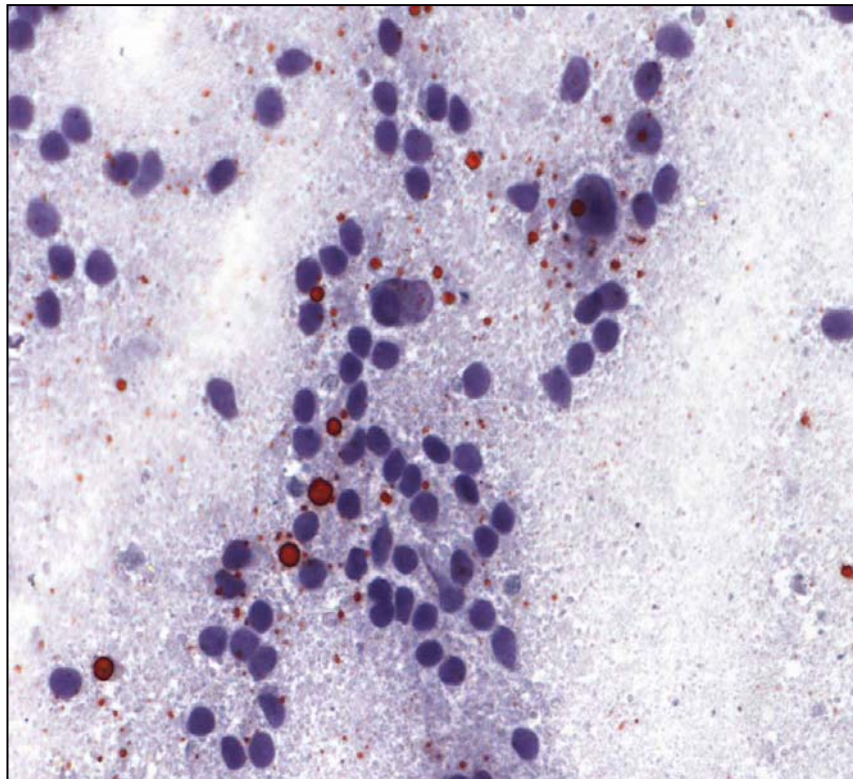




DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Services
Centers for Disease Control and Prevention
Rabies Section

Standard Operating Procedure
For
The Direct Rapid Immunohistochemistry Test (DRIT)
for the detection of rabies virus antigen



**Protocol for the Direct Rapid Immunohistochemistry Test (DRIT)
for the detection of rabies virus antigen**

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1. Introduction

Rabies virus causes an acute encephalitis in all warm-blooded hosts, including humans, and the outcome is almost always fatal. Although all species of mammals are susceptible to rabies virus infection, only a few species are important as reservoirs for the disease. In the United States, several distinct rabies virus variants have been identified in terrestrial mammals, including raccoons, skunks, foxes, and coyotes. In addition to these terrestrial reservoirs, several species of insectivorous bats are also reservoirs for rabies.

Transmission of rabies virus usually begins when infected saliva of a host is passed to an uninfected animal. Various routes of transmission have been documented and include contamination of mucous membranes (i.e., eyes, nose, mouth), aerosol transmission, and corneal transplantations. The most common mode of rabies virus transmission is through the bite and virus-containing saliva of an infected host. Following primary infection the virus enters an eclipse phase in which it cannot be easily detected within the host. This phase may last for several days or months. The uptake of virus into peripheral nerves is important for progressive infection to occur. After uptake into peripheral nerves, rabies virus is transported to the central nervous system (CNS). Typically this occurs via sensory and motor nerves at the initial site of infection. The incubation period is the time from exposure to onset of clinical signs of disease. The incubation period may vary from a few days to several years, but is typically 1 to 3 months. Dissemination of virus within the CNS is rapid, and includes early involvement of limbic system neurons. Active cerebral infection is followed by passive centrifugal spread of virus to peripheral nerves. The amplification of infection within the CNS occurs through cycles of viral replication and cell-to-cell transfer of progeny virus. Centrifugal spread of virus may lead to the invasion of highly innervated sites of various tissues, including the salivary glands. During this period of cerebral infection, the classic behavioral changes associated with rabies develop.

