Journal of Visualized Experiments

Immunohistochemistry test for the lyssavirus antigen detection from formalin-fixed tissues

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60138R2
Full Title:	Immunohistochemistry test for the lyssavirus antigen detection from formalin-fixed tissues
Keywords:	Rabies diagnostics; lyssavirus antigen detection; formalin-fixed
Corresponding Author:	Subbian Satheshkumar Panayampalli UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	xdv3@cdc.gov
Order of Authors:	Michael Niezgoda
	Panayampalli Subbian Satheshkumar
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Atlanta, GA, USA

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Centers for Disease Control
and Prevention (CDC)
Atlanta GA 30333

Journal of Visualized Experiments

June 17th, 2019

To the Editors,

We have revised the manuscript entitled **"Immunohistochemistry test for lyssavirus antigen detection from formalin-fixed tissues"** as per the reviewer's comments for consideration in Journal of Visualized Experiments for the special rabies diagnostics issue. I hope the revised version will be favorably reviewed.

This manuscript is original and is not under consideration elsewhere, nor have its contents been previously published. All authors have approved the manuscript and its submission to *Journal of Visualized Experiments*.

Sincerely,

Mike Niezgoda,
Immunology and Proteomics Team,
Poxvirus and Rabies Branch, DHCPP, NCEZID,
Centers for Disease Control and Prevention,
Atlanta, GA 30329.

Email : man6@cdc.gov
Phone : 404-639-1068

TITLE:

Immunohistochemistry Test for the Lyssavirus Antigen Detection from Formalin-Fixed Tissues

AUTHORS AND AFFILIATIONS:

Michael Niezgoda[1], Panayampalli Subbian Satheshkumar[1]

[1]Poxvirus and Rabies Branch, Division of High-Consequence Pathogens and Pathology, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA

Corresponding author:
Michael Niezgoda (man6@cdc.gov)

Email address of the co-author:
Panayampalli Subbian Satheshkumar (xdv3@cdc.gov)

KEYWORDS:

Rabies, Lyssavirus, Immunohistochemistry, Formalin fixation, Antigen detection, Diagnosis

SUMMARY:

Here, we present an immunohistochemistry test protocol for the detection of rabies virus antigen as an alternative diagnostic test for formalin-fixed tissues.

ABSTRACT:

One of the primary diagnostic modalities for rabies is the detection of viral ribonucleoprotein (RNP) complex (antigen) in the infected tissue samples. While the direct fluorescent antibody (DFA) test or the direct rapid immunohistochemical test (DRIT) are most commonly utilized for the antigen detection, both tests require fresh and/or frozen tissues for impressions on slides prior to the antigen detection using antibodies. If samples are collected and fixed in formalin, neither test is optimal for the antigen detection, however, testing can be performed by conventional immunohistochemistry (IHC) after embedding in paraffin blocks and sectioning. With this IHC method, tissues are stained with anti-rabies antibodies, sections are deparaffinized, antigen retrieved by partial proteolysis or other methods, and incubated with primary and secondary antibodies. Antigens are stained using horseradish peroxidase / amino ethyl carbazole and counterstained with hematoxylin for the visualization using a light microscope. In addition to the specific antigen detection, formalin fixation offers other advantages like the determination of histological changes, relaxed conditions for specimen storage and transport (under ambient temperatures), ability to test retrospective cases and improved biological safety through the inactivation of infectious agents.

INTRODUCTION:

Rabies is an acute progressive encephalitis caused by the negative sense RNA viruses belonging to the genus lyssavirus[1]. Nearly 99% of all human deaths caused by the infection with rabies virus (RABV), the type member of the genus, is transmitted by dogs[2]. Rabies diagnosis of

suspect animals relies on the detection of antigen (primarily viral encoded nucleoprotein, N protein) in complex with genomic RNA (ribonucleoprotein complex, RNP) in the brain tissue[3]. The antigen detection by the direct fluorescent antibody (DFA) test is considered the gold standard for rabies diagnosis[4]. The method utilizes fresh or fresh frozen brain material, a touch impression on a slide, fixation in acetone, staining using commercially available fluorescent isothiocyanate (FITC) labeled monoclonal or polyclonal antibodies (mAbs/pAbs) and read by the fluorescence microscopy[5]. The DFA test is rapid, sensitive, and specific for rabies antigen detection in fresh brain tissue. Recently, a direct rapid immunohistochemical test (DRIT), modified immunohistochemistry (IHC) technique, was demonstrated to exhibit similar sensitivity to DFA but offers the advantage of light microscopy for visualization[6]. While the detection method used in DRIT, is similar to IHC, the initial step utilizes fresh or frozen tissues to generate touch impressions of the sample followed by fixation in formalin.

IHC is a widely used technique to determine histological changes and detection of proteins using specific antibodies in formalin-fixed tissues embedded in paraffin blocks. IHC is an established alternative test for the rabies antigen detection in the tissue sections[7]. IHC has been particularly utilized for the diagnosis of retrospective cases that exhibited neurological diseases to determine the burden of rabies[8]. Paraffin-embedded formalin-fixed tissues preserve the proteins for the detection even after several years when stored at ambient temperature[9]. Formalin treatment modifies proteins by cross-linking and altering the amino acid side chains, which might make the epitopes no longer reactive against antibodies[10]. While the IHC test for rabies antigen detection involves either mAbs or pAbs, the latter is advantageous as multiple epitopes and divergent lyssaviruses can be detected[11].

The standard steps involved in IHC are formalin fixation of tissues, embedding in paraffin blocks, sectioning of tissues, deparaffinization and hydration, epitope recovery, reactivity against primary and secondary antibodies, and the development using chromogenic substrates. This manuscript describes a detailed account of the protocol for rabies diagnosis. For rabies antigen detection, mouse serum immunized with RABV (pAbs) generated at the U.S. Centers for Disease Control and Prevention (CDC) Atlanta, Georgia, in combination with biotinylated anti-mouse secondary antibodies are utilized. Biotinylated Abs are detected by the addition of streptavidin-horseradish peroxidase (HRP) complex followed by the color development with amino-ethylcarbazole substrate.

PROTOCOL:

While the IHC protocol was performed on formalin-fixed tissues, which inactivates RABV if present, appropriate biosafety protocols should be properly followed. All biosafety procedures are described in the Biosafety in Microbiological and Biomedical Laboratories (BMBL) 5th Edition (https://www.cdc.gov/biosafety/publications/bmbl5/index.htm), including wearing proper personal protective equipment (PPE), and vaccination requirement as described[12]. In addition, proper containment and handling of hazardous chemicals (like formalin, AEC and xylene), should be followed (e.g., fume hoods).

1. Formalin fixation of tissues

1.1. Place 3-5 mm brain tissues collected after necropsy[13] into 10% phosphate buffered formalin solution (1:20 to 1:50 tissue/formalin ratio) for 24-72 h.

