United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Neutralization and Passage of Master Seed in Cell Cultures

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Neutralization and Passage of Master Seed in Cell Cultures

1. Introduction

This testing protocol (PRO) describes the neutralization and passage of viral and obligate intracellular parasite master seeds (MS) in cell culture for the detection of extraneous agents as described in title 9, *Code of Federal Regulations* (9 CFR), section 113.55. The identification of extraneous agents is determined by methods described in the 9 CFR 113.46 and 113.47.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Incubator, 36°± 2°C, humidified

2.1.2 Water bath, 36°± 2°C

2.1.3 Microscope, inverted bright light

2.1.4 Vortex mixer

2.1.5 Pipette-aid automatic pipettor (Drummond Scientific) or equivalent

2.1.6 Eppendorf micropipettors (1000-µL, 200-µL, 100-µL) or equivalent

2.1.7 Centrifuge and rotor, low speed

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 Pipette tips

2.2.2 75-cm^2 tissue culture flasks, sterile

2.2.3 25-cm^2 tissue culture flasks, sterile

2.2.4 Glass slides, 2-chamber

2.2.5 Tissue culture monolayers meeting the requirements in the 9 CFR 113.51 and 113.52

2.2.6 Positive control viruses

2.2.7 Monospecific antisera to neutralize MS

2.2.8 Minimum essential medium (MEM) (National Centers for Animal Health (NCAH) Media #20030)

1. 9.61 g MEM with Earles salts without bicarbonate

2. 2.2 g sodium bicarbonate ($NaHCO_3$)

3. Dissolve reagents in **Steps 1 and 2** with 900 mL deionized water (DI)

4. Add 5 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to 60°± 2°C until dissolved. Add to **Step 3** with constant stirring.

5. Q.S. to 1000 mL with DI; adjust pH to 6.8-6.9 with 2 N hydrochloric acid (HCl)

6. Sterilize through a 0.22-µm filter

7. Aseptically add:

a. 10 mL L-glutamine (200 mM stock) (Replenish appropriate volume every 2 weeks.)

b. 50 µg/mL gentamicin sulfate

8. Store at 4°± 2°C

2.2.9 Growth Medium

1. 900 mL of MEM

2. Aseptically add 50-100 mL gamma-irradiated fetal bovine serum (FBS)

3. Store at 4°± 2°C

2.2.10 Maintenance Medium

1. 900 mL of MEM

2. Aseptically add 50-200 mL gamma-irradiated FBS

3. Store at 4°± 2°C

2.2.11 0.01 M Phosphate buffered saline (PBS) (NCAH Media #30054)

1. 1.33 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2. 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)

3. 8.5 g sodium chloride (NaCl)

4. Q.S. to 1000 mL with DI.

5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 2 N HCl.

6. Sterilize by autoclaving at 15 psi, 121°± 2°C for 35 ± 5 minutes.

7. Store at 4°± 2°C.

2.2.12 Trypsin-versene solution (NCAH Media #20005)

1. 8 g sodium chloride (NaCl)

2. 0.4 g potassium chloride (KCl)

3. 1.0 g dextrose

4. 0.58 g sodium bicarbonate ($NaHCO_3$)

5. 0.5 g irradiated trypsin

6. 0.2 g Versene (EDTA)

7. 0.4 mL phenol red (0.5%)

8. Q.S. to 1000 mL with DI.

2.2.13 Chloroform (Fisher #C298-500)

2.2.14 Cell culture plate, 96-well

2.2.15 12 x 75-mm tubes

2.2.16 Serological pipette, 10-mL

2.2.17 Graduated cylinders, 25-mL, 50-mL, 100-mL, and 250-mL, sterile

2.2.18 Current version of **VIRTWS2001**, *Master Seed Virus Extraneous Agent Testing – Tested According to VIRSOP2007*

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience in the preparation and maintenance of cell culture, propagation and maintenance of animal viruses, and evaluation of virus infectivity by CPE. Personnel must be familiar with use of test reagents and biological materials. Personnel must have knowledge of safe operating procedures, polices, and guidelines. Training in the operation of the instruments and equipment used is required for performance of this test. Personnel should maintain aseptic techniques throughout these procedures.

Technician staff and the VMO/Micro Agent Contact should meet and plan testing strategy for each MS presented for testing. Technician staff must keep the VMO/Micro Agent Contact informed of testing progress and provide updates as agreed upon during the planning phase.