The direct fluorescent antibody test (dFA) is the test most frequently used to diagnose rabies. The direct rapid immunohistochemistry test (DRIT) is an unlicensed procedure designed for consideration as a potential confirmatory measure of the direct fluorescent antibody test, according to the national standard operating procedure for the diagnosis of rabies in animals (http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm). In addition, the RIT may be used to enhance field surveillance among suspect wildlife, particularly in support of national, regional, state, or local oral vaccination programs. The RIT is not to be used for public health surveillance, in those situations in which human or veterinary exposure has occurred or is suspected, state public health authority or other officials should be contacted for immediate and appropriate diagnostic testing.

2. Safety

All persons involved in rabies testing should receive pre-exposure immunization with regular serologic tests and booster immunizations as necessary ([CDC, MMWR, 48: 1-22, 1999](#)). Unimmunized individuals should not enter laboratories or areas where rabies work is conducted. All tissues processed must be disposed of as medical waste and all activities related to the handling of animals and samples for rabies diagnosis should be performed using appropriate biosafety practices to avoid direct contact with potentially infected tissues or fluids (CDC and National Institutes of Health, Biosafety in Microbiological and Biomedical Laboratories, 4th edition, U.S. Government Printing Office, 1999). Personnel working with rabid animals and tissues from rabid animals e.g. brain, are at risk of rabies infection through accidental injection or contamination of mucous membranes with rabies virus contaminated material and by exposure to aerosols of rabies infected material. All manipulations of tissues and slides should be conducted in a manner that does not aerosolize liquids or produce airborne particles. Barrier protection is required for safe removal of brain tissue from animals submitted for rabies testing. At a minimum, barrier protection during field necropsy should include eye protection e.g. safety glasses, face shield; and surgical gloves. When processing multiple animals in a central location Personal Protective Equipment (PPE) should include: heavy rubber gloves, laboratory gown and waterproof apron, boots, surgical masks, protective sleeves, and a face shield. Fume hoods or biosafety hoods are not required, but they provide additional protection from odor, ectoparasites, and bone fragments.

3. Brainstem/cranial cord collection for rabies testing

Make a ventral midline incision from the symphysis of the mandible to several centimeters caudally beyond the larynx. Sever the musculature attachments of the tongue rostrally and on both sides, proceeding caudally to free the larynx, trachea, and esophagus (as if preparing to remove the 'pluck' or tongue, esophagus, trachea, lungs, and heart in one piece) and retract to expose the ventral surface of the spinal column and associated musculature. Palpate to identify the atlanto-occipital joint and dissect to expose the tough connective tissue located on the ventral surface of the joint. Although tough, the connective tissue is thin and directly overlays cerebrospinal and the spinal cord. With the tip of the scalpel blade, carefully cut through the connective tissue (but not the spinal cord) and work the tip of the scalpel down both sides of the joint, while flexing the joint to gain better access. The exposed brainstem/spinal cord tissue may then be severed as far caudally and rostrally as possible to yield central nervous system tissue suitable for rabies testing. Samples may be placed in screw cap vials, preferably unbreakable (i.e. not glass), or other suitable containers, such as ointment tins. Consideration should be

given to adequate sample information (such as species, a unique identifying number, date, animal location, etc.). Samples may be refrigerated until testing if they will be tested within several days. Otherwise, the samples should be frozen, and kept frozen during storage and shipment, until they are tested. To avoid cross contamination of samples; each specimen should be handled on a clean work surface with new disposable gloves. All instruments used during necropsy, dissection, and slide preparation must be properly disposed. Instruments not in use should be kept in closed storage. Only those instruments in use for processing a single sample should be exposed. Maintain test samples for 3 months. Representative positives can be subsampled and used as controls. All positive brain samples should be sent to the CDC for epidemiologic typing, and for other purposes, along with 10% of negatives for confirmation.