CAUTION: Formalin is a toxic fixative.

1.2. Record the approximate tissue weight (size), tissue type (brain areas) and volume of formalin. It is important for laboratories to document and maintain records on fixation times.

1.3. For longer tissue storage after formalin fixation and prior to processing, place the tissue in 70% ethanol.

2. Tissue processing

2.1. Following the specimen fixation, dissect the tissue to include the important brain areas e.g., cross sections of the brainstem, cerebellum (3 lobes), or the hippocampus (both hippocampi) each cut 3 to 5 mm thick and placed into processing cassettes.

2.2. Process the tissue cassettes for paraffin wax infiltration, embedded into paraffin blocks and sectioned (3 to 6 µm) on a microtome.

3. Preparation of Materials / Staining dishes

3.1. Set up the staining dish[14](**Table of Materials**) as shown in **Figure 1**. Fill each dish with 250 mL of the solution.

3.2. Preparation of the 3-Amino-9-ethylcarbizole (AEC) substrate stock solution

3.2.1. Dissolve one 20 mg tablet of 3-amino 9-ethylcarbazole (AEC) into 5 mL of N,N, dimethylformamide using a glass pipette.

CAUTION: AEC is a carcinogen.

3.3. Preparation of the protease (e.g., Pronase) stock solution for the antigen retrieval

3.3.1. Dissolve 7 mg of the protease in 200 mL of PBS.

3.4. Preparation of the rinse buffer PBS-T

3.4.1. Add 10 mL of Tween 80 to 990 mL of PBS. Mix it well to form a homogenous solution.

4. Deparaffinization and tissue rehydration

4.1. Make 5 µm paraffin section using a microtome, float it on a water bath at 38 ˚C and collect

on to glass slides. Label the slides with a reagent resistant pen/marker.

4.2. Place the slides onto a tray and melt in a 55-60 ˚C oven for 1 h. Do not raise the temperature above 60 ˚C as it can destroy the viral antigen.

4.3. Remove slides from the oven and immediately deparaffinize in 3 consecutive xylene rinses of 5 min each in the dishes 1, 2 and 3.

4.4. Rehydrate the sections on the slide by sequential immersions in decreasing dilution of ethanol to deionized water: (4 through 11 is a dip rinse) dish 4: xylene/100% ethanol (1:1); dish 5: 100% ethanol; dish 6: 100% ethanol; dish 7: 95% ethanol; dish 8: 95% ethanol; dish 9: 80% ethanol; dish 10: 70% ethanol; dish 11: deionized water (**Figure 1**. Dish set-up).

CAUTION: Xylene is a hazardous chemical and work should be conducted in a fume hood.

5. Proteolytic antigen retrieval

5.1. Treat slides with the protease (2.5 µg/mL of PBS) for 30 min for proteolytic antigen retrieval in dish 12.

5.2. Then rinse in PBS-T for 10 min (dish 13).

5.3. Treat with 3% hydrogen peroxide for 10 min (dish 14).

5.4. Again, wash with PBS-T for 10 min (dish 15).

6. Staining procedure

6.1. Handle slides one at a time keeping the remaining slides submerged in the buffer (do not remove the whole slide holder - keep slides wet). Remove one slide and blot off excess buffer (using a paper towel) from around the tissue section being careful not to disturb the tissue section. Incubate slides in a humidity chamber, made by placing moistened paper towels, on the lab bench top[14] at room temperature with normal goat serum (blocking) for 15 min.

6.2. Incubate with the optimal pre-determined dilution primary anti-rabies antibody (1:250 dilution of mouse anti-rabies serum, unpublished) (positive control) and negative control antibodies at room temperature same as above (step 6.1.) for 60 min with no washes in between.

6.3. After 60 min, wash with PBS-T for 10 min (dish 16).

6.4. Incubate with the biotinylated antibody (species specific) in a humidity chamber at room temperature for 15 min (handling same as step 6.1.).

6.5. Wash with PBS-T for 10 min (dish 16).

6.6. Incubate with Streptavidin-HRP complex in a humidity chamber at room temperature for 15 min (handling same as 6.1.).

6.7. Wash with PBS-T for 10 min (dish 16).

6.8. Incubate with peroxidase substrate, amino-ethylcarbazole (AEC), in a humidity chamber at room temperature for 10 min. Make AEC just prior to use. To do so, add 1 mL of AEC stock solution to 14 mL of 0.1M acetate buffer, pH 5.2. Add 0.15 mL of 3% H_2O_2. Filter the mixture just before use (0.45 µm filter).

NOTE: The working solution of AEC is only stable for 2-3 h. The AEC stock solution can be stored in the refrigerator for longer periods.

6.9. Wash in deionized water 10 min (dish 17)

6.10. Counterstained with Gill's Hematoxylin diluted 1:2 with deionized water for 2 min (dish 18).

6.11. Rinse off excess Hematoxylin with deionized water dip rinse (dish 19 and 20).

6.12. Rinse in Scott's Tap water 30 s (bluing solution) dish 21.

6.13. Wash in deionized water 10 min (dish 22)

6.14. Remove slides one at a time - mount with water-soluble mounting medium.

6.15. Read slides on a light microscope.

REPRESENTATIVE RESULTS:

Figure 2 demonstrates representative IHC staining results of positive and negative control samples in different brain tissues tested. **Figure 2A,D,G** represent positive samples at 200x, while **Figure 2B,E,H** correspond to 400x magnification, respectively. **Figure 2A-C** correspond to the brainstem; **Figure 2D-F** correspond to the cerebellum and Purkinje cells; and **Figure 2G-I** correspond to the hippocampus. **Figure 2C,F,I** are negative control samples. The magenta red staining demonstrates the color development using AEC substrate in the blue background (Hematoxylin counterstain) due to the reactivity of antibodies against rabies antigen. AEC is a peroxidase substrate, which upon oxidation reaction, catalyzed by HRP, results in water insoluble precipitate observable under a light microscope.

A positive result in IHC corresponds to magenta red staining in tissue sections. The staining of cytoplasmic inclusions and granular inclusions of varying size are indicative of samples positive for RABV infections. Samples are deemed negative if no specific red staining or only the blue

background due to hematoxylin was observed. In addition to the positive staining, the distribution of inclusions could provide indirect quantification of levels of rabies antigen in the sample, which might correspond to the viral load of the tissue samples. Irrespective of the levels of distribution, any specific staining will classify the sample as positive for RABV antigen detection.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow chart indicating different steps for IHC testing.

