Training Course and Workshop for Laboratory Diagnosis of Rabies
Bureau of Animal Industry (BAI), Quezon City, Philippines
July 1-5, 2019
Summary

For this training, the OIE welcomed a team from the OIE Reference Laboratory for Rabies, the Changchun Veterinary Research Institute (CVRI) laboratory in China, led by Dr Changchun Tu. We also welcomed Dr Maria Nerissa Dominguez, Technical Officer from the WHO Philippines office in Manila, to introduce human health updates during the seminar on the first day and Dr Ronello Abila, the OIE sub-Regional Representative for South-East Asia, to discuss risk assessment for the spread of rabies on the final day. Dr Ronnie D Domingo, Director of BAI, opened the training.

The objective of this course was to provide an overview of the principles, concepts and applications of PCR-based methods for the detection of rabies virus RNA. It also offered the opportunity to have hands-on training in the laboratory under the supervision of experts. Best strategies and practices for effective sample preparation, efficient reverse transcription, generation of high-quality PCR data and optimal qPCR assay design were discussed. In addition, theory and appropriate practical experience were provided on other diagnostic techniques such as brain tissue collection using the straw method, direct fluorescent antibody test (DFAT) and enzyme-linked immunosorbent assay (ELISA) for the detection of rabies.

For the seminar sessions, 35 participants attended; seven participants from laboratories across the country undertook the hands-on laboratory training. This core group of participants demonstrated increased knowledge of rabies diagnostics over the course of the week and learned several laboratory techniques which have strengthened capacity within the Philippines for rabies diagnosis. Dr Tu was helpful in making suggestions to improve laboratory efficiency and safety. Since the training, collaboration between CVRI and BAI has continued, including sequence analysis of Philippine rabies isolates which is extremely useful in understanding epidemiology of the disease in this country.

WORLD ORGANISATION FOR ANIMAL HEALTH
Protecting animals, preserving our future

DRAFT PROGRAMME FOR THE TRAINING COURSE AND WORKSHOP FOR LABORATORY DIAGNOSIS OF RABIES

Quezon City, Philippines
July 1-5, 2019

Course Agenda

The objective of this course is to provide an overview of the principles, concepts and applications of PCR-based methods for the detection of rabies virus RNA. It also offers the opportunity to have hands-on training in the laboratory under the supervision of experts. Best strategies and practices for effective sample preparation, efficient reverse transcription, generation of high-quality PCR data and optimal qPCR assay design will be discussed. In addition, theory and appropriate practical experience will also be provided on other diagnostic techniques such as brain tissue collection using the straw method, direct fluorescent antibody test (DFAT) and enzyme-linked immunosorbent assay (ELISA) for the detection of rabies.

Course Program

DAY 0-June 30, 2019

TIME	ACTIVITY	FACILITATOR	VENUE
Whole Day	Arrival of training participants, experts, and guests Local transfer from airport to accommodation		

DAY 1- July 01, 2019

TIME	ACTIVITY	FACILITATOR/ SPEAKER	VENUE
08:30 – 09:00	Registration of participants	ADDRL Staff	BAI-AHTIC Conference Room
09:00 – 09:30	**Opening Program** • Opening prayer • Philippine National Anthem • Welcome Remarks – **Dr. Ronnie D. Domingo** Director, BAI • Opening Message – **Dr. Enrico P. Garzon Jr.** Assistant Secretary for Livestock, Department of Agriculture • Keynote Message - **Dr. Lesa Thompson**, OIE RRAP Introduction of speakers and participants **Group photo**	ADDRL, DA & OIE	
09:30 – 09:45	Snacks/ Coffee break (Workshop participants submit pre-training quiz)		
09:45 – 10:15	**Global/Regional Updates on Rabies by the FAO/OIE/WHO Tripartite Collaboration**	Dr Lesa Thompson, OIE RRAP Dr Maria Nerissa Dominguez, WHO Philippines	BAI-AHTIC Conference Room
10:15 – 10:35	**OIE Standards on Rabies Diagnosis**	Professor Changchun Tu, CVRI, China (OIE reference lab)	
10:35 – 11.20	Open forum and closing		
11:20	End of morning seminar		

11:20 – 13:00	**Lectures:** Nucleic acid isolation • Introduction to principles and sample preparation techniques • RNA extraction from different samples • RNA quality control Introduction to the principles of PCR and qPCR • Principles of primer and probe design • Reverse transcription: guidelines for cDNA synthesis • Conventional and real-time quantitative PCR (qPCR) guidelines	CVRI	BAI-AHTIC Conference Room
13:00 – 14:00	Lunch break		
14:00 – 17:30	**Practical Session I:** Hands-on training on different sample preparation and extraction methods • Sample preparation • RNA extraction • Primer and probe preparation • 1st step of RT-nPCR: cDNA synthesis • qPCR	CVRI (Trainer/Expert) & ADDRL Staff	Virology lab

DAY 2- July 02, 2019

TIME	ACTIVITY	FACILITATOR	VENUE
08:30 – 12:00	**Practical Session II:** Hands-on training on conventional and real-time PCR and analysis of PCR results • 2nd step of RT-nPCR: 1st round PCR • Analysis of qPCR results *option for 15-minute snack/coffee break in between certain topics	CVRI (Trainer/Expert) & ADDRL Staff	ADDRL conference room
12:00 – 13:00	Lunch break		
13:00 – 17:00	**Practical Session III:** Hands-on training on conventional PCR and real-time PCR • 3rd step of RT-nPCR: 2nd round PCR **Quiz:** Real-time PCR (independent performance by participants)	CVRI (Trainer/Expert) & ADDRL Staff	Virology lab

DAY 3- July 03, 2019

TIME	ACTIVITY	FACILITATOR	VENUE
08:30 – 12:00	**Practical Session IV:** Hands-on training for conventional PCR and analysis of PCR results • 4th step of RT-nPCR: gel electrophoresis • Analysis of RT-nPCR results	CVRI (Trainer/Expert) & ADDRL Staff	Virology lab
12:00 – 13:00	Lunch break		
13:00 – 16:00	**Practical Session V:** Sample collection • Collection of brain tissue sample: straw method • Application of FTA cards	CVRI (Trainer/Expert) & ADDRL Staff	Virology lab

16:00-16:30	**Lecture:** Enzyme-linked Immunosorbent Assay (ELISA)	CVRI	ADDRL conference room
16:30- 18:00	**Practical Session VI:** Hands-on training on ELISA: dilution of sera and incubation over night	CVRI (Trainer/Expert) & ADDRL Staff	Virology lab

DAY 4- July 04, 2019

TIME	ACTIVITY	FACILITATOR	VENUE
08:30 – 12:00	**Practical Session VII:** Hands-on training on ELISA: test	CVRI (Trainer/Expert) & ADDRL Staff	Virology lab
12:00 – 13:00	Lunch break		
13:00- 14:00	**Practical Session VIII:** Calculating and interpretation of ELISA result	CVRI (Trainer/Expert) & ADDRL Staff	ADDRL conference room
14:00- 15:00	Presentation of current ADDRL practice on dFAT	ADDRL staff	
15:00-16:30	Assessment of current ADDRL practice on dFAT **Lecture:** Direct Fluorescent Antibody Test • Sample collection techniques • Preparation of test and control slides • Microscopy and Interpretation • Quality control and Quality assurance • Updates and General guidelines: Introduction of dRIT	CVRI (Trainer/Expert)	

