Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing

A Minimum Standard for Rabies Diagnosis in the United States

I. Introduction

Among the findings of the National Working Group on Rabies Prevention and Control was the need for a minimum national standard for the laboratory diagnosis of rabies (Hanlon et al., JAVMA, 215:1444-1446, 1999). In response to this recommendation, a committee was formed from representatives of national and state public health laboratories (*Appendix 1*) to evaluate procedures employed by rabies diagnostic laboratories in the United States. Both the National Working Group and this committee have as their goal the improvement of the overall quality of rabies testing through the formulation of guidelines and standards for equipment, reagents, training, laboratory protocols, quality assurance, and laboratory policy for rabies diagnosis.

As a first step to attaining this outcome, the committee prepared a standardized protocol for the analytical phase of rabies testing using the direct fluorescent antibody (DFA) test and evaluated the protocol by comparison testing of 435 samples submitted to public health laboratories for rabies diagnosis (*Appendix 2*) . Later documents will address other elements that can affect the quality of laboratory testing such as the pre-analytical steps (rationale for specimen submission), the post-analytical phase (result reporting and confirmatory testing), laboratory policy, and quality assurance.

The standardized protocol was developed from published procedures and the collective laboratory experience of the committee members. The group recognizes that a range of possible methods may achieve the desired outcome for some of the less critical steps in the diagnosis of rabies and that laboratory policy may be regionally defined in some cases; however, the goal of the group was to establish a single protocol by which all other methods could be validated by comparison. The recommendations included in this document should be closely followed to ensure a test of highest sensitivity and specificity. Modifications or short cuts in procedures often lead to false positive and false negative results and non-specific or uninterpretable reactions. A laboratory wishing to incorporate elements of test methods other than those presented in this document should validate and confirm those methods by consultation with one of the laboratories listed in *Appendix 1*.

The standard protocol for DFA will be made available to each rabies testing laboratory by postal or electronic mail and by participation in a training workshop. In addition, the protocol and other documents will be placed on the web sites maintained by the Centers for Disease Control and Prevention (CDC) at www.cdc.gov/ncidod/dvrd/rabies and the Association of Public Health Laboratories (APHL) at www.aphl.org. A listserve is planned so that an interactive dialogue may be established to address questions and disseminate information about rabies testing.

In addition to the procedural aspects detailed in this document, each testing laboratory must maintain the competency of its employees. There is no substitute for constant practice and experience in performing DFA testing. All new employees should be trained in all aspects of the procedure, and competency should be evaluated by the senior technologist on a routine basis. Competency can be assessed by observation of all procedural aspects on a routine basis, as well as performance on proficiency test samples, and testing on internal blind samples. All training should be documented throughout the training period, and observations of breakdown in technique or procedures should be noted and corrected before the lead technologist can be assured that the trainee can perform the procedure in a competent and reliable manner. All laboratories performing this DFA test for rabies should participate in national rabies virus proficiency testing, available through the Wisconsin State Laboratory of Hygiene. Enrollment information may be obtained through their web site (www.slh.wisc.edu/pt) or by calling 1-800–462–5261.

At least once every 6 years, each laboratory should send a representative to the National Laboratory Training Network sponsored course "Laboratory Methods for Detecting Rabies Virus". The course, held at least every 2 years, details all aspects of rabies testing and provides an opportunity for diagnosticians to meet colleagues from other states and discuss common problems and their solutions. Advances in rabies diagnosis and research are presented at the Rabies in the Americas meeting, held every year at locations in North and South America.

Bench training and consultation on basic aspects of rabies diagnosis are available at the state public health laboratories in New York, California, Texas, Wisconsin, and Ohio and at the national rabies laboratory at CDC (*Appendix 1*). These laboratories can be contacted at any time with questions or requests for consultation and training. Laboratories that annually process <100 samples may have particular difficulty maintaining rabies diagnostic proficiency and may want to work closely with larger laboratories where additional resources are available. Sources of funding are being investigated for those states that have no travel budget to attend meetings, workshops, or seminars. Potential sources that are being explored include ASM, APHL, or the Epidemiology and Laboratory Capacity (ELC) program at CDC.

II. 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 where rabies work is conducted. All tissues processed in an infectious disease laboratory 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 in rabies laboratories 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 necropsy should include the following as Personal Protective Equipment (PPE): 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. Glass chips and shards from slide manipulations are also potential sources of exposure to rabies. Care should be taken to protect eyes and hands during manipulation and staining of slides and during clean up of the microscope and surrounding area. A microscope adaptor is available to provide eye protection from any glass slivers produced when slides are moved across the microscope stage. Ergonomic equipment (fatigue mat, microscope controls) should be used to prevent fatigue-related injuries to employees during lengthy necropsy and slide-reading procedures.

III. Equipment and Reagents

(Use of trade names and commercial sources is for identification only and does not imply endorsement.)

A. Equipment

1. Necropsy instruments should be of sufficient quantity for 1 set per sample to prevent cross transfer of infected tissue between samples.

2. Autoclave and/or instrument sterilizer. All instruments should be cleaned and sterilized before reuse.

3. Specimen storage containers must be large enough that reserved portions of brain stem and cerebellum (and hippocampus, if tested) remain as recognizably separate pieces. Because of the risk of breakage, glass vials and tubes are unacceptable for specimen storage. Wide mouth, screw cap, polypropylene jars or sample bottles are used in many laboratories (e.g., Nalgene 2118 or 2189).

4. Refrigerated storage. An explosion proof -20°C freezer is required for fixation of impression / smear slides and storage of acetone and other reagents; long term sample storage requires a freezer at -70°C. Frost-free freezers should **not** be used. Heat cycles in frost-free freezers will denature proteins in reagents and specimens and may compromise test results.

5. Microscope slides should be of highest quality with coverslip matched to the lens working distance. Teflon or pre-ringed well slides can be used to denote stained areas for touch impression slides. User-specified templated slides can be ordered through Cel-Line/ERIE Scientific Co. (1-800-258-0834). Wells of 14 mm or 15 mm diameter are adequate for rabies tests. Marking instruments that contact tissue (e.g., wax pencil or Martex pen) should **not** be used to denote stained regions of the slides, because this process can transfer infected tissue between slides. Slip smears cannot be made on pre-ringed slides. A 16 mm area to be stained on a slip smear can be marked by dipping the rim of a 16 x 100 mm test tube into a small pool of nail polish poured onto a square of aluminum foil. A well is made on the slip smear by lightly touching the test tube rim to the surface of the smear. To avoid cross-contamination between specimens, a separate test tube and pool of nail polish is used for each specimen submitted for testing. Best contrast is obtained with red pigmented nail polish. Alternatively, separate slides can be made for staining with each reagent. The addition of a counter stain such as Evans Blue to the conjugate will also denote stained areas.

6. Acetone fixation and post-stain rinse containers should be in sufficient quantity such that slides made from each test animal are processed in a container separate from the slides from other animals. Positive control slides are processed in a container separate from test clides. A false-positive test may result from cross transfer of tissue between slides from different animals if a common container is used for fixation or washing.

7. Syringe filters (0.45um). Anti-rabies conjugates should be filtered to remove dissociated fluorescein isothiocyanate (FITC) and protein aggregates that may bind non-specifically to tissue. Conjugates should be filtered only once and should be evaluated for the effect of filtration on the titer of the working dilution (see section VI). Syringe filters allow the conjugates to be filtered as they are added to test slides. Similarly, syringe filters will remove contaminants and precipitates as mountants are added to slides. Filters for the conjugate must be low protein binding (e.g., cellulose acetate) to prevent loss of labeled antibody from the conjugate. Filter materials that bind proteins with great avidity (e.g., mixed cellulose esters) should not be used for conjugate filtration. Both large volume, mid-volume, and small volume filters are available (Schleicher & Schuell, Uniflo; Millipore, Millex-HV). Smaller volume filters avoid dead volume loss to the filter membrane. Evans Blue counterstain also binds to the membrane, but the filter is saturated quickly. To avoid slide to slide difference in counter stain, laboratories that employ Evans Blue counterstain in the conjugate should discard the first three drops of conjugate expressed though the filter.

8. Incubator (37°C) and humidified staining tray or chamber. Constant humidity must be maintained during the staining process. Conjugate dried on the slides during the staining process may be mistaken for specific staining, resulting in a false positive test, or may obscure specific staining, resulting in a false negative test.

9. Fluorescence microscope. The quality of the fluorescence microscope is critical to the sensitivity of the DFA test. Manufacturers offer many equipment options. *Appendix 3* contains a discussion of oculars, objective lens, filter sets, and light sources and their effects on instrument performance. At a minimum, all rabies diagnostic laboratories should have a reflected light (incident light) fluorescence microscope with high quality objective lenses. Both magnification and and numerical aperture (NA) must be considered in lens selection. Although image size increases with magnification, both resolution and image brightness are related to the NA of the objective lens, and brightness decreases with magnification. For example, a high quality 20X dry objective with a NA of 0.75 provides a brighter image over a larger field of view with no loss of resolution as compared to a dry 40X objective with an NA of 0.75. (See the discussion of magnification, image brightness and resolution in *Appendix 3*.) The use of immersion oil increases image brightness by preventing the loss of emitted light in the airspace between coverslip and dry objective. Although not every slide must be observed with a 40X oil objective of high NA, resolution of very fine dust-like inclusions and recognition of some types of non-specific staining is aided by examination with this type of lens. An oil immersion lens requires a high quality immersion oil. An oil should be chosen that produces the least autofluorescence and thus the best contrast between FITC and tissue (e.g.,Cargille type DF). The oil should have the same refractive index as glass (1.515).

B. Reagents

1. Acetone. Only American Chemical Society (ACS) Reagent Grade acetone should be employed and the acetone should not be reused.

2. FITC-conjugated anti-rabies antibodies. Reagents presently available commercially are listed in *Appendix 4*. Every laboratory should maintain stocks of anti-rabies conjugates from two different sources. The two sources should be two different monoclonal antibody pools or one monoclonal antibody conjugate and one hyperimmune serum conjugate. (Note: Centocor FITC-Anti-rabies Monoclonal Globulin and Light Diagnostics Rabies DFA II contain the same antibody pools and are not considered different sources.) Antigen presentation and antibody avidity and affinity vary with different rabies virus samples, and viral inclusions may appear quite different when stained with different reagents. Although hyperimmune serum reagents contain many different anti-rabies antibodies that can that produce a wide-spectrum of rabies virus recognition, these reagents also contain extraneous antibodies that can produce several types of non-specific reactions (*Appendix 5*). Extraneous antibodies are not a problem with monoclonal antibody reagents but the fine specificity of these reagents may result in non-recognition of some variants of rabies virus. The use of two reagents prepared from different pools of monoclonal antibodies greatly reduces the risk of non-recognition of any one variant.

FITC-labeled conjugates should not be used past the manufacturer's expiration date. Upon receipt, a conjugate should be reconstituted (if lyophilized) and titrated to determine a working dilution (section VI). Stock solutions of conjugate are stored as frozen aliquots in a non-frost-free freezer at -20°C or below (<-30°C is preferred). Conjugate diluted to the working dilution is stored at +4°C and discarded after 7 days. The working dilution of the conjugate is filtered as it is added to the test slide. Excess diluted conjugate can be stored at +4°C in the syringe and attached syringe-filter unit used for dispensing it, if care is taken to remove any remaining droplets from the filter outlet and the outlet is sealed against drying with a syringe tip or plastic wrap.

3. Specificity controls. *Appendix 4* lists commercially available control reagents. The specificity control for an FITC-labeled hyperimmune serum rabies reagent is an FITC-labeled serum reagent produced in the same animal host as the rabies reagent (typically goat or horse), but either as normal serum or hyperimmune serum directed to an agent other than rabies virus (e.g., FITC-labeled anti-distemper reagents). The control reagent should be diluted to the same mg protein concentration as the rabies reagent. Similarly, the specificity control for a rabies reagent prepared from a mouse monoclonal antibody is an FITC-labeled mouse monoclonal antibody of the same isotype and protein concentration as the rabies reagent but directed to an agent other than rabies virus. Suspensions of Normal Mouse Brain (NMB) and Rabid Mouse Brain (RMB) are no longer used as specificity controls. Historically, RMB and NMB were used as diluents for FITC-labeled hyperimmune serum conjugates to control for the presence in the conjugate of extraneous antibodies present in animals used for the production of rabies hyperimmune serum as a result of a natural exposure. False positive reactions were identified when staining was observed both with a conjugate adsorbed with NMB (which should have no effect on any antibody in the conjugate) and a conjugate adsorbed with RMB (which should remove only the rabies antibodies and have no effect on antibodies to other infectious agents). Because monoclonal antibody reagents contain only antibodies reactive with antigens on rabies virus, it is not possible for these reagents to contain extraneous antibodies and adsorption controls for these reagents are meaningless. A more lengthy explanation of control reagent use is found in Section VII and in *Appendix 5*.

4. Conjugate diluent should be 0.01M phosphate buffered isotonic saline solution at pH 7.4 to 7.6 (e.g., phosphate buffered saline (PBS) Sigma P3813 is 0.01 M phosphate buffer, pH 7.4, with 0.138 M NaCl and 0.0027 M KCl). No protein stabilizer (e.g., bovine serum albumin) is needed in the diluent.

5. Counterstains added to the working dilution of the conjugate provide contrast and lower background and also serve as a marker for accidental omission of the diagnostic reagent. Counterstain use is optional. Evans Blue counterstain (0.5% in PBS, Sigma, Product #E-0133, for use in immunofluorescent assays) can be aliquoted and stored at +4°C for up to 6 months and indefinitely at -20°C. The amount of counterstain added to a conjugate is determined by titration when the working dilution of the conjugate is determined (section VI). Due to counterstain, the tissue will be noticeably red, but should not be so strongly red as to diminish the specific green fluorescence of rabies virus proteins. An Evans Blue concentration of 0.00125% works in many laboratories. This concentration is prepared by adding 2.5 microliters of 0.5% stock dye solution per ml of conjugate diluent.

6. Rinse / soak buffer. A PBS formula of the same pH and molarity as the conjugate diluent is used as the rinse/soak buffer (e.g., Sigma P3813 is 0.01 M phosphate buffer, pH 7.4, with 0.138 M NaCl and 0.0027 M KCl). Carboys or other large containers used for storage and dispensing of rinse buffers should be disinfected by autoclaving on a regular schedule.

7. Mountant (0.05 M Tris-buffered saline pH 9.0 with 20% glycerol). Prepare 0.05 M Tris / 0.15 M NaCl solution by dissolving 0.623 grams of Trizma pre-set crystals and 0.85 g NaCl in a total volume of 100 ml distilled water. Filter (0.45 um) and store at room temperature. Remake at least once per year; check pH quarterly. Prepare a one month supply of mountant by mixing 4 parts Tris-saline pH 9.0 with 1 part glycerol. Store at room temperature. Glycerol concentrations above 20% affect the antigen binding capacity of some antibodies and should not be used. Glycerol should be replaced at yearly intervals because the pH changes slowly with time. The mountant should be remade or the pH tested once a month. The mountant is added to the coverslip by dispensing with a syringe fitted with a 0.45 um syringe filter. (Trizma pre-set crystals, Sigma catalog # T6003 ; ACS Reagent Grade glycerol, Sigma catalog #G7893)

8. Immersion oil should be formulated specifically for fluorescence applications (e.g., Cargille DF). Immersion oils formulated for general microscopy may produce significant auto-fluorescence.

IV. Sample Collection and Handling

A. Rationale for sample collection. When an animal develops rabies (most often when the bite of another animal transfers rabies-virus-laden saliva to the wound), rabies virus moves transneuronally from the site of entry to the spinal cord and brain. Patterns of virus spread within the central nervous system suggest that a thorough examination of the brain stem is critical to rabies diagnosis. Viral antigen is widespread in the brain of most animals positive for rabies, but because spread may also be unilateral, especially in larger animals (Figure 1), a negative finding for rabies can be made only if a complete cross-section of the brain stem is examined. Examination may be made at the level of the pons, medulla, or midbrain.

Cerebellar tissue should also be included in a rabies test. Although brain stem is the tissue most reliably found to contain viral antigen, the characteristic size and shape of intracytoplasmic inclusions produced as rabies virus accumulates in the large neurons of foliar regions of the cerebellum are easily detected and recognized by DFA. Inclusion of this tissue yields a more confident diagnosis than examination of brain stem alone. Although the hippocampus was once the tissue of choice for histologic tests for Negri bodies, hippocampus is of limited additional value when brain stem and cerebellum are examined. If the cerebellum is missing from tissue submitted for rabies testing, however, a negative finding may be made from examination of brain stem and hippocampus. While a negative finding for rabies can be made only if brain stem tissue is among the tissues examined, incomplete specimens should be tested, if possible. Specific staining in any tissue reacted with anti-rabies antibody is diagnostic of rabies infection.

Virus is present in the saliva of an infected animal only after virus proliferation in the central nervous system and subsequent centrifugal spread from the brain to the salivary glands. A negative DFA test for the presence of rabies virus in brain tissue assures that contact with saliva of a biting animal could not have transmitted rabies. Because virus may not spread to all salivary glands and may be present only intermittently in saliva, negative tests of salivary glands or saliva cannot rule out rabies infection.

B. Shipment of samples. Because rabies prophylaxis is usually delayed pending a laboratory report, specimen transit time to the laboratory should be as short as possible, preferably within 48 hours. A fresh, unfixed brain sample is critical to a rapid and accurate diagnosis of rabies. Refrigeration will preserve a sample for at least 48 hours. Freezing of the sample for transit will not reduce the sensitivity of the test, but may introduce additional testing delays and impede recognition and dissection of appropriate test samples. Repeated freeze-thaw cycles may reduce test sensitivity and should be avoided. Biocontainment during specimen transport is critical, to prevent both contamination of the outside of the package and cross-contamination between samples within the package.

C. Unacceptable deterioration or decomposition of a sample is a qualitative assessment of the condition of each sample upon arrival in the lab. 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.

D. Chemical fixation (e.g., formalin) can alter tissue to make a sample unsuitable for testing. Methods for testing fixed tissue exist, but these tests are performed by other than standard DFA methods on tissues prepared by controlled fixation procedures. These methods are not available in all laboratories and referral to reference laboratories will delay test results.

E. Laboratory handling of samples. Samples submitted to the laboratory may be a complete carcass, an intact head, or dissected brain tissue. Dissected brain tissue must include a complete cross section of the brain stem and either cerebellum or hippocampus. All material submitted with a sample, including the carcass, should be held frozen until the test is completed and results reported. A single additional freeze-thaw cycle will have no effect on rabies-specific staining if repeat testing of the sample should be required, and the freeze-thaw cycle may eliminate or reduce some non-specific staining. Retention of the carcass is necessary to verify the identity of an animal in the case of unusual test results, and to identify a wild animal to species. Sample identifiers (accession numbers) should be used to label boxes and all items accompanying the sample. All necropsy and tissue processing must include proper identification of each sample and avoidance of any practice that could lead to 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 thoroughly disinfected by boiling or autoclaving followed by thorough washing before reuse. Instruments not in use should be kept in closed storage. Only those instruments in use for processing a single sample should be exposed. Frozen reference material taken at necropsy should be retained in the laboratory for all test samples. Most laboratories maintain test samples for 2 to 6 months, but representative positive samples should be maintained for longer periods for use as controls, for epidemiologic typing, and for other purposes. Storage containers for reference material must be large enough that reserved portions of brain stem and cerebellum (and hippocampus, if desired) remain as recognizably separate pieces and allow complete cross sections to be made if repeat testing is required. A video describing necropsy procedures is available from the CDC.

V. Preparation of Impression Slides / Smears

A. Sampling. While a positive finding of rabies virus antigen in any tissue is diagnostic of rabies infection, a negative finding for rabies can be made only if the diagnostic test includes examination of at least two areas of the brain: brain stem and preferably cerebellum (Figures 2,3,4,5,6,7,8,9,10,11,12,13). A complete cross section of the brain stem is required. If brain stem is unavailable and other brain tissues are negative,the sample must be considered **unsatisfactory** for testing. If cerebellum is unavailable, a diagnosis may be made by examination of brain stem and hippocampus (Figures 14 and 15). Each brain area is tested with FITC-labeled anti-rabies reagents prepared from two different sources (i.e., two different monoclonal antibody reagents or a monoclonal antibody reagent and a hyperimmune serum reagent; see note in section III.B.2). For most animals a diagnostic test will include 4 observations: a smear or impression made from brain stem stained with reagent 1 and a smear or impression made from brain stem stained with reagent 2; a smear or impression made from cerebellum stained with reagent 1 and a smear or impression made from cerebellum stained with reagent 2. Each sampled brain area should be of a size sufficient for examination of 40 separate views (microscopic fields) at a magnification of approximately 200X. With most microscopes an impression made in a 15 mm diameter well or a smear of 10 mm^2 meets this recommendation (Figures 7-8 and 12-13).

1. Brain stem. The brainstem is anterior to the cerebellum and continuous with the spinal cord (Figures 2, 3 and 4). The uppermost portion of the brain stem is the midbrain; the hindbrain portion of the brain stem is composed of pons and medulla oblongata. A cross (transverse) section of one of these areas is necessary for rabies testing. Slides are prepared from the cut surface of the cross section to expose multiple ascending and descending nerve tracts (Figures 4, 5, and 6). For many animals (e.g., dog-sized or smaller animals), a complete cross section will fit in a 15 mm diameter well. A duplicate impression of the cross section is made for staining with the second reagent. If the cut surface of the cross section is >15 mm, but $\leq$30 mm, two impressions are made from the cut surface by dissecting the tissue (Figures 6, 7, 8). One impression / area is stained with each reagent. If the cut surface of the cross section is >30 mm, one impression is made from each of four tissue sections dissected from different areas of the lateral surface of the cross section (two impressions / areas are stained with each reagent.)

Slip smears are also made from the cut surface of a cross section, but are made from dissected tissue. The two different reagents are reacted with tissue on separate slides or in wells created on the tissue by stamping with nail polish (section III.A.5).

Cattle are often tested for both rabies and bovine spongiform encephalopathy (BSE). Tests for BSE require only the obex (Figure 9), Care should be taken in selecting tissue for BSE testing so that sufficient brain stem tissue is reserved for rabies testing.

2. Cerebellum. The visible portion of the cerebellum, the cerebellar cortex, is a thin layer of highly convoluted gray matter (Figure 2). The core of the cerebellum contains the white matter (Figure 5). The cerebellum may be broadly divided into the midline structure called the vermis ("worm") and two lateral cerebellar hemispheres. A rabies test should include examination of tissue from a cross section through the hemispheres and the vermis (Figures 5 and 10). For many animals (e.g., dog-sized or smaller animals), a complete cross section will fit in a 15 mm diameter well. A duplicate impression of the cross section is made for staining with the second reagent. If the cut surface of the cross section is >15 mm, but $\leq$30 mm, two impressions are made from the cut surface by dissecting the tissue (Figures 11, 12, 13). One impression / area is stained with each reagent. If the cut surface of the cross section is >30 mm, one impression is made from each of four tissue sections dissected from different areas of the lateral surface of the cross section (two impressions / areas are stained with each reagent.) If only one hemisphere is available because the brain has been bissected for other tests, slides can be made by sampling multiple areas of the same hemisphere so that the total surface area examined is equivalent to that examined for a complete cerebellum.

Slip smears are also made from the cut surface of a cross section, but are made from dissected tissue. The two different reagents are reacted with tissue on separate slides or in wells created on the tissue by stamping with nail polish (section III.A.5).

3. Hippocampus. The hippocampus is buried deep in the temporal lobe near the center of the brain and is only visible when the brain is dissected (Figure 14). The lateral horn-shaped protrusions of the hippocampus are the reason for its alternative name, Ammon's horn. If used for rabies testing, a cross section including areas of both horns is needed (Figure 15). For small animals (e.g., dog-sized or smaller animals), a complete cross section will fit in a 15 mm diameter well. A duplicate impression of the cross section is made for staining with the second reagent. For larger animals, one impression is made from each of the two horns (one impression is stained with each reagent). If the cut surface of the cross section is >30 mm, one impression is made from each of two sections dissected from each horn (two impressions / areas are stained with each reagent.)

Slip smear slides are made similarly from dissected tissue representing the cut surface of the two horns.

4. Combined areas for very small animals like bats. Slides are prepared from tissue cut through the brain at the point at which the cerebral hemispheres overlay the cerebellum. If the cut is made properly, the section will include parts of the cerebellum and midbrain, as well as both cerebral hemispheres (Figure 4). A duplicate slide is made for staining with the second reagent.

5. Control slides. Control slides are prepared in the same manner as test slides, but without acetone fixation. Positive and negative control slides are fixed in acetone at the same time as test slides to control for the effect of acetone fixation on test performance. Control brain material for slide preparation should be retained for this purpose from animals naturally infected with the most common variant of rabies virus in the submitting area for the laboratory. Slides are stored frozen at -20°C for up to one month or at -70°C for one year. Slides may be made from either brain stem or cerebellum. See section VI for discussion of the selection of brain material for control slides for conjugate titration.

B. Drying and fixation. Impressions/smears must dry completely at room temperature prior to the fixation step. This may take 15 to 30 minutes. Do not use ovens or a hot air source to dry slides as this may denature antigen. Unless a biocontainment hood is available, do not direct air from fans over slides as this may create airborne tissue particles. When the tissue no longer appears wet and glistening, slides from an individual test animal are combined in one container for fixation. Do not combine slides from different animals or combine test slides with control slides in the same container. Slides should be fixed for a minimum of 1 hr to overnight at -20°C.

C. Staining, rinsing, and mounting. After the acetone-fixed impression / smear control and test slides are air dried at room temperature, each anti-rabies conjugate is added by dispensing through a syringe fitted with a 0.45 um low protein binding filter. The slides are arranged so that the positive control slide is the first to receive the conjugate (to control for any unexpected removal of antibody by filtration) and the negative control slide is the last to receive the conjugate (to control for adequate removal of excess fluorescein by the filter throughout conjugate application). The slides are then incubated for 30 minutes at 37°C in a high humidity chamber. After staining, excess conjugate is drained from the slides or wicked onto absorbent paper and the slides are given a brief rinse under a stream of PBS, then immersed and soaked in PBS for 3 to 5 minutes (control slides and slides from each test animal in a separate rinse container). The PBS is discarded and replaced and the slides soaked for a second 3 to 5 minute interval. No distilled water rinse is necessary. Slides are carefully blotted to remove excess liquid, then briefly air dried before mounting. Slides are mounted by dropping a small amount of 20% glycerol - Tris buffered saline pH 9.0 onto coverslips arranged on absorbent paper. Stained slides are inverted over the coverslips. Excess mountant is wicked into the absorbent paper by applying light pressure to the back of the slides. A small volume syringe fitted with a 0.45 um filter and small bore rubber tubing can be used to dispense the mountant in small droplets onto the coverslips. Slides should be read within 2 hours of mounting. Rabies-specific staining should be stable for at least 2 hours, and stained slides can be preserved for reference for weeks to months at refrigerator temperature or below.

D. Reading. A sample can be considered negative for rabies only when each area of the brain stained with each anti-rabies conjugate is scanned over approximately 40 fields at a magnification of approximately 200X or greater for fluorescing inclusions. Fluorescence suggestive of rabies is examined at a 400X magnification (see section III.A.9 and *Appendix 3* for a discussion of magnification and resolution). Although not every slide must be observed with a 40X oil objective of high NA, resolution of very fine dust-like inclusions and recognition of some types of non-specific staining are aided by examination with this type of lens. A minimum of 160 total fields are examined: 40 fields in the brain stem stained with reagent 1, 40 fields in the brain stem stained with reagent 2, 40 fields in the cerebellum stained with reagent 1, and 40 fields in the cerebellum stained with reagent 2. Slides from each test animal are read by 2 microscopists (e.g., one microscopist reads all brain stem slides or all slides stained with reagent 1).

VI. Conjugate Titration.

A determination of a working dilution for a conjugate must be made exactly as the diagnostic reagent will be used for test samples (same diluent, tubes, syringe filters, and counterstain concentration). Serial twofold dilutions (e.g., 1:10, 1:20, 1:40) are prepared for all prospective conjugates. Two or more slides are stained for each dilution. Each microscopist reads the titration and records results independently. The consensus of the last dilution providing crisp +4 staining with minimal background fluorescence is the end-point dilution of the reagent. (See section VII for a definition of +4 staining). A more precise working dilution can be obtained by preparing limited dilutions around the end-point dilution. For example, if the end-point of the initial titration is 1:80, the conjugate is retested at dilutions of 1:70, 1:80, 1:90, 1:100, 1:110, and 1:120, and so on. The working stock of conjugate should be two steps more concentrated than the first dilution at which a fall-off in staining is observed (e.g., if the amount of antigen stained or the intensity of the stained antigen is diminished at a 1:110 dilution, the working dilution of the conjugate would be 1:90).

Because antigen presentation and antibody avidity and affinity vary with different rabies virus samples, and viral inclusions may appear quite different when stained with different reagents, titration results are confirmed by reaction with slides made from a second variant of rabies virus. Although positive control slides made from a single source are adequate for day to day test controls, an accurate reagent evaluation requires multiple observations. Control material for conjugate titrations should be retained for this purpose from animals naturally infected with the most common variant of rabies virus in the submitting area for the laboratory and from animals infected with a variant that is known not to share a high degree of genetic homology with the common variant. For example, laboratories in the eastern United States might want to titrate their conjugate for recognition of rabies infected raccoons and retest the working dilution by comparison to staining of a rabies positive raccoon and a rabies positive bat. Tissue from naturally infected animals is a better control material than mouse passaged virus. If adequate tissue is unavailable within a state, material should be obtained from other states or from the reference laboratories listed in *Appendix 1*. Control material should be stored in aliquots to avoid repeated freeze-thaw cycles and maintained at -40°C or below.

VII. Test Results

A. Staining intensity / antigen distribution. Rabies virus in the brains of infected animals produces intracytoplasmic inclusions of various shapes. A single microscopic field may contain dust-like particles of <1 um in diameter and large, round to oval masses and strings 2 to 10 um in diameter. When specifically stained with an FITC-labeled antibody, these inclusions appear smooth, with very bright margins, and a somewhat less intensely stained central area. Observations made for each test slide are recorded as staining intensity/antigen distribution (e.g., +4/+2).

Staining intensity is graded from +4 to +1. Positive control slides in all tests should always contain staining of +4 intensity (a glaring, apple green brilliance). Slightly diminished staining intensity (a slight loss of glare) 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 area of the 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).

B. Test interpretation. If the tissue sample submitted for testing 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 incomplete**) based on observed patterns of staining in test and control slides.

1. Test complete / reportable result. Test results are reported to the submittor as complete, samples of brain material are stored for reference, and the carcass and other material discarded, 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: Required brain areas were present and no tissue deterioration or alteration was noted when slides were prepared (section IV.C and D). 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 cerebellum or hippocampus).

2. Test incomplete / results not reported until test is repeated and/or result confirmed in reference laboratory. Sparse or weakly staining inclusions may indicate an unusual clinical course, the presence of an unusual variant of rabies virus, or non-specific or undesired binding of FITC-labeled antibody (*Appendix 5*). The following observations warrant repeat testing for confirmation of staining specificity:

a) Inclusions typical of rabies virus infection, but present in no more than 10% of examined fields or present in >10% of fields but with an extremely sparse distribution (i.e., 1 to 2 inclusions per field).

b) Inclusions typical of rabies virus infection, but staining with uncharacteristically weak intensity (<+3).

c) Inclusion morphology not typical of rabies infection, but staining with +4 intensity (e.g., regularly sized, uniform textured structures).

d) Fluorescing bacteria that might mask small amounts of rabies specific staining.

e) Particulate or free fluorescein that might mask small amounts of rabies-specific staining.

f) Discordant results for observations made with two reagents or between two readers.

C. Repeating the test and determining the specificity of staining. Do not report a sample as provisionally positive or negative. The DFA test is repeated by returning to the original brain tissue and remaking slides from brain tissue reserved at necropsy. If for any reason the test cannot be repeated, the test report should state that the condition of the sample is such that tests cannot rule out the presence of rabies in the specimen. Slides are made as in Section V, but at least 3 impressions or smears are needed for each brain area in the repeat test so that two diagnostic reagents and a specificity control reagent for at least one of the diagnostic reagents can be used. The specificity control for an FITC-labeled hyperimmune serum rabies reagent is an FITC-labeled serum reagent produced in the same animal host as the rabies reagent (typically goat or horse) but as normal serum or hyperimmune serum directed to an agent other than rabies virus. Similarly, the specificity control for a rabies reagent prepared from a mouse monoclonal antibody is an FITC-labeled mouse monoclonal antibody of the same isotype as the rabies reagent but directed to an agent other than rabies virus. The control reagent should be diluted to the same mg protein concentration as the rabies reagent. A more confident result can be obtained for samples with extremely limited amounts of rabies virus antigen if multiple slides are tested from each brain area. (For example, samples from large animals (Figure 1) are more likely than any other sample to contain rabies virus antigen limited to a few nerve tracts of the brain stem.) Assuming results for positive and negative control slides for the repeat test are as expected, several outcomes are possible in the repeat test:

1. If all test slides are negative, the animal is considered negative for rabies.

2. If additional test slides repeat the sparse staining observed with the first test and the reagent control indicates that the staining is specific (no staining of the specimen with the reagent control), the sample is considered positive for rabies. Observations of this type should be rare. A reference laboratory should be contacted if multiple instances of sparsely distributed rabies virus antigen are noted in test samples.

