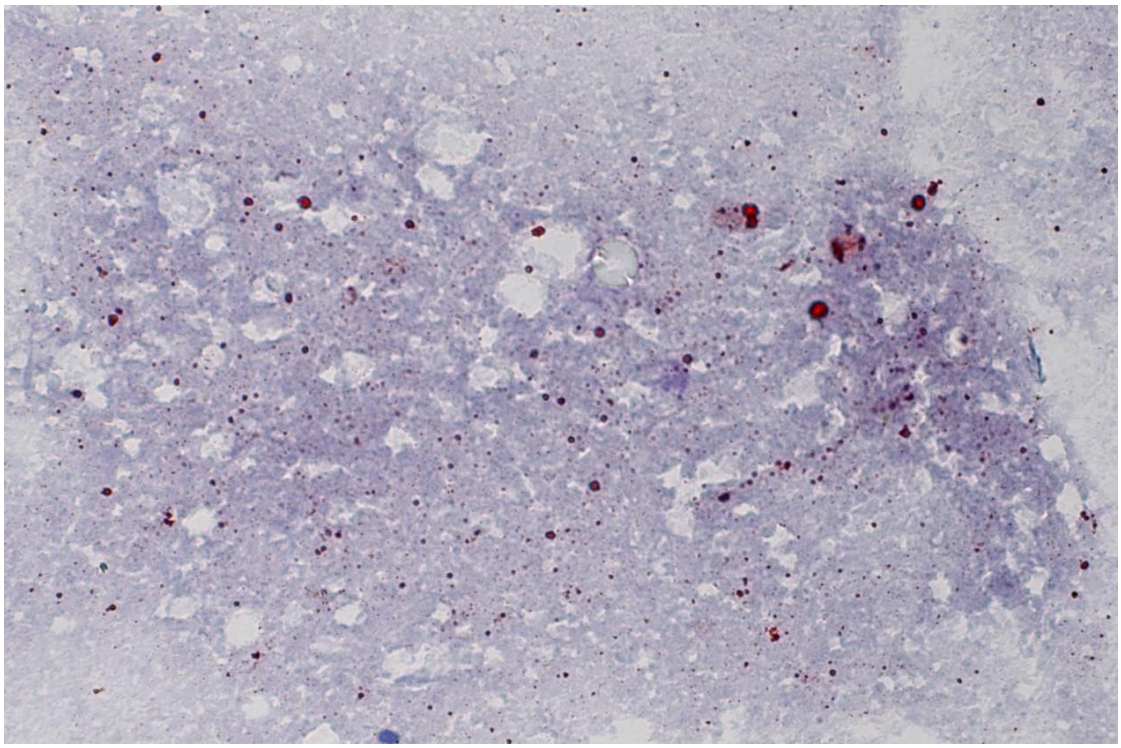
Direct, Rapid Immunohistochemical Test (DRIT)

- Official training manual and standard operating procedure -
- 4th Edition -



Global Alliance for Rabies Control

Direct, Rapid Immunohistochemical Test (DRIT)

Official training manual and standard operating procedure

4th Edition

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Introduction

Rupprecht & Niezgoda developed the direct, rapid immunohistochemical test (DRIT), in the early 2000's, while at the U.S. Centers for Disease Control and Prevention (CDC). The development of the DRIT assay, as it is currently known, is based on a simplified version of the standard avidin-biotin-complex immunohistochemical (IHC) diagnostic assay [1–3]. The main differences between DRIT and standard IHC tests is that the original IHC test takes many hours to conduct, requires a linker-antibody conjugated to a biotin moiety, and is performed upon cut sections of fixed tissue, while the DRIT is accomplished in less than 1 hour, utilizes either a polyclonal or monoclonal antibody that is directly labelled with a biotin-moiety, and is performed upon a simple touch impression of brain tissue, allowing an anatomic-pathologic appreciation of viral inclusions in neurons. Additionally, the need for a linker antibody is negated by the subsequent addition of a streptavidin that has a high affinity for the biotin moiety. The streptavidin is tagged to a reporter enzyme that is used to catalyze the formation of a colored precipitate once the antibodies are bound to the rabies virus (RABV) antigens in the presence of an appropriate substrate (H_2O_2) and chromogen (AEC).

Since its development in 2006, various studies investigating the benefit and applicability of the diagnostic assay have been published. All of the studies provided similar results stating that the DRIT not only has a diagnostic efficacy equal to that of the gold standard direct fluorescent antibody (DFA) test, but is also quicker, cheaper and easier to interpret by an inexperienced reader. These findings highlight the versatility of the DRIT as a potential World Health Organization (WHO) and World Organisation for Animal Health (OIE) accredited rabies diagnostic assay [4–11].

DRIT training programme

Training premise

The DRIT training programme will enable diagnostic technicians to perform and interpret the DRIT using the equipment and reagents located at their own diagnostic facility. This training method will ensure that an effective diagnostic assay is implemented at each of the participating laboratories, which in turn will improve the quality of the diagnostic testing performed at the respective facilities.

The aim of the training programme and accompanying manual is to ensure that a working diagnostic assay is applied, and also ensures that good laboratory practices are applied to guarantee the safety of all the diagnostic technicians.

The training programme is designed to last five full days, after which the trainee(s) should be competent to perform and interpret the DRIT assay. In addition, the participating laboratories will have to undergo annual proficiency testing in order to ensure that a high standard of diagnostic efficacy is maintained. The DRIT training program will be executed as follows:

Training schedule

Day 1: Lecture regarding the Standard Operating Procedure (SOP) of the DRIT assay using the information provided in the training manual. An in-depth discussion will follow the lecture in order to answer any questions regarding the DRIT.

Day 2: Demonstration of the DRIT assay. The working concentration of the biotinylated antibody preparation will be determined in this session by demonstrating the DRIT assay to the trainee(s).

