**Section 3.4.1**), making tenfold dilutions through 10^{-6}.

3.4.3 Combination NDV-IBV vaccine

Prepare the same as for the NDV vaccine. For titration of the Newcastle disease virus fraction, the dilutions are inoculated without further treatment. For titration of the infectious bronchitis virus fraction, neutralize the NDV by adding 0.8 mL of each dilution, 10^{-2} through 10^{-6}, to an equal volume (0.8 mL) of anti-NDV serum. Mix well, and incubate in an ice bath for 30 minutes prior to inoculation.

Table 1

Number of doses in 1 vial	Reconstituted Volume of Vaccine	Additional dilution to reach 1 dose/0.1mL inoculation (or 0.2mL of product + antiserum)
100	10.0 mL	none
500	50.0 mL	none
1,000	30.0 mL	1.5 mL vaccine plus 3.5 mL diluent
2,000	60.0 mL	1.5 mL vaccine plus 3.5 mL diluent
2,500	50.0 mL	2.0 mL vaccine plus 8.0 mL diluent
5,000	100.0 mL	2.0 mL vaccine plus 8.0 mL diluent
10,000	100.0 mL	1.0 mL vaccine plus 9.0 mL diluent
15,000	100.0 mL	1.0 mL vaccine plus 14.0 mL diluent
20,000	100.0 mL	1.0 mL vaccine plus 19.0 mL diluent
25,000	100.0 mL	1.0 mL vaccine plus 24.0 mL diluents

4. Performance of the Test

4.1 Egg inoculation

Prior to reconstituting the vaccine, prepare and label the appropriate number of eggs for allantoic cavity inoculation.

4.1.1 NDV inoculation

Inoculate the 10^{-4} through 10^{-8} virus dilutions of the NDV or NDV-IBV vaccine. Use 5, 9- to 11-day-old embryonated chick eggs per dilution (25 eggs total), and inoculate 0.1 mL of the appropriate dilution per embryo.

4.1.2 IBV inoculation

Inoculate the 10^{-2} through 10^{-6} dilutions of the IBV or the NDV-neutralized NDV-IBV vaccines. Use 6, 9- to 11-day-old embryonated chick eggs (30 eggs total). Inoculate 0.1 mL of the appropriate dilution of the IBV vaccine or 0.2 mL of the appropriate dilution of the NDV-neutralized NDV-IBV vaccine per embryo.

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4.2 Incubation

Incubate the eggs for 7 days candling daily with adjustments made for weekends. Deaths occurring the first 24 hours shall be considered due to trauma and not used in calculations. At least 4 embryos per dilution must be viable at 24 hours postinoculation for a valid test.

5. Interpretation of the Test Results

5.1 Controls

Titrate a known positive reference virus with each group of titrations. The titer of the positive reference virus must be within the established range for the test results to be valid.

5.2 NDV

Record all deaths occurring after 24 hours as positive. On the seventh day postinoculation, open all remaining eggs and examine the embryos. All obviously stunted embryos are considered positive.

5.3 IBV

Record all deaths occurring after 24 hours as positive. On the seventh day postinoculation, open all remaining eggs and examine the embryos for IBV lesions. An embryo exhibiting 1 or more lesions is considered positive.

1. Massachusetts type

Check for stunting, curling, and clubbed down.

2. Other types

Check for stunting, curling, and clubbed down. Open the embryos and check for bile stasis (dark green liver) and kidney urates.

5.4 Calculations

Determine the log 10 EID_{50} titer using the method of Reed-Muench. This dilution and inoculation procedure allow for a direct readout on a per dose basis. Round to 1 decimal.

5.5 Retests

Conduct retests as required by title 9, *Code of Federal Regulations* (9 CFR), section 113.8(b), and requirements of minimum release in firm's current Outline of Production, Part V.

5.6 Evaluation of test results

5.5.1 The 9 CFR 113.8(b) defines the criteria for a satisfactory/unsatisfactory serial.

5.5.2 The firm's requirements of minimum release/stability titers for each vaccine are listed in the current Outline of Production, Part V, for the specific product code.

6. Report of Test Results

Titers are reported out as EID_{50} per bird dose.

7. References

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

7.2 Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493-497.

8. Summary of Revisions

Version .06

- Minor changes to dilution nomenclature.
- Table 1 updated.

Version .05

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for the document.

Version .04

- The Contact information has been updated.

Version .03

- The document number has been changed from VIRSAM0411 to SAM 411.
- The Contact information has been updated.

Version .02

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:

- **2.2.12(2)** 15.775 g of penicillin has been changed to 500 units/ml and 100 g of streptomycin has been changed to 2 mg/ml.
- **2.2.12(3)** The "Normal Saline" formula has been deleted.
- **3.4.3** Table 1 has been updated to provide more clarification.
- **4.2** Clarification has been made to allow for adjustments on weekends.
- **7.1** The reference of the 9CFR was added to the list of References.