LN34 pan-lyssavirus real-time RT-PCR for post-mortem diagnosis of rabies in animals

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Abstract

Purpose

To describe the LN34 real-time RT-PCR assay procedure used for the qualitative detection of lyssavirus RNA in whole RNA extracted from brain tissue samples. The assay detects RNA from diverse lyssaviruses at varying concentrations [1].

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Guidelines

Purpose

To describe the LN34 real-time RT-PCR assay procedure used for the qualitative detection of lyssavirus RNA in whole RNA extracted from brain tissue samples. The assay detects RNA from diverse lyssaviruses at varying concentrations [1].

Responsibility

A trained technician will perform the assay according to the procedural protocol, return samples to storage, and report results to respective supervisors.

Specimens

RNA extracted from tissue representing a full cross section of brain stem

Before start

Definitions/Keywords

PPE – Personal Protective Equipment

SDS – Safety data sheet

NTC – No template control

Ct – Cycle threshold

PCR – Polymerase Chain Reaction
RT-PCR –Reverse Transcription-Polymerase Chain Reaction

RNA – Ribonucleic Acid

RNase – Ribonuclease

βA – Beta-actin
Room Temperature – Temperature range of 20°C to 25°C (68°F to 77°F)

DFA – Direct Fluorescent Antibody Test (also known as FAT)

dRIT – Direct, Rapid Immunohistochemical Test

Reagent Preparation

1. Acceptable surface decontaminants
    1. RNase Away (Fisher Scientific)
    2. RNaseZAP (Sigma-Aldrich)
    3. 70% EtOH
2. Reagent Preparation: Vortex and centrifuge all buffers, primers, and probes for a minimum of 5 seconds before use.
3. Primers and Probes
4. Preparation of single-use aliquots is recommended.
5. Store at -20°C in the dark.
6. Frozen reagents may be used until quality failure or until kit expires
7. Ag-Path 2X RT-PCR Buffer (Life Technologies): Have thawed and ready for use
8. 25X RT-PCR Enzyme Mix (Life Technologies): Keep on ice or in a cooling block
9. It is recommended that PCR mastermix reagents be thawed and prepared separately from RNA samples that will be tested
10. RNA samples
    - Recommended: Use freshly extracted RNA, store on ice until use
    - Thaw any frozen RNA samples on ice
    - Thaw single-use aliquot of positive control RNA on ice
11. Positive control RNA
    - Positive control RNA will be produced and validated at CDC, then shipped to testing laboratories. No dilution is necessary.
    - Positive control RNA should be stored in single-use aliquots at -80 °C until use. Avoid freeze-thaw.

- Positive control RNA should not be used if the Ct value is out of the expected range determined by CDC for a given lot. Between runs, the Ct value of the positive control RNA from the same batch should not differ by more than 5 Ct.

Quality Control/Corrective Action

- All issues, comments, concerns, or deviations from the protocol are to be documented and brought to the attention of the lab supervisor or technical supervisor immediately if they are thought to affect the quality of the test results in any way.
- Technical supervisor will review results prior to issuance of results.
- Temperature logs are kept to ensure refrigerators and freezers stay within acceptable temperature range for reagents. Real-time PCR machines are to undergo normal preventative maintenance and quality assessments, as suggested by the manufacturer.
- Because of the sensitivity of the fluorogenic 5'nuclease assay, special precautions must be taken to avoid false positive amplification. We recommend the following steps to avoid sample contamination:
  - Wear clean gloves (not previously worn when handling extracted RNA or PCR products) when setting up assays.
  - Change or decontaminate gloves whenever you suspect they are contaminated.
  - Keep tubes capped as much as possible.
  - Before setting up assays and after handling extracted RNA or PCR products, clean equipment and lab benches.
  - Use aerosol barrier (filter) pipette tips.
  - Use a clean room or cabinet system to minimize contamination, if possible.

