# Rabies LN34 PCR:
## Frequently Asked Questions

FAQ-CDC LN34 for Molecular Detection of Rabies Virus • August 2026

## Contents



## Considerations for Implementation of Molecular Rabies Testing

### Q: When can laboratories expect to be able to replace DFA with NAAT?

**A:** DFA testing should not be discontinued for the laboratory diagnosis of rabies virus. At this time, NAAT testing is meant to be an additional tool to aid in the diagnosis of rabies virus for laboratories with capacity. APHL does not yet recommend it as a primary diagnostic method based on the lack of an established external assessment program and minimum standard protocol.

### Q: What is the best workflow for laboratories as they implement rabies NAAT and maintain DFA to ensure timely results?

**A:** Each laboratory will need to evaluate their capacity, sample volume and testing needs to establish a workflow that supports each of these while optimizing cost savings and expedient turnaround times. Some proposed workflows

from most labor intensive to least: 1) perform DFA and NAAT in parallel for all samples received to gain confidence in the method and generate data 2) perform DFA for all samples received and perform NAAT as an additional assay for positive samples or 3) perform DFA for all samples and perform NAAT in cases where DFA does not provide a definitive result (i.e. nonspecific staining, unsatisfactory result). In this last case, only positive NAAT results should be reported (See APHL Rabies Molecular Testing Recommendations for more details).

# CDC Support for LN34

## Q: Where is the LN34 protocol available?

**A:** Publications describing the LN34 assay and modified LN34 assay are publicly available. Two versions (Short Protocol and Long Protocol) of the original LN34 assay protocol are available on APHL's website for public health laboratory staff receiving member benefits. If you have trouble accessing ask your supervisor/director or email infectious.diseases@aphl.org.

## Q: Will CDC provide primers and probes for the LN34 assay?

**A:** CDC will share primers and probes with laboratories to validate the existing LN34 assay, subject to staff availability and funding. If a laboratory purchases primers and probes from a commercial source, they should be verified against CDC-provided primers and probes to ensure equivalent performance. Requests can be made by contacting CDC.

## Q: Is CDC distributing the original LN34 primers or the recently published primers for multiplexing?

**A:** As of June 2026, CDC is currently distributing the original primers for the rabies target and β-actin primers which were updated in 2018.

## Q: Will CDC provide ongoing support for laboratories following validation and implementation of the LN34 assay?

**A:** CDC will continue to provide technical support and consultation, primers and probes, positive controls and an inactivated samples (~approximately 5 samples; mix of positive and negative) for assay validation to laboratories validating the LN34 assay, subject to availability. RNA may also be available to evaluate the PCR. CDC does not provide panels for proficiency testing, extraction kits or enzymes. Orders can be placed by contacting CDC.

# Proficiency Testing

## Q: Will a PT program be developed?

**A:** APHL along with the Wisconsin State Laboratory of Hygiene piloted an external performance evaluation program to complement the current DFA evaluation program. As of August 2026, Wisconsin's PT program has announced a performance evaluation exercise for the molecular detection of rabies. At this time the panel will consist of TRIzol-inactivated positive and negative samples which will require extraction. For questions about the exercise, you may contact: tiffany.curia@slh.wisc.edu. *It is not currently listed on the website/catalog for Fall 2026 but will be in future catalogs*.

# Assay Standardization

## Q: How will consistency and standardization in results interpretation and reporting be ensured across laboratories using different methods for rabies NAAT?

**A:** Ideally, like the DFA there would be a minimum standard protocol for rabies NAAT that includes the required performance characteristics that must be met by each laboratory. Additionally, there is a need for an external evaluation program to assess assay performance in each laboratory and between laboratories. APHL and CDC are currently working to develop these resources to help ensure consistency and standardization.

