Animal and Plant Health Inspection Service

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Fluorescent Antibody Staining Procedure for Detection of Viral Antigens

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Notes:

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Table of Contents

1. Introduction

This testing protocol (PRO) describes a fluorescent antibody staining method for detection of viral antigens. The protocol uses fluorescein isothiocyanate (FITC) conjugated antibodies for visualization of viral antigen-antibody complexes by ultraviolet (UV) light microscopy. The direct fluorescent antibody (FA) method uses a specific FITC-labeled antibody which binds to the viral antigen directly. The indirect fluorescent antibody (IFA) method uses two specific antibodies: 1) unlabeled primary antibody which binds to a specific viral antigen, and 2) FITC-labeled anti-species secondary antibody which binds to the primary antibody-antigen complex. These methods may be used for:

- Detection of extraneous agents in master seed viruses (MSV)
- Detection of extraneous agent in master cells stocks (MCS)
- Determining MSV neutralization
- Performance of viral titrations
- Performance of identity testing
- Detection of extraneous agents in ingredients of animal origin (FBS. Etc.,)
- Reagent evaluation

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°± 2°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 reagents listed below.

2.2.1 Deionized water (DI)

2.2.2 Acetone (100% or 80% in DI) will be referenced throughout this document; however, other fixatives may be used as appropriate.

2.2.3 Phosphate buffered saline (PBS), 0.01M, pH 7.2, National Centers for Animal Health (NCAH) Media #30054

2.2.4 FA rinse buffer NCAH Media #30446

2.2.5 Evan's blue biological stain (EBBS), 0.1% stock solution in DI

2.2.6 Antigen-specific FITC-conjugated polyclonal antiserum or conjugated monoclonal antibody (direct FA)

2.2.7 Primary antibody, polyclonal antiserum or monoclonal antibody, specific to viral antigens (IFA)

2.2.8 FITC-conjugated secondary antibody or anti-species conjugate specific to the species in which the primary antibody was produced (IFA)

2.2.9 Chambered slides: 1-, 2-, 4-, or 8-well

2.2.10 Cell culture plates, polystyrene: 24-, 48-, or 96-well

2.2.11 Microscope slide staining dish with rack or Coplin staining jar

2.2.12 Miscellaneous lab supplies: beakers, pipettes, wash bottle

2.2.13 Mounting medium (50% glycerol and 50% FA rinse buffer)

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel performing this protocol 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.

3.4 Fixation of cell cultures

Caution: Use fixative in a fume hood or biological safety cabinet. Gloves must be worn to avoid skin contact. Fixatives may be flammable; keep away from sources of heat or flame. Collect used acetone for proper disposal.

3.4.1 Chamber Slides

1. Decant media from chamber slides into an autoclavable container and remove the plastic walls, leaving slides with the gasket attached. Handle and dispose of all viral fluids according to the current version of **CVB-SOP-0137**, *Disposal of Live Agents*.

2. Place slides in a slide rack and immerse the loaded slide rack in a staining dish filled with PBS, wash for 10 ± 5 minutes.

Note: The decision to rinse slides with PBS is optional and is based upon the condition of the cell culture monolayer as observed by the technical staff.

3. Remove slides from 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.

4. If cell preparations are not immediately stained, store monolayers at -20°± 2°C or colder until use. Length of acceptable storage will vary for each antigen. Adequacy for use is monitored by FA or IFA staining.

3.4.2 Cell culture plates or dishes

1. Decant the media from plate/dish into an autoclavable container. Handle and dispose of all viral fluids according to the current version of **CVB-SOP-0137**.

2. With a wash bottle, gently rinse cell monolayer with PBS and decant.

Note: The decision to rinse slides with PBS is optional and is based upon the condition of the cell culture monolayer as observed by the technical staff.

3. If a PBS rinse is used, fill the plate/dish with PBS and allow cell culture layer to soak for 10 ± 5 minutes.

4. Decant PBS from plate/dish.

5. Fix cell monolayer with 80% acetone for 10 ± 2 minutes.

6. Decant acetone into a suitable container; gently blot vessel onto an absorbent surface and allow to air dry.

7. If cell preparations are not immediately stained, store vessels at -20°± 2°C or colder until use. Length of acceptable storage will vary for each antigen. Adequacy for use is monitored by FA or IFA staining.

4. Performance of the Test

4.1 Direct FA/Indirect FA

4.1.1 Apply appropriate volume of specific fluorescein-labeled antibody (direct FA) or primary antibody (IFA) to cover the fixed cell monolayer (**Appendix**). Monolayers should not be allowed to dry during staining process.