3.2 Determination of culture system

According to 9 CFR 113.55, each MS should be tested on monolayers of:

- African green monkey kidney (Vero) cells
- Cells of the species for which the vaccine is recommended
- Cells of the species for which the MS was isolated and/or propagated

In addition, each MS should be tested on monolayers of:

- Cells of the species from which the virus was obtained

Detection of extraneous agents on cell monolayers following passage must be performed according to 9 CFR 113.46 and 113.47. According to 9 CFR 113.47, the most permissive cell monolayer will be tested for rabies virus, reovirus, bovine viral diarrhea virus (BVD), and bluetongue virus (BT). See **Appendices I and II** for the appropriate culture systems to use with each virus.

Example: A BVD MS derived from an infected cow was isolated in swine kidney cells and propagated in canine kidney cells. In addition to being tested for reovirus, rabies, BVD, and BT, the MS should also be tested for extraneous agents from bovine, porcine, and canine species.

Note: All MS which were isolated and/or propagated in cell cultures must be tested according to 9 CFR 113.55. However, MS which have been propagated exclusively in eggs are exempt from 9 CFR 113.55. In addition, all MS of avian origin or MS that have been egg propagated will also be tested according to 9 CFR 113.31, 113.34, and Veterinary Services Memorandums No. 800.88 and 800.89 for:

- Avian lymphoid leukosis (**VIRPRO0415**, *Detecting Extraneous Avian Leukosis Virus in Biologic Products by p27 ELISA*),
- Hemagglutinating viruses (**VIRPRO0096**, *Detection of Hemagglutinating Viruses*),
- Reticuloendotheliosis virus (**VIRPRO0119**, *Polymerase Chain Reaction Assay for Detection and Identity of Extraneous Reticuloendotheliosis Virus (REV)*), and
- Chicken anemia virus (**VIRPRO0118**, *Polymerase Chain Reaction Assay for Detection and Identity of Extraneous Chicken Anemia Virus (CAV)*)

All cells used in MS testing must be satisfactory when tested as prescribed in 9 CFR 113.51 and 9 CFR 113.52. Embryonated eggs must also meet the purity and quality requirements as prescribed in Veterinary Services Memorandum No. 800.65.

3.3 Preparation and neutralization of MS

If the MS is capable of replicating in the cell or culture system in which it is to be tested, the MS must be neutralized (monospecific antiserum, monoclonal antibody, or alternative method, i.e., chloroform, tetracycline) prior to testing for extraneous agents. If the MS is not capable of replicating in the culture system, then the MS may not require neutralization.

Requirements for monospecific neutralizing antiserum:

- Antiserum should be prepared using antigen that is not derived from any passage level of the virus isolate giving rise to the MS to be tested.
- Antiserum must be free of antibodies to potential contaminants of the seed virus.
- Antiserum must be free of any non-specific inhibitors and complement which would prevent infection of extraneous agents in cell or culture systems.

3.3.1 Record information on current version of **VIRTWS2001**.

3.3.2 Rapidly thaw frozen MS in a 36°± 2°C water bath or rehydrate lyophilized MS according to manufacturer's instructions. Rehydrated MS should be allowed to equilibrate at room temperature for 15 ± 2 minutes prior to neutralization.

3.3.3 Transfer 1 mL into a labeled appropriate sized sterile tube of MS for each cell monolayer type used for testing.

3.3.4 Add an equal volume (1 mL) of heat-inactivated (56°± 2°C for 30 ± 5 minutes) MS monospecific antiserum to tube containing MS.

Note: Based on the antiserum neutralizing activity, additional antiserum may be required to fully neutralize MS.

3.3.5 Gently vortex the MS-antiserum mixture.

3.3.6 Incubate the MS-antiserum mixture at 36°± 2°C for 60-120 minutes to allow for neutralization of the MS.

3.4 Alternative neutralization methods - Chloroform treatment

Chloroform treatment may be used to test for specific extraneous agents corresponding to extraneous antibodies present in the neutralizing antiserum.

For the treatment to be effective the following criteria must be met:

- MS must be an enveloped virus;
- Neutralizing antiserum contains extraneous antibodies specific for non-enveloped viruses;
- MS should be tested for specific extraneous agents corresponding extraneous antibodies present in the neutralizing antiserum.

3.4.1 Transfer 0.1 mL of chloroform per mL of MS to a **glass** centrifuge tube using **glass** pipettes.

3.4.2 Vortex MS-chloroform mixture for 5 ± 1 minutes at room temperature.

3.4.3 Centrifuge at 1295 × g (2500 rpm in Beckman Avanti J-E centrifuge with a JS 5.3 rotor or equivalent) for 15 minutes at 4°± 2°C.