Figure 2: Immunohistochemical staining of positive and negative rabies brain tissue. (**A**) Intracytoplasmic viral inclusions and rabies virus antigen detection in the brainstem 200x total magnification; (**B**) positive brainstem 400x; (**C**) brainstem negative control 200x; (**D**) rabies virus inclusions within the cerebellum 200x; (**E**) cerebellum and Purkinje cells 400x; (**F**) cerebellum negative control; (**G**) Viral inclusions within hippocampus 200x; (**H**) hippocampus 400x; hippocampus negative control 200x. The red stain indicates the presence of rabies virus antigen using the Streptavidin-biotin complex staining method (AEC substrate). Hematoxylin counterstain (blue).

DISCUSSION:

Due to the high fatality rate of rabies after the symptom onset, the diagnosis of suspect animals for RABV infection is extremely critical for an appropriate post-exposure prophylactic treatment. Rabies diagnosis primarily depends on DFA, DRIT, and PCR-based techniques using fresh or frozen tissues. For testing of formalin-fixed tissues, the IHC test provides an alternative method for the sensitive and specific detection of RABV antigen. While the tissues fixed in formalin have proteins stabilized due to the modification of side chains like cross-linking, the samples need to be processed before the antigen detection. In this protocol, the epitopes were recovered through the partial proteolytic digestion by the protease (e.g., Pronase) to enable the binding of primary antibodies to the RNP complex. While mAbs reactive against N protein are predominantly relied on in DFA and DRIT, pAbs that are reactive against multiple epitopes on N protein would be preferred for an IHC test. In addition, the reactivity or pAbs could be broader against different RABV variants and against non-rabies lyssaviruses as compared to mAbs.

One of the major limitations of IHC test is the protocol involves several sequential steps and takes about 6 hours for completion. If the tissue needs to be fixed in formalin and embedded in paraffin blocks, it requires an additional 1 – 2 days before the tissue could be stained. Another limitation is the non-availability of commercial primary anti-rabies antibodies for the IHC test. However, IHC does provide an option to perform rabies diagnosis when only FF tissues are available for testing. The IHC test is particularly important for testing rabies cases if one half of the tissues are stored in formalin (and other unfixed tissues tested by DFA) and it was necessary to test complete cross section of the brainstem and other tissues, as required for diagnosis. Rabies antigen detection by IHC test can be utilized for human post-mortem brain samples for the diagnosis and / or retrospective analysis of suspect cases based on the clinical symptoms. While IHC was not approved as a primary or confirmatory test for rabies diagnosis, like DFA, the method detects antigen using rabies specific antibodies. Comparison of DFA using fresh/frozen

vs FF tissues provided similar sensitivity and specificity[15]. Unless antibodies are directly conjugated to FITC (the requirement for DFA test), HRP labeled antibodies can be used in IHC for staining rabies antigen. The advantage with HRP based detection is the ability to use a light microscope for the observation. The current commercially available DFA reagents, FITC conjugated rabies specific antibodies (mAbs) does not detect antigen after formalin fixation due to the modification of epitopes. However, if FITC conjugated rabies specific pAbs are available, it can be used as a staining method, as recommended by World Health Organization[16]. In addition to antigen detection, FF tissues can be subjected to RNA isolation followed by PCR and sequencing using specific primers for confirming the presence of RABV genomic RNA.

The other advantages of formalin-fixed tissues include determination of histological changes by hematoxylin and eosin staining method. While the formalin treatment preserves protein, it completely inactivates most pathogens in the sample due to the extensive crosslinking of proteins and degradation or modification of nucleic acids. Thus, the method improves the safety of biological sample handling, shipping and testing compared to DFA. The acetone fixation step in DFA does not inactivate RABV and should be handled with appropriate PPE[17]. The samples after formalin fixation are stable and can be stored at ambient temperature, which is suited for low-resource areas where access to a cold storage is limited. Similarly, paraffin-embedded formalin-fixed tissues can be considered for the long-term storage at ambient temperatures without losing the antibody reactivity against proteins.

ACKNOWLEDGMENTS:

We thank the laboratorians, epidemiologists, and affiliates with public health departments for sample submissions to the Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names and commercial sources are for identification only and do not imply endorsement by the Centers for Disease Control and Prevention.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Rupprecht, C., Kuzmin, I., Meslin, F. Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies. *F1000Research.* **6,** 184, doi:10.12688/f1000research.10416.1, (2017).

2 Fooks, A. R. et al. Current status of rabies and prospects for elimination. *Lancet.* **384** (9951), 1389-1399, doi:10.1016/S0140-6736(13)62707-5, (2014).

3 Finke, S., Brzozka, K., Conzelmann, K. K. Tracking fluorescence-labeled rabies virus: enhanced green fluorescent protein-tagged phosphoprotein P supports virus gene expression and formation of infectious particles. *Journal of Virology.* **78** (22), 12333-12343, doi:10.1128/JVI.78.22.12333-12343.2004, (2004).

4 WHO. WHO Expert Consulation on Rabies, Third Report. *WHO Technical Report Series.* **1012** ,1 - 195 (2018).

5 Goldwasser, R. A. & Kissling, R. E. Fluorescent antibody staining of street and fixed rabies virus antigens. *Proceedings of the Society for Experimental Biology and Medicine.* **98** (2), 219-223 (1958).
6 Lembo, T. et al. Evaluation of a direct, rapid immunohistochemical test for rabies diagnosis. *Emerging Infectious Diseases.* **12** (2), 310-313, doi:10.3201/eid1202.050812, (2006).
7 Fekadu, M., Greer, P. W., Chandler, F. W., Sanderlin, D. W. Use of the avidin-biotin peroxidase system to detect rabies antigen in formalin-fixed paraffin-embedded tissues. *Journal of Virological Methods.* **19** (2), 91-96 (1988).
8 Hamir, A. N., Moser, G., Rupprecht, C. E. A five year (1985-1989) retrospective study of equine neurological diseases with special reference to rabies. *Journal of Comparative Pathology.* **106** (4), 411-421 (1992).
9 Inoue, S. et al. Cross-reactive antigenicity of nucleoproteins of lyssaviruses recognized by a monospecific antirabies virus nucleoprotein antiserum on paraffin sections of formalin-fixed tissues. *Pathology International.* **53** (8), 525-533 (2003).
10 Webster, J. D., Miller, M. A., Dusold, D., Ramos-Vara, J. Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. *Journal of Histochemistry and Cytochemistry.* **57** (8), 753-761, doi:10.1369/jhc.2009.953877, (2009).
11 Feiden, W. et al. Immunohistochemical staining of rabies virus antigen with monoclonal and polyclonal antibodies in paraffin tissue sections. *Zentralblatt fur Veterinarmedizin Reihe B.* **35** (4), 247-255 (1988).
12 Manning, S. E. et al. Human rabies prevention--United States, 2008: recommendations of the Advisory Committee on Immunization Practices. *Morbidity and Mortality Weekly Reports Recommendations and Reports.* **57** (RR-3), 1-28 (2008).
13 WHO. Laboraotry techniques in rabies. **1** (Fifth edition), 67-72 (2018).
14 Patrick, E. M. et al. Enhanced Rabies Surveillance Using a Direct Rapid Immunohistochemical Test. *Journal of Visualized Experiments.* (146), doi:10.3791/59416, (2019).
15 Whitfield, S. G. et al. A comparative study of the fluorescent antibody test for rabies diagnosis in fresh and formalin-fixed brain tissue specimens. *Journal of Virology Methods.* **95** (1-2), 145-151 (2001).
16 WHO. Diagnostic procedures for antigen detection. https://www.who.int/rabies/about/antigendetection/en/.
17 Jarvis, J. A., Franke, M. A., Davis, A. D. Rabies direct fluorescent antibody test does not inactivate rabies or eastern equine encephalitis viruses. *Journal of Virology Methods.* **234,** 52-53, doi:10.1016/j.jviromet.2016.04.005, (2016).