4.1.2 Incubate at 36°± 2°C for 25 to 65 minutes in a humidified chamber.

4.1.3 Decant antibody from monolayers. Rinse at least twice in FA rinse buffer or PBS for 5 ± 2 minutes per rinse. After final rinse, gently tap vessel onto absorbent material to remove excess buffer or PBS buffer.

4.1.4 For IFA, repeat **Sections 4.1.1 through 4.1.3** using a secondary FITC-labeled antibody.

4.1.5 Gently tap excess FA rinse buffer from monolayers.

4.1.6 Monolayers may be rinsed with DI water to remove buffer salts.

Note: Counterstaining FITC-stained monolayers is often desirable to quench nonspecific background fluorescence or to heighten contrast between specific staining and background. Refer to Section 4.1.7.

4.1.7 (Optional) For counterstaining, dilute EBBS stock solution directly in conjugate (1:1000-1200) or in DI (1:1000). Apply EBBS diluted in DI to monolayers and incubate at 36°± 2°C for 20 ± 10 minutes in a humidified chamber. Decant EBBS and quickly rinse with DI. Gently tap vessel onto an absorbent surface.

4.2 Reading

4.2.1 Monolayers should be read wet and immediately after staining. Vessels are retained for review and shall be stored in the dark at 4°± 2°C for up to 7 days. Prior to storage, monolayers must be air dried or overlaid with mounting medium. Air dried monolayers must be re-moistened using mounting medium, FA rinse buffer or DI water before review.

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

4.2.3 Record results at the time of observation on the appropriate paperwork.

5. Interpretation of the Test Results

5.1 After staining, each group of monolayers shall be examined for the presence of specific fluorescence attributable to the presence of extraneous viruses. Cells exhibiting specific apple-green fluorescence are considered positive. Non-infected controls should have little nonspecific background fluorescence and be distinguishable from infected controls.

Note: The above are general guidelines for evaluating stained monolayers. The Agent Contact shall review monolayers to confirm results.

5.2 Specifically for MSV testing, a **VALID** test requires:

5.2.1 The negative control monolayers are free from specific fluorescence. If the negative control monolayers contain specific fluorescence, this indicates possible viral cross contamination, the test is INVALID, and must be repeated.

5.2.2 The positive control monolayers exhibit the expected fluorescence. If specific fluorescence is absent in the positive monolayers, the test is INVALID, and must be repeated.

Note: The positive 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.

5.2.3 The MSV inoculated cells are free from specific fluorescence to the MSV. If the MS inoculated cells contain specific fluorescence to the MSV, this indicates incomplete neutralization and/or replication of MSV, the test is INVALID, and must be repeated.

5.2.4 If the test is valid and the MSV test monolayers are equivocal, then the test is declared INCONCLUSIVE, and must be repeated.

5.2.5 If the test is valid and the MSV test monolayers are negative for specific fluorescence, then the MSV is SATISFACTORY.

5.2.6 If the test is valid and specific fluorescence is found in the MSV test monolayers, then the test MSV is UNSATISFACTORY. At supervisory discretion, the test may be repeated to confirm result.

6. Report of Test Results

All records are kept in accordance with the current recordkeeping practices. Test results will be reviewed and signed by knowledgeable staff (such as the Agent Contact, Microbiologist, or Lead Technician). 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*, sections 113.47 and 113.309, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version CVB-PRO-0040.02

- The document identification number has been changed from VIRPRO1014 to CVB-PRO-0040.
- Lot numbers changed on several viruses as positive control in table.

Version VIRPRO1014.06

- The FA use list has been updated to include reagents used to detect and test for Seneca Valley virus.

Version VIRPRO1014.05

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

Version VIRPRO1014.04

- The Contact information has been updated.
- **1:** The FA use list has been expanded to include MCS and ingredients of animal origin.
- **2.2:** Redundant equipment has been removed from the list.
- **3.4:** An optional PBS wash has been included.
- **4.1.2:** A time range for the incubation time of fluorescein-labeled antibody or primary antibody has been added.
- **4.1.3:** The antibody rinse of slides or plates has been changed from 3 to 2. This reflects the common practice which is in place at the CVB. Also added the option of using PBS to rinse plates or slides.
- **4.1.6:** The option of using a DI rinse to remove residual salts has been added.
- **4.2.1:** The option of using wetting slides or plates with either PBS or DI water, prior to reading has been added.
- **6:** The description of staff able to review results has been updated.
- **Appendix I:** Volume ranges are now included.
- **Appendix II:** A table which includes the recommended positive control viruses, and their use dilutions, and the test detection reagents and applicable use dilutions has been added to the document.