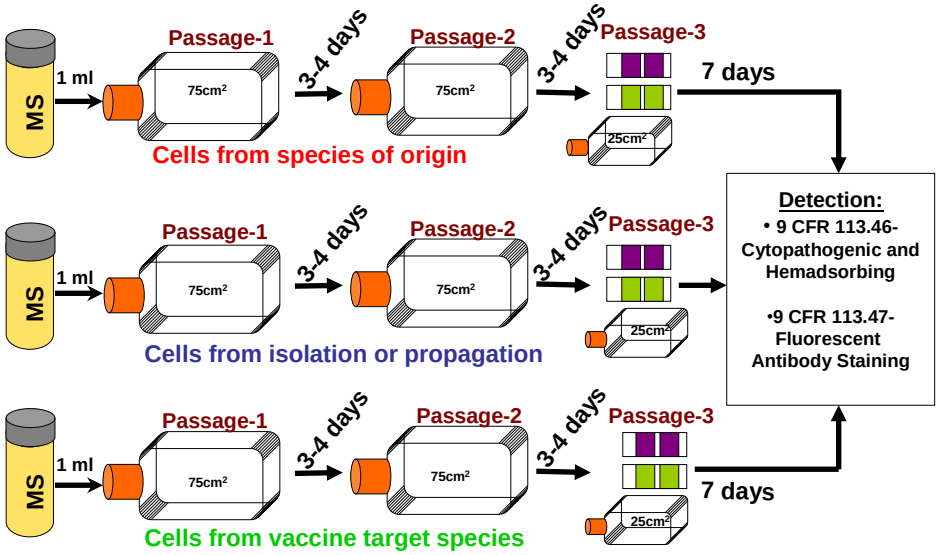
3.4.4 Harvest supernatant for inoculation (Refer to **Section 4.1**)

Note: Chloroform is a U-List hazard waste and should be disposed of in accordance with SAFSOP0005, *Hazardous Waste Satellite Accumulation Areas,* and SAFSOP0006, *Hazardous Waste Identification*.

4. Passage of MS in Permissive Cell Culture Substrate

For each type of cell monolayer used for testing, seed two 75-cm^2 cell culture flasks with a permissive cell type monolayer at a cell count that will produce a ≥ 75% confluent monolayer within 24 to 48 hours. Inoculate one flask with MS-antiserum mixture or non-neutralized MS (inoculum). Reserve one uninoculated flask as a negative control. For the inoculated flask, the 9 CFR 113.55 requires each cell monolayer to be inoculated with at least 1 mL of test MS. If the MS requires neutralization, a flask will be inoculated with 2 mL of test MS-antiserum mixture. If a greater volume of neutralizing antiserum was required to accomplish MS neutralization, the amount of MS-antiserum mixture added to the flask(s) needs to be adjusted accordingly. For example, if 2 mL of antiserum is required to neutralize 1 mL of MS, then 3 mL of MS-antiserum mixture must be added to each cell monolayer. An overview of MS testing for extraneous viral agents by serial passages in cell culture is illustrated in **Diagram 1**.

Diagram 1. Passage and detection of extraneous agents in MS
(9 CFR 113.55)



4.1 Inoculation of permissive cells (First MS passage)

4.1.1 Discard the growth medium from all cell monolayers.

4.1.2 Wash all cell monolayers with 5 mL warm MEM. Discard MEM.

4.1.3 Inoculate each MS test monolayer with the inoculum, and allow for adsorption at 36°± 2ºC for 1 hour. Sham inoculate each negative control monolayer with an equivalent volume of MEM.

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Note: Exceptions listed in footnotes of Appendices I and II.

4.1.4 Rock inoculated flask mechanically or manually at 15-minute intervals during incubation to ensure virus adsorption.

4.1.5 Discard inoculum following one hour adsorption to reduce serum toxicity and remove possible unneutralized residual MS virus.

4.1.6 Wash each monolayer once with 5 mL warm MEM.

Note: MS that were not treated with antiserum and the corresponding negative control do not require post adsorption discard and wash step.

4.1.7 Add 25 mL of maintenance medium containing 5-20% of FBS to each flask.

4.1.8 Incubate inoculated monolayer flask at 36°± 2ºC for 3 to 4 days.

4.1.9 Examine inoculated monolayer routinely during the 3- to 4-day incubation period for evidence of cytopathic effect (CPE).

4.2 First subculture of inoculated cells (Second MS passage)

4.2.1 Aseptically remove the maintenance medium from all cell monolayers at 3 to 4 days postinoculation.

4.2.2 Add 5 mL of warm trypsin-versene solution to each flask; rinse rapidly each monolayer and aseptically remove the fluid from the flask. Repeat if needed.

