Detection of Lyssavirus RNA by CONVENTIONAL hnRT-PCR

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CONTENTS

FOREWORD

This protocol has been validated by:

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INTRODUCTION

This Standard Operating Procedure (SOP) describes the detection of lyssavirus RNA by conventional hnRT-PCR for diagnostic purpose that fulfills the requirements of the ISO/IEC 17025 standard and the AFNOR NF U47-600 standard.

This protocol should not be considered transferable as it stands. It shall be adapted by each user for its working conditions and shall be validated with reference materials or samples of known status (as performed in the frame of rabies diagnosis proficiency testing by example).

The assay validation should meet the OIE Standard for validation (Chapter 1.1.6. "Principles and methods of validation of diagnostic assays for infectious diseases") [1] and should be able to detect a broad spectrum of globally circulating rabies virus strain (Chapter 3.1.17: "Rabies infection with rabies virus and other lyssaviruses") [2].

All commercial references described in this SOP are given for information. Other equivalent reagents or equipment could be used as far as it does not affect the results.

1. PURPOSE AND SCOPE

This test describes the generic detection of lyssavirus RNA for diagnostic purpose.
The test is based on a conventional (gel-based) hemi-nested RT-PCR assay, pan-Lyssavirus primers [3] and allows the detection of all ICTV recognized lyssavirus species.
The SOP has been fully validated following the NF U47-600 standard (www.afnor.fr) [4].

2. REFERENCE DOCUMENTS

1. OIE. Chapter 1.1.6. Principles and Methods of validation of diagnostic assays for infectious diseases. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Paris, France;World Organisation for Animal Health.
2. OIE. Chapter 3.1.17. Rabies (Infection with rabies virus and other Lyssaviruses). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019 Paris, France;World Organisation for Animal Health.
3. Heaton, P.R., Johnstone, P., McElhinney, L.M., Cowley, R., O'Sullivan, E. and Whitby, J.E., 1997. Heminested PCR assay for detection of six genotypes of rabies and rabies-related viruses. J Clin Microbiol 35, 2762-6.
4. AFNOR, 2015. "Méthodes d'analyses en santé animale PCR (Réaction de polymérisation en chaîne)-Partie 2: exigences et recommandations pour le développement et la validation de la PCR en santé animale, XP U 47-600-2. Normes & Recueils.
5. Robardet Robardet E, Picard-Meyer E, Andrieu S, Servat A, Cliquet F. International interlaboratory trials on rabies diagnosis: an overview of results and variation in reference diagnosis techniques (fluorescent antibody test, rabies tissue culture infection test, mouse inoculation test) and molecular biology techniques. J Virol Methods 2011 Oct;177(1):15-25.
6. Wakeley PR, Johnson N, McElhinney LM, Marston D, Sawyer J, Fooks AR. Development of a real-time, TaqMan reverse transcription-PCR assay for detection and differentiation of lyssavirus genotypes 1, 5, and 6. J Clin Microbiol. 2005 Jun;43(6):2786-92.
7. Darryl L. Davis, Edward P. O'Brie, and Catherine M. Bentzley. Analysis of the degradation of oligonucleotide strands during the freezing/thawing processes using MALDI-MS. Anal Chem 2000 Oct 15;72(20):5092-6.

3. TERMS, ABBREVIATIONS AND DEFINITIONS

bp	base pair
hnRT-PCR	hemi-nested reverse transcriptase polymerase chain reaction
ICTV	International Committee on Taxonomy of Viruses
N	Nucleoprotein gene
nPCR	nested PCR
RABV	Classical rabies virus
RNA	Ribonucleic Acid

4. PRINCIPLE OF THE METHOD

The test described herein is based on a **one-step RT-PCR approach** in order to reduce the risks of contamination during manipulation and opening of tubes [5]. The two enzymatic reactions (the reverse transcription of the RNA into complementary cDNA and PCR amplification) are performed successively in the same tube. **A second round amplification** (hemi nested [hn] PCR) is performed to increase the sensitivity of the method.

The hnRT-PCR targets the Nucleoprotein (N) gene and is performed with pan-Lyssavirus primers JW12 (forward) and JW6 (reverse) previously described by Heaton et al. [3] than detect a 606 base pair (bp) region of the N gene from all ICTV recognized lyssavirus species. The reverse primer JW10 which lies within the sequence of the first round 606 bp RT-PCR products are used in combination with the primer JW12 in the second round of the hnRT-PCR giving PCR products of 589 bp.

