United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 315

Supplemental Assay Method for the Detection of Serum Neutralizing Antibodies to Rabies Virus using the Rapid Fluorescent Focus Inhibition Test (RFFIT)

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Table of Contents

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1. Introduction

This Supplemental Assay Method (SAM) describes the Rapid Fluorescent Focus Inhibition Test (RFFIT) which is a serum neutralization method for assaying rabies antibody titers of animals used in rabies immunogenicity tests. The RFFIT utilizes fivefold dilutions of serum and a constant amount of virus inoculated onto baby hamster kidney cells (BHK_{21}). The cells are incubated 20 to 24 hours and stained by the fluorescent antibody technique. The titer of a serum is the dilution at which 50% of the examined microscopic fields contain at least 1 fluorescing cell.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.2 Cabinet, laboratory biosafety level-2

2.1.3 Water bath, 56°± 2°C

2.1.4 Micropipettor, 200-µL and 1000-µL single channel, 5- to 50-µL x 12-channel and tips

2.1.5 Centrifuge and rotor (Model J6-B centrifuge and Model JS-4.0 rotor), Beckman Instruments Inc.

2.1.6 Microscope (fluorescent)

2.2 Cell culture

A baby hamster kidney suspension cell line designated BHK_{21}, 13-S is used.

2.3 Growth medium and diluent

Glasgow minimum essential medium supplemented with 10% tryptose phosphate broth and 10% fetal bovine serum (pH 7.0-7.1) is used to grow cells. The same medium without fetal bovine serum is used as diluent for serum and virus.

2.3.1 Glasgow minimum essential medium (MEM) 20013

1. 12.5 g Glasgow MEM

2. 2.75 g sodium bicarbonate (NA_2HCO_2)

3. 2.0 g tryptose phosphate broth

4. Q.S. to 1000 mL with deionized water (DI).

5. Adjust pH to 6.8

6. Sterilize through a 0.22-µm filter.

7. Store at 2°- 7°C.

2.3.2 Diethylaminoethyl (DEAE) dextran (1%) (National Centers for Animal Health (NCAH) Media #30163)

1. 1.0 g DEAE dextran

2. Q.S. to 100 mL with DI.

3. Sterilize through a 0.22-µm filter

2.3.3 Heat-inactivated, rabies antibody-free horse serum (10 mL)

2.3.4 Kaolin (25%)

1. 25 g kaolin

2. Q.S. to 100 mL with DI.

3. Sterilize through a 0.22-µm filter

2.4 Virus

CVS-11 strain of rabies virus.

2.5 Serum controls

A negative reference serum and a low positive reference serum are assayed in each test.

2.6 Other materials

2.6.1 Polystyrene tubes 12 x 75-mm

2.6.2 1% diethylaminoethyl (DEAE) dextran in growth media

2.6.3 Rabies conjugate, current lot

2.6.4 Plastic 8-chamber tissue culture slides

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience in laboratory dilution techniques and the handling and discarding of human pathogens,

CAUTION: Live rabies is a potential deadly human pathogen! To work with live rabies, personnel must be vaccinated for rabies and monitored for a minimum rabies titer acceptable to appropriate human health officials. The use of appropriate biological safety cabinets is required for dilution of challenge material. All discarded challenge materials should be considered potentially infective and handled in a manner consistent with safe laboratory practices and in accordance with the recommendations of the Centers for Disease Control and Prevention (CDC) and the National Institutes of Health (NIH).

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at 56°± 2°C.

3.3 Preparation of reagents/control procedures

Serum to be tested is heat-inactivated at 56°C for 30 minutes. Equal volumes of serum and 25% kaolin are mixed and allowed to stand at room temperature for 20 minutes with frequent agitation. The mixture is then centrifuged at 1500 x g for 15 minutes to remove the kaolin. Treated serum is considered to be diluted 1:2.