4.2.3 Add 5 mL of fresh trypsin-versene to each cell monolayer. Incubate at 36°± 2°C for approximately 10 minutes or until the cell detachment from the flask is complete.

4.2.4 Add 2-3 mL MEM with 5% FBS using a sterile 10-mL syringe fitted with a sterile cannula or disposable pipette to inactivate the trypsin. Aspirate and dispense the cell suspension against the wall of the cell culture flask a few times until a homogeneous cell suspension is obtained. Avoid creating excessive foam.

4.2.5 Depending on cell type, dispense a portion of the total homogenous cell suspension into new sterile 75-cm^2 tissue culture flasks containing 25 mL of maintenance medium. The remaining of the cell suspension is maintained in the original flask or transferred into a tube and kept frozen at -70°C or colder until MS testing is concluded.

4.2.6 Monospecific antiserum (2-10%) may be added to the maintenance medium in the flask to prevent MS breakthrough.

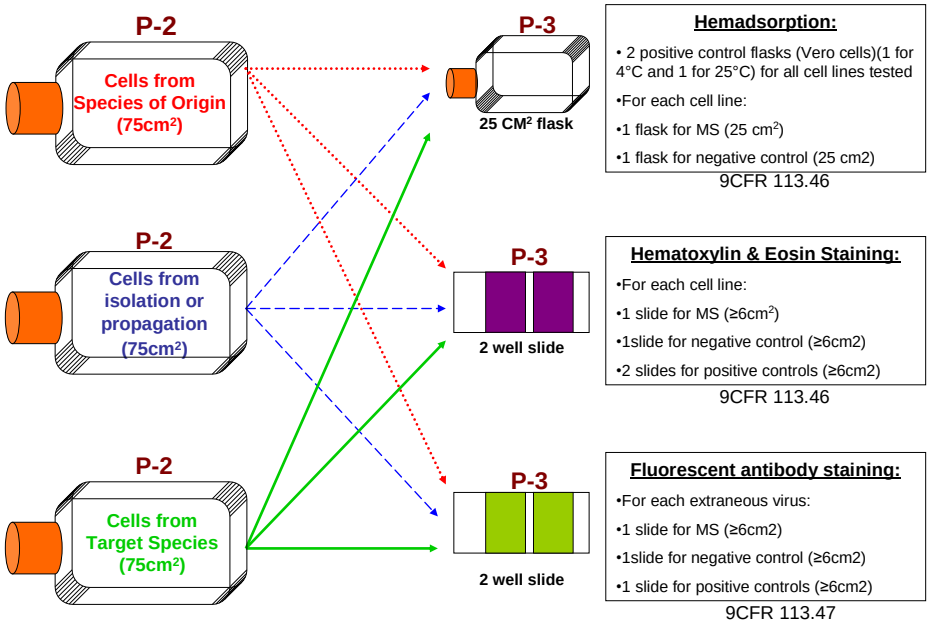
4.2.7 Incubate inoculated monolayer flask at 36°± 2ºC for 3 to 4 days.

4.2.8 Examine the inoculated monolayer routinely during the 3- to 4-day incubation period for evidence of CPE.

4.3 Second subculture of inoculated cells (Third MS passage)

If CPE is not observed, then MS inoculated cells are sub-cultured for a second time in the same manner as the first subculture except that this subculture must be dispensed into sufficient vessels to detect extraneous agents according to 9 CFR 113.46 and 113.47 (**Diagram 2**).

Diagram 2. Passage and detection of extraneous agents in MS



Note: Numbers of slides and vessels listed in the diagram are the minimum numbers required for testing. Additional slides and flasks are often prepared to ensure adequate third passage material for testing.

4.3.1 Hemadsorption (**SAM 313**, *Supplemental Assay Method for the Detection of Extraneous Hemadsorbing Agents in Master Seed Virus***)**: For each cell line, seed at least one 25-cm^2 flask with MS inoculated cells and at least one 25-cm^2 flask with uninoculated cells to be tested sequentially at 4°C and 25°C. In addition, seed two 25-cm^2 flasks of uninoculated Vero cells to serve as positive

controls, one flask for each temperature. The positive control flasks are seed-inoculated with bovine parainfluenza virus 3 at the time of the last subculture.