DAY 5- July 5, 2019

TIME	ACTIVITY	FACILITATOR	VENUE
08:30 – 09:45	**Open discussion and practical recommendations** • Laboratory setup • Instruments, software and reagents • Biosafety • QC and QA *up to 30min extension as needed	CVRI & ADDRL	ADDRL conference room
09:45 – 10:15	Training evaluation and post-training quiz	CVRI & OIE	
10:15 – 10:30	Snacks/ coffee break		
10:30 – 11:15	**Lecture:** Application of Spatial Risk Assessment in understanding the spread of rabies and evaluation on the impact of vaccination - Dr. Ronello Abila, OIE-SRR SEA	ADDRL	AHTIC Conference Room
11:15 – 12:00	**Awarding of Training Certificates** **Closing Ceremonies** • BAI/DA • OIE - Dr. Ronello C. Abila, OIE-SRR-SEA	ADDRL staff/ OIE/BAI /CVRI	
12:00 – 13:00	Lunch break		
	Departure of Participants, trainers and guests		

THE TRAINING COURSE AND WORKSHOP FOR

LABORATORY DIAGNOSIS OF RABIES

Quezon City, Philippines

July 1-5, 2019

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Introduction

Rabies is caused by neurotropic viruses of the genus *Lyssavirus* in the family *Rhabdoviridae* and is transmissible to all mammals. As the viruses are transmissible to humans, all suspect infected human material must be handled under the appropriate safety conditions specified by the World Health Organization. Laboratories working with lyssaviruses or suspect animal material must comply with national biocontainment and biosafety regulations and as well as following appropriate biosafety and containment procedures as determined by biorisk analysis.

Based on nucleotide sequences of the nucleoprotein gene (N), the lyssairuses are divided into 16 species by ICTV 2018, comprising classical rabies virus (RABV) and the rabies-related viruses (RRV): Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Australian bat lyssavirus (ABLV), Irkut virus (IRKV), West Caucasian bat virus (WCBV), Khujand virus (KHUV), Aravan virus (ARAV), Shimoni bat virus (SHIBV), Bokeloh bat lyssavirus (BBLV) and Ikoma virus (IKOV), Lleida bat lyssavirus (LLEBV) and Gannoruwa bat lyssavirus (GBLV). Recently, two novel lyssavirus species were identified as tentative species – Kotalahti bat lyssavirus (KBLV) isolated from a Brandt's bat (Myotis brandtii) in Finland in 2017 and Taiwan bat lyssavirus (TWBLV) isolated from a Japanese pipistrelle (Pipistrellus abramus) in Taiwan, China in 2016-2017.

RABV is found worldwide and is responsible for the overwhelming majority of reported animal and human rabies cases. Other lyssaviruses appear to have more restricted geographical and host range, with the majority having been isolated from bats with limited public and animal health implications. However, all lyssaviruses tested cause clinical disease indistinguishable from RABV.

The lyssaviruses have been divided into at least three phylogroups with distinct pathogenicity and immunogenicity. For phylogroup 1 (comprising RABV, ABLV, EBLV1, EBLV2, BBLV, ARAV, DUVV, KHUV, IRKV, and GBLV), conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination. Rabies virus vaccines, however, may not provide adequate cross-protection against all genetically divergent lyssaviruses. Little or no cross-protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed against lyssaviruses of phylogroups 2 and 3.

Purpose/scope of this manual

The diagnoses methods in this document are written according to the OIE “Manual of Diagnostic Tests and Vaccines for Terrestrial Animals” (2018) and the WHO “Laboratory Techniques in Rabies” (Fiveth Edition, 2018).

The rabies diagnosis chain

The chain of rabies diagnosis starts in the field with the collection of specimens and ends with a report to the sender. The positive and negative results obtained in the laboratory should then be published in “rabies bulletins” and integrated into national and international statistics.

The steps of this process are therefore:

1. Collection of specimens and epidemiological data in the field
2. Transport of the specimen to the laboratory
3. Choice of test(s)
4. Laboratory diagnosis
5. Disease analysed and reported

Safety

- Any staff performing this procedure in a rabies high security unit must first be vaccinated, with a demonstrated titre of 1.0 IU/ml or greater.
- Apparatus (tabletops, socket forceps, scissors, forceps and grinder, etc.) used in the post-mortem room must be cleaned between specimens.
- A sterile set of instruments should be used for each diagnosis and contaminated instruments should be decontaminated by boiling, autoclaving or adapted chemical treatment.
- All biological waste material should be decontaminated by incineration. Where this is not possible the waste may be buried in quicklime in an isolated place and at a depth that cannot be reached by carnivores.
- Potentially infectious material such as brain smears should be fixed before removal from the post-mortem room.

1. Collection of specimens and epidemiological data

Reliable diagnosis of rabies can only be performed from the brain in a laboratory.

Of the parts of the brain, the brainstem is the most reliable for the detection of antigen, followed by the thalamus and hippocampus. Other tissues can also be used for rabies diagnosis, although not all are as reliable as brainstem. These include: spinal cord, peripheral nerve tracts and salivary glands.

Whatever the specimen collected, it must be stressed that brain tissue is required for a reliable rabies diagnosis.

Which information should be collected?

Every specimen submitted for rabies diagnosis should be accompanied by a submission form containing the following data:

1. Specimen description
    a. Species
    b. Date of death and date of collection
    c. Date of shipment
    d. Location: name, geographical coordinates
2. Name and address of the owner or of the person who found the animal
3. Possible human exposure
    a. Date of exposure
    b. Type of exposure
    c. Location of the wound(s)
    d. Name and addresses of exposed people
4. Other information
    a. Age, sex and vaccination status of the suspect animal
    b. Clinical signs
    c. Animal contacts

2. Shipment of specimens

Whole carcasses, heads, brains or brain biopsies can be transported to the laboratory.

Ideally, fresh material should be transported to the specialised laboratory rapidly and under cold conditions.

If refrigerated/frozen shipment of samples is not possible, other preservation techniques may be used.
The choice of the preservative is dependent on the tests to be used for diagnosis:
i) Formalin-fixed specimens
Formalin fixation (10% [w/v] solution in phosphate buffered saline [PBS]) allows testing with FAT, immunohistochemistry, conventional and real-time PCR; however, modifications may be required and tests can be less sensitive compared to using fresh specimens. Formalin fixation inactivates the virus thus preventing virus isolation.
ii) Glycerol/phosphate buffered saline (PBS)
For transportation of specimens, infectivity may be extended for several days if diagnostic specimens are kept in a mixture of 50% glycerol in PBS. Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. Since rabies virus is thermolabile, this method does not prevent a decline in the viral load of the specimen. Under routine transport conditions in regions with high temperatures (above 30°C), this protection may only be effective for a matter of several days. Therefore, whenever possible specimens in glycerol/PBS should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used to test these specimens.
iii) Preservation for molecular techniques
For molecular techniques, lysis buffers for nucleic acid extraction and RNA preservation buffers impregnated onto filter paper can be used. These buffers preserve rabies virus RNA and allow transport of specimens at ambient temperature without specific biohazard precautions for detection of viral RNA and further genetic characterisation of rabies virus strains.

3. Choice of tests to be used

Whenever possible, all specimens should be examined using at least two different techniques: for example, the fluorescent antibody test (FAT) or the direct rapid immunohistochemistry test (dRIT) to detect antigen, the mouse inoculation test (MIT) or the rabies tissue culture inoculation test (RTCIT) as a back-up. (Molecular testing is also good practice in a capable laboratory, providing the operators have considerable experience and the results are interpreted with caution.)