Unacceptable deterioration or decomposition of a sample is a qualitative assessment of the condition of each sample upon arrival in the laboratory or at the time of testing. Observation results should be recorded on the RIT Result Sheet (page 14).

Good: Optimum brainstem/cranial cord, fresh, no tissue decomposition.

Fair: Slight tissue decomposition but identifiable as brainstem/cranial cord; may be some discoloration around the periphery but stable tissue at the core of the sample.

Poor: Substantial green color, discoloration, liquefaction, desiccation, or an unrecognizable gross anatomy.

Substantial green color, liquefaction, desiccation, or an unrecognizable gross anatomy can indicate an unsatisfactory sample. A substantial loss of tissue during staining and washing or the presence of bacteria on the stained slide may also indicate sample deterioration. If negative results are obtained on deteriorated tissue, the test report should state only that the condition of the sample is such that tests cannot rule out the presence of rabies virus in the specimen. The negative findings should not be mentioned, since this is often misinterpreted as a negative diagnosis. Positive test results are reported as such. Samples with indeterminate results and all positive samples should be send to the CDC in Atlanta for confirmation and typing.

4. Equipment and Reagents (The use of trade names is for laboratory comparison only and does not imply endorsement by PHS)

Equipment/Supplies:

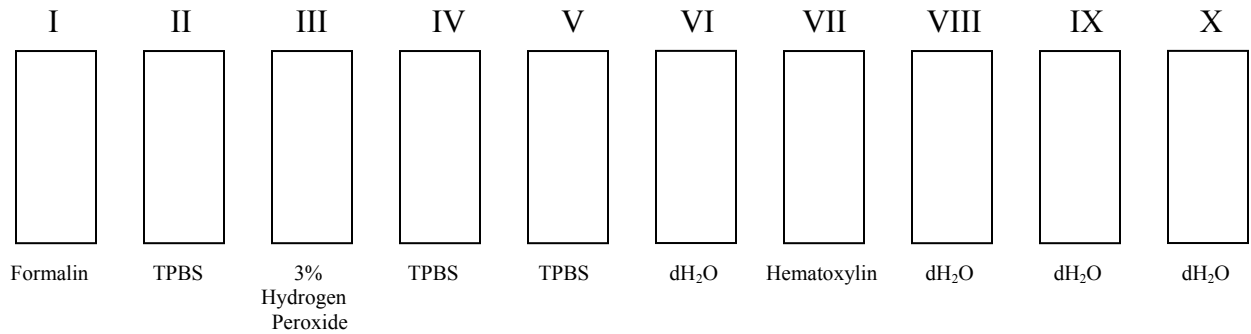
1. Light Microscope with 20x and 40x objectives.
2. Tissue-Tek slide staining kit; Fisher Cat.No.NC9479355 (T-Tek #25608902)
3. Slide holder, 24-place; Fisher Cat.No.NC9418050 (T-Tek #25608868)
4. Fisher Brand syringe 25mm 0.45 μ m filter, Fisher Scientific Cat. No. 09-719D.
5. Wheaton glass vials 8ml, Fisher Scientific Cat. No. 06-408BC.
6. BD 10cc syringe 209604, Fisher Scientific Cat. No. 14-823-2A.
7. Pipettor 200 μ l Rainin: <http://www.rainin.com/> P200 Pipetman pipet 50-200 μ l.
8. Corning cover glass 24x60, VWR Scientific Cat. No. 48396-160.
9. Microscope slides white frosted, Erie Scientific Co. Order No. 10-226.
10. Centrifuge tube (15 ml), polypropylene Falcon 352097 Fisher Scientific Cat. No. 14-959-70C.
11. Tips-universal (1 - 200 μ l vol.) Corning-Costar, Fisher Scientific Cat. No. 07-200-300.
12. VWR Pipet Bulb (0.05 - 100 ml), VWR Scientific Cat. No. 53497-055.
13. Serological pipet (1.0 ml), Pyrex Corning 7078D-1, VWR Scientific Cat. No. 53222-259.
14. Serological pipet (5.0 ml), Pyrex Corning 7078D-5, VWR Scientific Cat. No. 53222-281.
15. Serological pipet (10.0 ml), Plastic, VWR Scientific Cat. No. 20171-042.
16. Tissue culture plate lids (50/case), BD Falcon, Fisher Scientific, Cat. No. 08-772-2B.