Materials

- ✓ Disposable gloves, nitrile by Contributed by users
- ✓ Marking pen by Contributed by users
- ✓ Sterile polypropylene microcentrifuge tubes (RNase/DNase free) by Contributed by users
- ✓ Aerosol (filter) barrier tips (RNase/DNase free) by Contributed by users
- ✓ Optical 96-well Reaction Plates by Contributed by users
- ✓ Optical Adhesive covers by Contributed by users
- ✓ Ice by Contributed by users
- ✓ LN34 assay primer (10 µM) and probe (5 µM) set by Contributed by users
- ✓ β-actin assay primer (10 µM) and probe (5 µM) set by Contributed by users

  Ag-Path ID One-Step RT-PCR Kit AM1005 by Life Technologies
- ✓ Artificial RNA Template Control by Contributed by users
- ✓ Microcentrifuge by Contributed by users
- ✓ Real-time PCR machine capable of detecting FAM and VIC/HEX ABI ViiA 7, 7500, 7500 Fast, or by Contributed by users
- ✓ Freezer by Contributed by users

Protocol

General Guidelines

Step 1.

- Each RNA sample must be run in triplicate in **both** the LN34 and β-actin (βA) real-time PCR assays (6 reactions per sample).
- Three no template control reactions must be run in **both** the LN34 and β-actin (βA) real-time PCR assays (6 reactions per plate).
- Three positive control RNA reactions **must** be run in the LN34 real-time RT-PCR assay (3 reactions per plate). Running and monitoring the Ct value of the LN34 positive control is critical to assuring assay sensitivity within a laboratory over time.
- Three positive control reactions should be run in the βA real-time PCR assay (3 reactions per plate). Positive control RNA for the βA assay can be a previously tested extracted whole RNA sample or commercially available β-actin RNA may be purchased.
- Reaction assay mixtures (master mixes) containing all components except template should be made as cocktails and dispensed into the reaction plate or tubes. RNA, positive control RNA or water should then be added to the respective test and control wells

General Guidelines

Step 2.

Name	Sequence
LN34 Forward Primer 1	ACGCTTAACAACCAGATCAAAGAA
LN34 Forward Primer 2	ACGCTTAACAACAAAATCADAGAAG
LN34 Reverse Primer	CMGGGTAYTTRTAYTCATAYTGRTC
LN34 Probe	(FAM) AA+C+ACCY+C+T+ACA+A+TGGA (BHQ1)
LN34 Lagos Probe	(FAM) AA +C +ACTA +C +T +ACA +A +TGGA (BHQ1)
β-Actin Forward Primer	CGATGAAGATCAAGATCATTGC
β-Actin Reverse Primer	AAGCATTTGCGGTGGAC
β-Actin Probe	(HEX)-TCC ACC TTC CAG CAG ATG TGG ATC A-(BHQ1)
Positive Control RNA	GCA CAG GGT ACT TGT ACT CAT ACT GAT CTG AAT CCA TTG TAG AGG TGT TAG AGC ACG ACA GGT TTC CCG ACT GGA TCT TTC TTT GAT CTG GTT GTT AAG CGT TCG CCC TAT AGT GAG TCG TAT TAC A

Primers and probes used in the LN34 real-time RT-PCR assay. LN34 probe is labeled by fluorescent FAM at the 5′end and Black Hole quencher (BHQ1) at the 3′end. β-Actin probe is labeled by fluorescent HEX 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). β-Actin assay is adapted from [2]. Positive control RNA is adapted from [3].

Prepare Master Mixes for LN34 and βA Assays

Step 3.

Label one microcentrifuge tube per assay (LN34 and βA).

Prepare Master Mixes for LN34 and βA Assays

Step 4.

Determine the number of reactions (N) to set up per assay.

1. Calculate the number of reactions for the LN34 assay by multiplying the number of samples by three and add 6 for control reaction wells.
2. Calculate the number of reactions for the βA assay by multiplying the number of samples and add 6 for control reaction wells.

Prepare Master Mixes for LN34 and βA Assays

Step 5.

Fill in the table below by multiplying the "µL/Reaction" by the number of reactions for each reagent. Make 1-5 extra reactions to account for volume lost during pipetting.

