Detection of *Lyssavirus* RNA by TaqMan® Real Time RT-PCR (n)LN34

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FOREWORD

This protocol has been validated by:

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INTRODUCTION

This Standard Operating Procedure (SOP) describes the detection of *Lyssavirus* RNA by TaqMan® RT-qPCR (n)LN34 for diagnostic purpose.

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 WOAH Standard for validation (Chapter 1.1.6. "Validation of diagnostic assays for infectious diseases of terrestrial animals" and Chapter 3.1.19 "Rabies : infection with rabies virus and other *Lyssaviruses*") [1, 2] and should be able to detect a broad spectrum of globally circulating rabies virus strain [3].

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 TaqMan® pan-lyssavirus real-time RT-PCR method [4-6] and allows the detection of all ICTV recognized *Lyssavirus* RNA species [3].

2. REFERENCE DOCUMENTS

1. WOAH: Chapter 3.1.19 Rabies (infection with rabies virus and other Lyssaviruses). In: *WOAH Terrestrial Manual 2023.* 2023.
2. WOAH: CHAPTER 1.1.6. Validation of diagnostic assays for infectious diseases of terrestrial animals. . In: *WOAH Terrestrial Manual 2023.* 2023.
3. Virus Taxonomy: The ICTV Report on Virus Classification and Taxon Nomenclature [https://ictv.global/report/chapter/rhabdoviridae/rhabdoviridae/lyssavirus]
4. Wadhwa A, Wilkins K, Gao J, Condori Condori RE, Gigante CM, Zhao H, Ma X, Ellison JA, Greenberg L, Velasco-Villa A *et al*: A Pan-Lyssavirus Taqman Real-Time RT-PCR Assay for the Detection of Highly Variable Rabies virus and Other Lyssaviruses. *PLoS Neglected Tropical Diseases* 2017, 11(1).
5. Gigante CM, Dettinger L, Powell JW, Seiders M, Condori REC, Griesser R, Okogi K, Carlos M, Pesko K, Breckenridge M *et al*: Multi-site evaluation of the LN34 pan-lyssavirus real-time RT-PCR assay for post-mortem rabies diagnostics. *PLoS One* 2018, 13(5):e0197074.
6. Drzewnioková P, Marciano S, Leopardi S, Panzarin V, De Benedictis P: Comparison of Pan-Lyssavirus RT-PCRs and Development of an Improved Protocol for Surveillance of Non-RABV Lyssaviruses. *Viruses* 2023, 15(3).
7. Wakeley PR, Johnson N, McElhinney LM, Marston D, Sawyer J, Fooks AR: Development of a real-time, differential RT-PCR TaqMan assay for lyssavirus genotypes 1, 5 and 6. *Dev Biol (Basel)* 2006, 126:227-236; discussion 326-227.
8. Davis DL, O'Brien EP, Bentzley CM: Analysis of the degradation of oligonucleotide strands during the freezing/thawing processes using MALDI-MS. *Anal Chem* 2000, 72(20):5092-5096.

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
RT-qPCR	real-time RT-PCR
LoD	Limit of Detection

4. PRINCIPLE OF THE METHOD

The test described here is based on a one-step RT-PCR approach in order to minimise the risk of contamination during sample manipulation and tube opening. Both enzyme steps (reverse transcription of RNA into complementary cDNA and PCR amplification) are performed successively in the same closed tube.

This procedure is based on the **TaqMan RT-qPCR New LN34 assay** (referred to as **(n)LN34**) revised by **Drzewnioková *et al.* in 2023** [6], which is based on the original TaqMan RT-qPCR LN34 assay developed by Wadhwa *et al.* in 2017 and Gigante *et al.* in 2018 [4, 5]. The TaqMan RT-qPCR (n)LN34 assay targets a 164 nucleotide region including the leader sequence, the transcription initiation signal, and part of the coding sequence of the nucleoprotein (N) gene of all *Lyssavirus* species recognised by the ICTV [3]. This assay uses a combination of five pan-lyssavirus forward primers, three reverse primers, and two TaqMan® probes, as described in the referenced literature [6].

For each sample tested, a parallel assay targeting the endogenous *β-actin* gene (153 bp) is performed on the extracted RNA to check extraction efficiency and ensure the absence of PCR inhibitors. This control is performed using a **TaqMan RT-qPCR *β-actin* assay** using two primers and a single probe, previously described by Wakeley *et al.* in 2006 [7], and slightly modified by **Wadhwa *et al.* in 2017** [4].