Day 3: The trainee(s) will perform the DRIT diagnosis while being physically assisted by the trained technician.

Day 4: Trainee(s) will perform the DRIT diagnosis while being verbally assisted by the qualified technician.

Day 5: The trainee(s) will perform the DRIT on a panel of blind samples. The trainee(s) will not be assisted during this part of the training and the accuracy of the diagnosis will be investigated after completion of diagnosis.

Safety

Personnel

All the personnel participating in the DRIT training programme should receive pre-exposure prophylaxis, or booster immunization if needed, in order to ensure the safety of all laboratory staff. Individuals that have not been immunized should not enter the areas in the laboratory where any rabies work is performed. The trainee(s) should use Personal Protective Equipment (PPE) such as Heavy rubber gloves, laboratory gowns and closed shoes at all times in order to prevent accidental exposure [3].

Personal protective equipment (PPE)

The handling of tissues should be performed while wearing appropriate protective clothing in order to avoid direct contact with potentially infected tissue or fluids. While physically handling tissue samples derived from potentially rabid animals, the personnel have to manipulate the tissue in a manner that does not aerosolize liquids or produce airborne particles. Any tissue samples that have been processed must be disposed of as "medical waste" according to the predetermined procedure(s) of the diagnostic facility [3].

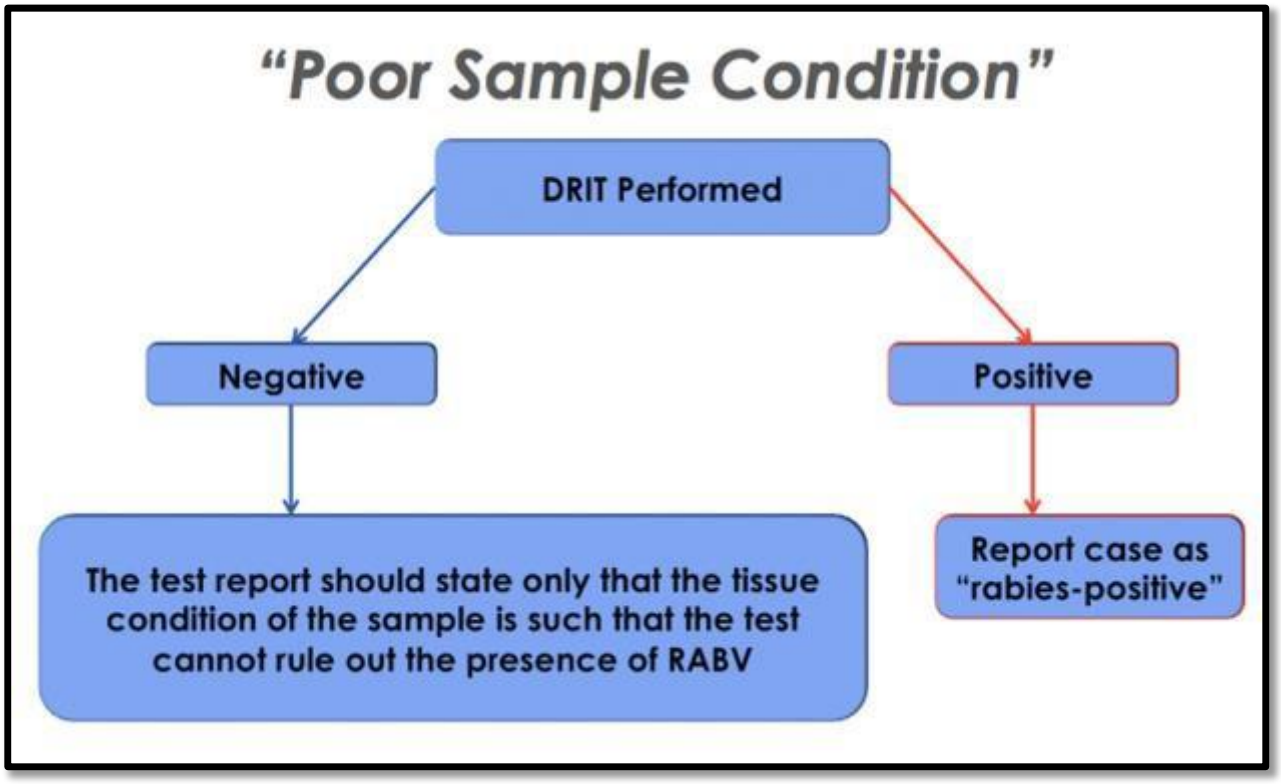
Laboratory technicians are wearing laboratory coats, latex gloves and long trousers.

Biological material - Deterioration or decomposition

The DRIT diagnostic assay relies on the detection of viral antigens that are present in the central nervous system (CNS) tissue. As such, the physical condition of the tissue is very important, with tissue being graded according to the following criteria [3]:

- Good: Tissue is fresh and shows no signs of decomposition.
- Fair: Slight tissue decomposition can be observed. Although slight discolouration might be observed, the tissue still has clearly identifiable anatomical features.
- Poor: The tissue shows signs of discoloration (substantial green colour), liquefaction, desiccation or an unrecognized gross anatomy.

If negative results are obtained from a tissue sample in “poor” condition, the test report should state only that the tissue condition of the sample is such that the test cannot rule out the presence of RABV in the specimen – the sample may thus not be reported as rabies-negative [3].



Diagnosing sample in “poor” condition

Reagents and consumables

Required reagents and consumables

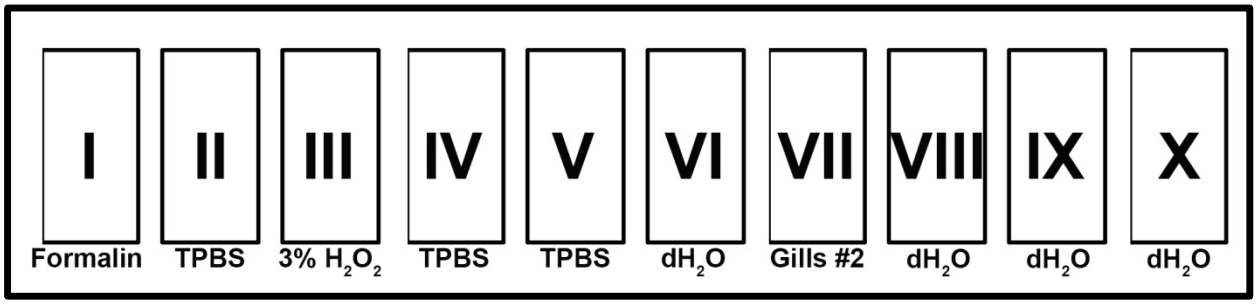
A pre-assembled package of diagnostic reagents and consumables is supplied to the diagnostic facility that partakes in the DRIT training programme. The package contains all the reagents required to perform the DRIT on a pre-determined number of CNS tissue samples.

Each package contains the following reagents that are appropriately marked:

- Phosphate buffered saline (1xPBS) – store at room temperature
- Tween 80 – store at room temperature
- Hydrogen peroxide (3%) – store at room temperature
- Biotinylated antibody preparation (Highly concentrated stock solution) – store at -20°C
- Streptavidin-peroxidase - store at 4°C
- Amino-ethylcarbazole (AEC) chromogen kit - store at 4°C
- Gills formulation #2 – store at room temperature
- Gel mounting medium – store at room temperature
- Distilled water – store at room temperature
- Single edge, frosted microscope slides – store at room temperature
- Glass cover slips – store at room temperature

Staining dish set-up for reagents

The staining dish set-up has been designed to simply the diagnostic process [2]. The preparation of the staining dishes should be done before starting the diagnostic procedure. The contents of each staining dish can be used for different time periods and care should be taken to label the dishes with the date when they were filled and make sure that reagents are replaced when necessary as described in the next section.



Staining dish set-up for DRIT

(Image modified from Niezgoda and Rupprecht, 2006 [2])

Reagent preparation for DRIT

Preparing of reagents that are aliquoted into coplin jars/staining dishes.

Because the slides are placed within the coplin jars/staining dishes, the volume of required reagents have to be sufficient to ensure that the slides will be fully submerged at all times.

- Formalin, 10%: ready-to-use. [*Change out after two batches of samples tested or once a week, whichever comes first*]
- TPBS buffer: Add 990 ml 1xPBS and 10 ml Tween 80. Shake well until the Tween 80 is completely dissolved. [*Change out after each batch of samples tested*]
- 3% Hydrogen peroxide: Ready-to-use. [*Change out after each batch of samples tested*]]
- Distilled water: Ready-to-use. [*Change out after each batch of samples tested*]]
- Gills formulation #2: The Gills #2 formulation is diluted 1:1 in distilled water [*Change out once a week*]

Preparing of reagents that are dropped onto the tissue impression

Each sample requires approx. 70-100 microlitres of each solution, but extra should be prepared in order to ensure that sufficient quantities are ready for use.

- Biotinylated antibody preparation (working concentration): prepare the working concentration of biotinylated antibody according to the section below.
- Streptavidin-peroxidase: ready-to-use
- Amino-ethylcarbazole (AEC) chromogen kit: The staining kit contains three dropper bottles (vials) filled with the required reagents. Create the working solution immediately before use as follows: *Mix 4 ml distilled water, 2 drops of vial 1 (Acetate buffer, 2.5 M, pH 5.0), 1 drop of vial 2 (3-amino-9-ethylcarbazole in N,N-dimethylformamide) and 1 drop of vial 3 (3% hydrogen peroxide in distilled water).* ***The reaction mixture is stable for four hours at room temperature, after which the mixture must be discarded.***
- Gel mounting medium: ready-to-use

Preparation of working concentration of the polyclonal antibody

Although the trainer will provide the working concentration of the biotinylated antibody stock to you, the ideal working concentration can be worked out according to the following method:

From the highly concentrated stock of biotinylated antibody preparation, prepare Serial twofold dilutions (e.g., 1:10, 1:20, 1:40). Two or more slides of positive control CNS tissue should be stained with each dilution. Two microscopists should read the titration and records results independently, and the last dilution providing crisp +4 staining is the end-point dilution of the reagent. 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, etc. 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) [12].

The following formula can be used to determine the required quantity of stock concentration of the biotinylated antibody and 1xPBS solution that should be mixed in order to prepare a specific working solution.

$$\left(\frac{1}{Required\ concentration}\right) x\ required\ volume = volume\ of\ antibody\ required\ in\ the\ final\ volume$$

and

$$Final\ volume\ of\ working\ stock - volume\ of\ antibody\ required = volume\ of\ 1xPBS\ to\ add\ to\ container$$

It is recommended that the **working solution of the biotinylated antibody preparation** be prepared in larger quantities (approximately 50ml, which can stored in a refrigerator at 4°C) in order to avoid repeated thawing of the **stock concentration of the biotinylated antibody preparation**, which should be stored at at-least -20°C at all times. The working solution of the DRIT PAb should be stored 4°C for the duration of its use (no longer than 3 months).

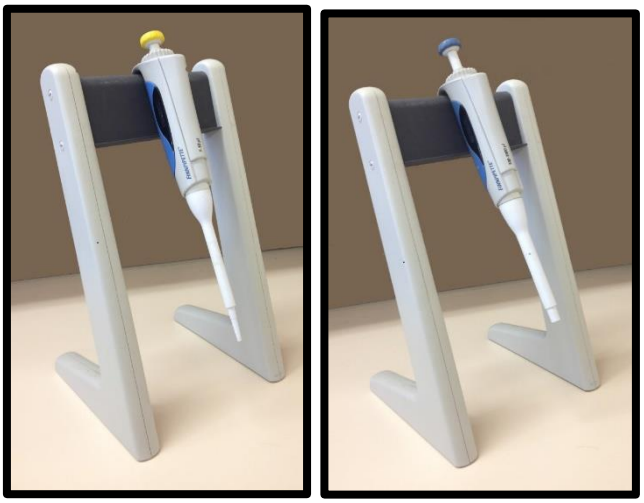
Equipment requirements

The equipment required for the DRIT is supplied by the diagnostic facility where the training takes place. A list of the basic equipment is as follows:
(Please note that the images of the equipment are for reference only, and appearance may vary)

- Coplin jars or individual glass staining dishes

- Humidity chamber for slides (standard ELISA lids can be lined with moist paper towel in order to act as humidity chambers)

- Pipettor: 10-100µl and 100-1000µl



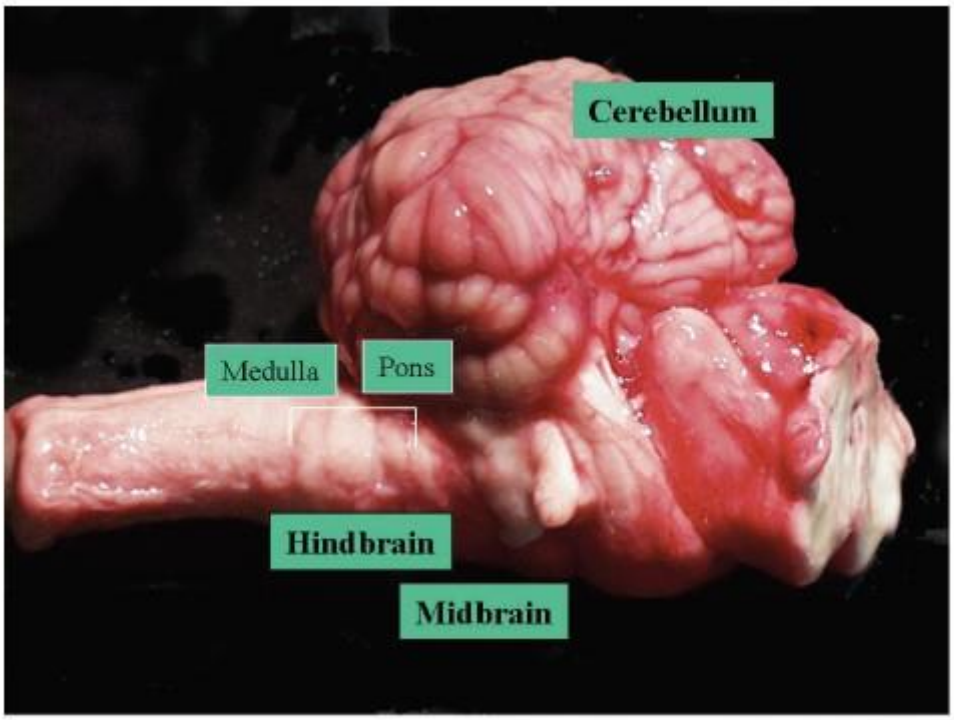
- Light microscope with 20x and 40x objectives

- Plastic dropper bottle and slide holder (optional)

What sections of the brain should be collected for DRIT testing

A fresh section of cerebellum or medulla oblongata (or any other available brain tissue) should always be tested.

If available, a cross-section of the brain stem (consisting of either the mid-brain, pons or medulla oblongata) me should always be include in the material being tested.



Lateral view of whole CNS tissue

(Image modified from Protocol for Post-mortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing [12])

Standard operating procedure

1. Create a tissue impression of a fresh section of cerebellum or medulla oblongata (or any other available brain tissue) (Page 13) by lightly pressing a degreased microscope slide on the brain pieces. The slides with tissue impressions are subsequently dried on a piece of absorbent tissue paper and left to dry at room temperature for 5 minutes. *Positive and negative control slides (freshly prepared from stored CNS tissue of known rabies infection status) must be included in every staining session. These control slides should be fixed and stained alongside the samples, using the same reagents.*
2. Immerse the tissue impressions in 10% neutral buffered formalin for 10 minutes (Dish I).
3. Remove tissue impressions from the 10% neutral buffered formalin and dip-rinse slides several times in fresh TPBS buffer (Dish II).
4. Immerse the tissue impressions in 3% hydrogen peroxide for 10 minutes (Dish III).
5. Remove excess hydrogen peroxide by dip-rinsing the slides in fresh TPBS buffer (Dish IV).
6. Immerse all tissue impressions in fresh TPBS buffer (Dish V)

Work with one slide at a time while leaving the remaining tissue impressions submerged in the TPBS buffer.

7. Remove one slide from the immersed TPBS buffer and shake off the buffer. After shaking off the buffer, the excess buffer can be blotted from the sides surrounding the tissue impression.
8. Place tissue impression in a humidity chamber (the tissue culture plate lid can be lined with moistened paper towel and used to replace commercially available humidity chambers). Add the biotinylated antibody preparation drop-by-drop until the entire tissue impression is covered. Repeat steps 7-8 for all slides before continuing to the next step..
9. After all of the slides have been prepared, place the lid on the humidity chamber and incubate for 10 minutes at room temperature (the incubation can be done on a bench top in the laboratory).
10. Shake off the biotinylated antibody after incubation and immerse the tissue impressions in TPBS buffer (Dish V; this TPBS buffer can be used until step 16 in the diagnostic process).

Work with one slide at a time while leaving the remaining tissue impressions submerged in the TPBS buffer.

11. Remove one slide from the immersed TPBS buffer and shake off the buffer. After shaking off the buffer, the excess buffer can be blotted from the sides surrounding the tissue impression.

12. Place tissue impressions in a humidity chamber and add the ready-to-use streptavidin-peroxidase to each tissue impression until it is completely covered. Repeat steps 11-12 for all slides before continuing to the next step.
13. After all of the slides have been prepared, place the lid on the humidity chamber and incubate for 10 minutes at room temperature (the incubation can be done on a bench top in the laboratory).
14. Shake off the streptavidin-peroxidase after incubation and immerse the tissue impressions in TPBS buffer (Dish V).

Work with one slide at a time while leaving the remaining tissue impressions submerged in the TPBS buffer.

15. Remove one slide from the immersed TPBS buffer and shake off the buffer. After shaking off the buffer, the excess buffer can be blotted from the sides surrounding the tissue impression.
16. Place tissue impressions in a humidity chamber and add the working solution of AEC chromogen to each tissue impression until it is completely covered. Repeat steps 15 -16 for all slides before continuing to the next step.
17. After all of the slides have been prepared, place the lid on the humidity chamber and incubate for 10 minutes at room temperature (the incubation can be done on a bench top in the laboratory).
18. Shake off the excess AEC chromogen after incubation and immerse the tissue impressions in distilled water (Dish VI) for 5 minutes.
19. Immerse tissue impression in counterstain of Gills #2 Hematoxylin (diluted 1:1 with distilled water) for 2 minutes (Dish VII).
20. Immediately remove the excess counterstain from tissue impressions by dip rinsing the slides in distilled water (Dish VIII). Perform a second dip-rinse in fresh distilled water (Dish IX) to ensure that all the excess counterstain is removed from the tissue impression.
21. Immerse slides in fresh distilled water (Dish X)

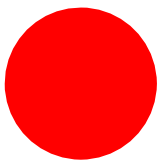
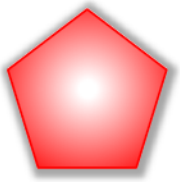
Work with one slide at a time.

22. Shake off distilled water and blot excess distilled water from the sides surrounding the tissue impression.
23. Mount the slides with a water-soluble mounting medium and cover glass, taking care not to allow the tissue impressions to dry out.
24. View the slides by light microscopy. Use a 20x objective to scan the field, and a 40x objective for the closer inspection of rose-red inclusion bodies present on the blue neuronal background.
25. Record results.

Reading and recording of DRIT results

How to read DRIT slides

Although the DRIT does not produce large amounts of background staining, the chromogen can sometimes form single faintly coloured deposits on the tissue. Chromogen flakes are never seen all over the impression and are often single particles. True positive signals and chromogen deposits can easily be distinguished if certain key elements are defined:

Criteria	**Positive signal**	**Chromogen deposit**
i) Shape	Positive signals have smooth rounded edges that are not jagged.	Chromogen deposits have sharp edges and are not rounded. Usually very big.
ii) Position and focus	Positive signals are located within the tissue impression. If the positive signal is in perfect focus, the surrounding tissue should also be in focus	Chromogen deposits are located on top of the tissue impression. If the chromogen flake is in focus, the surrounding tissue should be dull and out of focus.
iii) Colour	Positive signals have a uniform red colour that is the same throughout the entire shape	Chromogen deposits do not have a uniform colour distribution and is often lighter in colour (pink/orange) with no colour in the centre of the chromogen flake
	 Smooth edges Within tissue impression Bright red	 Jagged edges On top of tissue impression Colour is dull & not uniform

How to interpret the DRIT slides

Negative results: A sample is considered negative if more than 40 fields are viewed at a magnification of at least 200x and no rose-red inclusion bodies are visible on the blue neuronal background.
A definitive “rabies-negative” can only be made if a cross-section of the brain stem has been included in the material tested for viral antigen.

Positive results: Staining intensity and antigen distribution is based on the presence of the rose-red inclusion bodies than can manifest in the form of round or oval masses of varying sizes.

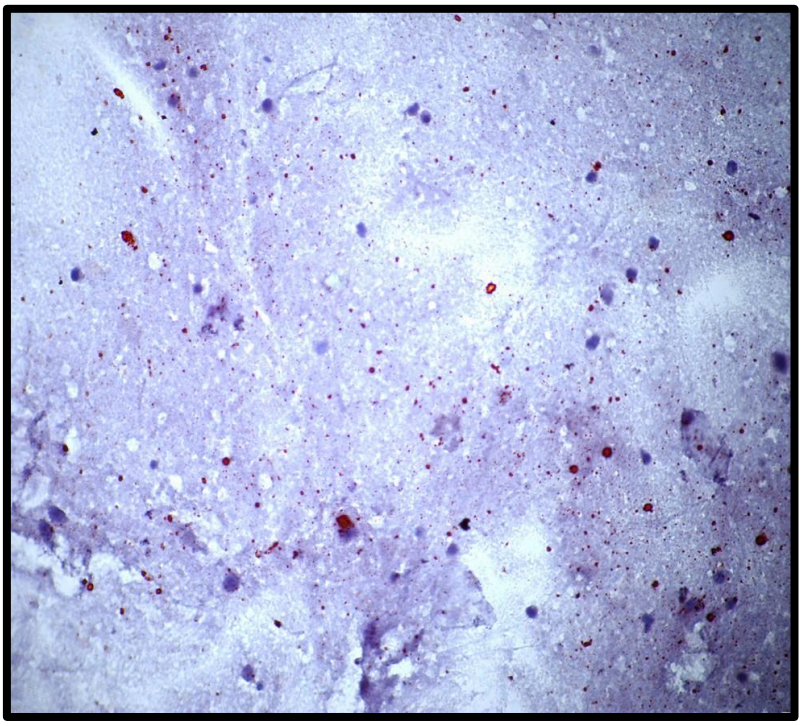
- *Staining intensity*

The staining intensity is graded from +1 to +4 and the positive control slides should always contain a tissue impression with a staining intensity of +4. If a slight loss of colour is observed the staining intensity is graded as +3, while noticeably dull stains are graded as +2 or +1.

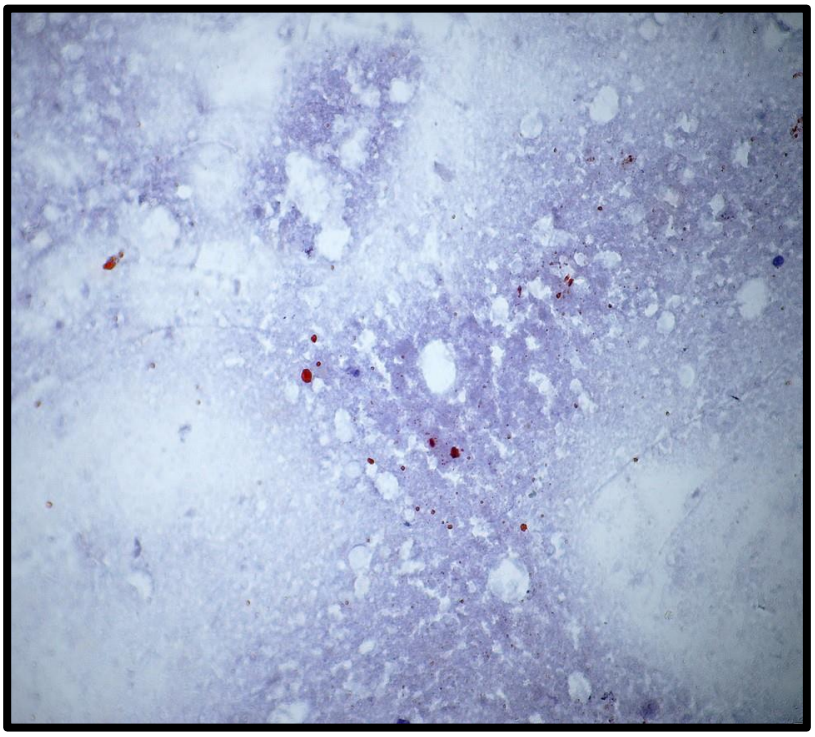
- *Antigen distribution*

The antigen distribution is graded by the amount of observed antigen and is scored as follows:

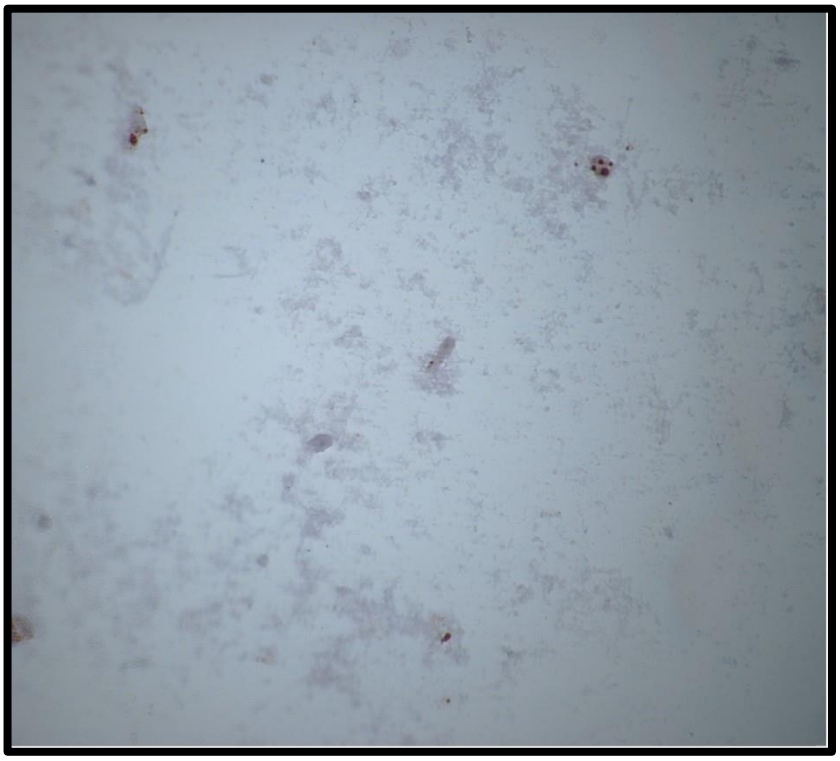
+4: large numbers of both small and large inclusions of varying shapes are visible in almost every field of the impression.



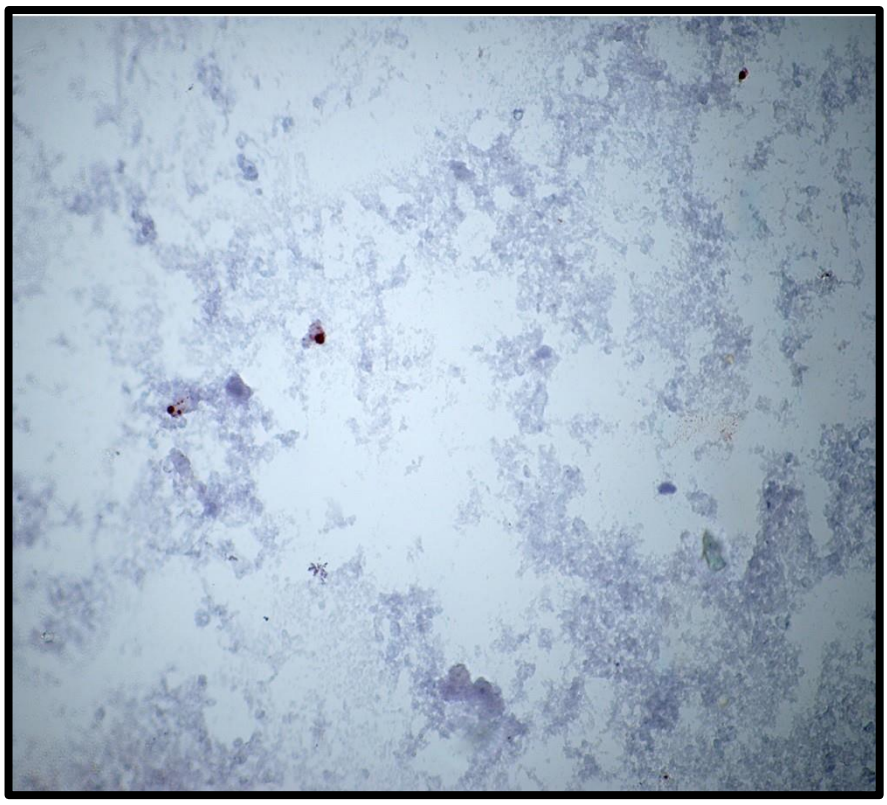
+3: numerous inclusions, of varying sizes and shapes, are found in almost every microscopic field.



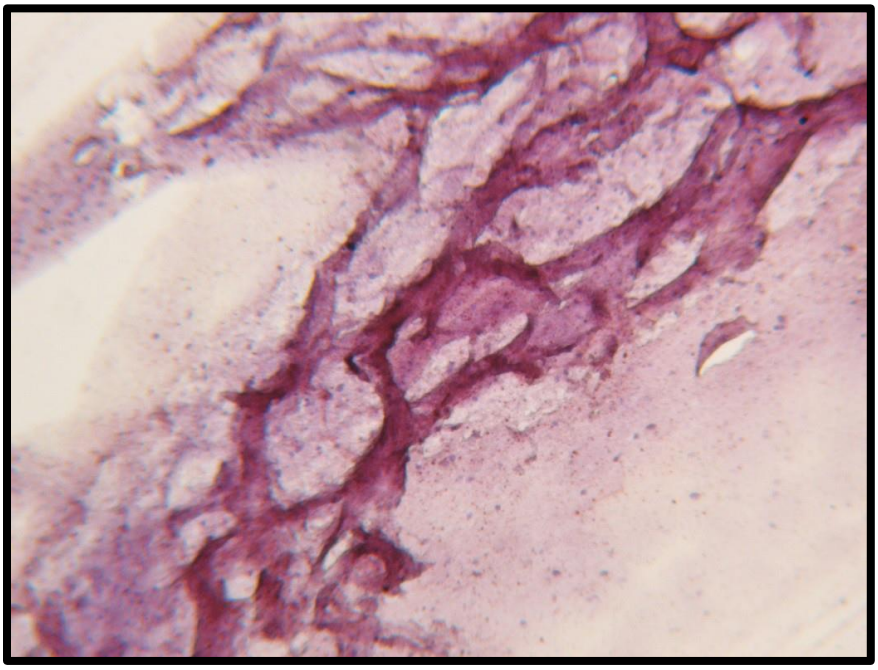
+2: 10% - 50% of the observed fields contain a few inclusions of varying sizes and shapes.



+1: Very few (usually one or two) inclusions of varying sizes and shapes are visible in less than 10% of the observed fields.



Background/non-specific staining: No distinct inclusions observed. The chromogen has produced coloured smears on the background, which makes the diagnosis inconclusive. If background staining is observed, the test has to be repeated.



What information should be collected along with each sample that will be tested

Although the testing of potentially rabies-infected samples is extremely important, the routine collection of information for the sample should not be neglected.
Although each country is different with regards to the information that will be collected, certain key indicators should always be included.

These specific data elements are the following:

- Date sample submitted, and date of testing (if not same)
- Type of animal
- Owner's details
- Clinical signs
- Location of the animal (GPS coordinates or nearest town)
- Possible human exposure and relevant details

A template of an ideal sample collection sheet can be found at the end of the training manual and digital copies can be provided if required.

Troubleshooting

Problem	**Solution(s)**
There was little/no tissue left in the impression made in the first step of the SOP. Or No tissue impression is visible on the slide	Make tissue impressions that are slightly thicker by using more CNS material when making the actual impression. Allow tissue impressions to dry at room temperature for 5 minutes before fixing the impressions in formalin.
I am not sure whether I am looking at red inclusions or a chromogen flake	Look at the possible inclusion and check for the following: Are the edges round? If the inclusion is in perfect focus, is the surrounding tissue also in focus? Is the colour a bright red? If the answers for all of the above questions are “yes”, the inclusion is a positive signal.
No rose-red inclusions were observed in either the positive control or any of the samples tested.	(Steps listed in order of priority) Repeat SOP with a fresh working solution of AEC chromogen. Create a new working solution of biotinylated antibody (using a new batch of stock solution would be advised). Ensure slides are submerged in distilled water after applying the AEC chromogen as alcohol will dissolve the rose-red precipitate. Use a new kit of AEC chromogen. Use a new bottle of Streptavidin- peroxidase solution.

The positive control slide had inclusions, but did not have a staining intensity of +4.	Samples with a +3 or +4 staining intensity can be reported as RABV positive, but samples with a reduced staining intensity will have to be repeated with a new positive control slide.
My tissue impression did not have a blue background once viewed under the microscope.	The counterstain step was not performed as discussed in the provided SOP. Fresh counterstain needs to be prepared by diluting Gills #2 formulation as discussed in the “reagent preparation” heading.
Intense background staining observed.	Prepare a fresh working solution of 3% hydrogen peroxide. Reduce the incubation time with the AEC chromogen. Test for presence of naturally occurring biotin in the tissue by repeating the SOP without the addition of the biotinylated antibody preparation. The presence of rose-red inclusions indicates that the tissue contains traces of the naturally occurring biotin and a commercial endogenous biotin-blocking agent will have to be applied.