The following tests can be performed according to the available specimens:

Specimen	Test
Fresh or frozen nervous tissue specimen	FAT or dRIT on smears, biological tests in mouse or mouse neuroblastoma cell cultures, RT-nPCR and One-step Real time-qPCR
Glycerol-preserved specimen, after removing excess glycerol	FAT or dRIT on smears, biological tests in mouse or mouse neuroblastoma cell cultures, RT-nPCR and One-step Real time-qPCR
Formalin-fixed nervous tissue specimens embedded in paraffin	FAT or dRIT following enzyme digestion (1), RT-nPCR and One-step Real time-qPCR

(1) Not recommended as a routine procedure

4. Laboratory diagnosis of rabies

Identification of the agent:

For the reliable diagnosis of rabies, the brain is required. Of the parts of the brain, the brainstem is the most reliable for the detection of antigen. Other tissues can also be used for rabies diagnosis, although not all are as reliable as brainstem. These include spinal cord, peripheral nerve tracts and salivary glands. The ante-mortem diagnosis of human rabies requires skin biopsies from the head region and corneal impression smears.

This section describes the gold standard test of rabies diagnosis: the fluorescent antibody test (FAT).

The direct rapid immunohistochemistry test (dRIT) can be used as an alternative method of FAT in routine rabies diagnosis as the tests have similar sensitivity and specificity, and this test can be used in laboratories without a fluorescent microscope.

Various molecular diagnostic tests, e.g. detection of viral RNA by reverse transcription PCR (RT-PCR) or real-time PCR, are used as rapid and sensitive additional techniques for rabies diagnosis. Although those molecular tests have the highest level of sensitivity, their use is currently not recommended for routine post-mortem diagnosis of rabies due to high levels of false positive or false negative results without standardisation and very stringent quality control.

- Collection of brain samples
- Fluorescent antibody test
- Molecular tests：

RT-nPCR

Real-time PCR

4.1 Identification of the agent:

4.1.1 Collection of brain samples: drinking-straw method

Ideally, the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected, preferably brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata.

In field conditions, in cases where animals cannot be brought to the laboratory or when there is no access to laboratory facilities, it is possible to collect brain samples without opening the skull.

Precautions should be taken when handling central nervous system tissues from suspected rabies cases. Protective personal equipment (such as gloves, mask) should always be worn and precautions must be taken to prevent aerosols. Cutting tools, scissors and scalpels, should be used with care to prevent injury and contamination.

a. MATERIALS

Equipment/consumables

Protective clothing: gown, gloves and mask

Drinking straws or disposable plastic pipettes

Scissors and scalpel

Collection tube

b. PROCEDURE/METHOD

Drinking-straw method for brain sampling:

1. Bend the head of the animal so that the nose is touching the chest between the forelegs

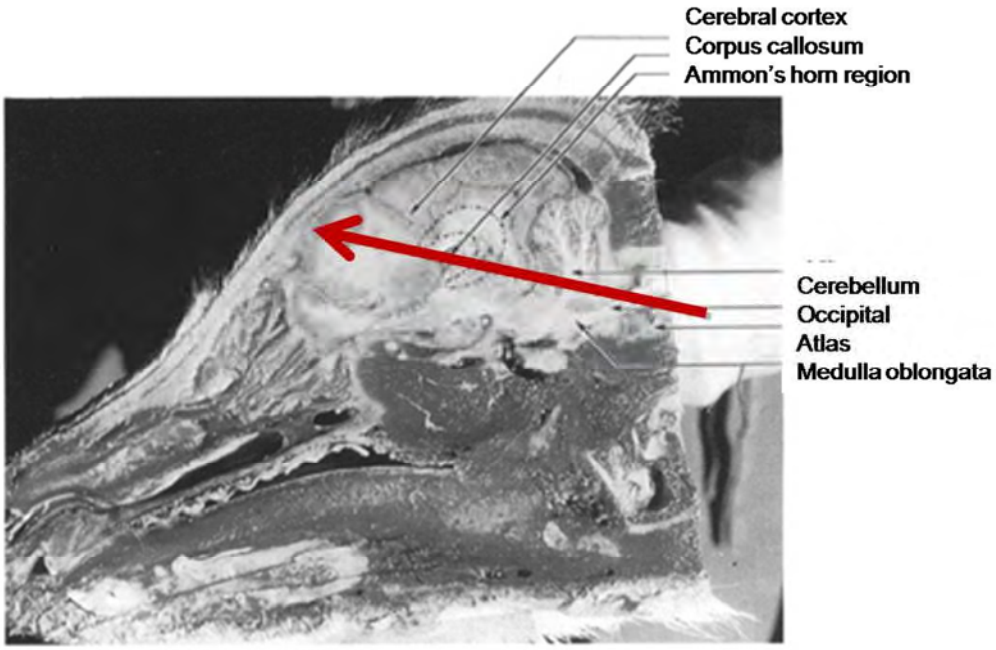
2. This gives access to the occipital foramen

3. The straw is then introduced and inserted towards an eye

Area sampled by the straw

A 5 mm drinking straw or a 2 ml disposable plastic pipette is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata. When using a straw it should be pinched between the fingers to prevent material escaping when withdrawing.

The different portions sampled with the straw are illustrated in this diagram:



4.1.2 Fluorescent antibody test (FAT)

The direct fluorescent antibody test is recommended by both WHO and OIE as **the gold standard in experimental diagnosis**.

It is a rapid, sensitive and specific test that is not very expensive. It involves the staining of a tissue smear (usually brain) with an FITC-labelled anti-rabies immunoglobulin, following acetone fixation. The stained smear is washed in buffer and read under blue-light fluorescence microscope to detect the characteristic green fluorescence associated with rabies antigen corpuscules.

a. MATERIALS

Chemicals and reagents

FITC Conjugate (Bio-rad)

High-grade acetone, stored at -20°C

Distilled water

0.1M PBS pH 7.4

Tween-20

Equipment/consumables

Bio-safety cabinet

Freezer: -20°C and -80°C

Humidified incubator: 37°C (+/- 2%)

Fluorescent microscope

Waste container

Coplin jars

Wooden applicator sticks (tongue depressors, for lifting and retracting the brain)

Microscope slides

Micropipette tips

Slide boxes

Humidified box

Permanent marker pen

b. PROCEDURE/METHOD

1. Label the microscope slides. Label one slide with the date and the sample number.

2. Group small pieces of the selected brain sections on a wooden applicator stick and make an impression smear directly onto the slide.

The routine use of positive and negative controls is essential.