A parallel assay is carried out for each tested sample to detect in the extracted tissular RNA fraction of the endogenous β-actin gene (153 bp) in order to **control the proper achievement of the RNA extraction step and the absence of PCR inhibitors.** It is performed with multi species β-actin primers, β-actin intronic (forward) and β-actin rev (reverse) previously described by Wakeley et al. [6].

Primers (pan-Lyssavirus and β-actin) are described in the OIE Chapter 3.1.17. [2].

5. REAGENTS[1]

- RT-PCR kit: Qiagen One step RT-PCR kit (Qiagen, France)
- Nested PCR: Platinium Taq polymerase and its associated buffer (Invitrogen, France)
- RNAse Out 40 U/µl
- dNTP 100mM
- Molecular grade water DNAse-RNase free
- Electrophoresis: Loading dye buffer, agarose, nucleic acid gel staining

[1] All commercial references described in this document are given for information. Other equivalent reagents or equipment can be used. Each user must validate its protocol in its working conditions (reagents, equipment).

6. EQUIPMENT AND MATERIALS

6.1. EQUIPMENT

- Dedicated and separated laboratory rooms or workstations for the preparation of primers/master mix, extraction of RNA, PCR amplification and post PCR
- PCR workstations with specific dedicated micropipettes
- Spin micro-centrifuges, refrigerated centrifuges
- Vortex
- Electrophoresis apparatus
- Conventional thermocycler

6.2. CONSUMABLES

- DNAse-RNase free, sterile and single use plastic consumables: microtubes, filter tips, …
- Single use gloves

6.3. PRIMERS

The molecular models used for the detection of **Lyssavirus and Beta actin genes** are described in the Table below:

Molecular models used:

Primers	Function	Sequences (5'-3')	Localisation	Usage	Target
JW12	Primer forward	ATG TAA CAC CYC TAC AAT G	55-73	RT-PCR and nPCR	Lyssavirus
JW6.1	Primer reverse	CAATTCGCACACATTTTGTG	660-641	RT-PCR	Lyssavirus
JW6.2		CAGTTGGCACACATCTTGTG			
JW6.3		CAGTTAGCGCACATCTTATG			
JW10.1	Primer reverse	GTC ATC AAA GTG TGR TGC TC	636-617	nPCR	Lyssavirus
JW10.2		GTC ATC AAT GTG TGR TGT TC			
JW10.3		GTC ATT AGA GTA TGG TGT TC			
β-actine_int	Primer forward	CGA TGA AGA TCA ART CAT TGC	1051–1072	RT-PCR	Beta actin
β-actine_rev	Primer reverse	AAA GCA TTT GCG GTG GAC	1204–1188		

All the masters stocks are at 100µM, aliquoted and stored at < -16°C. Working primers are at 20µM, aliquoted and stored at < -16°C.

Repeated freeze/thaw cycles should be avoided [7]. In the EURL, the number of freezing / thawing is limited to 5 times in order to preserve the characteristics of primers.

7. PROCEDURE

Follow the usual recommendations to avoid any contamination.

7.1. PREPARATION OF RNA SAMPLES

Extract viral RNA from suspected specimens with the chosen relevant method ensuring quality of the nucleic acids extracted.

There is a number of specialised methods using commercial columns-based extraction kits or magnetic bead-based extraction kits available either as manual or automated systems for robotic workstations.

For each run, include negative and positive controls as follows:

- A **negative extraction control** (as Phosphate Buffer Saline Solution or clarified brain suspension tested negative for the target[2] as naïve mouse or birds) extracted at the same time as samples, which allows to check the presence of possible contamination during the extraction steps and which ensures the absence of inter-sample contamination ;
- A **positive extraction control** = well-characterised sample that contains target RNA, which confirms the correct extraction of RNA.

The assay validated by the EURL is based on the manual extraction method with commercial based columns extracted kits (QIAMP viral RNA mini kit, Qiagen, France).

Viral RNA is extracted from 140µL of supernatant of a brain homogenate and eluted in a final volume of 60µL. The extraction step is performed according to the manufacturer's instructions.

[2] RNA extracted from a clarified suspension tested negative for the Lyssavirus can be used as the same time as a negative control for RT-PCR Lyssavirus and as a positive control for the RT-PCR Beta actin.

