

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

SAM 314

**Fluorescent Antibody Staining Procedure for Detection of
Extraneous Agents in Master Seeds**

Date: **November 13, 2014**

Number: SAM 314.03

Supersedes: SAM 314.02, June 20, 2011

Standard Requirement: 9 CFR 113.47

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Fluorescent Antibody Staining Procedure for Detection of Extraneous Agents in Master Seeds

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Fluorescent Antibody Staining Procedure for Detection of Extraneous Agents in Master Seeds

1. Introduction

This Supplemental Assay Method (SAM) describes fluorescent antibody staining techniques for detection of extraneous agents in Master Seeds (MS). The procedure utilizes either the direct or indirect method, and uses fluorescein isothiocyanate (FITC) conjugated specific antibody which enables visualization of viral antigen-antibody complexes by ultraviolet (UV) light microscopy. For the direct fluorescent antibody (FA) method, a specific fluorescein-labeled specific antibody binds to the viral antigen. The indirect fluorescent antibody (IFA) staining employs two specific antibodies: 1) The unlabeled primary antibody binds to a specific viral antigen if present in the infected cell; and 2) The fluorescein-labeled anti-species secondary antibody binds to the primary antibody-antigen complex. The antigen-antibody complexes can be visualized with a UV light microscope.

FA or IFA testing is conducted to detect a variety of extraneous agents, and is also used to determine if the MS was adequately neutralized.

The procedures described here are performed on third pass materials generated from MS-inoculated cell cultures and controls as described in the current version of **VIRPRO1013**, *Neutralization and Passage of Master Seed in Cell Cultures*.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Aerobic incubator, $36^{\circ} \pm 2^{\circ}\text{C}$, humidified

2.1.2 Laminar flow biological safety cabinet

2.1.3 Ultraviolet (UV) light microscope

2.1.4 Chemical fume hood

2.2 Reagents/supplies

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

2.2.1 Cultures prepared in chamber slides or other vessels in accordance with the current version of **VIRPRO1013**.

2.2.2 Deionized water (DI)

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- 2.2.3** Acetone (100% or 80% in DI) will be referenced throughout this document; however other fixatives may be used as appropriate.
- 2.2.4** Phosphate buffered saline (PBS), 0.01M, pH 7.2, National Centers for Animal Health (NCAH) Media #30054.
- 2.2.5** Evan's blue biological stain (EBBS), 1.0% stock solution in DI
- 2.2.6** Antigen-specific conjugated polyclonal antiserum or conjugated monoclonal antibody (direct FA)
- 2.2.7** Primary antibody, polyclonal antiserum or monoclonal antibody, specific to the virus (IFA)
- 2.2.8** Conjugated secondary antibody or anti-species conjugate specific to the species in which the primary antibody was produced (IFA)
- 2.2.9** Microscope slide glass staining dish with rack or Coplin staining jar
- 2.2.10** Miscellaneous lab supplies: Beakers, pipettes, wash bottle
- 2.2.11** Mounting medium (e.g., 10% glycerol, 50% glycerol, etc.)

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel performing this procedure must have experience or training in: aseptic biological laboratory techniques, cell culture, preparation, handling, and disposal of biological agents and chemicals. Personnel must also have knowledge of safe operating procedures and training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

Turn on biological safety cabinet at least 15 minutes before use.

3.3 Preparation of reagents/control procedures

On the day of the FA staining, dilute reagents in **Sections 2.2.6 through 2.2.8** with PBS to a previously determined optimal concentration for the detection of antigens.

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3.4 Preparation of the sample

The following procedures are followed for cell cultures grown in chambered slides, plates, or dishes.

Cell cultures are prepared for FA staining according to the current version of **VIRSOP2007**, *Master Seed Testing in the Virology Section*. Seven days after the last subculturing, MS inoculated and control monolayers are fixed and FA stained. Positive and negative control slides, prepared concurrently with the MS slides, are used for verification of the assay. To ensure or enhance fluorescence detection, additional MS and positive control monolayers may be fixed before day 7. Regardless of when monolayers are fixed, they shall be stained concurrently.

Previously prepared rabies virus positive control slides are available through the Center for Veterinary Biologics (CVB).

3.5 Fixation of cell cultures

Caution: Use fixative in a fume hood or biological safety cabinet. Gloves will be worn to avoid skin contact. Fixatives may be flammable; keep away from sources of heat or flame.