5. EQUIPMENT, MATERIALS AND REAGENTS [1]

5.1. 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
- Micro-centrifuges
- Vortex
- Real-time PCR thermocycler
- Freezers < -16 °C and < -65 °C

5.2. CONSUMABLES

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

[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).

5.3. REAGENT

- AgPath-id one-step RT-PCR reagents (Applied-Biosystem)
- Molecular grade water DNAse-RNAse free

5.4. PRIMERS

The TaqMan® RT-qPCR is performed with HPLC purified primers and probes.
The molecular models used for the detection of *Lyssavirus* and the endogenous *β-actin* gene are described in the Tables below:

List of primers and probes used for TaqMan (n)LN34 RT-qPCR:

Primer	Function	Sequences (5'-3')	Position *
(n)LN34 FW1	Primer forward	ACGCTTAACAACMARATCAAAGAA	1-24
(n)LN34 FW2	Primer forward	ACGCTTAACAACAAAATCADARAAG	1-25
(n)LN34 FW3	Primer forward	ACGCTTAACGACAAAAHCAGARAAG	1-25
(n)LN34 FW4	Primer forward	ACGCTTAACAGCTAAAAACYAGAAG	1-25
(n)LN34 FW5	Primer forward	ACGCTTAACARCAAAATCTTATAAG	1-25
(n)LN34 RV1	Primer reverse	CMGGGTAYTTRTAYTCATAYTGRTC	164-140
(n)LN34 RV2	Primer reverse	CTGGATATTTGTAYTCATAYTGATC	164-140
(n)LN34 RV3	Primer reverse	CAGGATATTTATATTCATACTGGTC	164-140
(n)LN34 Probe1	TaqMan Probe	(FAM) AA+C+ACCY+C+T+ACA+A+TGGA (BHQ1)	59-75
(n)LN34 Probe2	TaqMan Probe	(FAM) AA+C+ACTA+C+T+ACA+A+TGGA (BHQ1)	59-75

** The primer and probe positions are given relative the Lyssavirus full genome NC_001542.*
The (n)LN34 probes is labeled by fluorescent FAM at the 5'end and Black Hole quencher (BHQ1) at the 3'end. Locked nucleotide modified bases are indicated by a plus preceding the base in the sequence (e.g. +A, +G, +C, +T).

List of primers and probe used for TaqMan *β-actin* RT-qPCR:

Primer	Function	Sequences (5'-3')	Position *
β-actin FW	Primer forward	CGATGAAGATCAARTCATTGC	1051-1072
β-actin RV	Primer reverse	AAGCATTTGCGGTGGAC	1204-1188
β-actin Probe	TaqMan Probe	(HEX) TCCACCTTCCAGCAGATGTGGATCA (BHQ1)	1128-1157

** The primer and probe positions are given relative to the sequence of the mouse β-actin gene (accession number NM 007393).*

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 and probes.

6. PROCEDURE

Follow the usual recommendations to avoid any contamination.

6.1. PREPARATION OF RNA SAMPLES

Extract viral RNA from suspected specimens using an appropriate and validated method to ensure the quality of the nucleic acids extracted. Several specialised extraction techniques are available, including commercial kits based on silica columns or magnetic beads, which can be run manually or on automated systems.

For each extraction run, include the following controls to ensure the reliability of the process:

- **Negative extraction control**: This control allows for the detection of any potential cross-contamination during the extraction procedure (e.g. a sample of brain material free of the target sequence) ;
- **Positive extraction control** (also known as a process sentinel): This control is a characterised sample containing a known quantity of target RNA. Its successful amplification confirms that the extraction and subsequent detection steps have been performed correctly. In accordance with NF U47-600, this control should be calibrated at a concentration between 10 and 100 times the PCR limit of detection (LoD) to effectively monitor the overall efficiency of the analytical process.

6.2. TAQMAN RT-QPCR

6.2.1. PREPARATION OF THE REACTION MIX[2]

Prepare the reaction mix in a clean, dedicated pre-PCR laboratory or area, ideally separate from post-amplification zones, to prevent cross-contamination with target RNA or amplicons. Use RNase-free

[2] There are numerous available commercially reagents dedicated for TaqMan® 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.

consumables and reagents to minimise RNA degradation. All pipetting should be done with calibrated pipettes and aerosol-resistant filter tips.