7.2. RT-PCR

7.2.1. PREPARATION OF THE MASTER MIX[3]

1. Thaw all frozen reagents (i.e. Qiagen One step RT-PCR, primers, molecular grade water, dNTPs). Mix the individual solutions and place them on ice until the addition of the enzyme mix.
2. Prepare the master mix as follows:

RT-PCR LYSSAVIRUS :

Master mix	Final concentration	Volume (in µL) for 1 tube
Molecular grade water		4.25
Qiagen One step RT-PCR (5X)	1X	3
dNTPs (10mM)	0.4mM	0.6
JW12 (20µM)	0.7	0.525
JW6 (20µM of each primer)	2	0.525
Qiagen RT-PCR Ez mix		0.6
RNAse Inibitor (40 U/µl)	20 U	0.5

ENDOGENOUS GENE (RT-PCR BETA-ACTIN):

Master mix	Final concentration	Volume (in µL) for 1 tube
Molecular grade water		4.1
Qiagen One step RT-PCR (5X)	1X	3
dNTPs (10mM)	0.4mM	0.6
β-actine_int (20µM)	0.8	0.6
β-actine_rev (20µM)	0.8	0.6
Qiagen RT-PCR Ez mix		0.6
RNAse Inibitor (40 U/µl)	20 U	0.5

3. Mix the reaction thoroughly.
4. Dispense a volume of 10µL of master mix into PCR tubes.

[3] There are numerous available commercially reagents dedicated for RT-PCR and PCR. Optimisation of PCR (master mix formulation and cycling parameters) should be carried out by each user according to the enzyme mix used and the thermocycler used under his own working conditions.

7.2.2. ADDITION OF TEMPLATE

5. Add 5µL of RNA samples/control RNA(s) to the individual PCR tubes containing the master mix and close the tubes.
6. Transfer the PCR tubes to the thermocycler for thermal cycling.

For each run, include positive and negative controls as follows:

- A **PCR negative control** = ultrapure water instead of sample to validate the no-contamination of the PCR.
- A **PCR positive control** = known target RNA previously extracted and included to ensure the good running of PCR.
- This control must be calibrated around the limit of detection of the PCR (LD PCR) to act as a low positive control.

7.2.3. SETTING UP OF THERMOCYCLER[4]

7. Load the samples into the machine.
8. Program the thermocycler as follows:

RT-PCR LYSSAVIRUS :

STEP	Temperature	Time	Number of cycles
Reverse transcription	50°C	30 min	1
Polymerase activation	95°C	15 min	1
Denaturation	94°C	30 sec	45x
Annealing	55°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	1

ENDOGENOUS GENE RT-PCR BETA-ACTIN :

STEP	Temperature	Time	Number of cycles
Reverse transcription	50°C	30 min	1
Polymerase activation	95°C	15 min	1
Denaturation	94°C	30 sec	45x
Annealing	50°C	30 sec	
Extension	72°C	1 min	

[4] The cycling parameters have been validated on the conventional thermocycler Eppendorf Mastercycler Gradient. Thermal profiles may be subject to optimization depending on used PCR equipment and kits.

Final extension	72°C	10 min	1

Storage: Lyssavirus RT-PCR products are used to implement nPCR. They are stored at 5±3°C if used during the day or at <-16±3°C for longer storage.
Beta actin RT-PCR products are stored at 5±3°C until electrophoresis.

7.3. NESTED PCR

7.3.1. PREPARATION OF THE MASTER MIX

9. Mix the individual solutions and place them on ice.
10. Prepare the master mix as follows:

Master mix : Lyssavirus	Final concentration	Volume (in µL) for 1 tube
Molecular grade water		14.45
PCR Buffer without $MgCl_2$ (10X)	1X	2
$MgCl_2$ (50mM)	1.5 mM	0.6
dNTPs (10mM)	0.1mM	0.2
JW12 (20µM)	0.25	0.25
JW10 (20µM of each primer)	0.75	0.25
Taq Platinium (5U/µl)	1.25U	0.25

11. Mix the reaction thoroughly.
12. Dispense a volume of 18µL of master mix into PCR tubes.

7.3.2. ADDITION OF TEMPLATE

13. Add 2µL of the RT-PCR Lyssavirus products to the individual PCR tubes containing the master mix and close the tubes.
14. Transfer the PCR tubes to the thermocycler for thermal cycling.

7.3.3. SETTING UP OF CONVENTIONAL THERMOCYCLER

15. Load the samples into the machine.
16. Program the thermocycler as follows:

STEP	Temperature	Time	Number of cycles
Polymerase activation	94°C	2 min	1
Denaturation	94°C	30 sec	30x
Annealing	55°C	30 sec	
Extension	72°C	1 min	

Final extension	72°C	10 min	1

17. Transfer the PCR tubes to a room dedicated to electrophoresis.

Storage: Nested PCR products are stored at 5±3°C until the realization of the electrophoresis. PCR products are stored at <-16±3°C awaiting final results.

