United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Hematoxylin and Eosin Staining Procedure for Detection of Cytopathic Extraneous Agents in Master Seeds

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1. Introduction

The hematoxylin and eosin (H-E) staining is a histological staining procedure used by pathologists for the study of viral infections in tissue sections. In agreement with the literature, the Center for Veterinary Biologics (CVB) Virology Laboratory has adapted this H-E staining technique for staining cell cultures when testing for the presence of extraneous agents in master seeds (MS), master cell stocks (MCS), and ingredients of animal origin. This adapted (modified) H-E method described in this protocol allows for the detection/visualization of cytopathological changes (i.e., inclusion bodies, giant cells, etc.) induced by extraneous agents present in MS.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 CO_2 incubator (36°C± 2ºC)

2.1.2 Bright light microscope

2.1.3 Automatic slide stainer (optional) (located at the Pathobiology Laboratory, National Veterinary Services Laboratories (NVSL))

2.2 Reagents/supplies

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

2.2.1 Reagents/supplies for fixation (CVB Virology Laboratory)

2.2.2 Coplin jars

1. Slide racks (metal or glass) to hold slides being fixed

2. Glass dishes

3. Cell culture glass slides (2 chambered Lab-Tek) (Nalge Nunc International)

Note: Other multi-chamber glass slides may be used; however, a sufficient number must be inoculated to encompass a 6 cm^2 surface area.

4. Sterile distilled water

5. Phosphate buffer saline 0.01M (PBS) (National Centers for Animal Health (NCAH) Media #30054) 8.5 g sodium chloride

a. 0.22 g sodium phosphate monobasic

b. 1.19 g sodium phosphate dibasic

c. QS to 1000 mL with deionized water (DI) water and adjust pH to 7.2 ± 0.1 with 2 N hydrochloric acid (HCl)

6. Fixative (NCAH Media #41057)

a. 90.0 mL 80% Ethanol (80% ethanol is prepared by mixing 20.95 mL DI with 100 mL 95% ethanol)

b. 5.0 mL glacial acetic acid

c. 5.0 mL neutral buffered formalin

2.2.3 Reagents/supplies for H-E staining (Pathobiology Lab, NVSL)

1. Hematoxylin

2. Eosin Y (alcoholic)

3. Potassium Acetate (1%)

a. 7.5 g potassium acetate

b. 750 mL DI

4. Clarifier

a. 1000 mL 95% ethyl alcohol

b. 1000 mL isopropyl alcohol

c. 10 mL acetic acid

d. Combine **a. through c**. and store at room temperature (22°C-25ºC)

2.2.4 Glass coverslips

2.2.5 Permount mounting fluid

2.2.6 Cardboard slide holders for transporting stained slide cell preparations

2.2.7 Current version of **CVB-TWS-0126**, *Master Seed Virus Extraneous Testing* - Tested According to **CVB-SOP-0149,** *Master Seed Testing in the Virology Section*

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience in cell culture techniques, including the performance of viable cell counts and determination of optimal cell density for serial cell passage. Personnel reading the test should also be knowledgeable about and trained in the detection of cytopathic viruses in cells cultures by means of cytological staining techniques.

3.2 Preparation of reagents/control procedures

The CVB Laboratory prepares the formalin-acetic acid-ethanol fixative for the modified H-E stain. The H-E stains and additional reagents used for this staining procedure are prepared at the NVSL Pathobiology Laboratory.

3.3 Preparation of the sample

Cell cultures are prepared for H-E staining according to the current version of **CVB-PRO-0039,** *Neutralization and Passage of Master Seed in Cell Cultures*. Positive and negative control slides, prepared concurrently with the MS/MCS slides, are used for verification of the assay. Seven days after the last subculturing, MS/MCS inoculated and control cell monolayers are fixed for H-E staining. Fixed preparations are then transported to the NVSL Pathobiology Laboratory where they are stained and mounted according to **Section 4**. All information pertaining to H-E fixation procedure must be recorded on CVB-TWS-0126.

3.3.1 Decant cell culture media from the MS/MCS inoculated and control cell monolayers into an autoclavable container. Remove slide chamber wall and place the slides in a slide rack. If there are five or fewer slides, the fixation procedure can be performed in a Coplin jar.