Figure 1

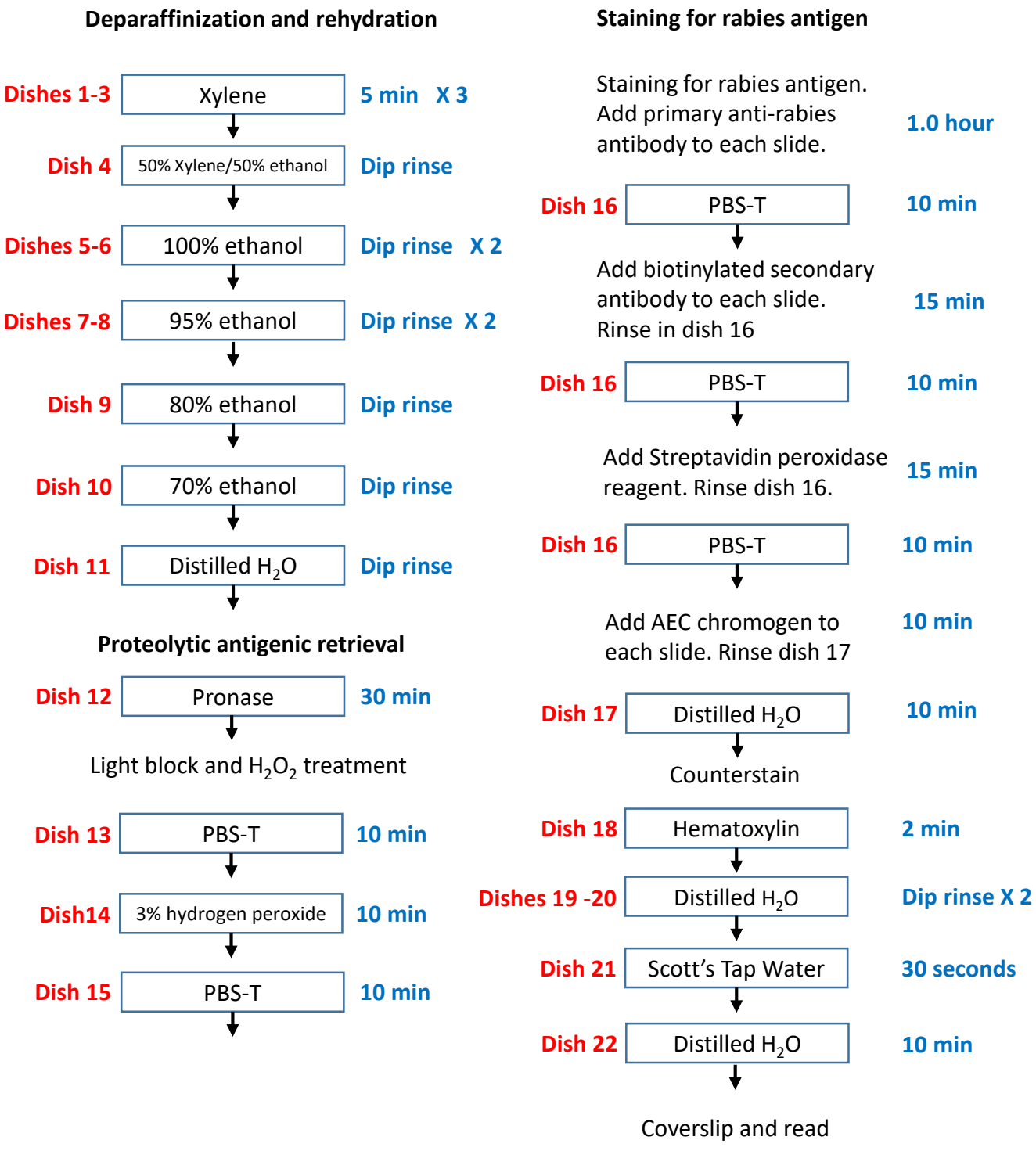
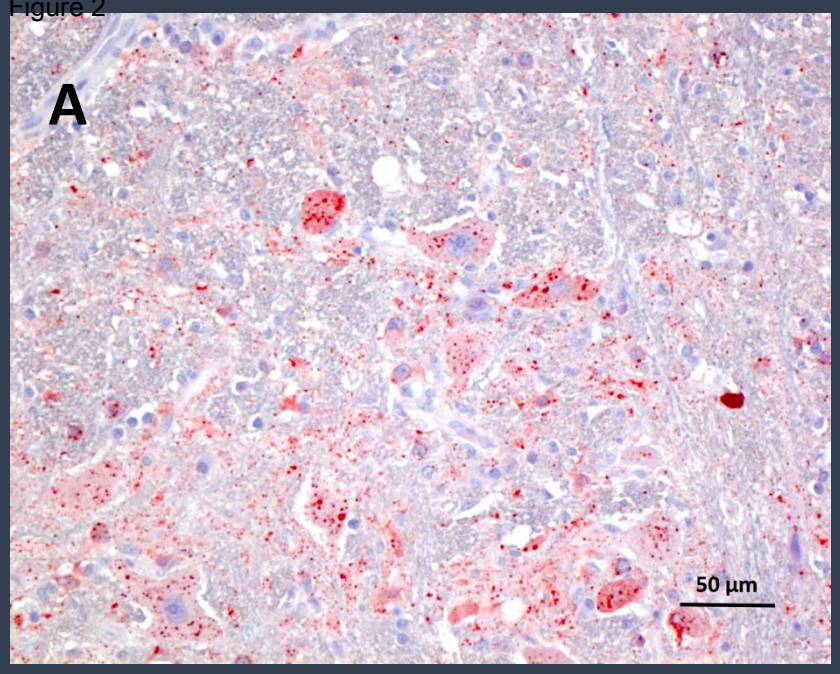