Version VIRPRO1014.03

- The Contact information has been updated.
- National Veterinary Services Laboratories (NVSL) has been changed to National Centers for Animal Health (NCAH) throughout the document.

Version VIRPRO1014.02

- The title of this document has been revised and the document has been rewritten to superseded VIRSOP1500 and VIRPRO1010.
- An appendix has been added to include volumes of reagents used for various vessels.

Appendix I
Volume of Antibody to be used for FA Staining per Vessel

Vessel	Volume/chamber or well (µL)
2-chamber slides	200-300
4-chamber slides	100-150
8-chamber slides	50-75
24-well plates	200-300
48-well plates	100-150
96-well plates	50-75
60 mm plates	2000

Appendix II

Agent	Detection Method	Primary antibody	Concentration	Conjugate identification	Conjugate concentration	Cell line	Required by 9CFR	Positive control (PC)	Lot Titer	PC concentration (log)
BVDV-1	*FA (Direct)*	*N/a*	*N/a*	CJ-F-BVD-10ML	*Undiluted*	BT	√	00-17	$10^{6.6}$/2mL	3 -4 (1:1000)
BVDV-2	*FA (Direct)*	*N/a*	*N/a*	CJ-F-BVD-10ML	*Undiluted*	BT	√	05-15	$10^{6.3}$/2mL	3 -4 (1:1000)
Blue Tongue Serotype 10	*FA (Direct)*	*N/a*	*N/a*	CJ-FA-BTV-MAB-10ML	*Undiluted*	BT	√	85-9	$10^{4.5}$/1mL	2.5 (1:100)
Rabies	*FA (Direct)*	*N/a*	*N/a*	*Fujireloio 800-092*	*1:100*	*Any*	√	*Virology Slides*	*N/a*	*N/a*
Reovirus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-REO-10ML	*Undiluted*	Vero	√	01-17	$10^{5.0}$/1mL	3 (1:100)
Bovine										
Bovine adenovirus type 1	*FA (Direct)*	*N/a*	*N/a*	CJ-F-BAV1-10ML	*Undiluted*	MDBK-A	√	06-39	$10^{5.7}$/2mL	3 (1:100)
Bovine adenovirus type 5	*FA (Direct)*	*N/a*	*N/a*	CJ-F-BAV5-10ML	*Undiluted*	BT	√	04-04		2-3 (1:100)
Bovine coronavirus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-BCV-10ML	*Undiluted*	MDBK	N/a	05-01		2
Bovine parvovirus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-BPV-10ML	*Undiluted*	BT	√	99-09	$10^{5.2}$/1mL	4 (1:10)
Bovine respiratory syncytial virus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-BRSV-10ML	*Undiluted*	BT	√	88-16	$10^{5.4}$/2mL	3 (1:100)
Infectious bovine rhinotracheitis virus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-IBR-10ML	*Undiluted*	BT	N/a	84-5, 92-12	$10^{6.2}$/1mL	4 (1;100)
Parainfluenza virus type 3	*FA (Direct)*	*N/a*	*N/a*	CJ-F-PI#-10ML	*Undiluted*	BT	N/a	84-6	$10^{7.0}$/1mL	4 (1:1000)
Porcine										
Swine adenovirus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-PAV-10ML	*Undiluted*	*B-ST/PK-15*	√	*Strain NADC lot 85-17*	$10^{6.3}$/1mL	3-4 (1:100-1:1000)
Porcine parvovirus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-PPV-10ML	*Undiluted*	*B-ST/PK-15*	√	*Strain NADL lot 87-6*	$10^{4.5}$/1mL	2-3 (1:100)