3.3.2 Fully submerged slide rack in a glass dish containing PBS and wash for 10 ± 1 minutes at room temperature.

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.

3.3.3 Remove slide rack from the PBS wash and fully submerge in a glass dish containing formalin-acetic acid-ethanol fixative. Fix slide cell monolayers for 30 to 45 minutes at room temperature.

3.3.4 Using nitrile or vinyl gloves, remove slide rack from the fixative and fully submerge in a glass dish containing sterile DI. Remove single slides from slide rack by means of sterile forceps and remove slide gaskets. Store slides in sufficient sterile DI in sterile Coplin jars. Transport slides in the Coplin jars to the NVSL Pathobiology receiving center for transfer to NVSL Pathology Laboratory for staining.

Note: Fixed slides should not be stored in DI longer than 5 days. Complete VS Form 10-4, *Specimen submission*, and request that CVB H&E staining procedure is run on samples (Form FM-PL-0032, *Request for Histology Services*) prior to delivering the fixed slide cultures to the Pathology receiving center.

4. Performance of Staining Procedure

The H-E staining is performed by Pathobiology Laboratory personnel. Pathobiology personnel are responsible for the preparation, maintenance, and operation of the automatic slide stainer used for performing the H-E staining. If the automatic slide stainer is not available, H-E staining of the slide cell cultures can be performed manually by following the steps below.

In the original H-E staining procedure, the first six stations (steps) include removal of the paraffin from the tissue sections by xylene treatment followed by xylene removal with ethanol which is not necessary for slide cell cultures.

Note: Remind Pathobiology Laboratory personnel to start the staining method (automatic or manual) on Station #7 and continue until the completion of procedure as described below:

Station 7 - Tap water, 1 minute
Station 8 - Hematoxylin, 1.5 minutes
Station 9 - Running water, 2 minutes
Station 10 - Clarifier, 1 minute
Station 11 - Running water, 2 minutes
Station 12 - 1% Potassium Acetate, 1 minute
Station 13 - Tap water, 1 minute
Station 14 - 95% ethyl alcohol, 1 minute
Station 15 - Eosin, 1.5 minutes
Station 16 and 17 - 95% ethyl alcohol, 1 minute each station
Station 18 and 19 - 100% ethyl alcohol, 1 minute each station
Station 20 and 21 - Xylene, 1 minute each station
Stained slides are coverslipped with permount

5. Reading and Interpretation of the Test Results

Examine entire H-E-stained cell monolayers under a bright light microscope using 100-400X magnification. Record results of reading and interpretation on CVB-TWS-0126.

5.1 For a **VALID** test:

5.1.1 The negative control slides are free from viral inclusion bodies and related virus-induced cellular changes such as multinucleated giant cell formation.

5.1.2 The positive control slides exhibit the expected intra-cytoplasmic and/or intranuclear inclusion bodies and related virus-induced cytological changes.

5.2 A test is **INVALID** if:

5.2.1 The negative control slides contain either intra-cytoplasmic and/ or intra-nuclear inclusion bodies or giant multinucleated cells, this indicates possible viral cross contamination and must be repeated.

5.2.2 Specific viral inclusion bodies are absent in the POSITIVE CONTROL slides; the test must be repeated.

5.2.3 Viral inclusion bodies in the positive control slides are equivocal, and/or if the negative control slides show equivocal viral inclusion bodies indicating possible viral cross contamination; the test must be repeated.

5.3 If the test is valid and the MS/MCS test slides are negative for viral inclusion bodies and related virus-induced cytological changes, then the test MS/MCS is SATISFACTORY.

5.4 If the test is valid and intra-cytoplasmic or intra-nuclear viral inclusion bodies and/or giant multinucleated cells are found in the MS/MCS test slides, then the test MS/MCS 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 the agent contact or designee. Test results are then entered into the current reporting system and released to the reviewer for distribution to the firm.

7. References

Kuchler RJ. Biochemical Methods in Cell Culture and Virology. Dowden, Hutchinson & Ross, Inc. Stroudsburg, Pennsylvania. 1977, pg. 130.