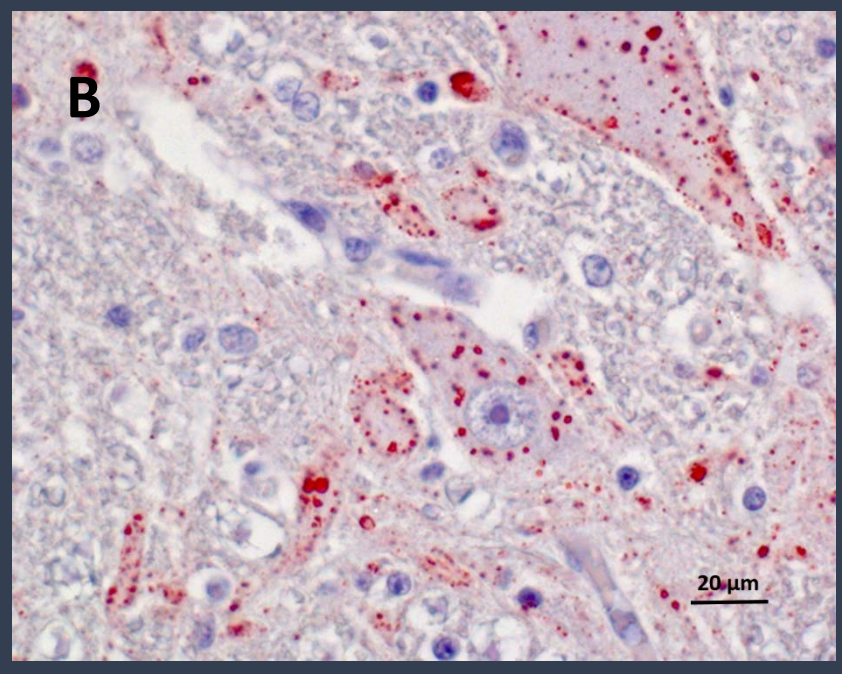
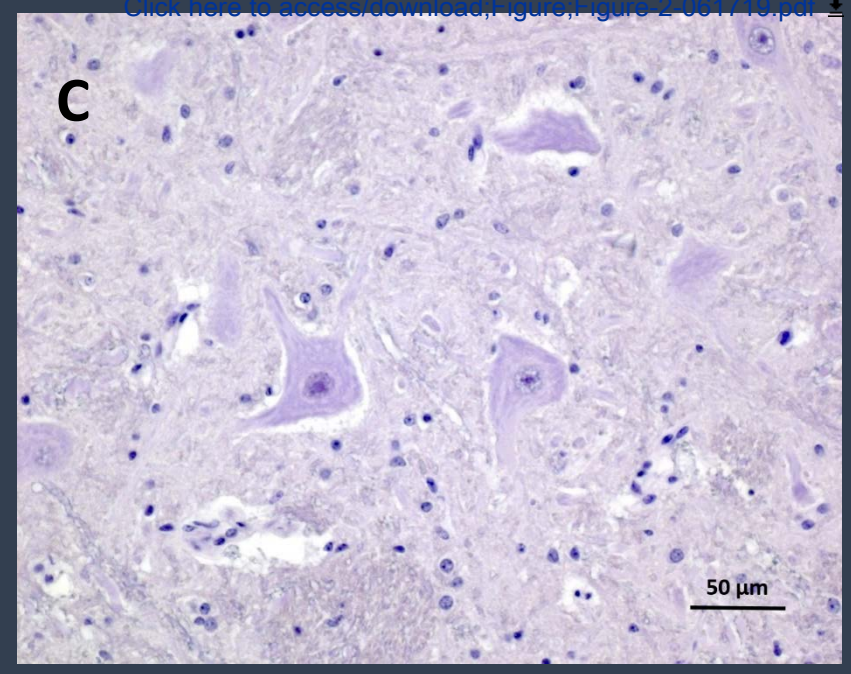
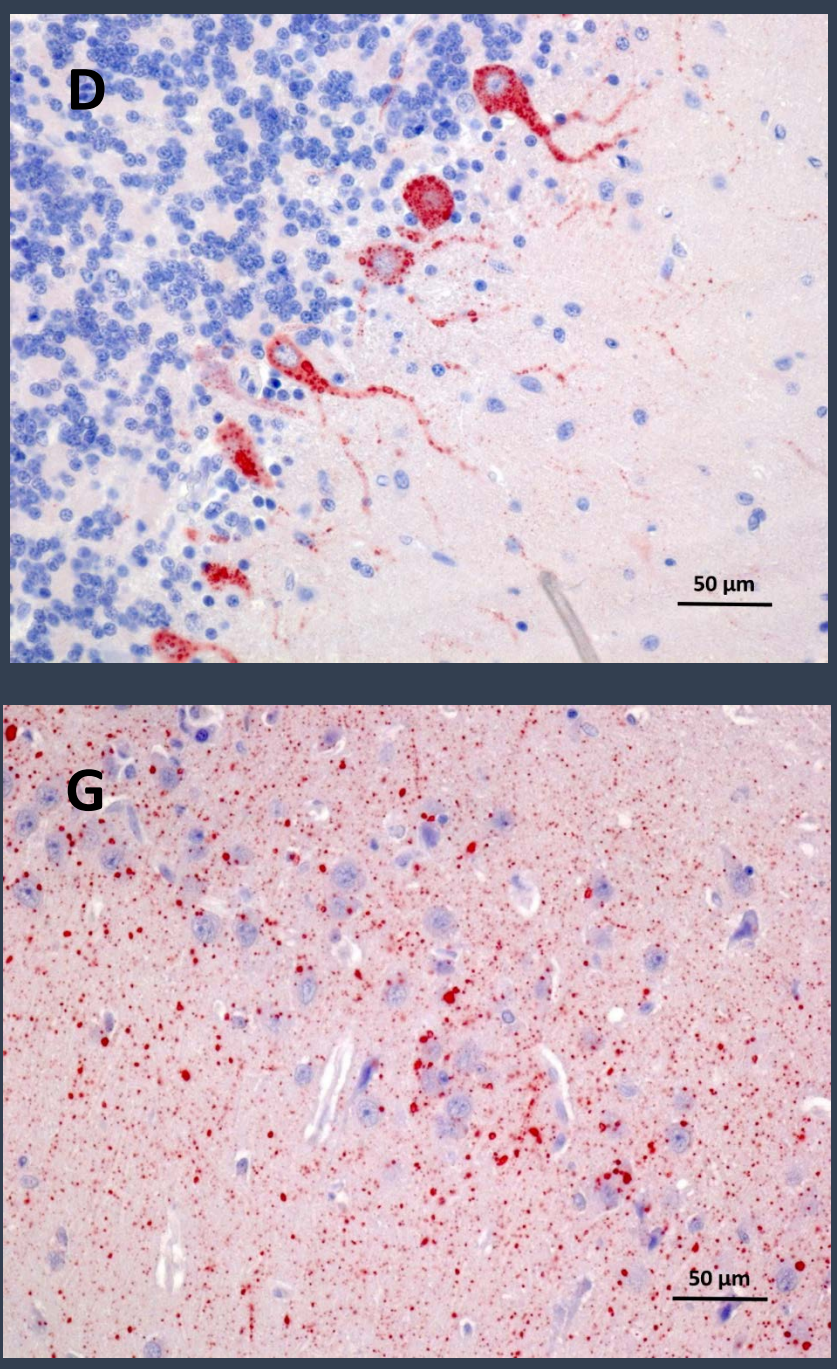