3. Air dry the smear thoroughly.

4. Place all slides into a Coplin jar containing cold acetone within the freezer for 30 min.

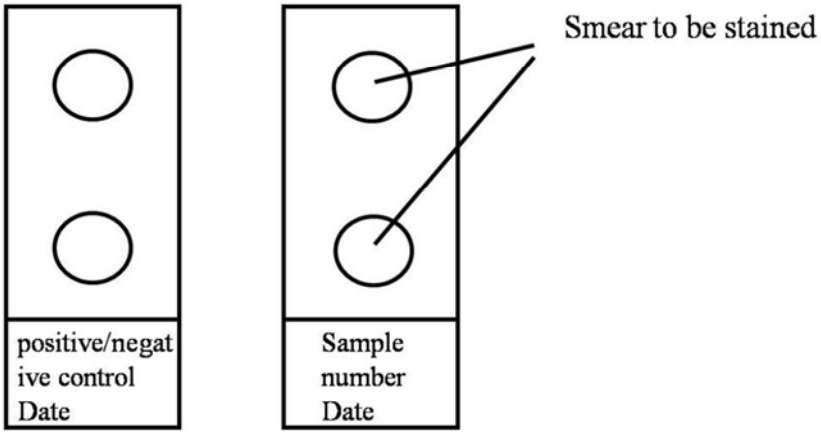
5. Remove the slides from the Coplin jar and rinse in PBST (1000mL PBS+0.5mL Tween-20) for 3 times.

6. Add 40µL of (Mixture of Bio-Rad Conjugate and 1% Evan's Blue) per slide.

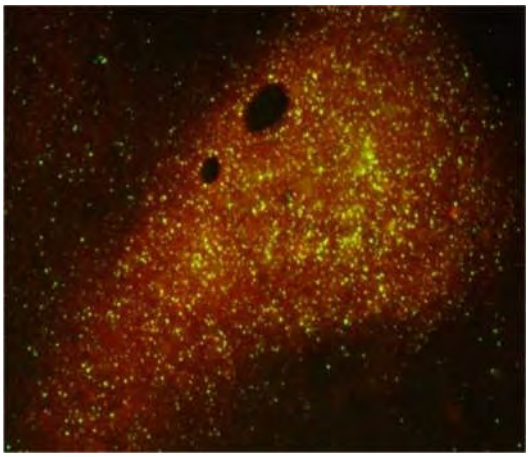
7. Incubate at 35-37°C for 30-45 min in a humidified chamber (i.e. incubator).

8. Wash 2 times with PBST.

9. Air dry and apply cover slip, using mounting medium for fluorescence.



c. Reading

	
Positive brain tissue	Negative brain tissue

The slides should then be examined for specific fluorescence using an inverted fluorescence microscope filtered for the appropriate wavelength (490 nm and re-emits at 510 nm).

Slides are examined using a x10 eye piece and a x10 objective.

Generally, every slide should be examined independently by two experienced technicians who compare their results at the end of the reading session.

d. Result

Controls are read first, both positive (above left image) and negative (above right image) controls must be validated for tests.

Infected tissue appears as bright 'apple' green fluorescent inclusions. Dull green or red/green auto-fluorescent granules should not be counted as positive antigen.

4.1.3 Molecular test

4.1.3.1. RNA Extraction with QIAGEN Virus RNA Kit

Procedure is used for the extraction of viral RNA from both infected animal brain tissue samples and infected tissue culture. The extracted RNA is used in the nested RT-PCR and one-step real time PCR to detect rabies virus.

a. Materials

Chemicals and reagents

0.1M PBS pH7.4

QIAGEN Virus RNA Kit (Cat No#52904)

Equipment/consumables

Bio-safety cabinet

Pipettes (range from 10μL up to 1000μL)

Centrifuge (4°C centrifuge is recommended)

1.5 ml centrifuge tube (RNase and DNase free)

b. Procedure

i. Sample preparation

Portions of brain collected from the hippocampus, thalamus, medulla oblongata and cortex are crushed with a grinder and grinding rod to obtain a 10%(w/v) suspension in PBS. Centrifuge at 10,000 rpm/min for 5 min. Transfer the supernatant into a new 1.5 ml tube.

Cell culture suspension is also available for RNA extraction if it is requested.

The same protocol should be used to prepare negative control with mouse brains and positive controls with pieces of positive brains of animals naturally-infected with RABVs.

ii. Isolation of RNA

Advance preparation in PCR Clean Room：

1. Add the appropriate amount of absolute ethanol (as indicated on the bottle) to Buffer AW1 and Buffer AW2 concentrate to obtain a working solution. Indicate on bottle cap that this step has been done.

In Virus Isolation Room:

2. Add 310µL of Buffer AVE to a tube containing 310 µg carrier RNA to obtain solution of 1µg/µL. Dissolve RNA carrier thoroughly, divide it into conveniently sized aliquots, and store at -30°C to -15°C.

3. Calculate volume of Buffer AVL-carrier RNA mix needed per batch of samples (Refer to Table 1 at end of procedure).

4. Before commencing sample processing, dispense 560µL of Buffer AVL-Carrier RNA mix into a microcentrifuge tube.

5. Add 140µL of processed material (lysate) to the buffer mix and vortex briefly for 15 seconds.

6. Incubate at room temperature for 10 minutes.

7. Briefly centrifuge to remove drops from inside of the lid.

8. Carefully remove supernatant by pipetting.

9. Put in zipper bag and transfer/send to Extraction Room.

In Extraction Room:

10. Add 560µL of absolute ethanol to the lysate (prepared in Virus Isolation Room) to give a total volume of 1120µL and pulse–vortex for 15 seconds. Briefly centrifuge to remove drops from the inside of the lid.

11. Carefully transfer 630µL of the lysate to the QIAmp mini spin column without wetting the rim.

12. Close the lid gently and centrifuge at 8,000 rpm for 1 minute. Discard flow- through and place QIAmp spin column into a clean 2 ml collection tube.

13. Add remaining lysate to QIAmp spin column and repeat the process.

14. Add 500µL of Buffer AW1 to spin column and centrifuge at 8,000 rpm for 1 minute.

15. Discard flow-through and replace the spin column.

16. Transfer RNeasy spin column to a clean 2 ml tube.

17. Add 500µL of Buffer AW2 and spin for 3 minutes at 14,000 rpm.

18. Discard flow-through and place the spin column in a new collection tube and centrifuge at 14,000 rpm for 1 minute.

19. Transfer spin column to microcentrifuge tube and add 50µL of Buffer AVE directly to the spin column membrane.

20. Let stand for 1 minute at room temperature.

21. Centrifuge for 1 minute at 8,000 rpm to elute the RNA.

22. Discard spin column and cap the microcentrifuge tube. Label.

23. Store eluted RNA at 4°C or -20°C until use. For long-term storage, keep at -70°C.

Table 1. Volumes of Buffer AVL and carrier RNA–Buffer AVE mix required for the QIAamp Viral RNA Mini procedure

No. samples	Buffer AVL (ml)	Carrier RNA–AVE (µl)	No. samples	Buffer AVL (ml)	Carrier RNA–AVE (µl)
1	0.56	5.6	13	7.28	72.8
2	1.12	11.2	14	7.84	78.4
3	1.68	16.8	15	8.4	84.0
4	2.24	22.4	16	8.96	89.6
5	2.80	28.0	17	9.52	95.2
6	3.36	33.6	18	10.08	100.8
7	3.92	39.2	19	10.64	106.4
8	4.48	44.8	20	11.20	112.0
9	5.04	50.4	21	11.76	117.6
10	5.60	56.0	22	12.32	123.2
11	6.16	61.6	23	12.88	128.8
12	6.72	67.2	24	13.44	134.4

c. Results

The resulting RNA is then used as necessary in further applications.