4. Reagents

*Note: All reagents can be stored at room temperature unless noted otherwise.

1. Formalin, 10% buffered; Fisher Brand, Order no. SF 100-4 (4L)
2. Phosphate buffered saline (PBS); Fisher Scientific Cat. No. SH30256.02.
3. Hydrogen peroxide 3%. Use any commercially available 3% hydrogen peroxide e.g. Walgreen Drug Store; CVS Pharmacy, Grocery Store brand, etc.
4. *Primary antibody: CDC cocktail (50 ml) of mouse anti-rabies biotinylated monoclonal antibodies, store at 4°C. Supplied by CDC; ready-to-use.
5. *Streptavidin-Peroxidase: 50mL (71-00-38): Kirkegaard & Perry Laboratories Inc; ready-to-use, store at 4°C.
6. Amino-ethylcarbizole (AEC) substrate, SIGMA no. A6926.
7. N,N, Dimethyl formamide GR, EM Science, Cherry Hill, NJ. Thomas Scientific Order No. C279-A87.
8. Acetate Buffer, 0.1M, pH 5.2, Polyscientific, Bay Shore, NY, (516) 586-0400. Catalog No. S140.
9. Gills formulation #2, Fisher order no. CS401-4D, 4L. Diluted 1:2 in distilled water.
10. Gel/Mount, BioMeda Corp., Foster City CA, (aqueous/dry mounting) Fisher Scientific Cat. No. BM-M01, 20ml.
11. TWEEN 80, Polyethylene glycol, Sigma-Aldrich order no. P1754.
12. Deionized water, Fisher Scientific, Cat. No. S75232

5. Tissue-Tek staining tray set-up and reagent exchange.



Staining dish number:

- I. Formalin - change out after 2 runs or once a week.
- II. TPBS - change out with each test.
- III. 3% Hydrogen peroxide - change out with each test.
- IV. TPBS - change out with each test.
- V. TPBS - change out with each test.
- VI. Deionized/distilled water (dH₂O) - change out with each test.
- VII. Hematoxylin - change out once a week.
- VIII. dH₂O - change out with each test.
- IX. dH₂O - change out with each test.
- X. dH₂O - change out with each test.

6. Preparation of reagents for staining dish

- I. Formalin, 10% buffered; ready-to-use
- II. Phosphate buffered saline with 1% tween-80 (TPBS)

TPBS (PBS with 1% tween-80) = to 990 ml of PBS add 10 ml Tween-80.
Shake until tween-80 is completely into solution.

- III. 3% hydrogen peroxide; ready-to-use
- IV. TPBS
- V. TPBS
- VI. Deionized/distilled water (dH₂O); ready-to-use
- VII. Hematoxylin

Gills formulation #2 diluted 1:2 in distilled water. The staining dish will hold 250 ml of solution (125 ml hematoxylin + 125 ml deionized water).

- VIII. Deionized/distilled water (dH₂O)
- IX. Deionized/distilled water (dH₂O)
- X. Deionized/distilled water (dH₂O)

7. Protocol for the Direct Rapid Immunohistochemical Test (DRIT)

Streptavidin-biotin peroxidase staining technique for diagnosis of rabies virus.