3.5.1 Chamber Slides

1. Decant the media from the chamber slides into an autoclavable container and remove the plastic walls, leaving the slides with the gasket attached. Handle and dispose of all viral fluids according safe laboratory practices.
2. Place the slides in a slide rack and immerse the loaded slide rack in a staining dish filled with PBS, and wash for 10 ± 5 minutes
3. Remove the slides from the PBS and place in a staining dish filled with acetone for 10 ± 5 minutes. Use 100% acetone for glass slides and 80% acetone for plastic slides. Remove the slides and allow to air dry. Used acetone should be collected for proper disposal.
4. If cell preparations are not immediately FA stained, store the slides at -20°C or colder until use. Length of storage will vary for each antigen. Adequacy for use is monitored by FA staining.

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3.5.2 Cell culture plates or dishes

1. Decant the media from the plate/dish into an autoclavable container. Handle and dispose of all viral fluids according to safe laboratory practices.
2. Gently wash cell monolayer with PBS and decant.
3. Fix cell monolayer with 80% acetone for 10 ± 5 minutes.
4. Decant the acetone into a suitable container; gently blot the plate/dish onto an absorbent surface and allow the plate/dish to air dry. Used acetone should be collected for proper disposal.
5. Stain plate immediately or store at -20°C or colder until use. Length of storage will vary for each viral antigen. Adequacy for use is monitored by FA or IFA fluorescence of positive control.

4. Performance of the Test

Note: Volumes of reagents used in part 4.1 are for 2-chambered slides. When different vessels are stained, volumes may be adjusted to compensate for differences in cell monolayer area. Regardless of vessel used, a minimum surface area of 6 cm^2 is required for the final reading for both material under test and each of the controls.

4.1 Direct FA/Indirect FA

- 4.1.1 Apply $300 \pm 25\ \mu\text{L}$ of fluorescein-labeled antibody (FA) or primary antibody (IFA) to cover the fixed cell monolayer. Slides should not be allowed to dry during staining process.
- 4.1.2 Incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ for 40 ± 10 minutes in a humidified chamber.
- 4.1.3 Decant the antibody from the slides. Rinse slides three times in PBS for approximately 5 ± 2 minutes per rinse. After final rinse, gently tap slide onto absorbent material to remove excess PBS.
- 4.1.4 For IFA, repeat sections **4.1.1 through 4.1.3** using a secondary fluorescein-labeled antibody.
- 4.1.5 Following PBS rinses, stained slides should be quickly rinsed in DI.

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4.1.6 Gently tap the DI from the slides.

Note: Counterstaining fluorescein-stained cell culture is optional and sometimes desirable to quench nonspecific background fluorescence or to heighten contrast between specific staining and background refer to Section 4.1.7.

4.1.7 For counterstaining (**optional**), make a predetermined dilution of the EBBS stock solution in DI which is based on the requirements for individual viruses. Apply the diluted EBBS to slides and incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ for 20 ± 10 minutes in a humidified chamber. Decant the EBBS and quickly rinse with distilled water. Gently tap the slides onto an absorbent surface.

4.1.8 Every effort should be made to read the slides immediately after staining. Alternatively, slides may be stored in the dark at $4^{\circ} \pm 2^{\circ}\text{C}$ for up to 7 days. Prior to storage, slides must be air dried or overlaid with mounting medium. Air dried slides must be moistened using DI or mounting medium prior to reading.

4.2 Reading

4.2.1 The stained cell cultures are observed at 100X to 200X magnification in a darkened room with the use of a UV light microscope.

4.2.2 Results of observations to be recorded.

5. Interpretation of the Test Results

Cells exhibiting specific apple-green fluorescence are considered positive. For a valid test, the positive cell culture controls must contain a sufficient number of infected cells to easily determine positive status; however, non-infected cells should also be present in order to differentiate positive cells from negative cells. Negative control wells must be free of specific fluorescence. Additionally, the MS inoculated cell cultures will be stained to confirm neutralization and must remain free of specific fluorescence.

If agent specific fluorescence is observed in the MS inoculated cell monolayers, the MS will be determined to be unsatisfactory. The MS may be retested to confirm original results if warranted by the agent contact or reviewer.

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6. Report of Test Results

All records are kept in accordance with the current recordkeeping practices. Test results will be reviewed and signed by the agent contact. Test results are then entered into the current reporting system and released to the reviewer for distribution to the firm.

7. References

Title 9, *Code of Federal Regulations*, part 113.309, U.S. Government Printing Office, Washington, DC.

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

This is a complete revision of an existing document to reflect current practices in place at the Center for Veterinary Biologics.

- The Contact information has been updated.