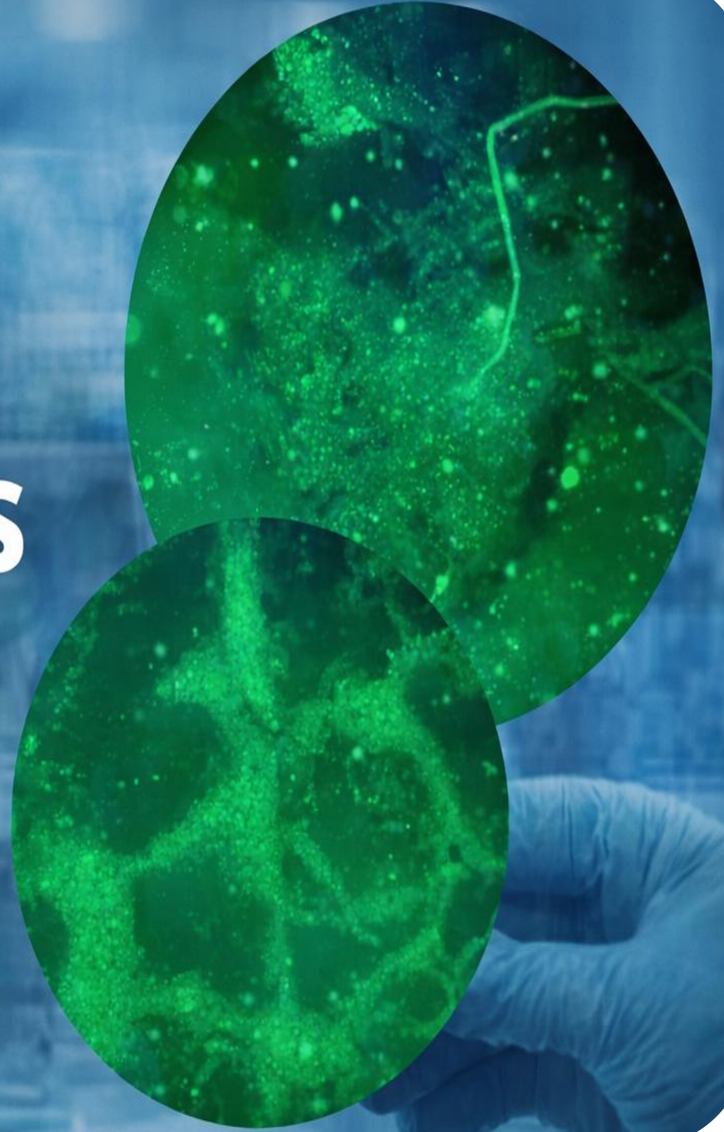
NATIONAL CENTRE FOR ANIMAL HEALTH
NATIONAL VETERINARY LABORATORY
STANDARD OPERATING PROCEDURE

FAT Rabies Diagnosis

Fluorescent Antibody Test for Rabies



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SOP No: NCAH/LSU/VIRO 01
Title: SOP on FAT for Rabies
Version No: 3, Total Pages:9
Issue Month/Effective Date: May 2026
Revision: Summary: Conjugate Dilution
Supersedes Version No: 2018.1
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Application/Distribution: NCAH/RVLs

1. SCOPE

The Fluorescent Antibody Test (FAT) is used for the qualitative detection of rabies virus antigen in animal brain tissue samples. It is applied for the laboratory confirmation of suspected rabies cases, primarily in post-mortem specimens such as hippocampus, cerebellum, and brain stem. This test is performed in veterinary diagnostic laboratories to support rabies diagnosis, surveillance, and reporting.

2. PRINCIPLE

The smears are incubated with (Fluorescent isothiocyanate) FITC-labelled anti-lyssavirus polyclonal antibody. Un-bound antibody is then removed by washing and smears are examined by fluorescence microscopy. In rabies positive specimens the antibodies bind to the antigen and produce apple-green fluorescing inclusion bodies or viral aggregates when viewed under a fluorescent microscope.