4.1.3.2 Nested RT-PCR

The reverse transcriptase (RT) polymerase chain reaction (PCR) is used to amplify a certain fragment of the virus genome (viral RNA). This protocol outlines the steps involved in the synthesis of *lyssaviruses* complementary DNA (cDNA) from viral RNA using the reverse transcriptase and amplification of viral cDNA for both diagnostic and research purposes.

a. Materials

Primer pairs

N127 (First round PCR) 5'-ATG TAA CNC CTC TAC AAT GG-3'

N829 (First round PCR) 5'-GCC CTG GTT CGA ACA TTC T-3'

RVN371F (Second round PCR) 5'-ACA ATG GAK KCT GAC AAR ATT G-3'

RVN371R (Second round PCR) 5'-CCT GYY WGA GCC CAG TTV CCY TC-3'

Chemicals and reagents

RNase free H_2O, dNTPs, Random Primer (6nt) (50pmol/μL), Oligo$(dT)_{15}$ (50pmol/μL), M-MuLV reverse transcriptase (200 U/μL), 10×M-MuLV buffer, RNase inhibitor (40 U/μL) (TAKARA), PCR Master mix (TIANGEN Biotech Co. Ltd.).

All PCR reagents are stored at –20°C.

Equipment/consumables

Pipettes (10-1000μL), vortex mixer, microcentrifuge, PCR system, microwave oven, gel mould, conical flask (250ml), gel electrophoresis tank/power pack, Gel Doc-IT imaging system, measuring cylinder

b. Procedure

i. Reverse Transcription of Rabies Viral RNA

1. Remove RT reagents from the freezer, keep them on ice, thaw and vortex them before use.

2. Prepare 12 μL RT reaction mix in a 0.2 mL PCR tube according to the reagents in table. Allow for pipetting variation by preparing a volume of master mix at least one reaction greater size than required.

Reagent	Volume Per Reaction (μL)
dNTPs (2.5 mM)	4
Random Primer (50 μM)	1.5
Oligo(dT) $_{15}$ (50 μM)	0.5
M-MuLV buffer (5×)	4
M-MuLV reverse transcriptase (200 IU/μL)	1
RNasin (40 IU/μL)	1
Total volume	12

3. Add 8 μL sample, positive control RNA or negative control to the RT reaction mix within a PCR workstation in template room. The RT positive control is RNA extracted from the cell culture infected with fixed RABV strain CVS-11 (Challenge Virus Standard-11) and stored at -80 ℃. The negative control contains RNase-free ddH2O.

4. Mix the contents of the RT tubes by vortexing, then centrifuge briefly.

5. Load the reaction tubes into a thermal cycler. Set up the cDNA synthesis program with the following conditions: 42 ℃ for 90 min, 95 ℃ for 5 min and 4 ℃ on hold. Set reaction volume to 20 μL. Start the RT run.

ii. First round PCR

1. Keep PCR reagents in table on ice in a clean room until use, then thaw and vortex them.

2. Prepare the first round PCR mix in a 0.2 mL PCR tube.

Reagent	Volume Per Reaction (μL)
PCR master mix	12.5
N127(20pmol/μL)	1
N829(20pmol/μL)	1
dd H_2O	9.5
Total	24

3. Add 1 µL sample cDNA into the first round PCR mix within a PCR workstation in template room. The PCR positive control is CVS-11 cDNA prepared in the above RT method. The PCR negative control is ddH2O.

4. Transfer the sealed tubes to a PCR thermal cycler and cycle using the parameters as follows.

94°C for 2 min	1 Cycle
94°C for 30sec	35 Cycles
56°C for 30sec	
72°C for 40sec	
72°C for 10 min	1 Cycle
Hold at 4°C	

iii. Second round PCR

1. Prepare the second round PCR mix in a 0.2 mL PCR tube using the reagents in table.

Reagent	Volume Per Reaction (µL)
PCR master mix	12.5
RVN371F(20 pmol/µL)	1
RVN371R(20 pmol/µL)	1
dd H_2O	9.5
Total	24

2. Add 1 µL first round PCR product into the second round PCR mix. In addition include ddH2O as a negative control of the second round PCR.

   Perform the PCR thermal cycler using the same parameters as the first round PCR.

iv. Gel Electrophoresis

1. Prepare a 1.5 % agarose gel by adding 1.5 g agarose into 100 mL Tris-acetate-EDTA (TAE) and dissolving thoroughly by heating in a microwave oven.

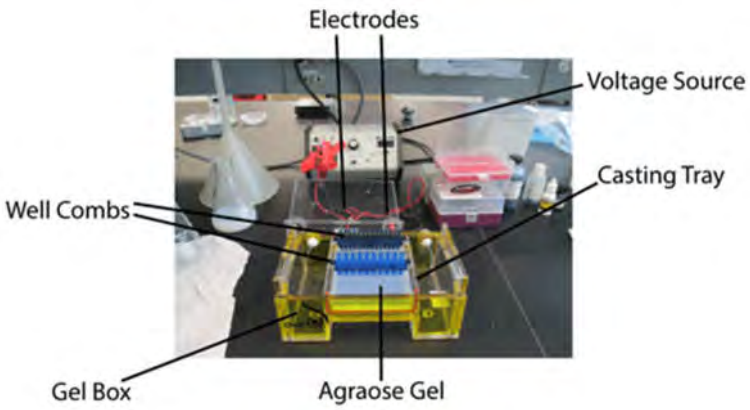
2. Add ethidium bromide (EB) (final concentration 0.01 %) or other commercial EB substitution. Pour gel into the mould and leave the gel to solidify at ambient temperature for at least 30 min.

3. Prepare the loading samples by mixing 5 µL of each PCR product with 1 µL 6 × loading buffer.

4. Load the samples and suitable DNA marker separately into the wells, and run the gel for approximately 30 - 45 min at 120 V until the dye line is approximately 75 - 80 % down the gel.

5. Turn off power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.

6. Use a UV gel documenting device to visualize and photograph the DNA fragments.



c. Results

A positive PCR result is observed in the form of a bright band; the size of the PCR product obtained can be confirmed by comparing its position against the bands of the marker, which are of known size (refer to data sheet provided with the marker for size of bands). The positive controls should also produce bands, whereas the negative controls should produce no band.

Let take the image blow for example, the marker provides the size of bands from 100bp to 2000bp. The first round PCR yields 845bp amplicons and the second round PCR produces 371bp amplicons.

It is a good practice to check if positive and negative controls are correct before making a judgment on the sample(s).

In the example above, for the first round PCR, the negative control (-ve) should produce no band while the positive control (+ve) obtains a bright band (between 750bp to 1000bp). The PCR result shows that sample 1 is negative and sample 2 is positive.

For the second round PCR, the negative control (-ve) should produce no band; the positive control (+ve) obtains a bright band (between 250bp to 500bp). So sample 5 is negative and sample 6 is positive.

Typically, if the first round PCR shows a positive, the second round PCR is usually positive. Then we confirm the sample as positive.

If the first round PCR doesn't show a positive but the second round PCR is positive, we can also confirm the sample is positive.

4.1.3.3 One-step Real-time qPCR

Rabies virus and rabies-related viruses are members of the *Rabdoviridae* family, genus *Lyssavirus*. The nucleic acid sequence of N protein (part of which is the target for this assay) displays a high degree of homology between species and is therefore, an excellent target for detection of the virus in field samples.

The reverse transcription (RT)-polymerase chain reaction (PCR) is used to amplify the N gene of the virus genome (viral RNA). Real-time PCR has been developed to increase sensitivity and to obtain results faster.