# Assay Validation

## Q: How have laboratories established limit of detection for the LN34 and the β-actin targets?

**A:** Laboratories have created a series of serial dilutions of known positive samples from their laboratory or utilized samples provided as part of a panel from CDC c to determine the limit of detection for the LN34 target. Alternatively, a commercial RNA sequence matching the sequence of the CDC-provided positive control can be ordered (See Appendix A for an example quote). Since β-actin is not the primary target in the assay the limit of detection does not need to be assessed.

# Sample and Processing

## Q: How do laboratories ensure proper sampling for both DFA and NAAT from small animal brains or samples in which distinct brain sections cannot be identified?

**A**: As stated in the Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing, a full cross-section of the brainstem and the cerebellum (or hippocampus) must be sampled for accurate results. Even in small brains there should be plenty of tissue available to perform the impression for DFA and to collect tissue into a tube for NAAT. In juvenile animals where it may be difficult to differentiate the brain sections, laboratories will often process the entire intact hindbrain. However, whether due to the brain size, age or development of the animal or decomposition of the brain, if it is not clear if the appropriate brain sections are present and have been sampled, negative results should not be reported. In that case, the result should be reported as unable to rule out rabies infection.

## Q: Have laboratories utilized any different sampling methods for NAAT as compared to DFA?

**A**: Yes, the Wisconsin State Laboratory of Hygiene has described a swabbing method that was thoroughly validated in their laboratory and compared to more typical sampling protocols. The process is described as rolling a Dacron or rayon swab over the entire surface of the required brain sections and then placing the swab into a tube with 600 µL of TRIzol. For large animals, they will take one swab per section and extract each separately (one brainstem and one cerebellum/hippocampus specimen per animal). PCR is also performed separately.

## Q: Should NAAT or DFA be performed for specimens a) after freeze/thaw, b) with advanced decomposition/autolysis or c) from formalin-fixed tissues?

**A**: As stated in the Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing, freezing the sample for transport will not reduce the sensitivity of the test but repeated freeze-thaw cycles should be avoided entirely or minimized. If freeze/thaw cycles are an unavoidable aspect of the workflow, consider including it in the validation plan. Formalin-fixed tissues and decomposed tissues are unacceptable sample types for both NAAT and DFA. Negative results from unsatisfactory samples should be reported as unable to rule out rabies.

## Q: What determines if an animal is “small” or “large” when it comes to brain dissection and sample processing?

**A**: For DFA it may depend on whether the cross section of brainstem can fit across the width of a slide. If it is bigger than the width of one slide a second slide can be used. For NAAT, generally a pea sized amount of tissue is extracted. For small brains, all required tissue (100 mg or less) can be added to 1 mL of homogenization buffer with beads. For larger brains, CDC recommends extracting the brainstem and cerebellum separately from one another using up to 1mL of buffer. The final volume should be adjusted to 100 mg tissue/1mL of buffer. Equal volumes of each should be extracted.

### Q: Should anything be added to the sample at the time of sample receipt to protect the RNA from degradation?

**A**: It is not necessary to add anything additional at the time of sample receipt to preserve the RNA as the commonly used buffers for inactivation and extraction (i.e., TRIzol, Zymo DNA/RNA Shield, Redbud Inactivation Buffer and Primestore MTM) will all protect the RNA. CDC shared that they have demonstrated that extracted rabies virus RNA has been shown to be stable at 4°C for up to six months. Zymo DNA/RNA shield has been shown to preserve rabies virus RNA for over 10 years when held at -80°C.

## Nucleic Acid Extraction and Virus Inactivation

### Q: Which extraction platforms and kits are used for LN34 or other rabies virus NAAT?

**A:** CDC performs manual extractions using either TRIzol or Zymo Directzol miniprep kit. For TRIzol extraction, 300 µL of TRIzol-homogenate is mixed with 300 µL of ethanol and added to a spin column. If Zymo DNA/RNA shield is used instead of TRIzol, ethanol should not be added to the homogenate. Successful extraction has also been demonstrated using automated extraction platforms and kits as shown below.