3. APPLICATION

The test is used for diagnosing Rabies in animals.

4. OBJECTIVE

To describe the FAT method in detecting rabies inclusion bodies (viral antigen) in cells of acetone-fixed brain smears.

5. SAMPLE COLLECTION

a. Materials and reagents required

- Personal Protective Equipment preferably coverall, gown, gloves, face shield and mask.
- Cutting tool
- Scissors
- Scalpels
- 50% Glycerol saline or PBS
- Thumb forceps
- Virkon or any other disinfectants
- Plastic straw
- Sterile leak proof vial

b. Preferred samples: brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata.

c. Sample collection Procedure

i. Occipital foramen route for brain sampling

- Position the carcass properly to access the foramen magnum (occipital foramen).
- Clip the hair, sterilize with spirit and make a deep incision using the scalpel.
- Insert a sterile plastic tube/plastic straw (approximately 5 mm in diameter) gently through the foramen magnum in the direction of the eye.
- Advance the tube carefully to collect brain tissue, ensuring sampling from key regions including the medulla oblongata, base of cerebellum, Ammon's horn (hippocampus), and

cerebral cortex.

- Withdraw the tube slowly and transfer the collected brain material into a clean, labeled container or 50% glycerine saline or PBS preservative
- Alternatively, open the foramen magnum using a scalpel and use thumb forceps to collect a complete cross-section of the brain stem.
- Collect additional portions of the cerebellum using a plastic straw.
- Place all collected specimens into properly labeled, leak-proof containers for laboratory testing.

ii. Retro-orbital route for brain sampling

- Position the animal carcass to allow access to the eye socket.
- Use a trocar to carefully make a small hole in the posterior wall of the eye socket.
- Introduce a biopsy needle through the hole into the brain.
- Advance the needle in the appropriate direction to collect brain tissue from regions including the cerebral cortex, hippocampus (Ammon's horn), cerebellum, and brain stem**.**
- Withdraw the needle slowly, taking care not to damage the tissue.
- Place the collected brain sample into a clean, labeled, leak-proof container or 50% Glycerol saline or PBS preservative
- Maintain the sample at 2–8°C and transport promptly to the laboratory for testing.

iii. Brain sample collection by opening the skull

- Place the animal carcass in dorsal recumbence and secure the head properly to allow safe handling.
- Make a midline skin incision over the head extending from the occipital region towards the muzzle and reflect the skin laterally to expose the skull.
- Using bone cutters or a saw, carefully open the skull without damaging the underlying brain tissue.
- Gently remove the skull cap to expose the brain.
- Using forceps and a spatula or scalpel, collect representative portions of brain tissue, including the cerebral cortex, hippocampus (Ammon's horn), cerebellum, and brain stem**.**
- Avoid contamination and excessive handling of the tissue during collection.
- Transfer the collected samples into clean, properly labeled, leak-proof containers or 50% Glycerol saline or PBS preservative.
- Preserve the samples under cold conditions (2–8°C) and transport promptly to the laboratory for testing.

iv. Packaging (Tripple packaging) and Shipment of Samples

- Place the brain tissue sample into a **leak-proof primary vial**.

- Ensure the vial is tightly closed and labeled with the animal ID, date of collection, and mark “Rabies Suspected Sample”.
- Wrap the primary vial in absorbent material (cotton, gauze, or tissue) to contain any accidental leakage.
- Place the wrapped vial into a **secondary container**, such as a sealable plastic bag or box.
- Add extra absorbent material inside the secondary container in case of leakage.
- Place the secondary container into cool box **(Tertiary)**
- Add ice packs or cold packs around the secondary container to maintain 2–8°C during transport.
- Seal the box with tape and label clearly with:

    - Biohazard symbol
    - “Infectious Substance – Category A” / Rabies Virus.
    - Sender and recipient details

- Transport the package promptly to the National Centre for Animal Health (NCAH) or Regional Veterinary Hospital & Epidemiology center (RVH &EC), following biosafety and biosecurity measures.
- Avoid rough handling; keep the package upright and maintain the cold chain.