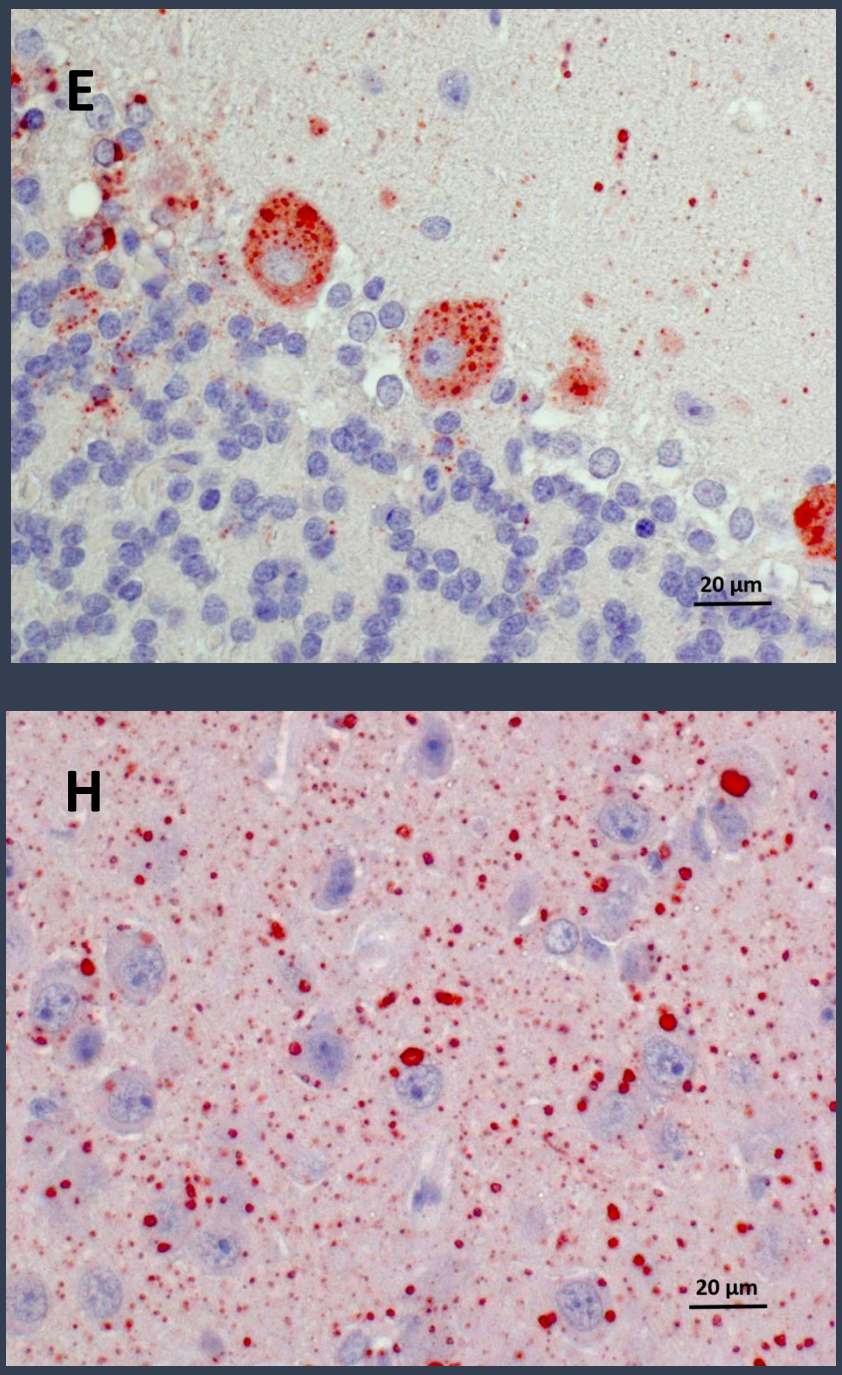
Figure 2

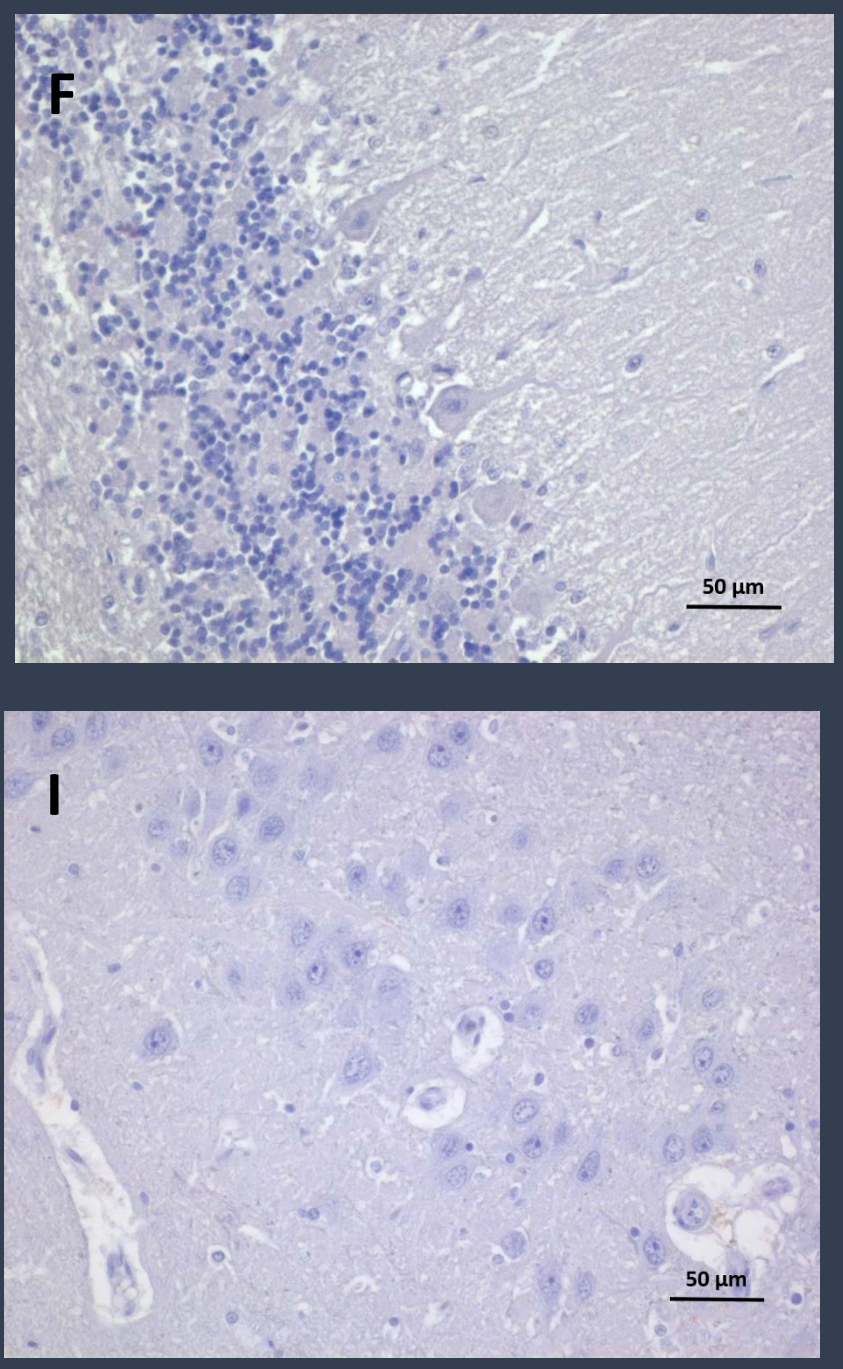












Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3% hydrogen peroxide	Pharamacy brands		Off the shelf 3% H2O2
3-Amino-9-ethylcarbazole (AEC)	Millipore Sigma	A6926	
Acetate Buffer pH 5.2	Poly Scientific R&D Corp.	s140	
Buffered Formalin 10% Phosphate Buffered	Fisher Scientific	SF100-4	Certified
Cover slips Corning	Fisher Scientific	12-553-471	24 X 50 mm
Ethanol 190 Proof	Pharmco-AAPER	111000190	
Ethanol 200 Proof	Pharmco-AAPER	111000200	
Gill's hematoxylin formulation #2	Fisher Scientific	CS401-1D	
HistoMark Biotin-Streptavidin Peroxidase Kit	seracare	71-00-18	Mouse Primary Antibody
ImmunoHistoMount	Millipore Sigma	i1161	Mounting media
N,N, Dimethyl formamide GR	Fisher Scientific	D119	
Phosphate Buffered Saline	HyClone	RR14440.01	01M, pH 7.2 (pH 7.2-7.6)
Plan-APOCHROMAT 40X/0.95 Objective	Multiple vendors		
Plan-APOCHROMATIC 20X/0.75 Objective	Multiple vendors		
Pronase	Millipore Sigma	53702	Protease, *Streptomyces griseus*
Scott's Tap Water	Poly Scientific R&D Corp.	s1887	
Tissue-Tek Slide stain set	Fisher Scientific	50-294-72	
TWEEN-80	Millipore Sigma	P1754	
Xylene	Fisher Scientific	X3S-4	Histological Grade
Zeiss Axioplan 2 imaging - microscope	Multiple vendors		

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Immunohistochemistry test for lyssavirus antigen detection from formalin-fixed tissues

Author(s): Michael Niezgoda, Panayampalli Subbian Satheshkumar

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☒ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☐ The Author is **NOT** a United States government employee.

☒ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

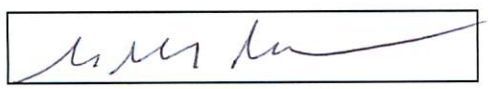
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Michael Niezgoda
Department:	Poxvirus and Rabies Branch
Institution:	Centers for Disease Control and Prevention
Title:	Microbiologist
Signature:	
Date:	16-Apr-2019

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Rephrased. "Here, we present an immunohistochemistry test protocol for detection of rabies virus antigen, an alternate diagnostic test for formalin-fixed tissues."

3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points throughout.

Formatted as per the requirements.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

5. The Protocol should contain only action items in complete sentences that direct the reader to do something.

6. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

7. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

9. 1.1. Please briefly describe how is this done.

Reformatted this section. "Place the tissues collected after necropsy[13] into 10% phosphate buffered formalin solution and record the approximate tissue weight (size), tissue type (brain areas) and volume of formalin. Generally, a 3-5 mm tissue section is fixed in formalin for 24-72 hours in a 1:20 to 1:50 tissue/formalin ratio. It is important for laboratories to document and maintain records on fixation times. For longer tissue storage after formalin fixation and prior to processing, place the tissue in 70% ethanol. CAUTION: Formalin is a toxic fixative."

10. 4: What kind of dishes? Amount of the solution used. Also please use complete sentences throughout. Actually, step 4.1 is redundant and can be removed.