Appendix I Cell types with corresponding virus recommendations

	Intracytoplasmic				**Intranuclear**			
	PI3[1] Lot 84-6 $10^{7.0}$/mL	Reo[2] Lot 01-17 $10^{5.0}$/mL	ICH[3] Lot 06-44 $10^{7.74}$/mL	IBR[4] Lot 86-2 $10^{6.2}$/mL	BAV5[5] Lot 04-04 $10^{6.0/mL}$	EHV1[6] Lot 5-85 $10^{6.28/mL}$	PRV[7] Lot 87-10 $10^{6.2}$PFU/mL	SAV[8] Lot 85-17 $10^{6.3}$/mL
BT 1:3	4 Logs co-inoc	5 Logs co-inoc			4 Logs co-inoc			
CRFK 1:5	4 Logs con-inoc	4 Logs co-inoc						5 Logs co-inoc
Eq Der 1:2	5 Logs co-inoc	4-5 Logs co-inoc				3 Logs 72hr inoc 1-2 day fix		
MDBK	4 Logs co-inoc 2 day fix			2-3 Logs 24hr inoc 2-3 day fix	5 Logs co-inoc			
MDCK 1:6	5 Logs co-inoc	4-5 Logs co-inoc	3 Logs co-inoc					
PK15 (CC2 slides)	1-3 Logs co-inoc	1-3 Logs co-inoc					3 Logs 24hr inoc	5 Logs co-inoc (4 Logs usable)
ST (CC2 slides)	1-3 Logs co-inoc	1-3 Logs co-inoc					3 Logs co-inoc	5 Logs co-inoc
Vero 1:4 or 1:6	5 Logs co-inoc	4-5 Logs co-inoc					3-4 Logs 24hr inoc	
MARC145	4-5 Logs co-inoc	4-5 Logs co-inoc						5 Logs co-inoc

[1]Bovine Parainfluenza Type 3, [2]Reovirus, [3]Infectious Canine Hepatitis, [4]Infectious Bovine Rhinotracheitis Virus, [5]Bovine adenovirus 5, [6]Equine herpesvirus [7]Pseudorabies, [8]Swine adenovirus.
[1]Paramyoxvirus, [2]Reovirus, [3]Adenovirus, [4]Herpesvirus, [5]Parvovirus

Fix positive control slides at 3 days unless otherwise noted

Appendix II

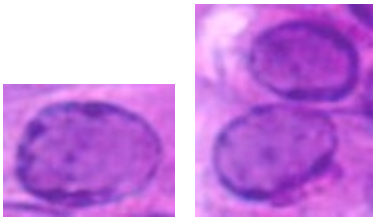
Intracytoplasmic inclusions

Figure 1

[1]PI3virus: Multinucleated cells, typically smaller multiple inclusions (speckled), bright pink
[2]Reovirus: Inclusions wrap nucleus, shaped, bright pink

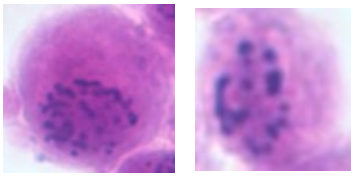
Intranuclear inclusions

Herpes viruses:

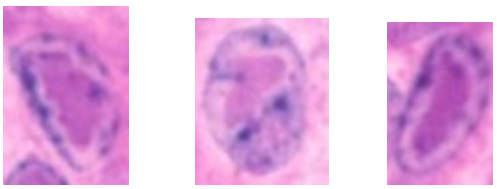


IBR: Misshapen nucleus and chromatin lined up along edge of nucleus; may have multiple inclusions per nucleus

EHV-1:



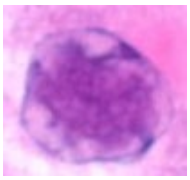
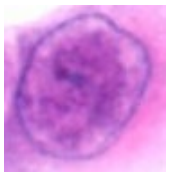
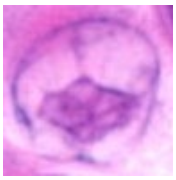
PRV:



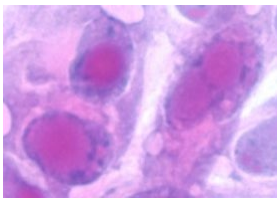
Adenoviruses:

One homogenous inclusion per nucleus with light color ring around inclusion; requires high doses

ICH (Infectious Canine Hepatitis): “Dough bubble” vacuolation, purple inclusions

BAV5:



SAV: