Detection of Lyssavirus RNA by SYBR Green Real Time RT-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 SYBR Green RT-qPCR 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

The method described in this SOP describes the generic detection of Lyssavirus RNA by real-time RT-PCR for diagnostic purpose.

The assay is based on the SYBR Green real-time RT-PCR method, pan-Lyssavirus primers [3,4] and allows the detection of all ICTV recognized Lyssavirus RNA species.

The SOP has been fully validated following the NF U47-600 standard (www.afnor.fr) [5].

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.
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4. 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.
5. AFNOR, 2015. « Méthodes d'analyse en santé animale - PCR (réaction de polymérisation en chaîne) - Partie 1 : exigences et recommandations pour la mise en oeuvre de la PCR en santé animale NF et Partie 2 : exigences et recommandations pour le développement et la validation de la PCR en santé animale. U47-600-1, Normes & Recueils.
6. 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.
7. Picard-Meyer E, Peytavin de Garam C, Schereffer J L, Marchal C, Robardet E, Cliquet F. Cross-platform evaluation of commercial real-time SYBR green RT-PCR kits for sensitive and rapid detection of European bat Lyssavirus type 1. Biomed Res Int 2015; 2015:839518.for the detection of Lyssaviruses. J Virol Methods. 2011 Oct;177(1):87-93.
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3. TERMS, ABBREVIATIONS AND DEFINITIONS

bp	base pair
N	Nucleoprotein gene
ICTV	International Committee on Taxonomy of Viruses
Ct	Cycle Threshold
HPLC	High pressure liquid chromatography
RABV	Classical rabies virus
RNA	Ribonucleic Acid
SYBR Green RT qPCR	SYBR® Green real-time RT-PCR
T_M	Melting temperature

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 [6,7]. The two enzymatic reactions (the reverse transcription of the RNA into complementary cDNA and PCR amplification) are performed successively in the same tube.

The SYBR Green RT-qPCR assay targets the Nucleoprotein (N) gene and is performed with pan-Lyssavirus primers JW12 (forward) and N165-146 (reverse) previously described by Wakeley et al. [4], that detect a 110 base pair (bp) region of the N gene from all ICTV recognized lyssavirus species [3,4].

A parallel assay is carried out for each tested sample to detect in the extracted tissular RNA fraction 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. [4]. Primers (pan-Lyssavirus and β-actin) are described in the OIE Chapter 3.1.17. [2].

5. REAGENTS[1]

- QuantiTect® SYBR® Green RT-PCR kit (QIAGEN, France)
- Molecular grade water DNAse-RNAse free

[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 and extraction of RNA/amplification
- PCR workstations with specific dedicated micropipettes
- Spin micro-centrifuges, refrigerated centrifuges
- Vortex
- Real-time PCR thermocycler

6.2. CONSUMABLES

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

6.3. PRIMERS

The SYBR Green RT-qPCR is performed with HPLC purified primers.
The molecular models used for the detection of **Lyssavirus and Beta actin genes** are described in the Table below:

Molecular models used:

Primer	Function	Sequences (5'-3')	Position
JW12	Primer forward	ATG TAA CAC CYC TAC AAT G	55-73
N165-146	Primer reverse	GCA GGG TAY TTR TAC TCA TA	165–146
Beta actin_int	Primer forward	CGATGAAGATCAARTCATTGC	1051–1072
Beta actin_rev	Primer reverse	A AAGCATTTGCGGTGGAC	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 [8]. 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.

7.2. SYBR GREEN RT-QPCR

7.2.1. PREPARATION OF THE MASTER MIX [3]

1. Thaw all frozen reagents (i.e. 2x QuantiTect SYBR Green Master mix, primers, molecular grade water). Mix the individual solutions (2x QuantiTect SYBR Green RT-PCR Master mix and primers) and place them on ice with the molecular grade water.

[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.
[3] There are numerous available commercially reagents dedicated for SYBR Green RT-qPCR. Optimisation of PCR should be carried out by each user according to the master mix used and PCR machine used under his own working conditions.

2. Prepare the master mix as follows:

LYSSAVIRUS RT-PCR:

MASTER MIX	Final concentration	Volume (in µL) for 1 tube
Molecular grade water		8.75
QuantiTect SG Master Mix (2x)	1x	12.5
JW12 (20µM)	0.6µM	0.75
N165-146 (20µM)	0.6µM	0.75
QuantiTect RT Mix		0.25

ENDOGENOUS GENE (BETA-ACTIN RT-PCR):

MASTER MIX	Final concentration	Volume (in µL) for 1 tube
Molecular grade water		8.75
QuantiTect SG Master Mix (2x)	1X	12.5
Primer Bact. Fw (20µM)	0.6µM	0.75
Primer Bact. Rv (20µM)	0.6µM	0.75
QuantiTect RT Mix		0.25

3. Mix the reaction thoroughly and dispense a volume of 23µL of master mix into PCR tubes

7.2.2. ADDITION OF TEMPLATE

4. Add 2µL of RNA samples/control RNA(s) to the individual PCR tubes containing the master mix and close the tubes.

For each PCR 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.

All test samples should be run at least in duplicate.

7.2.3. SETTING UP OF REAL-TIME CYCLER[4]

The real-time cycler optimized and validated for the method described here is programmed as follows:

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	
Annealing	55°C	30 sec	45x
Extension	72°C	30 sec*[b]	
Melt curve	Ramp from 65°C to 95°C , Increase of 1°C per step, Wait 5 sec by step		

*[b]: fluorescence data collection.
The cycling parameters described above have been validated on the Rotor-Gene Q MDx instrument (Qiagen).

7.2.4. READING OF FLUORESCENCE

5. Results are read in FAM on the "Green" channel.
6. For each run, check the baseline. The threshold line is placed above the background noise, towards the lower third/middle of the exponential phase.
7. Results are determined for each sample by its cycle threshold (Ct), that corresponds to the number of cycles required for the fluorescent signal to cross the threshold.

8. RESULTS

8.1. TEST VALIDATION

The tests are validated as follows:

- 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.

[4] Thermal profiles may be subject to optimization depending on used PCR equipment and kits, by especially testing different annealing temperatures.

8.2. VALIDATION OF SAMPLES

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

- The sample is considered **positive** when the C_T is obtained with a characteristic amplification curve (sigmoidal) and a T_M characteristic of the target. 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 the C_T is undetected, e.g. absence of characteristic amplification curve, no C_T or amplification of primer-dimer.

When the sample is considered negative for β-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 the C_T is obtained with a characteristic amplification curve (sigmoidal), a T_M characteristic of the target and a C_T value equal or less than the cut-off value of PCR.

- The sample is considered weak positive when the C_T is obtained with late CT values (< 45 and > to the cut-off value) and a T_M characteristic of the target.

- The sample is considered negative when the C_T is undetected : absence of characteristic amplification curve; No C_T or non characteristic curve (e.g. primer-dimer).

8.3. EXPRESSION OF RESULTS

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

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

- The result is reported as "Detected in LD_{PCR} with late Ct values" when a sample is declared weak positive using the RT-qPCR Lyssavirus and positive using the RT-qPCR Beta actin.

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

9. PERFORMANCE CHARACTERISTICS OF THE METHOD[5]

The table below describes the performance criteria of the described method 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	50 copies/µL
LD Method	≤ 1000 copies/µL	100 copies/µL
Diagnosis sensitivity	≥ 90%	100%
Diagnosis specificity	≥ 90%	100%

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