3. If one reagent is negative and the inclusions in test slides stained with the other reagent are shown to be non-specific (i.e., control for that reagent also contains inclusions), the sample is considered negative for rabies.

4. If inclusions are found in test slides stained with only one reagent and the reagent control indicates that the staining is specific (no staining of the specimen with the reagent control), the sample is considered positive for rabies. Rabies prophylaxis is given to persons exposed to the animal. Because this finding may indicate a new variant of rabies virus, the original brain tissue should be sent to a reference laboratory for confirmation and virus typing.

5. If all test slides, including the specificity control slides, contain inclusions that are indistinguishable from rabies virus inclusions or that might mask specific staining of rabies virus inclusions, the test is considered non-diagnostic because of non-specific reactions. Persons exposed to the animal are treated on the basis of clinical suspicion of rabies in the animal. Alternative tests (virus isolation, PCR) are conducted to rule out or confirm rabies in the sample.

D.Consultation by another laboratory. Unexpected outcomes should be confirmed by another laboratory (e.g., rabies in a new area or in an animal not often found rabid in the submitting area; inclusions of unusual morphology or distribution;failure of one reagent to react with a virus recognized by a second reagent). The public health laboratories listed in *Appendix 1* have agreed to accept samples from other laboratories for confirmation and reference typing. Portions of brain stem and cerebellum (and hippocampus, if included in the original test) must be recognizable as separate brain areas and sufficiently large that complete cross sections can be made by the reference laboratory.

Appendix 1. Reference diagnostic committee members.

Name	Affiliation	e-mail	Phone	FAX
Ronald Genevie	OH Department of Health	rgenevie@gw.odh.state.oh.us	614-644-4659	614-752-9863
Jim Powell	WI State Lab of Hygiene	jwp@slh.wisc.edu	608-262-7323	608-262-6807
Pushler Raj	Tx Department of Health	pushler.raj@tdh.state.tx.us	512-458-7491	512-458-7452
Robert Rudd	NY Department of Health	rjr06@health.state.ny.us	518-896-4527	518-869-6540
Charles Rupprecht	Centers for Disease Control	cyr5@cdc.gov	404-639-1050	404-639-1058
David Schnurr	CA Department of Health Services	dschnurr@dhs.ca.gov	510-307-8615	510-307-8599
Jean Smith	Centers for Disease Control	jss2@cdc.gov	404-639-1050	404-639-1058
Charles Trimarchi	NY Department of Health	trimarchi@wadsworth.org	518-869-4550	518-869-6540

Appendix 2. Test results for 435 diagnostic samples examined with the minimum standard protocol.

Five state public health laboratories (CA, NY, OH, TX, WI) evaluated the minimum standard protocol described in this document by reexamining 406 diagnostic samples previously tested in their laboratory. For each sample tested by the minimum standard protocol, the same diagnosis was reached as had been made for that sample tested by the laboratory's standard operating procedure for DFA testing. In addition the CDC laboratory used the minimum standard protocol to confirm or clarify test results obtained for 29 samples tested by DFA in 15 other state laboratories. Test 1, original test with standard operating procedure for the laboratory; Test 2, repeat testing with minimum standard protocol.

	Test Results		
Testing Laboratories	**Number of samples- Test 1 and Test 2 positive**	**Number of samples - Test 1 and Test 2 negative**	**Number of samples - Test 1 non-diagnostic because of undesired staining (Test 2 results and alternative test results, if available)**
NY	20	63	
TX	34	53	
WI	27	47	
OH	54	51	
CA	24	45	
GA / CDC	1		
NC / CDC			1 (Test 2 negative, PCR negative)
PA / CDC			1 (Test 2 negative, PCR negative)
ID / CDC	1		
VA / CDC	3		
MI / CDC	1		
WA / CDC	1		
MN / CDC	1		
WV / CDC	3	1	
MT / CDC			2 (Test 2 negative)
SD / CDC			1 (Test 2 negative, PCR negative)
AL / CDC	4	3	
CO / CDC	1		
DE / CDC			1 (Test 2 negative)
OK / CDC	1		

Appendix 3. Fluorescent microscopy.
Fluorescent microscopy makes use of the property of some molecules to absorb light of a particular wavelength and then to re-emit the light at longer wavelengths (fluorescence). The primary (exciting) light is separated from the secondary (emitted) light by filters (coated glass elements) in the microscope. The elements of the fluorescent microscope which determine the overall sensitivity of the instrument are discussed in this section.

Most recent technical developments in fluorescence microscopy relate to **reflected (incident) light** fluorescent microscopes in which the objective lens serves as both the condenser for the exciting light and the collector of the emitted light. In this system, light emitted by the lamp (Mercury or Xenon arc) passes to a filter cube containing an excitation filter, a dichroic mirror (beam splitter), and a barrier (emission) filter. The excitation filter selects and transmits light wavelengths suitable for excitation of FITC to a dichroic mirror oriented at a 45° angle to the light path. The mirror reflects the excitation light to the objective lens which condenses the light onto the test slide, exciting any FITC molecules bound to the specimen during the DFA test. The longer wavelength light emitted by the excitation (the fluorescence) is collected by the objective lens and passed back to the dichroic mirror. The reflected light then passes through a barrier filter to suppress stray excitation light, allowing only longer wave lengths of light to reach the oculars. Fluorescent microscopy is covered in detail at a web site maintained by Florida State University (http://micro.magnet.fsu.edu/primer/index.html). The web site sections entitled "Microscopy Primer" and "Fluorescence" were the sources for most of the information contained in the following discussion of lens, light sources, and filters.

The **objective lens** is by far the most important component of a good microscope. Lens types are defined by their degree of correction for optical aberrations. Apochromat lenses are the most corrected, followed by fluorite lenses. Least correction occurs with achromat lenses. Either fluorite or apochromat lenses should be used for rabies diagnosis. Although a high degree of optical correction is not essential in a lens used for rabies DFA, the smaller numerical aperture of an achromat lens makes it less useful than a fluorite or apochromat lens at the same magnification. Regardless of type, the objective lens should carry the prefix "plan", indicating that the lens provides a flat view to the edges of the microscope field. An example of the an objective lens with an interpretation of the labeling can be found at: http://micro.magnet.fsu.edu/primer/anatomy/specifications.html.