Provided the name of dishes, volume and referenced manuscript with similar set-up.

"3.1. Set up staining dishes (Tissue-Tek staining dish[14]) (Figure 1).
3.1.1. Fill dishes (each dish with 250 ml) 1 to 3 with Xylene."

11. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The protocol is currently 7 pages.

12. Please ensure that you discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

The reference for dish set-up from previous publication is provided.

"3.1. Set up staining dishes (Tissue-Tek staining dish[14])"

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

No copyright issues, these pictures were prepared for this protocol.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

15. Figure 2: Please include a scale bar.
Scale bar is included in the figure

16. Please alphabetically sort the materials table.
Materials updated alphabetically.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this paper, the authors describe a protocol for the immunohistochemistry (IHC) test for detection of rabies virus antigen in formalin-fixed tissues. The figures clearly indicate that this technique is a useful diagnostic alternative if DFA and dRIT are not available.

Major Concerns:

None

Minor Concerns:

This manuscript is well written and reader-friendly. I have only a few minor comments I want the authors to consider.

1) The protocol in itself provides sufficient detailed information to follow the procedure and establish the test under laboratory conditions. However, it would be good for the reader to put this technique better into context as far as the rabies diagnostic hierarchy is concerned. Although it is claimed that the IHC is an established alternative test for rabies antigen detection in tissue sections, it is no primary diagnostic and not even a confirmatory test in the frame rabies routine surveillance. It definitely has its legitimacy for testing of retrospective analysis of suspect cases, in particular for human post mortem brain samples based on the clinical symptoms and for in vivo studies.

Hierarchy – definitely DFA test is the primary diagnostic test to rule out rabies. IHC is considered only when there is no fresh tissue and formalin-fixed tissue is available. Previous studies demonstrated similar sensitivity for DFA on fresh or FF tissue. Included the following information.

"While IHC was not approved as a primary or confirmatory test for rabies diagnosis, like DFA, the method detects antigen using rabies specific antibodies. Comparison of DFA using fresh/frozen vs FF tissues provided similar sensitivity and specificity[15].Unless antibodies are directly conjugated to FITC (requirement for DFA test), HRP labelled antibodies can be used in IHC for staining rabies antigen. The advantage with HRP based detection is the ability to use light microscope for observation. The current commercially available DFA reagents, FITC conjugated rabies specific antibodies (mAbs) does not detect antigen after formalin fixation due to modification of epitopes. However, if FITC conjugated rabies specific pAbs are available, it can be used as a staining method, as recommended by World Health Organization (https://www.who.int/rabies/about/antigendetection/en)."