7.4. ELECTROPHORESIS

For each sample tested and each reaction control, 5 µL of amplified products "Lyssavirus" (e.g. nPCR products) is added to 1 µL of loading dye buffer (loading buffer is 6x concentrated). Same mix (addition of 1 µL of loading dye buffer) is realized for "Beta acxtin" (e.g. RT-PCR products). The mixture is then applied to a 1.5% agarose gel, to which an intercalant[5] has been added.

The nucleic acid gel stain used in the EURL is the SYBR safe at a final concentration of 1:10.000 (i.e. 1µl SYBR Safe per 10ml of agarose gel).

A well is reserved for the deposition of a DNA size marker in order to estimate the size of amplicons.

7.5. ANALYSIS OF PCR PRODUCTS

The result is positive when a DNA fragment is detected at the expected size (form a bright band):

- 606 bp for the first round of RT-PCR Lyssavirus (JW12-JW6).
- 589 bp for the nested PCR Lyssavirus (JW12-JW10).
- 153 bp for the RT-PCR Beta actin (β-actine_int- β-actine_rev).

The result is negative:

- when no DNA fragments are visible for the nested PCR Lyssavirus.

- when no bands are visible for the Beta actin RT-PCR.

8. RESULTS

8.1. TEST VALIDATION

The tests are validated as follows:

[5] Ethidium bromide has been used for decades to stain nucleic acids in agarose gels under UV light. However, because ethidium bromide has been shown to be mutagenic and potentially carcinogen, the safer SYBR-based options have been preferred by many users over the use of ethidium bromide.

- INTERNAL ENDOGENOUS GENE (RT-PCR Beta actin):

- if the positive extraction and PCR controls = positive.
- if the negative PCR control = negative.

- RT-PCR LYSSAVIRUS:

- if the positive extraction and PCR controls = positive.
- if the negative extraction and PCR controls = negative.

8.2. VALIDATION OF SAMPLES

- INTERNAL ENDOGENOUS GENE (RT-PCR BETA ACTIN):

- The sample is considered **positive** when results of RT-PCR Beta actin are **positive** (Presence of a DNA fragment of ≈ 153-bp). Positive and negative controls should fulfill the validation criteria (i.e. negative control(s) = negative ; positive control(s) = positive).

- The sample is considered **negative** when results of RT-PCR Beta actin are **negative** (Absence of a DNA fragment of ≈ 153-bp). Positive and negative controls should fulfill the validation criteria (i.e. negative control(s) = negative ; positive control(s) = positive).

When the sample is considered negative for Beta-actin, the absence of PCR inhibitor should be checked by diluting the sample (for example to 1/10).

- RT-PCR LYSSAVIRUS:

- Positive and negative controls shall fulfill the validation criteria (i.e. negative control(s) = negative ; positive control(s) = positive).

- The sample is considered **positive** when results of RT-PCR Lyssavirus (presence of a DNA fragment of ≈ 606-bp) or nPCR (presence of a DNA fragment of ≈ 589-bp) are **positive**. Positive and negative controls should fulfill the validation criteria (i.e. negative control(s) = negative ; positive control(s) = positive).

- The sample is considered **negative** when results of nPCR Lyssavirus are **negative** (absence of a DNA fragment of ≈ 589-bp). Positive and negative controls should fulfill the validation criteria (i.e. negative control(s) = negative ; positive control(s) = positive).

8.3. EXPRESSION OF RESULTS

- The result is reported as "Absence of detection of RNA Lyssavirus" when a sample is declared negative using the hnRT-PCR Lyssavirus and positive using the RT-PCR Beta actin.

- The result is reported as "Detection of RNA Lyssavirus" when a sample is declared positive using both the RT-PCR Lyssavirus and the RT-PCR Beta actin.

- The result is reported as "Not interpretable" when a sample is declared negative using the RT-PCR Lyssavirus and the RT-PCR Beta actin.

9. PERFORMANCE CHARACTERISTICS OF THE METHOD[6]

The table below describes the performance criteria of the described method (hnRT-PCR) validated by the EURL for rabies according to the NF U47-600 standard.

Criteria	Expected results	Observed results
Analytical specificity (inclusivity)	≥ 90%	100%
LD PCR (Limit of detection)	≤ 100 copies/µL	100 copies/µL
LD Method	≤ 10000 copies/µL	10000 copies/µL
Diagnosis sensitivity	≥ 90%	97%
Diagnosis specificity	≥ 90%	100%

[6] Each laboratory must determine its own performance characteristics under his working conditions (equipment, reagents, etc.).