**Extraction kits:**

- Zymo: Quick RNA Miniprep
- Qiagen RNAeasy
- QIAmp Viral RNA Mini Kit
- EZ 1&2 Virus Mini Kit v2.0
- EZ1&2 RNA Tissue Mini Kit

**Automated Extraction Platforms:**

- Qiagen EZ1 Advanced XL with EZ 1&2 RNA Tissue Mini Kit (most commonly used)
- Qiagen Qiacube Connect
- bioMerieux Nuclisens EMAG
- IndiMag 48 with Qiagen RNAeasy
- Redbud NA1

### Q: Can CDC share their virus inactivation data?

**A**. Yes, CDC will share their inactivation data upon request via email.

### Q: What method(s) should be used for inactivating rabies virus?

A: Laboratories have reported successful virus inactivation using Zymo DNA/RNA Shield, Redbud NA1 Inactivation buffer and Primestore MTM with appropriate processing while maintaining RNA integrity.

### Q: How can a laboratory demonstrate inactivation in the absence of performing viral culture in-house?

**A.** Obtaining and reviewing published or shared inactivation data is a great place to start and might be sufficient for your laboratory director/safety and quality teams along with in-house validation. However, some laboratories may require proof of inactivation within your own facility and with your own staff. With limited access to rabies viral culture this may prove challenging and it is something APHL and CDC continue to work on.

### Q: Are there laboratories that can perform cell culture to demonstrate virus inactivation?

**A**: Yes, but there are very few laboratories with this capacity for rabies virus. APHL suggests coordinating within the Regional Consortia or establishing partnerships with other public health laboratories that may have this capacity.

# LN34 Protocol

## Q: Which PCR Platforms have been validated by CDC for LN34?

**A:** The LN34 assay is a laboratory developed test (LDT) so each laboratory will need to perform their own validations/verifications. The CDC has validated the LN34 protocol on the ABI7500 and the Via7. Other laboratories have validated the Thermo Fisher QuantStudio 5.

## Q: Has anyone compared the performance of the LN34 on the ABI 7500 versus other instruments?

**A:** While no formal analysis has been published, several laboratories reported very similar performance between the ABI7500 and the QuantStudio 5.

## Q: What are the Ct value cutoffs for the LN34 assay?

**A**: In the original LN34 protocol original LN34 assay, the Ct cutoff value for the rabies targets and β-actin from post-mortem animal samples is 35. CDC reported using an increased cutoff of 40 for non-brain post-mortem samples, ante-mortem samples, formalin fixed and other degraded samples.

## Q: Can laboratories implement the LN34 multiplex assay instead of the original LN34 assay?

**A**: Yes, laboratories may utilize the modified LN34 protocol for multiplexing. Note that the β-actin has a higher Ct value compared to the single-plex assay and CDC recommends increasing the β-actin cutoff to 37. Additionally, CDC is finalizing a multi-site evaluation of the multiplex assay which will be published upon completion.

## Q: Does the LN34 assay have to be run in triplicate?

**A**: The original LN34 assay was published and evaluated being performed with the LN34 assay run in triplicate and internal controls in a single well and CDC maintains this practice. While each laboratory can assess the assay in their validation, it is NOT recommended to run in a single well especially if the results will drive patient management.

## Q: Have any laboratories added a second rabies target to prevent false positives?

**A:** CDC has evaluated many versions of this assay and noted that adding additional targets within the LN34 assay can result in decreased sensitivity. There are other rabies NAAT methods that include more than one rabies virus target.

If laboratories are interested in decreasing false positivity, they can consider adding additional probes with either more diversity or degeneracy but so far, CDC has not identified a rabies virus with a mutation in the LN34 target area as it appears to be required for virus survival. If a second target is desired, it should either identify MOST rabies viruses OR be very degenerate and run in a separate reaction from the LN34 assay.

## Q: What is the typical turnaround time from necropsy to results with LN34?

**A:** This will be laboratory dependent and will vary by the number of samples being tested and the workflow. A reasonable estimate for processing, amplification and results generation is 2.5-3.5 hours if the testing had to be prioritized.