4.3.2 Hematoxylin and Eosin Staining (**VIRPRO1012**, *Hematoxylin and Eosin Staining Procedure for Detection of Cytopathic Extraneous Agents in Master Seeds***)**: For each cell line, seed one slide (2-chambered) with cells from the MS inoculated monolayer and three slides (controls) with cells from the uninoculated monolayer. One control slide will serve as a negative control and two slides will serve as positive controls. At the time of the last subculture, independently prepare one positive control slide with a virus causing intra-cytoplasmic inclusions (**Appendix III**) and one positive control slide with a virus causing intra-nuclear inclusions (**Appendix IV**).

4.3.3 Fluorescent antibody staining (**VIRPRO1014**, *Fluorescent Antibody Staining Procedure for Detection of Viral Antigens*): See **Appendix I** for species specific requirements and the acceptable culture systems used in testing each extraneous agent. Regardless of vessel used, a minimum surface area of 6 cm^2 is required for the final reading test material and each of the controls for evaluation of MS. For each specified agent, seed one slide with cells from the MS inoculated monolayer and two slides (control) with cells from the uninoculated monolayer. Prepare the positive control slide with a positive control virus at the time of subculture (**Appendix V**). Additionally, seed one slide with cells from the MS to confirm neutralization.

Note: To ensure or enhance fluorescence detection, positive control monolayers may be fixed before Day 7. If positive control monolayers (slides) are fixed early, the corresponding MS inoculated monolayer must be fixed at this time. An additional MS inoculated monolayer must also be fixed on Day 7. Regardless of when monolayers are fixed, they shall all be stained concurrently. In this circumstance, seed two slides with cells from the MS inoculated monolayer in Section 4.3.3.

4.3.4 Examine the cell monolayers for evidence of CPE regularly throughout the 7-day incubation period at 36°± 2°C.

Note: If evidence of CPE or contamination is observed in inoculated or uninoculated monolayer during the 14-day test period, report to Vet/Micro for confirmation to determine if the MS is unsatisfactory.

4.3.5 At the conclusion of the incubation period perform detection of extraneous agents according to referenced test methods.

5. Interpretation of the Test Results

Interpret test results for detection of extraneous agents according to referenced test methods.

6. Report of Test Results

Report test results for detection of extraneous agents according to referenced test methods.

7. References

Title 9, *Code of Federal Regulations*, sections 113.31, 113.34, 113.46, 113.47, 113.51, 113.52, and 113.55, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .04

- The Contact information has been updated.
- **4.1.7 and 4.2.5:** Reference to ABRMSOP3200 deleted as the document has been inactivated.
- **Appendix IV:** BT Permissive Cells – lot updated to 04-04.
- **Appendix V:** Updated to reflect current information.

Version .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 .02

- **3.4.4:** USDASOP0003 has been replaced by SAFSOP005 and SAFSOP0006.
- **4.3.2/4.3.3:** The seed-inoculate has been changed to "prepared".
- **4.3.3:** "Regardless of vessel used, a minimum surface area of 6 cm^2 is required for the final reading test material and each of the controls for evaluation of MS" has been added.

- **Appendix III:** Changed “dilution” to “dose”.
- Amended Appendix III to Appendices III and IV.
- Appendix IV has now become Appendix V.
- Positive control slides are derived from the MS inoculated cells throughout the document.

Appendix I - 9 CFR Required Extraneous Agent Testing by Species

Agent	Culture System[1]	Species required testing
Avian Leukosis Virus (ALV)[a]	CEF cells	All avian MS
Chicken Anemia Virus (CAV)	MDCC MSB1 cells, Chicks	All avian MS
Reticuloendotheliosis (REV)	CEF cells	All avian MS
Hemagglutinating viruses	SPF eggs	All avian MS
Bluetongue virus (BT)	BT, SPF eggs, EBK, EBL, Vero	All mammalian MS
Bovine viral diarrhea virus (BVD)	BT, EBL, MDBK	All mammalian MS
Rabies virus	Vero	All mammalian MS
Reovirus	Vero	All mammalian MS
Bovine adenovirus (BAV)	BT, EBL, MDBK, Vero	Bovine, caprine, ovine
Bovine parvovirus	BT, EBL, Vero	Bovine, caprine, ovine
Bovine respiratory syncytial virus (BRSV)	BT, EBL, MDBK, Vero	Bovine, caprine, ovine
Canine coronavirus	CRFK, A-72, DKp	Canine
Canine distemper	MDCK, Vero, DKp	Canine
Canine parvovirus[b]	CRFK, DKp	Canine
Equine herpesvirus	EQ Der, Eq Lung, BT, EEK	Equine
Equine viral arteritis (EVA)	EQ Der, Vero, EEK	Equine
Feline infectious peritonitis virus (FIP)	CRFK, KKp	Feline
Feline panleukopenia (FPV)	CRFK, KKp	Feline
Porcine adenovirus	ST, PK-15, Vero, SKp	Porcine
Porcine hemagglutinating encephalitis virus (HEV)	ST, PK-15, Vero, SKp	Porcine
Porcine parvovirus	ST, PK-15, SKp	Porcine
Transmissible gastroenteritis virus (TGE)	ST, PK-15, SKp	Porcine