With this procedure, rabies virus RNA can be enzymatically amplified as DNA copies. Rabies RNA can be copied into a DNA molecule using reverse transcriptase (RT). The DNA copy of rabies can then be amplified using polymerase chain reaction (PCR) and the amount of DNA formed after each cycle detected with fluorescently-tagged oligonucleotide probes. This technique can confirm FAT results and can detect rabies virus in tissue samples, such as brain tissue, saliva, CSF (Cerebrospinal Fluid) and suspension of cell culture.

Standardisation and very stringent quality control are required. As PCR can produce false positive or false negative results it should only be used in combination with other conventional techniques.

The protocol has been validated to detect rabies viral RNA for genotype I.

a. Materials

Primer pairs and fluorescently-tagged oligonucleotide probes

RVFQ For: 5’-ATGTAACACCYCTACAATG-3’

RVFQ Rev: 5’-GCAGGG TAYTTRTACTCATA-3’

RVFQ Probe: FAM-5’-ACAAGATTGTATTCAAAGTCAATAATCAG-3’-TAMRA

Chemicals and reagents

dNTPs(2.5mM), $MgCl_2$(25mM), Ex-Taq (5U/µL), Triton X-100(10%v/v), RNase Inhibitor (40U/µL), Reverse Transcriptase M-MLV (200 U/µL) (TAKARA company)

Equipment/consumables

Pipettes (10-1000μL)

Vortex mixer

Microcentrifuge

Real-time PCR system

b. Procedure

i. Preparation of Real-time PCR master mix

1. Calculate the total number of reactions (including positive and negative controls).
2. Prepare a master mix containing the following reagents. (**Note**: all enzymes are handled on ice)

Reagent	**Volume Per Reaction (μL)**
RNase-free dH_2O	16.15
dNTPs(2.5mM)	0.5
10×Taq buffer (Mg^{2+} free)	5
$MgCl_2$(25mM)	12
Triton X-100(10%v/v)	1
RNase Inhibitor (40U/μL)	0.25
Ex-Taq (5U/μL)	0.5
Reverse Transcriptase M-MLV (200 U/μL)	0.6
RVFQ For Primer (20 pmol/μL)	2
RVFQ Rev Primer (20 pmol/μL)	1
Probe	1
Total volume	40

3. Cap the tube and invert or vortex it several times to mix the reaction components.
4. Centrifuge the tube briefly to remove the bubble.

ii. Load the tubes

1. Transfer 40 µL of PCR reaction mix into PCR tubes. Add 10 µL RNA for each tube.

2. Seal the plate with the appropriate lids.

3. Label (avoid label on the lid) the tubes clearly with date and sample identification. One of the samples will be positive mouse brain RNA (positive control) which was extracted at the same time as the test samples.

4. Centrifuge the plate briefly.

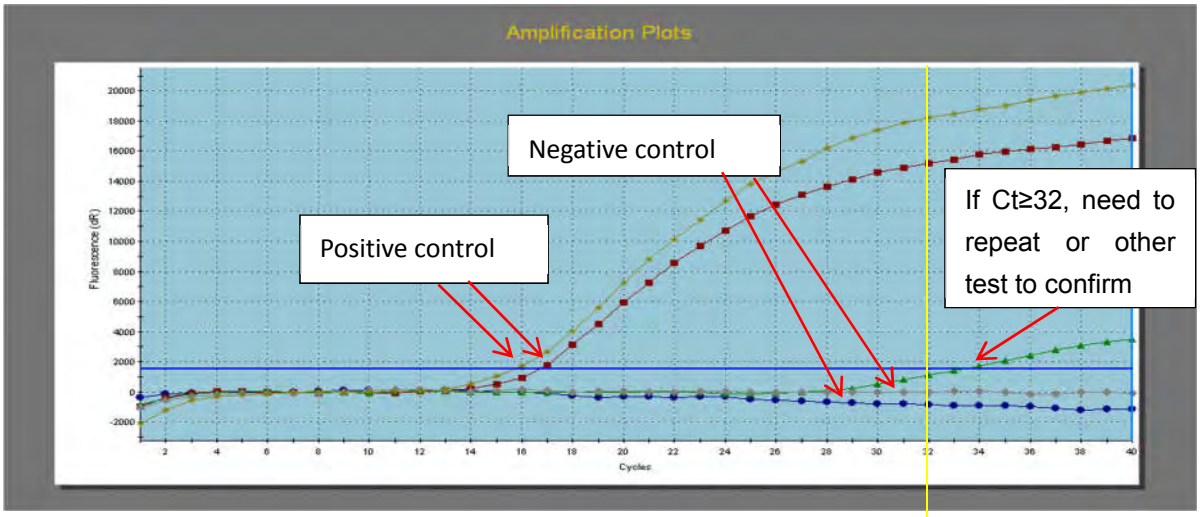
5. Load the plate into the instrument.

iii. Incubate the reactions using the following thermocycling profile

42°C for 30 min	1 cycle
92°C for 2 min	1 cycle
92°C for 30sec	40 cycles
55°C for 30sec	
72°C for 20sec **Collect the fluorescence**	
Hold at 4°C	

b. Results

For each FQ-PCR, a critical threshold cycle number (Ct) is determined corresponding to the PCR cycle number at which the fluorescence of the reaction exceeds a value determined to be statistically higher than background by software associated with the PCR system.



Reading results from the figure above shows:

Positive control: Ct<32,

Negative control: no Ct,

then the PCR works well and there is no contamination in the process of preparation of PCR master mix and template addition.

For reading the samples： 0＜Ct＜32 shows the sample is positive;

no Ct shows the sample is negative;

if 32≤Ct≤40, further confirmation tests are needed as there might be contamination.

4.2 Serological tests

The main application of serology for rabies is to determine responses to vaccination in domestic animals, particularly in connection with international travel, or for monitoring mass vaccination campaigns in dogs and other wildlife reservoir species. The measurement of rabies antibodies has typically involved virus neutralisation (VN) tests to detect rabies virus neutralising antibodies. ELISAs are now also recognised as acceptable tests to detect binding antibodies. Depending on the nature of the ELISA, there can be variable sensitivity and specificity. However, ELISAs are currently not applicable to international movement of animals or trade.

BioPro Rabies ELISA Ab Kit

Blocking ELISA for detection of Rabies virus antibodies in serum or plasma

480 reactions

Cat. No.: RAB01-05
Version: Rabies 1.1c

Instruction for use.

Manufacturer

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INTRODUCTION

Rabies is fatal viral disease occurring in humans and animals. Disease is caused by highly neurotropic Rabies virus. Virus is mostly transmitted through close contact with infected saliva of rabid animals via bites or scratches and then through neural path to the CNS causing encephalopathy and ultimately death.
Domesticated and wild carnivores belong to most infected animals. For eradication of Rabies in wild carnivores oral immunisation in many country is used. Estimation of seroprevalence in vaccinated population is one of the methods for evaluation of oral immunisation effectiveness.

ELISA Rabies kit was developed for detection of antibodies against Rabies in domesticated and wild carnivores and was validated with fox and dog serum samples. We recommend use this ELISA for detection of antibodies against Rabies in foxes. This method is rapid and simple and in comparison with "golden standard" methods like FAVN or RFFIT is more convenient because very often quality of fox serum samples may be decreased by bacterial contamination or autolysis.