Agent	Detection Method	Primary antibody	Concentration	Conjugate identification	Conjugate concentration	Cell line	Required by 9CFR	Positive control (PC)	Lot Titer	PC concentration (log)
Transmissible gastroenteritis virus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-TGE-10ML	*Undiluted*	*B-ST/PK-15*	√	*Strain M59S, lot M59S*		1.5
Porcine hemagglutinating encephalomyelitis virus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-PHEV-10 ML	*Undiluted*	*B-ST/PK-15*	√	*HEV e lot 16-02*		2
Porcine circovirus type 1							N/a			
Porcine circovirus type 2	*FA (Direct)*	*N/a*	*N/a*	CJ-F-PCV2-10ML	*Undiluted*	*B-ST/PK-15*	N/a	*PCV2/6 88 12-4-01*		*2.5-3*
Porcine respiratory and reproductive virus						Marc 104	N/a			
Swine influenza Virus						MDCK	N/a			
Seneca Valley Virus	FA (Indirect)	88-3669S 4A	1:20	KPL Goat anti-swine Catalog # 02-14-06	1:100	CEF	N/a	18-02	TBD	TBD
Equine										
Equine herpesvirus type 1	*FA (Direct)*	*N/a*	*N/a*	CJ-F-ERV-10ML	*Undiluted*	EQ Der	√	5-85		4 (1:10)
Equine herpesvirus type 4	*FA (Direct)*	*N/a*	*N/a*	CJ-F-ERV-10ML	*Undiluted*	EQ Der	√	01-03	$10^{4.8}$/2mL	3 (1:100)
Equine herpesvirus type 4 (confirmation)	*FA (Indirect)*					EQ Der				
Equine arteritis virus	FA (Indirect)	CVB	1:100	92-10	Secondary: KPL anti-equine (1:100)	EQ Der	√	94-10	$10^{6.1}$/2mL	5 (1:10)
Equine infectious anemia virus						EQ Der	N/a			
Equine influenza virus	FA (Indirect)	CVB	1:50-1:100	06-31	Secondary: NVSL anti-chicken 291 FA 860 2	MDCK	N/a	92-7 or 1-86 or 79	Vary	1:100

Agent	Detection Method	Primary antibody	Concentration	Conjugate identification	Conjugate concentration	Cell line	Required by 9CFR	Positive control (PC)	Lot Titer	PC concentration (log)
Canine										
Canine coronavirus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-CCV-10ML	*Undiluted*	*CrFK*	√	07-06	$10^{5.2}$/1mL	3 (1:100)
Canine distemper virus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-CDV-10Ml or CJ-F-CDV-MAB-10ML	*Undiluted*	*MDCK*	√	99-05	$10^{4.7}$/1mL	2.7 (1:100)
Canine parvovirus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-CPV-MAB-10Ml or CJ-F1-CPV-10ML	*Undiluted*	*CrFK*	√	96-05	$10^{6.4}$/1mL	4.4 (1:100)
Canine parainfluenza virus	FA (Direct)	*N/a*	*N/a*	CJ-F-CPI-10ML	*Undiluted*	*Vero*	N/a	96-16	$10^{8.5}$/1mL	3-4 (1:1000-1:10000
Canine herpes virus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-CHV-10ML	*Undiluted*	MDCK	N/a	VR552 or 97-16	$10^{6.79}$/1mL	5 (1:100)
Canine adenovirus type 2	*FA (Direct)*	*FA (Direct)*	*FA (Direct)*	CJ-F-CAV-10mL	*Undiluted*	*MDCK*	N/a	06-44	$10^{7.74}$/1mL	5 (1:1000)
Infectious canine hepatitis virus (CAV1)	*FA (Direct)*	*FA (Direct)*	*FA (Direct)*	CJ-F-CAV-10ML	*Undiluted*	*MDCK*	N/a	06-44	$10^{7.74}$/1mL	5 (1:1000)
Canine influenza virus						*MDCK*	N/a			
Feline										
Feline infectious peritonitis virus	*FA (Direct)*	*N/a*	*N/a*	CJ-F-FIP-10ML	*Undiluted*	CrFK	√	06-42		4
Feline parvovirus	*FA (Direct)*	N/a	N/a	CJ-F-FPL-10ML	*Undiluted*	CrFK	√	02-14	$10^{6.83}$/1mL	3 (1:1000)
Feline calicivirus	FA (Indirect)	*CVB*	*1:80*	*96-02*	*Secondary: KPL anti-cat (1:100)*	CrFK	N/a	96-14	$10^{5.2}$/1mL	3 (1:100)
Feline rhinotracheitis virus	*FA (Direct)*	*N/a*	*N/a*	*CJ-F-FVR-10ML*	*Undiluted*	CrFK	N/a	96-15	$10^{8.26}$/1mL	5 (1:1000)
Feline leukemia virus	FA (Indirect)	210-46-FeLV1	*Undiluted*	AB1-FELV and AB2-FELV	*Undiluted*	CrFK	N/a	95-09 (Challenge)	$10^{6.0}$/1mL	4 (1:100)