[1]African green monkey kidney (Vero), Bovine turbinate (BT), Canine Golden Retriever tumor (A-72), Chicken Embryo Fibroblast (CEF), Crandell Reese feline kidney (CRFK), Dog kidney primary (DKp), Embryonic bovine lung (EBL), Embryonic Equine kidney (EEK), Equine Dermal (EQ Der), Equine Lung (Eq Lung), Kitten kidney primary (KK_P), Madin-Darby Bovine Kidney (MDBK), Madin-Darby canine kidney (MDCK), Marek's disease chicken cells (MDCC MSB1), Porcine kidney PK-15, SPF=specific pathogen free, Swine kidney primary (SKp), Swine testicle (ST); [a] Does not need absorption step; [b]Seeded on actively dividing cells.

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Appendix II - Non-required Extraneous Agent Testing

Agent	Acceptable Culture System[1]
Avian Encephalomyelitis Virus (AEV)	SPF eggs
Avian Influenza Virus (AIV)	SPF eggs or MDCK cells
Avian Metapneumovirus (aMPV)	DF-1, BHK_{21}, tracheal cultures, SPF eggs
Avian Paramyxovirus 3 (APMV-3)	SPF eggs
Bovine Coronavirus (BCV)	BT, MDBK, Vero
Bovine Enterovirus (BEV)	EBK, BT, MDBK, Vero
Bovine Parainfluenza Type 3 (PI_3)	BT, MDBK, Vero
Bovine Rotavirus (BRV)	EBK, MA104, Vero
Canine Adenovirus (CAV)	DKp, MDCK
Canine Herpes Virus	DKp, MDCK
Canine Parainfluenza (CPI) Virus	DKp, MDCK, Vero
Chlamydophila felis	SPF Eggs, McCoy
Duck Viral Enteritis (DVE)	DEFp
Eastern Equine Encephalomyelitis(EEE)	EEK, BT, EQ Der,
Eimeria spp. – Coccidiosis	SPF chickens
Encephalomyocarditis (EMC)Virus	BHK_{21}, SKp
Epizootic Hemorrhagic Disease (EHD)	EEK, EBL, EQ Der
Equine Infectious Anemia (EIA)	EQ Der, Vero
Equine Influenza Virus (EIV)	SPF eggs, EQ Der
Feline Calicivirus (FCV)	KKp, CRFK
Feline Infectious Peritonitis (FIP) Virus	KKp, CRFK
Feline Rhinotracheitis Virus (FRV)	KKp, CRFK
Fowl Pox Virus	SPF eggs, CEF_p
Hemorrhagic Enteritis Virus of Turkeys (HEV)	MDTC RP-19 cells, turkeys-SPF
Infectious Bovine Rhinotracheitis Virus (IBR)	EBL, BT, MDBK
Infectious Bronchitis Virus (IBV)	SPF eggs
Infectious Bursal Disease Virus (IBDV)	SPF eggs

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Infectious Canine Hepatitis (ICH)	DKp, MDCK
Infectious Laryngotracheitis Virus (ILTV)	SPF eggs or CKp
Mareks Disease Virus (MDV)	CEF cells
Newcastle Disease Virus (NDV)	SPF eggs
Avian Paramyxovirus 1 (APMV-1)	SPF eggs
Porcine Circovirus (PCV)[a]	PK -15*
Porcine Respiratory and Reproductive Syndrome Virus (PRRSV)	MA104, MARC 145
Pseudorabies Virus (PRV)	SKp, BT, PK-15, MDBK
Swine influenza virus (SIV)	SPF eggs, ST, MDCK
Venezuelan Equine Encephalomyelitis Virus (VEE)	EEK, BT, EQ Der,
Viral Arthritis/Tenosynovitis (Reovirus)	SPF eggs or CKp
West Nile Virus	Vero
Western Equine Encephalomyelitis Virus (WEE)	EEK, BT, EQ Der