PRINCIPLE OF TEST

The wells of microplates are coated with Rabies antigen. Diluted samples are incubated in the wells. After washing biotinylated anti-Rabies antibody is added to wells. If investigated sample contain specific anti-Rabies antibodies, these block binding of biotinylated anti-Rabies antibodies with coated Rabies antigen. If investigated sample do not contain specific anti-Rabies antibodies, biotinylated anti-Rabies antibodies form antigen-biotinylated antibody complex. After washing Streptavidin peroxidase conjugate is added to wells. Streptavidin peroxidase will bind to antigen-biotinylated antibody complex. After another washing step substrate solution (TMB) is added to wells, forming blue compound becoming yellow after stopping reaction. Intensity of colour is read at 450 nm and decrease of the intensity compared against negative control is proportional to amount of blocking antibodies in investigated sample.

- Use new disposable tips for every investigated sample.
- Do not use reagents after expiration date and do not mix reagents from different lots.
- Stop solution contains acid (0,5M H_2SO_4), that can cause serious burns. In case of contact with skin and eyes, wash immediately with plenty of water and seek medical advice.
- Distilled or deionised water of good quality should be used for preparation of washing solution.
- Avoid foam and bubble formation by careful pipetting and washing.
- During the test procedure plate must never become dry. If the test procedure is discontinued for any reasons leave the wells of the plate filled with wash solution.
- Protect TMB Substrate from direct light and oxidising agents. Use only clean laboratory glass or plastic with TMB Substrate. Do not use TMB substrate showing blue colour before dispensing to the wells.
- Before discarding all used materials and disposables should be decontaminated either by immersion for at least 1 hour in freshly prepared 5% sodium hypochloride, or by autoclaving at 121°C for 30±1 minutes minimum.

PREPARATION OF REAGENTS

Preparation of microplates

Microplates should be allowed to come to room temperature (18 - 25°C) before use. Opened unused strips may be stored in closed plastic bag with desiccant bag at 2 - 8°C in the dark no more than one month.

Wash solution

Concentrated wash solution (10x) should be brought to room temperature before use. May form crystals at 2 - 8°C, which will disappear at the room temperature (18 - 25°C). If they do not disappear, solution should be left at the room temperature for longer time or it is possible to warm solution to 37±2 °C and gently shake till all the crystals will be dissolved.

To prepare wash solution mix 1 volume of concentrated wash solution (10x) with 9 volumes of distilled or deionised water (e.g. 50 ml of concentrated wash solution (10x) add to 450 ml of distilled or deionised water).

Biotinylated anti-Rabies antibody

Dilute concentrated biotinylated anti-Rabies antibody to 1/100 in Diluent for biotinylated antibody. (e.g. quantity needed for one plate is represented with mix of 110 µl concentrated biotinylated anti-Rabies antibody and 11 ml Diluent for biotinylated antibody).

Before preparation of working dilution, concentrated biotinylated anti-Rabies antibody must to be vortexed.

Prepared working dilution of antibody has to be used within 8 hours!

Streptavidin peroxidase conjugate

Dilute concentrated Streptavidin peroxidase conjugate to 1/100 in Diluent for Streptavidin peroxidase conjugate (e.g. quantity needed for one plate is represented with mix of 110 µl concentrated biotinylated Streptavidin peroxidase conjugate and 11 ml Diluent for Streptavidin peroxidase conjugate).

Before preparation of working dilution, concentrated Streptavidin peroxidase conjugate must to be vortexed.

Prepared working dilution of Streptavidin peroxidase conjugate has to be used within 8 hours!

PREPARATION OF SAMPLES

Investigated serum samples need to be diluted to 1/2 in Sample diluent (e.g. 60µl + 60µl) in a dummy plate or in microtubes. Positive control serum, negative control serum and control sera needs to be diluted in the same manner as serum samples.

Note:

1. As a dummy plate any low protein binding microplate can be used.
2. It is also possible dilute controls and serum samples directly in plate. Dispense 50µl of Sample diluent per well and then dispense 50 µl of positive control serum, negative control serum and control sera into appropriated wells. Then dispense 50µl of serum samples to the remaining wells.

TEST PROCEDURE

1. Incubation of investigated and control sera

Note: All the control sera have to be vortexed before dispensing.

1.1 Dispense 100µl of diluted positive control serum to A1 and B1 wells.
1.2 Dispense 100µl of diluted negative control serum to A2 and B2 wells.
1.3 Dispense 100µl of diluted control serum 1 to well C1.
1.4 Dispense 100µl of diluted control serum 2 to well D1.
1.5 Dispense 100µl of diluted control serum 3 to well E1.
1.6 Dispense 100µl of diluted serum samples to the remaining wells.
1.7 Cover the plate with adhesive foil and incubate overnight (18 - 24 hours) at 2 - 8°C with gently shaking on orbital shaker.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC	NC	10									
B	PC	NC	11									
C	CS1	4	12									
D	CS2	5	13									
E	CS3	6	…									
F	1	7										
G	2	8										
H	3	9										

2. Incubation with biotinylated anti-Rabies antibody

2.1 Remove foil from the plate, empty the content of the plate and wash 6 times with washing solution. Tap the plate firmly on the absorbent paper after last washing step.
2.2 Dispense diluted biotinylated anti-Rabies antibody 100µl per well.
2.3 Cover the plate with adhesive foil and incubate 30±1 minutes at 37± 2°C with gently shaking on orbital shaker.

3. Incubation with Streptavidin peroxidase conjugate

3.1 Remove foil from the plate, empty the content of the plate and wash 4 times with washing solution. Tap the plate firmly on the absorbent paper after last washing step.
3.2 Dispense diluted Streptavidin peroxidase conjugate 100µl per well.
3.3 Cover the plate with adhesive foil and incubate 30 minutes at 37± 2°C with gently shaking on orbital shaker.

4. Incubation with TMB Substrate

4.1 Remove foil from the plate, empty the content of the plate and wash 4 times with washing solution. Tap the plate firmly on the absorbent paper after last washing step.
4.2 Dispense 100µl of "ready to use" TMB Substrate per well.
4.3 Incubate the plate 15-30 minutes at room temperature (18 - 25°C) with gently shaking on orbital shaker, away from direct sunlight.

Note: In the case that shaking with orbital shaker during incubations is not available and all incubations were done without shaking, we propose to incubate the plate with TMB Substrate longer than 20 minutes.

5. Stopping the reaction

5.1 Dispense 50µl of Stop Solution per well

6. Reading

6.1 Read optical density (OD) at 450nm.

VALIDATION CRITERIA

- The OD of the negative control serum must be higher than 1,0.
- Difference between means of OD of negative and positive control serum must be equal or higher than 0,8.

If validation criteria are not met, test results of that specific test plate are invalid and samples have to be retested.

PANEL OF CONTROL SERA:

Should be used as a help for user to assure that test is working in optimal conditions. If the criteria mentioned bellow are not achievable, please contact producer.
Percentage of blocking for control serum 1 should be between 45% and 70%.
Percentage of blocking for control serum 2 should be between 25% and 45%.
Percentage of blocking for control serum 3 should be lover than 30%.

INTERPRETATION:

Calculate percentage of blocking (PB) for each sample:

$$PB\% = \frac{OD_{NC} - OD_{sample}}{OD_{NC} - OD_{PC}} \times 100$$

- Serum sample with **PB lower than 40%** is considered as negative for Rabies antibodies.
- Serum sample with **PB equal or higher than 40%** is considered as positive for Rabies antibodies.
- Serum sample with **PB equal or higher than 70%** is considered as serum sample with antibody level equal or higher than 0,5 IU/ml based on FAVN test.