1. Make routine touch impressions of suspect CNS tissues on labeled glass microscope slides (include standard positive and negative controls).
2. Air-dry slides for 5 minutes at room temperature.
3. Immerse slides in 10% buffered formalin at room temperature for 10 minutes. **Dish I.**
4. Remove and dip-rinse slides several times to wash off any excess fixative in wash buffer TPBS (PBS with 1% tween 80). **Dish II.**
5. Immerse slides in 3% hydrogen peroxide for 10 minutes. **Dish III.**
6. Remove excess hydrogen peroxide by dip-rinsing slides in TPBS, **Dish IV.** Transfer slides to the next rinse **Dish V** (after dipping, shake off excess buffer, and blot excess buffer from slide edges surrounding the impression). Work with one slide at a time leaving the remaining slides immersed within TPBS rinse.
7. Incubate slides in a humidity chamber (e.g. may use the plastic top to a 96-well plate or another simple cover over slides, on a moistened paper towel, on lab bench top) at room temperature with primary antibody - biotinylated anti-rabies mAb for 10 minutes (add enough of this primary antibody by drop to cover the impression).
8. After incubation shake off excess conjugate. Dip-rinse slides with TPBS, Dish V (shake off excess TPBS and blot buffer from slide edges surrounding the impression). Can use this same wash buffer through step 10.
9. Incubate slides with streptavidin-peroxidase complex (add enough of this complex to the slide by drop to cover the impression) in a humidity chamber at room temperature for 10 minutes. After incubation shake off excess.
10. Dip-rinse slides with TPBS, **Dish V** (shake off excess buffer and blot excess buffer from slide edges surrounding the impression).

11. Incubate slides with peroxidase substrate, amino-ethylcarbizole (AEC) – prepare the working dilution just prior to use (see page 10). Add enough of this substrate to the slide by drop to cover the impression in a humidity chamber at room temperature for 10 minutes. After incubation, shake off excess substrate.
12. Dip-rinse slides in deionized/distilled water, **Dish VI**.
13. Counterstain with Gills Hematoxylin (diluted 1:2 with deionized/distilled water) for 2 minutes, **Dish VII**.
14. Immediately dip-rinse this stain from slides with deionized/distilled water **Dish VIII**. Make a second dip-rinse of slides with fresh deionized/distilled water (**Dish IX**) to ensure removal of excess stain.
15. Transfer slides to fresh distilled water **Dish X**. Mount slides with water-soluble mounting medium and cover-slip. (work with one slide at a time, shake off excess deionized/distilled water and blot excess from slide edges surrounding the impression). Do not allow slides to air-dry prior to cover-slipping. If multiple slides are stained, they may sit in the deionized/distilled water rinse before cover-slipping.
16. View slides by light microscopy, using a 20x objective to scan the field, and a 40x objective for higher power inspection (rabies virus antigen appears as red inclusions against the blue neuronal background).
17. Record results.

**8. Preparation of peroxidase substrate: amino-ethylcarbazole (AEC),
Step 11, page 9.**

A. STOCK solution:

Reagents:

1. Amino-ethylcarbazole (AEC) substrate, SIGMA no. A6926.
2. N,N, Dimethyl formamide GR, EM Science

Supplies:

1. 5 ml Pyrex (glass) pipet
2. 8 ml Wheaton jar

To prepare AEC stock solution

- a. Dissolve one 20mg tablet of 3-amino 9-ethyl carbazole (AEC) in 5 ml of N,N, dimethylformamide in a glass Wheaton jar (label 'AEC stock' and date).

The AEC stock solution should be stored in the refrigerator (4 °C) for 1 to 2 months.

B. WORKING dilution; prepare fresh with each test just prior to staining slides:

Reagents:

1. Acetate buffer, 0.1M, pH 5.2
2. AEC Stock (above)
3. 3% Hydrogen peroxide

Supplies:

1. 1 ml Pyrex (glass) pipet
2. 10 ml plastic pipet
3. Pipettor (200 μ l)
4. Pipet tips (200 μ l)
5. 0.45 μ m syringe filter
6. 10 cc syringe
7. 15 ml centrifuge tube (2)

To prepare AEC working dilution:

- a. Add 7 ml of acetate buffer to a 15 ml centrifuge tube using a 10 ml plastic pipet.
- b. Add 0.5 ml of AEC stock solution (above) using a 1 ml Pyrex (glass) pipet.
- c. Add 0.075 ml (75 μ l) of 3% hydrogen peroxide.