Magnification: Lens magnification (e.g., 20X vs 40X) must be sufficient to visualize the very fine dust-like inclusions produced in some rabies infections. However, as magnification increases, the brightness of the image decreases rapidly and resolution of the image (see below) is directly related to the light gathering capacity of the lens. Although both dry and oil objectives can be used successfully for rabies diagnosis, immersion oil prevents the loss of emitted light that occurs in the airspace between coverslip and dry objective, thereby increasing overall resolution and the brightness of viral inclusions. When oil objectives are used, immersion oil with the same refractive index (1.515) as glass should be employed. The immersion oil should be formulated specifically for fluorescence applications and evaluated for autofluorescence and best contrast between FITC and tissue.

Numerical aperture: Numerical aperture (NA) refers to the light gathering capability of the lens, which directly affects image resolution. The larger the aperture, the more light collected. The theoretical NA for a 40X dry objective is 1.0. In reality, only an NA of 0.95 is achievable and at considerable cost. A more common NA for a reasonably priced 40X dry objective is 0.75. By comparison, most 40X oil objectives have an NA of 1.0 and may be as high as 1.3.

The effect of NA and magnification on relative brightness can be calculated using the following formula: $(NA)^4$ divided by $(magnification)^2$ = brightness. Sample values are compared in this table:

$(NA)^4$	$(magnification)^2$	brightness
$(0.65)^4$	$(40)^2$	0.0001100
$(0.75)^2$	$(40)^2$	0.0001970
$(0.95)^2$	$(40)^2$	0.0005091
$(0.75)^2$	$(40)^2$	0.0007910
$(1.3)^2$	$(40)^2$	0.0017850*

* oil objective

The four-fold relative greater relative brightness of a 20X vs 40X objective of the same NA (0.0001970 vs 0.0007910) illustrates the negative effect of magnification on image brightness.

Resolution: Resolution is defined as the smallest distance that two objects can be separated and still be recognized as two distinct objects. In reality, resolution is defined by "^{r}Airy ", the radius of the Airy disk produced by the diffraction image of an object in the specimen, and is a function of the wavelength of the light and the NA of the objective (^{r}Airy = 0.61(wavelength/NA)). Although shorter wavelengths of light provide better resolution, the rabies DFA test is restricted to the emission spectra of FITC (peak=525nm); therefore, for rabies DFA the resolving power of a lens is directly related to its NA. (The value 0.61 is a constant.) The resolving power of both a dry 20X or 40X objective lens with an NA of 0.75 is 427 nm. A 40X oil immersion lens with an NA of 1.3 has a resolving power of 246 nm.

Light sources (lamps): Suitable light sources for routine rabies diagnosis are either mercury (Hg) or xenon (Xe) gas arc lamps, with the short arc (HBO 100W) Hg lamp being most commonly used. Both light sources provide suitable amounts of light at wavelengths appropriate for FITC excitation. The short arc mercury lamp (HBO 100W) produces light with the greatest luminous density and thus the brightest image, and since it has the shortest arc, concentrates the greatest amount of light at the back of the objective lens. Since this configuration produces the greatest average brightness, some manufacturers optimize their microscope light paths for use with this lamp. A table comparing mercury and xenon lamps can be found at http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorosources.html.

Filter systems: The excitation filter, the dichroic mirror (beamsplitter) and the barrier (emission) filter mounted in the filter cube determine the band width of excitation light reaching the specimen and the appearance of the emitted fluorescence. A typical filter cube set is described at http://micro.magnet.fsu.edu/primer/techniques/fluorescence/filters.html. Although most FITC excitation filters will select for light in the blue portion of the visible spectrum, wider or narrower band excitation filters are available. The two most commonly used filter sets have either a realively wide excitation band at 450-490 nm or a narrower excitation band with a range between 475 to 490 nm and peak emission at 485 nm. Both filter cubes have a long pass (LP515) barrier filter which allows wavelengths above 515 nm to pass to the oculars. In general, wider excitation ranges are more likely to excite autofluorescence in tissue components with a resultant reduction in image contrast; however, the yellow and red wavelengths of light passed along with the green light emitted by FITC produce a sparkling, bright yellow/green fluorescence that is easy to discern from the dull green fluorescence often associated with non-specific staining. While a narrower excitation range will produce less tissue autofluorescence, darker tissue background, and better contrast, loss of image brightness and contrast occurs when narrow pass barrier filters are used.

As with any mechanical device, the microscope will function only as well as it is maintained. Annual maintenance should be performed by a certified technician. Routine maintenance should be performed according to the manufacturer's specifications. Lamp alignment and cleaning of lens and ocular optical surfaces, should be performed monthly or as needed. Lenses should not be cleaned more than necessary and never cleaned by rubbing with lens paper, as the paper may contain abrasive dust. Lenses should be cleaned by lightly swabbing the front lens surface with a clean cotton swab moistened with lens cleaner, then repeating the process with a clean swab. Though the determination is subjective, the microscopist needs to be aware of a decline in performance that might compromise test sensitivity. Even though the useful life of a lamp could be 200 to 400 hours, the performance may decline in as little as 50 hours. As with other equipment used in the direct fluorescent antibody test, complete maintenance records should be kept as part of a comprehensive quality assurance program.

Historically, the first fluorescence microscopes employed for rabies diagnosis used transmitted light, focused by either a brightfield or darkfield condenser. Because of the difficulty in separating the exciting light passed directly through the specimen to the objective from that of the FITC emission, brightfield was replaced by darkfield transmitted light fluorescence microscopy. Although darkfield condensers increased the contrast between the specimen and emitted light, they also decreased the brightness of the emitted signal. To maximize the intensity of the exciting light, darkfield oil condensers were added, using oil between the condenser and the bottom of the specimen slide. A precise alignment of the darkfield condenser was needed to ensure maximum transmission of the exciting light to the specimen. Oil objective lenses were also required to maximize the brightness of the emitted fluorescence. Because of the inconvenience and relatively low intensity of emitted light produced, transmitted light fluorescence microscopy is not recommended for routine rabies diagnosis. All laboratories performing rabies testing should be using reflected (incident) light and objectives maximized for fluorescent microscopy.

The web page of the National High Magnetic Field Laboratory at Florida State University (www.micro.magnet.fsu.edu/primer) contains comprehensive information regarding all aspects of microscopy. Sales representatives of the various microscope manufacturers can also provide more specific information and demonstrate the equipment. There is often a great deal of difference in the quality of fluorescent microscopes produced by different manufacturers and within the microscope line of a single manufacturer. Before purchasing new equipment, laboratories should ask sales representatives to set up a fluorescence microscope for evaluation using the reagents and protocols in use in the laboratory. Rabies reference testing laboratories in adjacent states will also have information on systems in use in their laboratories.