Note: For the purpose of evaluation of oral immunisation effectiveness we recommend to use first positive cut off (PB equal or higher than 40%). To use second positive cut off (PB equal or higher than 70%) for interpretation of results with fox sera is not recommended due to the most fox serum samples are in fact body fluids with unknown dilution factor, which make almost impossible to quantify the exact level of protection.

SCHEME OF TEST PROCEDURE

1. Dilute control sera and serum samples.
1.1 Dispense 100µl of diluted positive control serum to A1 and B1 wells.
1.2 Dispense 100µl of diluted negative control serum to A2 and B2 wells.
1.3 Dispense 100µl of diluted panel of control serum to wells A3-A5.
1.4 Dispense 100µl of diluted serum samples to remaining wells.
1.5 Incubate overnight at 2 - 8 °C, shake gently.
2. Prepare biotinylated anti-Rabies antibody.
2.1 Wash plates 6x time.
2.2 Dispense 100µl of biotinylated anti-Rabies antibody diluted to 1/100 to all wells.
2.3 Incubate 30 minutes at 37± 2°C, shake gently.
3. Prepare streptavidin peroxidase conjugate.
3.1 Wash plates 4x time.
3.2 Dispense 100µl of streptavidin peroxidase conjugate diluted to 1/100 to all wells.
3.3 Incubate 30 minutes at 37± 2°C, shake gently.
4.1 Wash plates 4x time.
4.2 Dispense 100 µl of TMB Substrate to all wells.
4.3 Incubate 15-30 minutes at room temperature (18 - 25°C), shake gently.
5.1 Stop reaction with 50 µl of Stop solution.
6.1 Read absorbance (OD) at 450 nm.
7.1 Validation criteria.
7.2 Interpretation of result.

APPENDIX 1 to BioPro Rabies ELISA Ab kit – Instruction for use,v 1.1
Date: 10.12.2013

Based on user inputs we have decided to add following information to Instruction for use of our BioPro Rabies ELISA Ab kit:

1. INSUFICIENT SAMPLE VOLUME

If there is not enough sera (less than 50µl) dilute such serum sample with PBS to achieve sample volume of 50µl (for example if you have 30 µl of serum add 20 µl of PBS). Mark such sample as diluted and follow standard test procedure, step 1.

2. OBTAINING SAMPLE FROM A BLOOD CLOT

Alternatively the sample can be obtained from a blood clot using following procedure:

2.1. FRESH BLOOD CLOT

- Cut the **fresh blood clot** (obtained from heart or major blood vessels) and transfer to 10-12 ml tube.
- Close the tube and leave at room temperature (18 25°C) or in incubator heated to 37±2°C for 2-3 hours.
- Transfer to refrigerator (4-8°C) for 12 hours ±30 minutes (overnight).
- Centrifuge the liquid part from blood clot at 5-10.000 rpm for 15±10mins and then test obtained liquid as serum following standard procedure, step 1.

2.2. DRIED BLOOD CLOT

- Cut the **dried blood clot**, put into 10-12ml tube, add PBS with antibiotics (PNC G 100000 U/l, STM 100mg/l, NEO 50 mg/l) maximally in 1:1 ratio, mark as diluted sample and follow according to point 2.1. of this appendix.

Contact

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5. Management of biological safety in the laboratory

Lyssaviruses are Group 3 pathogens i.e. “organisms that may cause severe human disease and present a serious hazard to laboratory workers; they may present a risk of spread in the community but there is usually effective prophylaxis or treatment available”.

Human deaths have resulted from infection with each of the *lyssavirus* species except Lagos bat virus, Irkut, Khujand, Aravan and the putative Shimoni; however even these viruses are known to cause an infection in other animals which is clinically indistinguishable from rabies. Therefore, it must be assumed to be capable of causing fatal encephalitis in humans.

The risk of acquiring a rabies virus infection in the laboratory is related to the techniques undertaken. Rabies diagnosis in field specimens may present a greater risk than the production of fixed strains of rabies on well-defined substrates, because field strains may have undetermined characteristics and also because such specimens may carry other unknown pathogens.

Every laboratory must develop its own working routine when dealing with pathogens, but there are some general principles that are summarised here. These risk limiting measures include:

5.1 Maintenance of integrity in "safe" and "high-risk" working areas

High-risk areas, which may comprise only the necropsy room or include different rooms where rabies diagnosis is performed, must be clearly separated from safe areas.

Access to the high-risk areas of the laboratory should be restricted only to those personnel who are vaccinated for rabies and who are familiar with the laboratory’s safety protocols.

Staff should not enter a high-risk area without wearing protective clothing. This protective clothing must not be worn in a “safe” area. Clothing from a contaminated area must be decontaminated (e.g. by autoclaving) before removal for laundry. One method of ensuring that security is not breached is to wear colour-coded laboratory clothing, i.e. white clothing for safe areas and a coloured tag for the high-risk area.

Contaminated paperwork associated with a rabies specimen may represent a hazard to unvaccinated staff. If packages are to be opened in the post-mortem room, registration documents should be completed and removed from the room before the commencement of necropsies.

Apparatus (tabletops, vice, socket forceps, scissors, forceps and grinder, etc.) used in the post-mortem room must be disinfected and cleaned between specimens. A sterile set of instruments should be used for each specimen and contaminated instruments should be disinfected by boiling, autoclaving or adapted chemical treatment. All contaminated surfaces and instruments should be washed and disinfected after every diagnostic session and the floor, walls and other structures of the post-mortem room should be disinfected and washed down daily (or routinely). Potentially infectious material such as brain smears should be fixed before removal from the post-mortem room, but it should be remembered that acetone fixation does not totally inactivate rabies virus.

5.2 Immunisation of staff

WHO recommends the preventive pre-exposure immunisation of staff that work with live rabies virus.

The immunisation protocol includes three injections at days 0, 7 and 28 and a booster injection one year later and then every 3 years in the absence of serological follow-up. Serological confirmation of protection should be carried out 10 days or more after the last injection. The subsequent testing of antibody level is recommended annually and staff whose antibody level declines below 1.0 IU/ml should be given a booster vaccination.

5.3 Physical protection from biohazardous agents

Good laboratory practice is the first line of defence against accidental infection.

Wearing of protective clothing, consisting of gowns, aprons, gloves, visors, masks, goggles and boots will protect the operator's skin and mucous membranes against splashing and spillage. Operation processed in the biosafety cabinet is recommended when dealing with tissue samples without opening the skull. All protective clothing is removed when leaving the rabies laboratory and stored near the exit.

Contaminated documents that contain information requiring transmission to other areas should be photocopied in a plastic envelope or decontaminated (for instance with ultra-violet light).

Storage and consumption of food or drink, smoking and applying cosmetics should be prohibited in the laboratories.

6. Disposal of biohazardous waste

Rabies virus is easily inactivated by heat or chemical treatment.

5% NaOH are some of the most commonly employed disinfectants.

Diagnostic operations produce waste that is infected or contaminated with rabies virus. Safe disposal of these wastes is an important aspect of laboratory management. Although incineration is the most effective means of destroying animal and biohazard waste, it requires a lot of energy. Waste can be autoclaved at 121℃ for 30 min before it is removed from the laboratory. An alternative is to bury this waste in quicklime in a pit inaccessible to the public and to scavengers, in compliance with local legislation.