Filter mixture using a 10 ml syringe with syringe filter (0.45 μ m) into separate 15 ml centrifuge tube

This mixture once made is only stable for 2-3 hours.

9. Reading and recording results

A test sample can be considered negative for rabies when brain stem/cord is scanned over approximately 40 fields at a magnification of approximately 200X or greater for inclusions.

Test Results

Staining intensity / antigen distribution. Rabies virus in the brains of infected animals produces intracytoplasmic inclusions of various shapes (Pictures B, C, and D, page 13). A single microscopic field may contain numerous sized round to oval masses and strings. When specifically stained with biotinylated antibody, the substrate 3-amino-9-ethylcarbazole (AEC), upon oxidation, forms a rose-red end product. Hematoxylin counterstain will produce a blue tissue and nuclear background. AEC is susceptible to excessive light and will fade in intensity. Storage in the dark is therefore recommended.

Observations made for each test slide are recorded as staining intensity/antigen distribution on result sheet.

Staining intensity is graded from +4 to +1. Positive control slides in all tests should optimally contain staining of +4 intensity. Slightly diminished staining intensity (a slight loss of color) is graded as +3 intensity and may occur in test samples positive for rabies when sample handling has not been optimal. Noticeably dull stain is graded +2 to +1 and cannot be considered as diagnostic for a rabies infection without confirmation of specificity. Even though diminished staining intensity may be the result of denaturation of rabies virus antigen, diminished staining may also result from non-specific binding of antibody to components of inflamed tissue or artifacts of tissue decomposition.

Antigen distribution. For each brain examined, staining is graded by the amount of antigen present as follows:

+4, a massive infiltration of large and small inclusions of varying shape in almost every area of the impression.

+3, inclusions of varying size and shape are found in almost every microscopic field, the number of inclusions per field varies, but inclusions are numerous in most fields.

+2, inclusions of varying size and shape are present in 10% to 50% of the microscopic fields and most fields contain only a few inclusions.

+1, inclusions of varying size and shape are present in <10% of the microscope fields and only a few inclusions are found per field (usually only one or two inclusions per field).

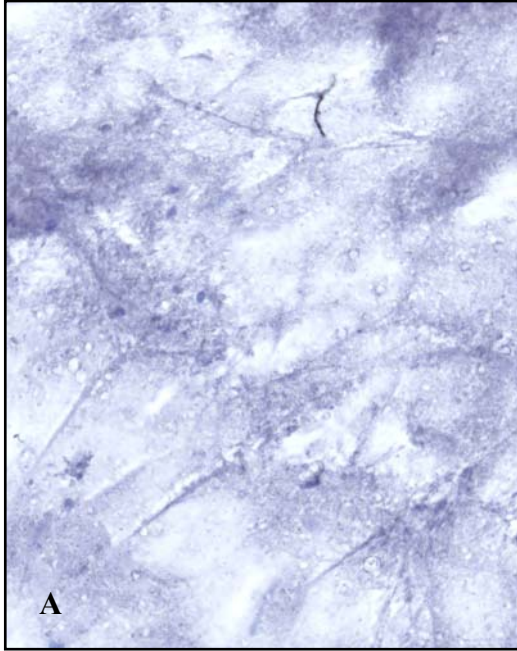
Test interpretation. If the tissue sample was adequate and suitable for rabies diagnosis, results for a test animal are reported as positive or negative for rabies (test complete) or non-diagnostic (test indeterminate) based on observed patterns of staining in test and control slides.