[1]African green monkey kidney (Vero), Baby Hamster kidney 21 (BHK_{21}), Bovine turbinate (BT), Chicken Embryo Fibroblast primaries (CEF_p) Chicken embryo fibroblasts continuous line (DF-1), Chicken kidney (CK_p), Clone of MA 104 (MARC-145), Crandell Reese feline kidney (CRFK), Dog kidney primary (DKp), Embryonic bovine lung (EBL), Duck embryo fibroblast primary (DEF_p), Embryonic Equine kidney (EEK), Equine Dermal (EQ Der), Equine Lung (Eq Lung), Kitten kidney primary (KKP), Madin-Darby Bovine Kidney (MDBK), Madin-Darby canine kidney (MDCK), Marek's Disease Turkey cell line (MDTC RP-19), Mouse Synovial cell (McCoy), Porcine kidney (PK-15* PCV free), SPF=specific pathogen free, Swine kidney primary (SKp), Swine testicle (ST); [a]Actively dividing cells should be used and treated with glucosamine (Tischer et. al. 1987).

Appendix III – Intra-cytoplasmic Positive Control Viruses for Hematoxylin and Eosin Staining

Permissive Cells	Split Ratio	Species of MS	Virus	Lot	Dose	Inoculation	Fixation
BT	1:3	All	PI3	84-6	4	Co-inoculate	3 day
Vero	1:4	All	PI3/Reo	84-6/88-12	5	Co-inoculate	3 day
MDBK	1:6	Bovine, Ovine Caprine, Ovine	PI3	84-6	4	Co-inoculate	2 day
MDCK	1:6	Canine	PI3	84-6	5	Co-inoculate	3 day
EQ Der	1:2	Equine	PI3	84-6	5	Co-inoculate	3 day
CRFK	1:5	Feline	PI3/Reo	84-6/88-12	4	Co-inoculate	3 day
MARC-145	1:4	Porcine	PI3	84-6	4-5	Co-inoculate	3 day
PK-15	1:4 or 1:5	Porcine	PI3/Reo	84-6/88-12	1-3	Co-inoculate	3 day
ST	1:3 or 1:4	Porcine	PI3/Reo	84-6/88-12	1-3	Co-inoculate	3 day

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Appendix IV – Intra-nuclear Positive Control Viruses for Hematoxylin and Eosin Staining

Permissive Cells	Split Ratio	Species of MS	Virus	Lot	Dose	Inoculation	Fixation
BT	1:3	All	BAV5	04-04	4	Co-inoculate	3 day
Vero	1:4	All	PRV	87-10	3-4	24 hr	3 day
MDBK	1:6	Bovine, Ovine, Caprine	IBR/BAV5	00-16/04-04	2-3/5	24 hr/Co-inoculate	2-3 day
MDCK	1:6	Canine	ICH	97-14	3	Co-inoculate	3 day
EQ Der	1:2	Equine	EHV1	5-85	3	72 hr	1-2 day
CRFK	1:5	Feline	SAV	85-17	5	Co-inoculate	3 day
MARC-145	1:4	Porcine	SAV	85-17	5	Co-inoculate	3 day
PK-15	1:4 or 1:5	Porcine	PRV	87-10	3	24 hr	3 day
ST	1:3 or 1:4	Porcine	PRV	87-10	3	Co-inoculate	3 day