3.4 Preparation of the sample

Each serum is diluted in the chambers of the plastic slide (4 chambers/serum). A 0.05 mL volume of diluent (Glasgow medium with 10% tryptose phosphate broth and without fetal bovine serum) is dispensed into the first chamber and 0.2 mL is dispensed into the next 3 chambers. An initial 1:2.5 dilution is made by adding 0.2 mL of treated serum

(considered a 1:2 dilution) to the 1st chamber. The contents of the chamber are mixed, 0.05 mL is transferred to the next chamber to make a fivefold dilution, and 0.1 mL from the 1st chamber is discarded. The fivefold dilution process is continued for the next 2 chambers with 0.1 mL being discarded from the 2nd and 3rd chambers and 0.15 mL being discarded from the 2nd and 3rd chambers and 0.15 mL being discarded from the 4th chamber. The final result is 4 fivefold dilutions (1:2.5, 1:12.5, 1:62.5, and 1:312.5) of 0.1 mL in each chamber.

4. Performance of the Test

4.1 Preparation and addition of virus

The CVS-11 strain of rabies virus is diluted to contain 30-100 fluorescing foci $dose_{50}$/0.1 mL. A fluorescing foci $dose_{50}$ (FFD_{50}) is the amount of virus necessary to produce at least 1 fluorescing cell in 50% of the microscopic fields examined. A 0.1 mL volume of diluted virus is added to each chamber. This addition results in final serum dilutions of 1:5 through 1:625. A backtitration (tenfold dilutions) of the indicator virus is performed in tubes and 0.1 mL of the working dilution, the 10^{-1} dilution, and the 10^{-2} dilution are added to chambers containing 0.1 mL of diluent. The backtitration and the virus-serum mixture are incubated in a 5% CO_2 incubator at 37°C for 90 minutes.

4.2 Preparation and addition of cells

Two- to 4-day-old monolayers of BHK_{21}, 13-S cells are split approximately 30 minutes prior to completion of the virus-serum mixture incubation period. The cells are standardized to 8×10^5 cells/mL in growth medium containing 10 µg/mL of DEAE-dextran. The cells are allowed to stand at room temperature for at least 10 minutes. At the conclusion of the 90 minute incubation period, the treated cells are dispensed onto the slides, 0.2 mL per chamber. The slides are then incubated at 37°C in a 5% CO_2 incubator for 20 to 24 hours.

4.3 Staining procedure

The plastic chambers are removed from the slide in such a way that the gasket is left attached to the slide. The slides are gently dipped in Dulbecco's phosphate buffered saline (PBS), fixed in cold (ºC) acetone for 10 minutes and dried. Rabies conjugate is diluted in PBS and dispensed onto each slide. The slides are incubated at 37°C for 30 minutes, placed in a PBS bath for 10 minutes, rinsed in deionized water, and dried.

5. Interpretation of the Test Results

Twenty microscopic fields at 130X are examined in each chamber and the number of microscopic fields containing at least 1 cell with specific rabies fluorescence is recorded. The virus neutralizing titer of a serum is the final dilution of serum in which 10 to 20 fields contain at least 1 fluorescing cell. The serum titer is calculated by the method of Spearman-Kärber.

The results of the serum controls and the virus backtitration are evaluated to determine the validity of the test. At least 15 of 20 fields of the 1:5 dilution of the negative reference should contain fluorescing cells and the titer of the positive control reference should be within 2.5-fold of its established geometric mean. The backtitration of the indicator virus should be within the following ranges:

Undiluted - 20 of 20 fields positive
10^{-1} dilution - $\geq$ 18 of 20 fields positive
10^{-2} dilution - $\leq$ 10 of 20 fields positive

6. Report of Test Results

The virus neutralizing titer of a serum is the final dilution of serum in which 10 to 20 fields contain at least 1 fluorescing cell.

7. References

Smith JS**,** Yager, PA, Baer, GM. A rapid fluorescent focus inhibition test (RFFIT) for determining rabies virus neutralizing antibody. In *Laboratory Techniques in Rabies, 4th ed.*, Meslin, FX, MM Kaplan, and H Koprowski, eds. World Health Organization, Geneva. 1996, Chpt 15.

8. Summary of Revisions

Version .03

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

Version .02 (February 11, 2011)

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:

- The Contact information has been updated.
- **2.1:** A list of test equipment has been added.
- **2.2:** Media formulations have been included in the document.
- **3.1:** Training requirements and associated hazards have been added.
- **3.2:** Criteria for water bath temperature have been added.
- **6:** Criteria for test reporting have been added
- **7:** Reference information has been added.