Test complete / reportable result. Test results are reported if the following observations are made:

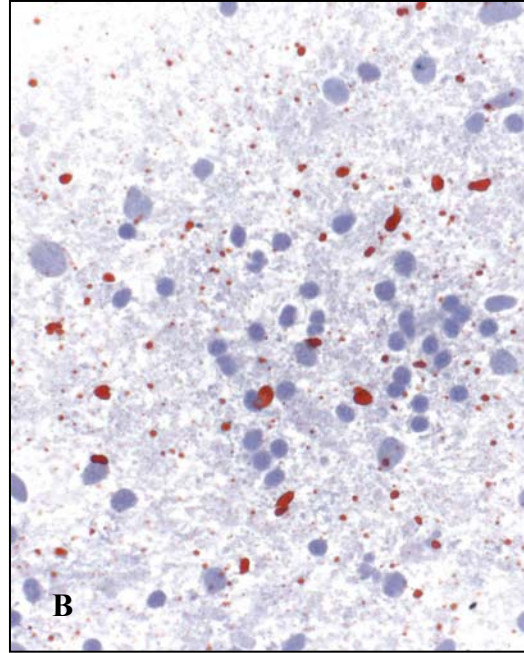
Test controls: Both large and small antigen accumulations in positive control slide stain with +4 intensity and +3 to +4 antigen distribution. No staining is present on negative control slide.

Test samples: No tissue deterioration or alteration was noted when slides were prepared. Samples are clearly negative (no specific staining in test slides) or clearly positive (at least +3 to +4 intensity and +2 to +4 distribution of antigen in slides made from brain stem and spinal cord).

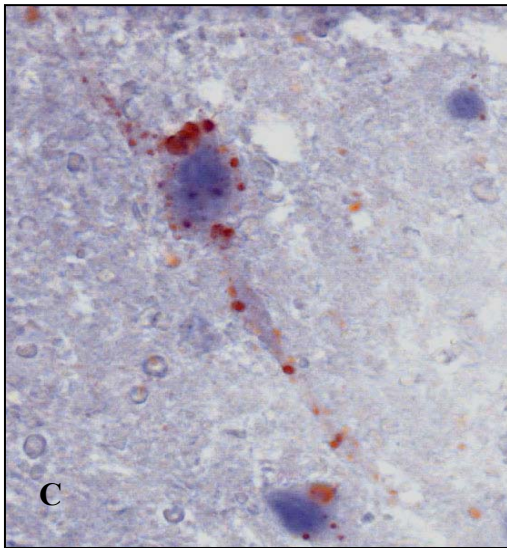
All samples positive for rabies virus should be sent to CDC in Atlanta for confirmation, along with 10% of negative samples.



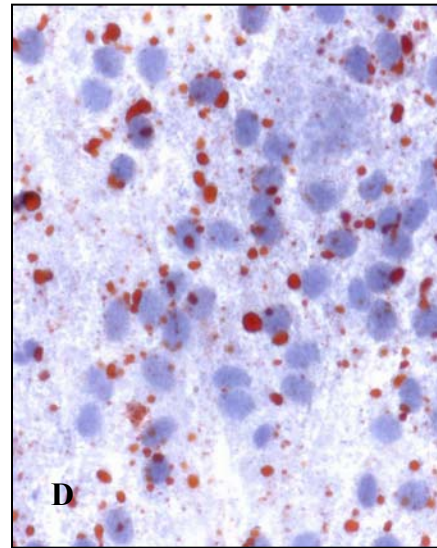
A. Negative brain touch impression



B. Positive reaction (detection of rabies virus nucleoprotein).



C. Observation of cell associated intracytoplasmic inclusions characteristic of rabies virus infection.



D. Positive reaction 400x total magnification.

10. Contact Information:

Dr. Charles E. Rupprecht
Email: cyr5@cdc.gov
Tele: (404) 639-1050

Michael Niezgoda
Email: man6@cdc.gov
Tele: (404) 639-1068

Shipping Address:

NCID/DVRD/VRZB/Rabies
Centers for Disease Control and Prevention
1600 Clifton Rd.
Mail Stop G33
Atlanta, GA 30333