For **(n)LN34 RT-qPCR**, prepare the reaction mix as follows:

Reaction MIX (n)LN34 RT-qPCR	**Final concentration**	**Volume (in µL) for 1 tube**
Molecular grade water		1
AgPath-ID™ RT-PCR Buffer (2x)	1x	12.5
(n)LN34 FW1 (20µM)	0.8 µM	1
(n)LN34 FW2 (20µM)	0.8 µM	1
(n)LN34 FW3 (20µM)	0.8 µM	1
(n)LN34 FW4 (20µM)	0.8 µM	1
(n)LN34 FW5 (20µM)	0.8 µM	1
(n)LN34 RV1 (20µM)	0.8 µM	1
(n)LN34 RV2 (20µM)	0.8 µM	1
(n)LN34 RV3 (20µM)	0.8 µM	1
(n)LN34 Probe1 (20µM)	0.2 µM	0.25
(n)LN34 Probe2 (20µM)	0.2 µM	0.25
AgPath-ID™ RT-PCR Enzyme Mix (25X)	1X	1

For ***β-actin*** **RT-qPCR**, prepare the reaction mix as follows:

Reaction MIX *β-actin* RT-qPCR	**Final concentration**	**Volume (in µL) for 1 tube**
Molecular grade water		8.25
AgPath-ID™ RT-PCR Buffer (2x)	1x	12.5
β-actin FW (20µM)	0.4 µM	0.5
β-actin RV (20µM)	0.4 µM	0.5
β-actin Probe (20µM)	0.2 µM	0.25
AgPath-ID™ RT-PCR Enzyme Mix (25X)	1X	1

Vortex the reaction mixes, centrifuge briefly, and dispense a volume of 23 µL into PCR tubes.

6.2.2. ADDITION OF TEMPLATE

Add 2µL of RNA samples or extraction controls to PCR tubes containing the reaction mix.
For each PCR run, the following controls must be included to ensure assay reliability:

- **Negative PCR control**: ultrapure, nuclease-free water, to check the absence of contamination in reagents and workflow.
- **Positive PCR control**: target RNA of known quantity, used to ensure that the PCR amplification is functioning correctly. In accordance with standard NF U47-600, this control is calibrated at a concentration between 3 and 10 times the PCR limit of detection (LoD), serving as a low positive control to effectively monitor the sensitivity of the PCR.

All samples should be analysed at least in duplicate to ensure result consistency and detect potential variability.

6.2.3. SETTING UP OF REAL-TIME CYCLER [3]

The cycling conditions optimized and validated for this method are programmed as follows:

STEP	Temperature	Time	Number of cycles
Reverse transcription	50°C	30 min	1
Polymerase activation	95°C	10 min	1
Denaturation	95°C	15 sec	45x
Annealing - Extension	56°C	30 sec*	

** Fluorescence data collection.*

Cycling conditions described above correspond to the EURL validated assay using the QuantStudio™ 5 instrument (Applied Biosystems).

6.2.4. READING OF FLUORESCENCE

The (n)LN34 assay results are read in the FAM channel, while the endogenous *β-actin* control is read in the HEX channel.

For each run, ensure that the baseline is correctly set. The threshold line should be positioned above the background noise (ideally in the lower third to middle of the exponential amplification phase).

Results are interpreted based on the cycle threshold (Ct) value for each sample, which corresponds to the number of cycles required for the fluorescence signal to cross the defined threshold.

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

7. RESULTS

7.1. VALIDATION OF THE EXTRACTION PROCESS

RNA extraction is valid if the negative and positive extraction controls fulfil the validation criteria.

The **negative extraction control**, performed on negative brain tissue or a blank matrix (e.g. water or PBS), must give a negative TaqMan (n)LN34 RT-qPCR result confirming absence of cross-contamination. If brain tissue is used, a positive β-actin result is also required to confirm RNA integrity and extraction efficiency

The **positive extraction control** (process sentinel) must show positive amplification in both the TaqMan (n)LN34 RT-qPCR and *β-actin* RT-qPCR assays. The Ct value obtained for the (n)LN34 assay must be in accordance with the expected value, which should be validated using a control card to approve the RNA extraction.

7.2. VALIDATION OF SAMPLES

7.2.1. ENDOGENOUS CONTROL

The absence of inhibitors in the samples is validated using the results from the TaqMan *β-actin* RT-qPCR assay. All samples must be positive for the endogenous *β-actin* gene.