2) While the advantages of IHC are highlighted in detail, I am missing a few words regarding its limitations, e.g. quality of samples, its relatively time consuming nature, sensitivity etc. For example in Chapter 3.1.17 on Rabies of the OIE Terrestrial Manual it is stated that techniques that stain sections of paraffin embedded brain tissues are time consuming, less sensitive and more expensive than DFA and dRIT.

We understand that there are limitations with IHC test. IHC test is not approved as primary or confirmatory test, but it does provide similar information like DFA on FF tissue for rabies antigen

detection. IHC takes requires multiple steps and about 6 hours for completion. Also, there are no commercial primary rabies antibodies available at present. Included the following information.

"One of the major limitations of IHC test is the protocol involves several sequential steps and takes about 6 hours for completion. If the tissue needs to be fixed in formalin and embedded in paraffin blocks, it requires additional 1 – 2 days before the tissue could be stained. Another limitation is non-availability of commercial primary anti-rabies antibodies for IHC test."

Line 22: Is the IHC really a confirmatory test in its true meaning, e.g. if other tests fail or obtaine indeterminate results?

We have removed confirmatory test sentence from the protocol; generally indeterminate of DFA is tested by PCR; there is also possibility to extract RNA and confirm nucleic acid by PCR and sequencing. Added this sentence about PCR.

"In addition to antigen detection, FF tissues can be subjected to RNA isolation followed by PCR and sequencing using specific primers for confirming the presence of RABV genomic RNA."

Line 25: I suggest modifying the sentence: '… is detection of viral antigen (ribonucleoprotein RNP complex) in the infected tissue samples' and than exclusively use the word 'antigen' throughout the text.
Replaced as antigen throughout the manuscript

Line 42: RV is not only transmitted by dogs.
Reworded the sentence.

"Nearly 99% of all human deaths caused by infection with rabies virus (RABV), the type member of the genus, is transmitted by dogs[2]."

Line 133: 3-Amino-9-ethylcarbizole (AEC): Although the component is not classifiable as to its carcinogenicity one should mention that it may cause congenital malformation in the fetus. It is toxic if inhaled and may cause respiratory tract irritation.
Added the information on toxicity.

"3.2. Preparation of the 3-Amino-9-ethylcarbizole (AEC) substrate stock solution
3.2.1. Dissolve one 20mg tablet of 3-amino 9-ethylcarbazole (AEC) into 5ml of N,N, dimethylformamide. Use glass pipette. CAUTION: AEC is a carcinogen."

Lines 170-172: What primary rabies antibodies are available or can be used and recommended?
Currently, no primary antibodies against rabies are commercially available. We have in-house produced antibody, which we are planning to publish and will make it available for collaborators.

Reviewer #2:
Manuscript Summary:
Need to add few more sentences regarding the advantages of IHC.

Advantages of IHC are added.

"The other advantages of formalin-fixed tissues include determination of histological changes by hematoxylin and eosin staining method. While the formalin treatment preserves protein, it completely inactivates most pathogens in the sample due to extensive crosslinking of proteins and degradation or modification of nucleic acids. Thus, the method improves the safety of biological sample handling, shipping and testing compared to DFA. The acetone fixation step in DFA does not inactivate RABV and should be handled with appropriate PPE[16]. The samples after formalin fixation are stable and can be stored at ambient temperature, which is suited for low-resourced areas where access to cold storage is limited. Similarly, paraffin-embedded formalin-fixed tissues can be considered for long-term storage at ambient temperatures without losing antibody reactivity against proteins."

Reviewer #3:

Manuscript Summary:

The manuscript describes an immunohistochemistry (IHC) protocol for detection of lyssavirus antigen in formalin-fixed tissues. The authors have succinctly but clearly outlined the benefits of using this procedure as an alternative to the gold standard direct fluorescent antibody (DFA) test or the direct rapid immunohistochemical test and reasonably argue that in some instances (i.e., formalin-fixed brain tissue is the only sample available) or for some laboratories (i.e., those which may not have consistent cold storage or expensive fluorescence microscopes), this method may be the best or only alternative for rapid diagnosis of suspect rabies cases. The method is clearly written and easily followed, with a couple of minor exceptions (highlighted below) where some additional detail would be of benefit. The procedure is accurate as written, easy to follow and the figures are very helpful. Overall publication of this manuscript and video of this procedure would be an important contribution for rabies diagnosis and well received. There are just a few minor comments below that might help improve the clarity of one particular series of steps (i.e., the Staining procedure).

Minor Concerns:

(1) Overall, the steps of the procedure are very clearly written and can be easily followed even without figures. The one exception is that the Step 7. Staining procedure seems to lack some detail that for anyone who does not already have a reasonable amount of experience in performing IHC is not very clear. All of the steps under 7. would benefit from a figure showing what the humidity chamber would look like (For example, the manuscript mentions a humidity chamber and defines this as "moistened paper towel on lab bench top", but does not specify if a cover is needed---this is assumed by the term chamber), and, for each of the steps, some brief statements detailing the addition of different reagents to the slides (i.e., normal goat serum, biotinylated antibody, Strepavidin-HRP complex, peroxidase substrate AEC & hematoxylin). For example, when adding the various reagents to the slides, how much volume is used for each? How are they dispensed? After each PBS-T wash, should the slides be blotted as in step 7.1?

Perhaps provide some specific detail on if and how slides can be processed together (e.g., circumstances when it is OK to wash multiple slides in a single wash dish and when not, if not). This additional information could be easily captured in a flow diagram figure of the staining steps and would provide the clarity needed for a less experienced laboratorian.

Humidity chamber reference is added.

"Handle slides one at a time keeping remaining slides submerged in buffer (do not remove whole slide holder - keep slides wet). Remove one slide and blot off excess buffer (using paper towel) from around tissue section being careful not to disturb the tissue section. Incubate slides in a humidity chamber at room temperature with normal goat serum (blocking) for 15 minutes (moistened paper towel on lab bench top[14]."

Volume and blotting details are added in the protocol.

Flow diagram including staining steps are added in Figure 1.

(2) Again, to assist those less experienced with IHC protocols, it would be helpful to include specific information on the equipment used (e.g., particularly what the washing/staining dishes look like, etc.) Would a figure of the full set up be useful? Perhaps in the video this will be much clearer and the authors can recommend alternative ways of setting up the system that might be helpful for those with less resources but not compromise the integrity of the procedure.

Reference for staining dishes from similar manuscript is added in the manuscript.

"3.1. Set up staining dishes (Tissue-Tek staining dish[14]) (Figure 1)."