Acknowledgments: Links to the illustrations used in the microscopy section were provided by Michael W. Davidson, National High Magnetic Field Laboratory, 1800 East Paul Dirac Drive, Florida State University, Tallahassee, FL 32306.

Appendix 4. List of commercially available reagents.

A. FITC-labeled anti-rabies and control reagents

Fujirubio Diagnostics, Inc. 1-800-342-9225
CENTOCOR FITC-Anti-Rabies Monoclonal Globulin Catalog #800-090. A mixture of two IgG2a monoclonal antibodies.

Chemicon International, Inc 1-800-437-7500
Light Diagnostics Rabies DFA Reagent (Monoclonal antibody FITC-conjugate) Catalog #5100. A mixture of two IgG1 monoclonal antibodies and one IgG2 monoclonal antibody.

Light Diagnostics Rabies Negative Control (Monoclonal antibody FITC-conjugate) Catalog #5102. A mixture of IgG1 and IgG2 monoclonal antibodies.

Light Diagnostics Rabies DFA Reagent II (Monoclonal antibody FITC-conjugate) Catalog # 5500. Contains the same two IgG2a monoclonal antibodies as Centocor #800-090.

Light Diagnostics Rabies Polyclonal DFA Reagent (Goat IgG FITC-conjugate) Catalog #5199. Goat hyperimmune serum (IgG fraction).

Light Diagnostics Rabies Negative Control Catalog #5202. FITC-labeled normal goat serum (IgG fraction).

B. Immersion oil

Fisher Scientific 1-800-766-7000
Cargille Laboratories Immersion Oil for Fluorescence Microscopy; Type DF

Appendix 5. Specificity control reagents.

Undesired or non-specific staining may be misidentified as rabies virus inclusions (false positive test) or may mask rabies virus inclusions in the test sample (false negative test). A reagent's lack of specificity may be the result of several factors, not all of which can be effectively controlled. Fortunately, commercial reagents presently available are of high quality and almost all tests for rabies yield clearly positive or clearly negative results. The inclusion of reagents from two different sources in every test will greatly aid in recognition of undesired staining, since different reagents are susceptible to different problems. Also, the simple step of repeating the test from frozen brain material will eliminate some non-specific reactions. The following discussion lists two types of undesired staining (specific staining related to extraneous antibodies and shared epitopes; non-specific staining related to mechanisms other than antigen recognition) and suggests possible control strategies for those few tests when a clear diagnostic finding cannot be made.

Extraneous antibodies are sometimes present in animals used for the production of rabies hyperimmune serum as a result of a natural exposure. When the same agent is also present in a test sample, a false positive diagnosis of rabies may result. The problem of extraneous antibodies is restricted to hyperimmune serum reagents, because monoclonal antibody reagents contain only antibodies reactive with antigens on rabies virus. Therefore, the inclusion of a monoclonal antibody reagent as one of the two reagents in every test is an effective control for recognition of extraneous antibodies. Extraneous antibodies should be removed from the reagent by purification or adsorption steps before the product is marketed. Observations of discordant results on a sample tested with hyperimmune serum and monoclonal antibody reagents should be confirmed and investigated by a reference laboratory and by the reagent's manufacturer so that improvements can be made to the product.

Although unrelated to non-specificity, a second and very important reason for submitting the problem test sample to a reference laboratory is that discordant results between hyperimmune serum and monoclonal antibody reagents may also be caused by nonrecognition of a virus variant by the monoclonal antibody reagent (a false negative test). If this should occur, new monoclonal antibody reagents may be necessary.

Non-specific staining may also occur because of antibody recognition of a **shared epitope** (antibody recognition site on an antigen) between rabies virus and another organism. Although both hyperimmune serum and monoclonal antibody reagents are susceptible to this type of problem, shared epitopes are uncommon and unlikely to be present in the antibodies comprising two different reagent sources. As above, the inclusion of two different reagents in every test is an effective control for recognition of shared epitopes, but the source of the problem should be investigated by sample submission to a reference laboratory.

The most common cause of undesired staining is the binding of antibody to a tissue by **mechanisms unrelated to antigen recognition**. For example, charged protein attraction increases with increasing FITC:protein ratios which may vary between different reagents and between different lots of the same reagent. Some tissues are more prone than others to undesired staining. For example, the myelin basic protein content and antibody binding capacity of an animal's brain increases with an animal's age; decomposing tissue becomes "sticky" to varying degrees. Antibodies may also be non-specifically bound to tissue by Fc receptors in the test sample. Fc receptors are present on gram positive cocci (staphylococcus and streptococcus) and some parasites (e.g., schistosomes) and are induced on the surface of cells infected by some viruses (e.g., herpes viruses). Fc receptors bind by attaching to the Fc portion of the antibody molecule, but the avidity of the binding varies with the antibody isotype (e.g., IgG1 vs IgG2) and with the type of animal used to produce the antibody (e.g., goat hyperimmune serum vs mouse monoclonal antibody). The binding can be quite strong and difficult to block effectively. Staphylococcus protein A and Streptococcus protein G are often used for purification of antibodies and in immunoassays in place of anti-antibody reagents.

The control for antibody binding unrelated to antigen recognition is the inclusion of FITC-labeled, rabies-negative control reagents in the test. Control reagents must be matched to each rabies diagnostic reagent as to the animal used to produce the antibody and diluted to the same mg protein concentration as the rabies reagent. For example, the specificity control for an FITC-labeled hyperimmune serum rabies reagent is an FITC labeled serum reagent produced in the same animal host as the rabies reagent (typically goat or horse), but either as normal serum or hyperimmune directed to an agent other than rabies virus (e.g., FITC-labeled anti-distemper reagents). Similarly, the specificity control for a rabies reagent prepared from a mouse monoclonal antibody is an FITC-labeled mouse monoclonal antibody of the same isotype and protein concentration as the rabies reagent but directed to an agent other than rabies virus (e.g., because both monoclonal reagents may contain both IgG 1 and 2 antibodies, a negative control antibody should contain both of these isotypes).

The inclusion of the negative control reagent in a repeat test allows a sample to be considered negative for rabies if one reagent is negative and the inclusions in test slides stained with the other reagent are shown to be non-specific (i.e., control for that reagent also contains inclusions). If all test slides, including the specificity control slides, contain inclusions that are indistinguishable from rabies virus inclusions or that might mask specific staining of rabies virus inclusions, the test is considered non-diagnostic and alternative tests (virus isolation, PCR) are performed to rule out or confirm rabies in the animal.

A discussion of the relative advantages and disadvantages of hyperimmune serum and monoclonal antibody reagents can be found in Herrmann, J.E. 1995. Immunoassays for the diagnosis of infectious diseases, p. 110-122. In Manual of Clinical Microbiology, 6th ed., (PR Murray, EJ Baron, MA Pfaller, FC Tenover, and RH Yolken, eds). ASM Press, Washington DC.