Reagent	µL/Reaction	LN34 (FAM/BHQ1)	βA (VIC/BHQ2)
Water	6.5		
2X RT Buffer	12.5		
25X RT-PCR Enzyme Mix	1		
Forward Primer [10µM]	1		
Reverse Primer [10µM]	1		
Probe [5µM]	1		
RNA	2		
Total	25		

Dispense Mastermix

Step 6.

Designate and label wells for each sample to run in triplicate for both the LN34 assay and βA assay

Dispense Mastermix

Step 7.

Dispense reagents for the LN34 assay into the LN34-labeled microcentrifuge tube. Briefly vortex and centrifuge prior to dispensing 23 µl of master mix into each LN34 assigned well. Avoid introducing bubbles.

Dispense Mastermix

Step 8.

Dispense reagents for βA assay into the βA-labeled microcentrifuge tube. Briefly vortex and centrifuge prior to dispensing 23 µl master mix into βA assigned wells.

Add Samples

Step 9.

Briefly vortex and centrifuge the tubes containing the RNA samples.

Add Samples

Step 10.

Pipette 2 µl of extracted RNA from the first sample into each well labeled for that sample. Avoid introducing bubbles.

Repeat for the remaining samples and the positive control RNA.

Add Samples

Step 11.

After the addition of the last sample/control, place the optical adhesive cover over the wells, being sure to cover all the wells and seal completely.

Optional: briefly vortex

Centrifuge at 500 × *g* for 1 min at room temperature or tap down samples on tabletop

Real-time RT-PCR

Step 12.

Place sealed plate into a real-time PCR instrument that can detect FAM and VIC/HEX.

Set to the conditions below. Be sure to use **STANDARD MODE** not FAST mode.

ViiA7 - Standard Mode

Step	Cycles	Temp	Time
Reverse Transcription	1	50°C	30 min
RT inactivation/initial denaturation	1	95°C	10 min
Amplification	45	95°C	1 sec
		56°C	20sec

ABI7500 - Standard Mode

Step	Cycles	Temp	Time
Reverse Transcription	1	50°C	30 min
RT inactivation/initial denaturation	1	95°C	10 min

Amplification	45	95°C	15 sec
		56°C	30sec

Real-time RT-PCR

Step 13.

Allow the instrument to analyze the results automatically, any manual threshold or baseline adjustments needs to be recorded and explained.

Sample Retention and Storage

Step 14.

- All samples should be stored until testing is complete and results are reported.
- Retention of the sample is necessary to re-run the test when required.
- RNA should be stored at -80°C for long term storage.

Interpretation of results

Step 15.

Use the table below to determine if a sample is positive, negative, or inconclusive

1. If the LN34 result is positive, even in the presence of a negative βA result, the result should be considered positive.
2. If LN34 amplicon is not detected, the βA Ct must be ≤33 for the sample to be considered negative.
3. βA Ct values determine the quality, suitability, and potential inhibition of the sample being tested. Some samples may fail to exhibit βA growth curves due to low concentration in the original clinical specimen. Failure to detect β-actin in any clinical sample may indicate:
    - Improper extraction of RNA from clinical materials resulting in loss of RNA or carryover of PCR inhibitors from clinical specimens
    - Improper assay set up and execution
    - Inadequate clinical sample
    - Reagent or equipment malfunction

Result	LN34 Ct	β-actin Ct	Action	Interpretation
Positive	**≤ 35**	**Any**	**None**	**Lyssavirus RNA present**
Negative	**Not detected**	**≤ 33**	**None**	**No Lyssavirus RNA present**
Inconclusive	35–45	Any	Repeat or additional testing required	Possible contamination, insufficient sample, inhibition, or low virus load
Inconclusive	Not detected	> 33 or Not detected	Repeat or additional testing required	Possible inhibition or insufficient sample

Interpretation of results

Step 16.