Appendix V - Positive Control Viruses for Fluorescent Antibody Staining

Agent	Lot number	Lot titer	Dose	Cell Type
Bluetongue virus 11	85-9	$10^{4.5}$/mL	10^{2}	BT, EBK, EBL, Vero
Bovine adenovirus 1	06-39	$10^{6.6}$/mL	$10^{5.6}$ or $10^{4.6}$	BT, EBL, MDBK, Vero
Bovine adenovirus 5	04-04	$10^{6.6}$/mL	$10^{5.6}$ or $10^{4.6}$	BT, EBL, MDBK, Vero
Bovine parvovirus	99-09	$10^{5.2}$/mL	$10^{3.2}$	BT, EBL
Bovine parvovirus	99-09	$10^{5.2}$/mL	$10^{4.2}$	Vero
Bovine respiratory syncytial virus	88-16	$10^{5.4}$/2mL	$10^{2.4}$	BT, EBL, MDBK
Bovine respiratory syncytial virus	88-16	$10^{5.4}$/2mL	$10^{3.4}$	Vero
Bovine viral diarrhea virus type 1	00-17	$10^{6.6}$/mL	$10^{2.6}$ or $10^{3.6}$	BT, EBL, MDBK
Bovine viral diarrhea virus type 2	05-15	$10^{6.3}$/mL	$10^{2.3}$ or $10^{3.3}$	BT
Bovine viral diarrhea virus type 2	05-15	$10^{6.3}$/mL	$10^{3.3}$	EBL, MDBK
Canine coronavirus	07-06A	$10^{5.3}$/mL	$10^{5.3}$	A72, CRFK, DKp
Canine distemper	99-05	$10^{3.6}$/mL	$10^{1.6}$	DKp, MDCK, Vero
Canine parvovirus	96-05	$10^{6.4}$/mL	$10^{2.4}$	CRFK, DKp
Equine herpesvirus 1	5-85	$10^{5.3}$/mL	$10^{2.3}$	BT
Equine herpesvirus 1	5-85	$10^{5.3}$/mL	$10^{1.3}$	EEK, EQ Der, Eq Lun
Equine herpesvirus 4	01-03	$10^{3.7}$/mL	$10^{1.7}$	BT, EEK, EQ Der, Eq Lun
Equine viral arteritis	94-10	$10^{6.1}$/mL	$10^{3.1}$	EQ Der, Vero
Feline infectious peritonitis virus	94-11	$10^{5.5}$/mL	$10^{0.5}$	CRFK, KKp
Feline panleukopenia	02-14	$10^{6.8}$/mL	$10^{3.8}$	CRFK, KKp
Porcine adenovirus	85-17	$10^{6.3}$/mL	$10^{4.3}$	PK-15, SKp, ST, Vero
Porcine hemagglutinating encephalitis virus	01-16	$10^{5.5}$/mL	$10^{3.5}$	PK-15, SKp, ST, Vero
Porcine parvovirus	87-6	$10^{4.5}$/mL	$10^{2.5}$	PK-15, SKp, ST
Rabies virus	Previously prepared			Vero
Reovirus	01-17/98-06	$10^{6.6}$/mL	$10^{2.6}$ to$10^{3.6}$	Vero
Transmissible gastroenteritis virus	88-20/M595	$10^{6.8}$/2mL	$10^{3.8}$	PK-15, SKp, ST
Bovine Coronavirus (BCV)	85-6	Unknown	Undiluted	BT, EBL, MDBK
Bovine Enterovirus (BEV)	83-1	$10^{6.2}$/2mL	$10^{2.2}$ to$10^{3.2}$	BT, EBL, MDBK
Bovine Parainfluenza Type 3 (PI_3)	84-6	$10^{6.0}$/2mL	$10^{2.0}$ to$10^{3.0}$	BT, EBL, MDBK, EBK

Neutralization and Passage of Master Seed Virus in Cell Cultures

Agent	Lot number	Lot titer	Dose	Cell Type
Bovine Rotavirus (BRV)*	84-9	$10^{7.9}$/mL	$10^{2.9}$	BT, EBL, MDBK
Canine Adenovirus (CAV)	01-07	$10^{7.9}$/mL	$10^{2.9}$	MDCK
Canine Herpes Virus	97-16	$10^{6.8}$/mL	$10^{2.8}$	MDCK
Canine Parainfluenza (CPI) Virus	96-16	$10^{8.5}$/mL	$10^{2.5}$	Vero, MDCK
Encephalomyocarditis (EMC)Virus	87-9	$10^{5.0}$/mL	$10^{2.0}$	Vero, SKp, BHK_{21}
Equine Infectious Anemia (EIA)	94-1	$10^{2.9}$/mL	$10^{1.9}$	EQ Der
Feline Calicivirus (FCV)	01-10	$10^{5.9}$/mL	$10^{0.9}$	CRFK
Feline Infectious Peritonitis (FIP) Virus	94-11	$10^{5.5}$/mL	$10^{2.5}$	CRFK
Feline Rhinotracheitis Virus (FRV)	96-15	$10^{8.5}$/mL	$10^{3.5}$	CRFK
Infectious Bovine Rhinotracheitis Virus (IBR)	00-16	$10^{9.3}$/2mL	$10^{3.3}$	MDBK, BT, EBK, EBL
Infectious Canine Hepatitis (ICH)	97-14	$10^{7.9}$/mL	$10^{2.9}$	MDCK
Porcine Respiratory and Reproductive Syndrome Virus	94-3	$10^{6.1}$/mL	$10^{3.1}$	MA104/MARC-145
Pseudorabies Virus (PRV)	87-10	$10^{6.6}$/mL	$10^{3.6}$	MDCK, PK-15
West Nile Virus	00-21 equine	$10^{5.1}$/mL	$10^{4.1}$	Vero
West Nile Virus	00-23 crow	$10^{7.8}$/mL	$10^{5.8}$	Vero

*Needs trypsin to replicate in cells