If a sample produces a negative result, the presence of PCR inhibitors should be checked by diluting the sample (e.g., 1:10 dilution).

The TaqMan *β-actin* RT-qPCR assay is considered valid if both positive and negative PCR controls are conform.

7.2.2. PAN-LYSSAVIRUS DETECTION

The TaqMan (n)LN34 RT-qPCR assay is validated if the negative PCR control gives no amplification (negative result), and the positive PCR control (using calibrated RNA) gives a positive result with a Ct value in the expected interval. Ct values should be validated using an internal control card to confirm the validity of each RT-qPCR run.

A sample is considered positive when a characteristic (sigmoidal) amplification curve is observed and the Ct value is below the established cut-off. For information, for the EURL for rabies, the cut-off is fixed at 36 for the RT-qPCR (n)LN34 assay. More specifically, the cut-off is defined based on the Ct values obtained from the lowest concentrations detected at the PCR limit of detection (LoD). This value generally corresponds to three Ct values above the Ct value obtained for the lowest RNA concentrations detected in RT-qPCR.

A sample is considered negative when no characteristic amplification curve is observed, no Ct value, or when a Ct value is obtained above the cut-off, indicating the absence of target RNA or a signal below the limit of detection.

7.3. EXPRESSION OF RESULTS

The results of the TaqMan (n)LN34 and *β-actin* RT-qPCR analysis are interpreted and expressed as follows:

(n)LN34 RT-qPCR	***β-actin* RT-qPCR**	**Results Interpretation**	**Expression of results**
+	+	*Lyssavirus* RNA detected Validation of RNA extraction	Detection of RNA *Lyssavirus*
–	+	No *Lyssavirus* RNA detected RNA extraction valid	Absence of detection of RNA *Lyssavirus*
+ (Ct near cut-off / weak signal)	+	Amplification detected at the limit of detection (LoD) *	Detection of RNA *Lyssavirus* at limit of detection
+	-	*Lyssavirus* RNA detected ; Absence of amplification of endogenous control **	Not interpretable
-	-	No amplification **	Not interpretable

***+** : Amplification detected (Ct value with a characteristic sigmoidal curve)*

***–** : No amplification (no Ct value detected)*

* In cases where a sample gives a positive result with a Ct value near the assay's limit of detection (LoD), the result should be interpreted with caution. If all replicates are positive with a characteristic amplification curve, the result may be considered as positive at the limit of detection. However, confirmation by repeat extraction and assays is recommended in cases of doubt about the analysis.

** If the sample is negative for the endogenous *β-actin* control, whatever the (n)LN34 RT-qPCR result, this may indicate PCR inhibition or RNA degradation. A 1:10 dilution of the RNA extract should be tested; if *β-actin* amplification is restored, inhibition is confirmed. Otherwise, a new extraction is required.

8. PERFORMANCE CHARACTERISTICS OF THE METHOD[4]

The validation of the (n)LN34 RT-qPCR assay was conducted at the EURL for rabies in accordance with the French standard NF U47-600 relative to PCR assay methods in animal health. The main performance characteristics were evaluated as follows:

- Analytical specificity: Assessed by testing a panel of variants representative of different *Lyssavirus* species (Inclusivity).
- PCR Limit of Detection at 95% (LoD 95%): This is the lowest concentration of target RNA detectable with at least 95% confidence. It is determined by testing a total of 24 replicates in three independent runs (8 replicates per run). The 95% LoD is defined as the lowest concentration for which at least 23 out of 24 replicates give a positive result.
- Diagnostic sensitivity is assessed by testing a panel of naturally infected samples. The method is considered sensitive if it correctly detects ≥95% of positive samples, calculated as the proportion of true positives among all tested positive samples. A diagnostic sensitivity of 95% is a commonly recognised criteria for reliable diagnostic performance [2].
- Diagnostic specificity is evaluated by testing a panel of known negative samples. The method is considered specific if it correctly identifies ≥95% of negative samples, calculated as the proportion of true negatives among all tested negative samples.

The table below lists the performance criteria of the described method by the EURL for rabies, in accordance with the NF U47-600 standard.

Criteria	Expected results	Observed results
Analytical specificity (inclusivity)	≥ 95%	100%
LD PCR (Limit of detection)	≤ 100 copies/µL	25 copies/µL
Diagnosis sensitivity	≥ 95%	100%
Diagnosis specificity	≥ 95%	100%

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