11. Equipment/Reagent check-list (The use of trade names is for laboratory comparison only and does not imply endorsement by PHS)

Poly Scientific: 1 (800) 645-5825

<u>Product</u>	<u>Cat No</u>	<u>Size</u>	<u>Cost</u>	
Acetate buffer 0.1M, 5.2 Ph	s140	32oz	\$41.50 ea	_____

Fisher Scientific: 1 (800) 766-7000

<u>Product</u>	<u>Cat No</u>	<u>Size</u>	<u>Cost</u>	
Gill Hematoxylin Stain	CS401-1D	1L	\$39.06 ea	_____
Gel/Mount media	BMMO1	20ml	\$27.20 ea	_____
N,N, Dimethyl formamide	D119-500	500ml	\$63.54 ea	_____
Fisher Brand syringe 25mm filter	09-719D	50/pk	\$134.00 pk	_____
Wheaton glass vials 8ml	06-408C	144/cs	\$68.49 cs	_____
BD 10cc syringe 209604	14-823-2A	100/pk	\$20.78 pk	_____
Centrifuge tube 15 ml	14-959-70C	500/cs	\$178.33	_____
Formalin 10% buffered	SF100-4	4L	\$51.95	_____
Phosphate buffered saline (PBS)	SH30256.02	12/cs	\$168.00	_____
Deionized water	S75232	5@1gal	\$36.00	_____
Tips-universal (1-200 μ l)	07-200-300	960/cs	\$48.84	_____
Tissue culture plate lids	08-772-2B	50/cs	\$88.00	_____
Tissue-Tek Staining Set 25608902	NC9479355	1 ea	\$253.91	_____
Slide holder 25608868	NC9418050	1 ea	\$28.24	_____

VWR Scientific: 1 (800) 932-5000

<u>Product</u>	<u>Cat No</u>	<u>Size</u>	<u>Cost</u>	
Corning cover glass 24x60	48396-160	10/cs	\$111.87	_____
Serological pipet Pyrex 1ml	53222-259	50/pk	\$20.14	_____
Serological pipet Pyrex 5ml	53222-281	25/pk	\$16.15	_____
Serological pipet Plastic 10ml	20171-042	200/cs	\$76.52	_____
VWR Pipet filler bulb	53497-055	1 ea	\$29.43	_____

Sigma-Aldrich: 1 (800) 325-3010

<u>Product</u>	<u>Cat No</u>	<u>Size</u>	<u>Cost</u>	
TWEEN 80, Polyethylene glycol	P1754	500ml	\$20.85 ea	_____
3-Amino 9-ethylcorbvazole	A6926	50 tablets	\$72.50 jar	_____

ERIE Scientific Company: 1 (800) 258-0834

<u>Product Cel-Line™</u>	<u>Cat No</u>	<u>Size</u>	<u>Cost</u>	
Microscope slides white frosted	10-226-H	15mm (2-well)	\$51.56/gross*	_____

* 1 gross = 144 sides

KPL (Kirkegaard & Perry Laboratories)

1 (800) 638-3167

<u>Product</u>	<u>Cat No</u>	<u>Size</u>	<u>Cost</u>	
Peroxidase Labeled Streptavidin	71-00-38	50ml	\$82.00	_____

Rainin: <http://www.rainin.com/>

<u>Cat No</u>	<u>Size</u>	<u>Cost</u>	
1 (800)-472-4646			
P200 Pipetman pipet 50-200ul	P200	1 each	\$259.50 _____

Other Items

Light microscope with 20x and 40x objectives	_____
3% Hydrogen peroxide (off-the-shelf pharmacy or grocery store brand)	_____
Lysol IC Quaternary disinfectant 1 gallon (Fisher Scientific)	_____
Clock-Timer (Fisher Scientific; VWR Scientific; have many varieties)	_____
Drummond Pipet-Aid (http://www.drummondsci.com/) - optional	_____
Primary biotinylated mouse antibody (50 ml); supplied by CDC (ready-to-use)	_____