Quality Controls **REQUIRED** for each run

1. The LN34 Ct value of the positive control RNA should be within the range provided by CDC for that lot of positive control RNA. If the positive control replicate reactions do not cross the threshold within the specified range, invalidate the run and repeat the assay. The positive control RNA will not amplify in the βA assay.
2. The no template control (NTC) reactions should not exhibit growth curves that cross the threshold line for either the LN34 or the βA assay. If amplification is observed in the NTC, it may indicate contamination. Invalidate the run and repeat testing for all samples.
3. All replicates for a given sample should amplify for a valid positive result. If only a subset of replicates amplify in either assay, the sample should be re-tested.
4. Any sample producing highly variable results (Ct value differences > 5 between replicates) should be considered invalid and the sample should be re-tested.
5. Negative sample or NTC reactions where one out of three replicates exhibits Ct values greater than 38 and the remainder of replicates showed no amplification can be considered negative/no amplification for that assay only for one sample per assay run.

Interpretation of results

Step 17.

IMPORTANT: For rabies rule-out, tissue representing a full cross section of non-fixed brain stem must be tested. For all other samples, we suggest reporting negative LN34 test result as INCONCLUSIVE for rabies, because viral presence in other tissues can be delayed, low abundance, intermittent, or not existent.

A positive LN34 test result can be used to identify the presence of virus in a variety of tissues and tissue conditions.

Interpretation of results

Step 18.

Inconclusive Samples

1. All inconclusive samples must be re-tested. If the sample is inconclusive upon repeat testing and all controls performed as expected, re-extraction of RNA is recommended.

2. Ct value from 33 – 45 in the βA assay may indicate PCR inhibition. In the case where LN34 is negative and βA Ct is between 33 and 45, dilute RNA samples and repeat testing.

3. Ct value from 33 – 45 or no amplification in the βA assay may indicate failed RNA extraction. Repeat extraction for such samples, then repeat LN34 assay.

4. Ct value from 35 – 45 in the LN34 assay may indicate contamination, low virus load, PCR inhibition, or failed extraction. Collect a new piece of brain stem from the original tissue, perform RNA extraction, and re-test the sample.

5. If a result remains inconclusive for a given sample after repeated testing, use a secondary method such as the DFA test, dRIT, or virus isolation.

6. A rabies positive sample extracted from properly collected and stored brain tissue is expected to have a Ct value less than 35 cycles for the LN34 assay. Samples with low viral RNA (LN34 Ct > 35) and very high total RNA (βA Ct < 25) may indicate potential problems with the assay, sample, or extraction, in particular sample contamination if positive rabies samples were processed on the same day. Re-extract and re-test the sample. If the issue persists, please share results and samples with CDC.

Interpretation of results

Step 19.

Limitations

- This assay does not differentiate between lyssaviruses
- If inhibitors are present in an RNA extraction, PCR assays may produce a false negative result.
- If inhibition of the β-actin control reactions is noted for a particular sample, extracted RNA should be tested at 2 or more dilutions (e.g., 1:10 and 1:100) to verify the result.

References

Step 20.

1. Wadhwa, A., et al., *A Pan-Lyssavirus Taqman Real-Time RT-PCR Assay for the Detection of Highly Variable Rabies virus and Other Lyssaviruses.* PLoS Negl Trop Dis, 2017. **11**(1): p. e0005258.
2. Wakeley, P.R., et al., *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. **43**(6): p. 2786-92.
3. Hoffmann, B., et al., *Improved safety for molecular diagnosis of classical rabies viruses by use of a TaqMan real-time reverse transcription-PCR 'double check' strategy.* J Clin Microbiol, 2010. **48**(11): p. 3970-8.

Warnings

Hazards and Safety Precautions

- Wear appropriate personal protective equipment (gloves, safety glasses, and lab coat).
- Follow procedures as demonstrated in the Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition: (https://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf)
- Samples may contain infectious agent(s). You should be aware of the health hazards presented by such agents and should use, store, and dispose of such samples in accordance with the required safety regulations.
- Pre-exposure rabies vaccination, regular serologic tests, and booster immunizations as necessary are required for all persons prior to working with lyssaviruses or with known or potentially infected specimen, or engaging in diagnostic, production, or research activities with these viruses.
- Some chemicals used with this assay may be hazardous or become hazardous; refer to the SDS as needed. Dispose of chemical waste as directed in the SDS and according to local regulations.