6. APPARATUS/TEST KIT

6.1 Fluorescent microscope, Zeiss or equivalent, with mercury vapour lamp, 50 or 100 watt (450- 490 nm excitation filters, and 510 nm stop filter).
6.2 Refrigerator (+2 to +8°C).
6.3 Freezers, chest or upright, with temperature of not less than -20°C (e.g. - 70°C).
6.4 Incubator, temperature capability of 37°C ± 2°C).
6.5 Double door autoclave, any brand.
6.6 Class II biological safety cabinets, any approved brand.
6.7 Single channel Micropipettor for drawing 100µl volumes.
6.8 Single channel Micropipettor for drawing 0.5-10µl volumes.
6.9 Vortex mixer.
6.10 pH meter.
6.11 Dark room.
6.12 Forceps.
6.13 Scissors.
6.14 Standard laboratory timers.
6.15 Closed plastic container for use as a humidified chamber
6.16 Microscope slides, clear, frosted one end, any brand, non-fluorescent
6.17 Cover slips 13 mm thick, any bran.
6.18 Petri dishes, disposable or other suitable container to place brain material
6.19 Non fluffing high quality absorbent paper towel.
6.20 Coplin staining jar

6.21 HB pencil for labelling slides.
6.22 Lens cleaning tissue
6.23 Squeeze/wash bottle with PBS
6.24 A suitable containers with virucidal disinfectant capable of killing rabies virus
6.25 PPE including Nitrile/Latex powder free disposable Gloves
6.26 Disposable tips (1-10 µl, 20-200 µl,)
6.27 Autoclavable bags

7. REAGENTS, SOLUTION AND BUFFER

7.1 0.01M Phosphate buffered saline (PBS), pH 7.2-7.4
7.2 Heat sterilized distilled or deionized water, or water of an equivalent purity.
7.3 High-grade (99.9 to 100%) acetone (CH3)2CO (MW 58.8), Assay by (GC) is minimum of 99.5%, ASC grade, or similar.
7.4 High grade glycerol
7.5 Anti-lyssavirus FITC polyclonal conjugate (can be obtained from the ARC- Onderstepoort Veterinary Institute (www.arc.agric.za), SANOFI Pasteur (www.sanofipasteur.com), Chemicon (www.chemicon.com) with a predetermined working dilution.
7.6 Lens cleaning fluid.
7.7 Mounting media/mountant.
7.8 Positive and negative control brain specimens
7.9 Fresh or 50% glycerol saline preserved test brain specimen.
7.10 Evans blue. Counterstain added to the working dilution of the conjugate is optional. Evans Blue counterstain (0.5%) can be aliquoted and stored at +4°C for up to 6 months and indefinitely at -20°C. The amount of counterstain added to the conjugate is determined by titration when the working dilution of the conjugate is determined. Due to counterstain, the cells will be noticeably red, but should not be strongly red as to diminish the specific green fluorescence. An Evans Blue concentration of 0.00125% works very well in many laboratories. This concentration is prepared by adding 2.5 µl of 0.5% stock dye solution per ml of conjugate diluent.

8. PROCEDURE

8.1. Sample preparation

- Using forceps take out the brain specimen from container and place on a petri dish or any other suitable container while exposing the different parts of the brain required for the test.
- In case the brain specimen is preserved in 50% glycerol, brain sample should be blotted on paper towel or washed with PBS to remove excess glycerol and then treated as fresh ones.