Commercial suppliers of DRIT-specific reagents

We have provided the names of the supplier(s) whose products we have tested and are familiar with in practice, but comparable products from other suppliers may also be available:

- Formalin, 10% neutral buffered (**Sigma-Aldrich, Cat # HT501128**)
- Phosphate buffered saline (PBS) (**Fisher Scientific, Cat # SH30256,02**)
- Tween 80 (**Sigma-Aldrich, Cat # P1754**)
- H2O2 (3%) (from any commercial supplier)
- Streptavidin-peroxidase (**Kirkegaard & Perry Laboratories Inc, Cat # 71-00-38**)
- AEC staining kit (**Sigma-Aldrich, Cat # AEC101**)
- Gills formulation #2 (**Sigma-Aldrich, Cat # GHS232**)
- Gel/mount (**Fisher Scientific, Cat # BM-M01**)
- Distilled water (**Fisher Scientific, Cat # S75232**)
- Biotinylated antibody preparation (**supplied by GARC**)

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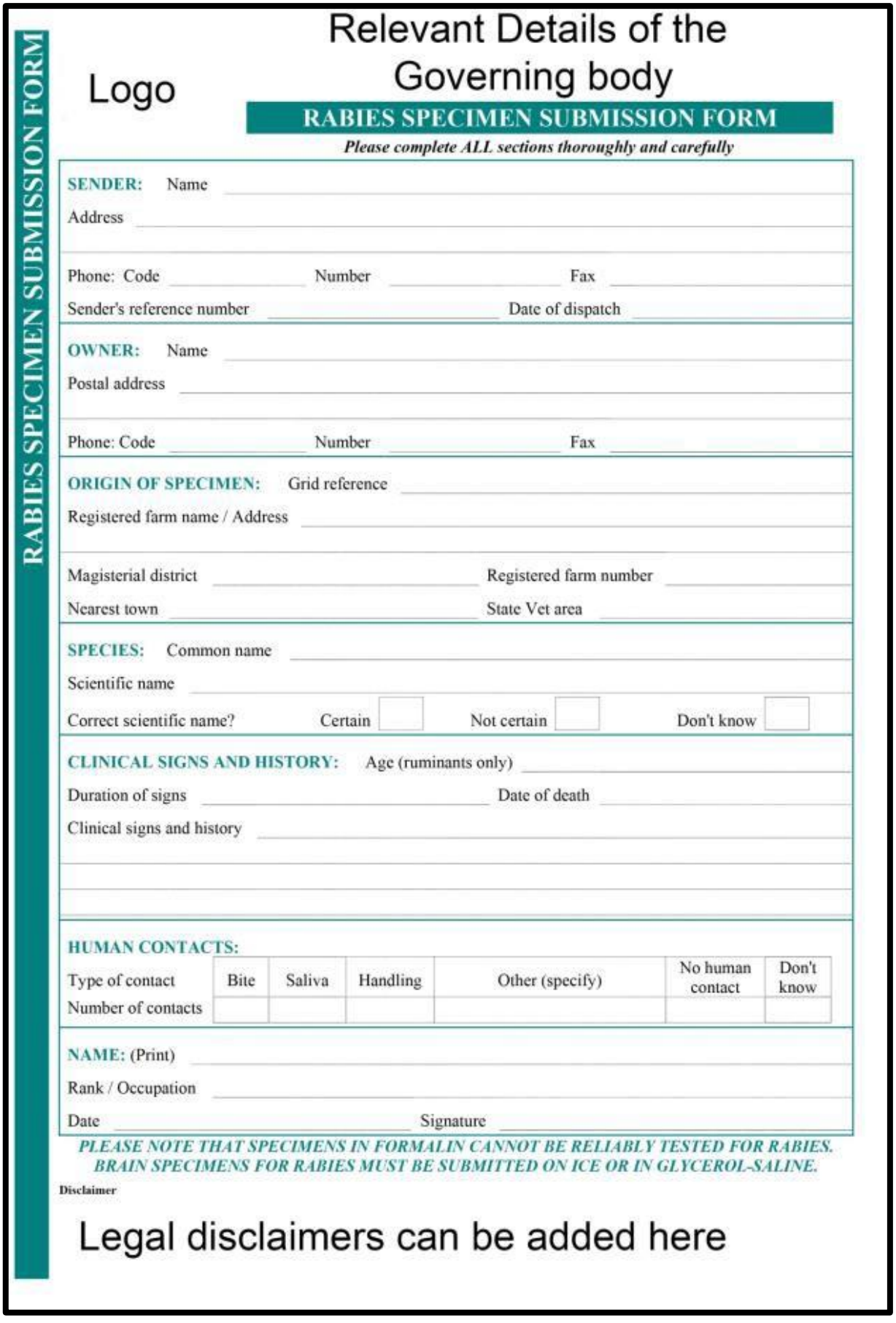
Rabies sample collection sheet



RABIES SPECIMEN SUBMISSION FORM

Logo

Relevant Details of the Governing body

RABIES SPECIMEN SUBMISSION FORM

Please complete ALL sections thoroughly and carefully

SENDER: Name

Address

Phone: Code Number Fax

Sender's reference number Date of dispatch

OWNER: Name

Postal address

Phone: Code Number Fax

ORIGIN OF SPECIMEN: Grid reference

Registered farm name / Address

Magisterial district Registered farm number

Nearest town State Vet area

SPECIES: Common name

Scientific name

Correct scientific name? Certain ☐ Not certain ☐ Don't know ☐

CLINICAL SIGNS AND HISTORY: Age (ruminants only)

Duration of signs Date of death

Clinical signs and history

HUMAN CONTACTS:

Type of contact	Bite	Saliva	Handling	Other (specify)	No human contact	Don't know
Number of contacts						

NAME: (Print)

Rank / Occupation

Date Signature

PLEASE NOTE THAT SPECIMENS IN FORMALIN CANNOT BE RELIABLY TESTED FOR RABIES. BRAIN SPECIMENS FOR RABIES MUST BE SUBMITTED ON ICE OR IN GLYCEROL-SALINE.

Disclaimer

Legal disclaimers can be added here