- Make impression smear by lightly pressing with the microscope slide on the end of the slide resulting 1cm in diameter smear
- Prepare the negative and positive controls in the accordingly and each time.
- Air dry the smears for five minutes at room temperature.
- Discard all the contaminated equipment/materials used during the process into a container/bin with appropriate disinfectant.

8.2. Fixation

- Immerse the prepared slides into fresh cold acetone and then keep them at -20°C for 20 minutes.
- Following that, air-dry the fixed slides for approximately 5 minutes in the class II biological safety cabinet until all traces of acetone and moisture have evaporated.

8.3. Preparation of Conjugate

Preparation of 1:10 conjugate Dilution (standardised working dilution at LSU, NCAH)

Calculation for one smear

- ✓ Total volume required = 150 µl
- ✓ Conjugate needed = 150÷10=15 µl
- ✓ PBS needed= 150-15=135 µl
- ✓ ***15 µl of conjugate + 135 PBS***

8.4. Staining

- Prepare enough (Rabies DFA reagent) conjugate according to conjugate dilution titration.
- Place the slides into a humidified chamber. The humidified chamber is prepared by placing absorbent paper in an enclosed container with a flat bottom, and then pouring some PBS on the paper to create moisture during the incubation step. The paper should not be too wet as this may interfere with conjugate.
- Apply freshly prepared working dilution conjugate to cover the smear (approximately 150 µl per smear will suffice for a 1 cm diameter of smear. Too little conjugate will result in excessive drying while too much of the conjugate will increase the possibility of the conjugate running off the slides and these could both produce undesirable results).
- Place lid on humidified chamber.
- Incubate the slides in the humidified chamber at 37°C for 45 minutes.
- At the end of the 45-mins incubation, remove excess conjugate and wash or rinse the slides in PBS three times and then blot dry them on paper. Please ensure that the slides are not completely dry!

Note: Always prepare the control slides at the last.

8.5.Mounting

- Place one drop of mounting media on the slide and place put a cover slip, ensuring that the mounting fluid is evenly distributed over the smear, while minimizing air bubbles

9. RESULT INTERPRETATION

a. Observe the slides under fluorescent microscope at magnification at 20X & 40X, starting with positive and negative controls. Rabies antigens will appear in positive control slide and test slides as fluorescent-apple-green intracytoplasmic inclusions (dust-like particles to large, round/oval pinhead shape), while such fluorescing inclusion bodies should not appear in negative control smears.

b. Grade the smear quantitatively with a score of +1 to +4, depending on the abundance of viral antigens and give a quantitative grade of +1 to +4 depending on the intensity of fluorescence and record the results in the appropriate test results recording system as Positive or Negative.

c. Results for a test sample is reported as positive or negative for rabies based on observed staining in test sample and provided that the test is validated: - The sample is positive if specific fluorescence is detected in the sample. - The sample is negative if no specific staining is detected in the sample.

10. WASTE DISPOSAL

Since the virus is zoonotic and infect humans, all specimens, prepared slides, and contaminated laboratory materials must be handled and disposed of in accordance with appropriate biosafety and biosecurity procedures. All infectious materials should be autoclaved prior to disposal to ensure complete inactivation of the pathogen. Disposal must follow standard laboratory waste management protocols to prevent exposure, cross-contamination, and environmental release, thereby ensuring the safety of personnel and the public.

11. RISK ASSESSMENT

11.1 All laboratory personnel who handle and work with suspected rabies virus infected tissue specimens must be well trained, competent and comply with national bio-containment and biosafety regulations to protect staff from contact with pathogens.

11.2 All personnel involved in rabies testing should receive pre-exposure immunization.

11.3 Only personnel who demonstrate an antibody titre of 0.5 international units (IU) per ml or higher should be allowed to handle the suspected rabies infected specimens.

11.4 Personnel should be routinely monitored every 6 months for adequate rabies neutralizing antibodies. Booster vaccinations must be given when the titre falls below

0.5 IU/ml. In the absence of serological monitoring, the vaccination regimen should consist of a booster vaccination at 1 year and thereafter every 1-3 years, depending on the vaccine.

11.5 Appropriate protective clothing must be worn at all times.

11.6 The specimens must only be processed in a Class II Biological Safety Cabinet.

11.7 Aerosols ±high speed centrifugation and any procedure that generates aerosols should be carried out in tightly closed containers and possibly under a negative draught hood.

11.8 All contaminated instruments and utensils must be sterilized autoclaving immediately after the test procedure is finished and before they are washed.

11.9 Disposable items must be placed into sterilizing bags and must be sterilized by autoclaving before disposal.

11.10 Disinfection of biological safety cabinets and used instruments should be done with disinfectants such as 1% Virkon and/or other virucidals efficient to kill lyssaviruses.

12. TROUBLESHOOTING

12.1 Each new lot of acetone must be checked to ensure that the acetone does not interfere with the staining of the brain smears.
Select tissue from 4 to 6 previous specimens that have stained both weakly and strongly positive for rabies virus

12.2 Prepare two slides from each specimen.

12.3 Fix one set of slides in the acetone in use and the other set in the new lot of acetone.

12.4 Follow the procedures for fixing and staining.

12.5 Read both sets of slides noting the amount of virus as well as intensity of staining.

12.6 Both sets of slides must have identical results. If the new acetone does not meet this criterion, discard and purchase another lot.

12.7 Similarly, the optimal working dilution of a new batch of conjugate must be determined by titration. Naturally, the working dilution may differ between laboratories depending on the microscope optimal system.

12.8 Select known positive and negative brain material to prepare controls.

12.9 Inoculate suckling mice with a 10% suspension of sample that previously tested positive with a score of +4. Pool brain samples from which succumb and test positive for rabies virus antigen. To prepare the negative control, inoculate suckling mice with PBS. Harvest brain tissues after 28 days, pool and place in vials.

12.10 Prepare two-fold dilutions of the conjugate in PBS starting from 1:80 to 1:2560.

12.11 Prepare two slides for each specimen.

12.12 Fix the slides as described in the protocol.

12.13 Follow the procedures for fixing and staining.

12.14 Read both sets of slides noting the amount of virus and intensity.

12.15 The least conjugate dilution that gives an excellent staining will be used as a working dilution of that batch.

12.16 Prevent conjugate contamination by dispensing 1-ml quantities of the diluted conjugate into eppendorf tubes and store at -20°C or lower.

12.17 External quality control will involve an annual participation by our laboratories in an FAT proficiency testing on a panel of rabies positive and negative samples.

13. REFERENCES

- Anonymous (2001). In: Rabies Diagnosis Manual. *(Edited by King, A. Bishop, G. Bingham, J., Wandeler, A. and Barrat, J.)* Southern and Eastern African Rabies Group, 22 pp

- Bingham, J. and van der Merwe. M. (2002). Distribution of rabies antigen in infected brain material: determining the reliability of different regions of the brain for the rabies fluorescent antibody test. *Journal of Virological Methods* 101: 85 - 94.

- Bishop, G.C., Durrheim, D.N., Kloeck, P.E., Godlonton, J.D., Bingham, J., Speare, R. and the Rabies Advisory group. (Eds.) (2002). Rabies: Guide for the medical, veterinary and allied professions. Department of Agriculture, Government Printer, Pretoria, South Africa. 77pp.

- Dean, D. and Abelseth, K. (1973) The Fluorescent Antibody Test. In: *Laboratory techniques in Rabies. (Edited by Kaplan, M. and Koprowski, H.)* Geneva: WHO. pp 73-84.

- Office International des Epizooties (2008) Rabies. In: *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Fifth Edition.* OIE. Paris de Prony.

- Melsin, F.X., Kaplan, M.M and Koprowski, H. 1996. Laboratory techniques in rabies, fourth edition. WHO, Geneva.

LSU NCAH 2026
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