# Resulting and Reporting

## Q: How should laboratories handle discrepant results between DFA and NAAT?

**A**: If the appropriate brain sections are present and sampled, this should be a rare scenario but can occur and additional consultation and testing is recommended. It is possible early in a rabies virus infection that RNA may be detectable but DFA was not able to detect viral antigen early in an infection (Dettinger et al. 2022). Similarly, if a laboratory has a DFA positive and NAAT negative, the laboratory should troubleshoot the NAAT method and request additional consultation and testing if needed.

## Q: Can a negative PCR result be reported on samples in which the DFA shows non-specific staining?

**A:** In the proposed interim rabies diagnostic algorithm (APHL Rabies Molecular Testing Recommendations) the suggested language for reporting this would be "Unable to rule out Rabies Infection." Specifically, the document states the following: "Positive NAAT results can be reported as positive, but any other NAAT result should be reported as unsatisfactory and/or with a statement that rabies infection cannot be ruled out. Due to the absence of a minimum standard protocol for NAAT and lack of a PT program, these should not be reported as negative for rabies."

# Quality Control and Environmental Monitoring

## Q: How can laboratories minimize false positives and ensure accurate NAAT results?

**A**: Risk can be mitigated during necropsy by ensuring the use of new, clean instruments, changing gloves and disinfecting workspaces between samples so as not to carry positive material to a negative sample. Good molecular laboratory practice such as maintaining unidirectional workflow and adherence to quality control is critical.

The LN34 assay has a built-in internal control (β-actin) to confirm amplification occurred in a rabies-negative sample. Including a no-template and/or negative template control (NTC) with every run can help identify false positive results in the run.

## Q: What controls should be included for nucleic acid extractions and for each PCR setup?

**A**: Laboratories should follow best practices for molecular testing in your laboratory. This could include negative controls such as a no-template control (NTC), a known negative sample at extraction and PCR setup as well as a positive control such as a low positive sample (Ct 28-30) or a different target to demonstrate successful extraction and/or PCR amplification. Follow your laboratories standard practices for molecular testing and workflows.

## Q: Should laboratories perform environmental monitoring of their molecular/rabies laboratory?

**A:** Yes, it is good laboratory practice to perform environmental monitoring/swipe tests routinely (monthly or quarterly) to ensure there has been no contamination. Additionally, laboratory surfaces and instruments should be cleaned and disinfected regularly (see next question). Areas where molecular testing is set up or handled can also be treated with bleach or commercial products such as RNase away followed by ethanol after each PCR run to minimize the risk of environmental contamination from nucleic acids.

## Q: What disinfectants are utilized in the rabies laboratory?

**A**: CDC uses 1% Virkon. Other appropriate disinfectants are Micro-chem Plus by NCL (diluted 1:64, 2-minute disinfection), 10% chlorine bleach (sodium hypochlorite) solution made daily, Vesphene and Bacdown are also used in other laboratories.

# Staff Vaccination

## Q: Are molecular staff who perform rabies PCR outside the rabies laboratory required to be vaccinated and to comply with regular titer checks?

**A**: All laboratories should follow the recommendations in the Use of a Modified Preexposure Prophylaxis Vaccination Schedule to Prevent Human Rabies: Recommendations of the Advisory Committee on Immunization Practices for staff working with potential rabies positive samples. For staff performing PCR on inactivated samples outside of the rabies laboratory, vaccination should be assessed based on a comprehensive risk assessment and consultation with the biosafety and leadership team(s).

# Miscellaneous

## Q: Are laboratories returning samples to submitters or owners when testing is complete?

**A**: Samples are not usually returned to submitters or owners due to biosafety concerns. Samples are sometimes released through a third party (i.e., crematorium, animal protective foundation) after testing is complete and rabies has been ruled out.

This project was 100% funded with federal funds from a federal program of $2,178,609. This publication was supported by Cooperative Agreement #NU60OE000104 from the US Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of CDC.

# Appendix A: Rabies RNA Control

# Quotation Offer

**Seller**
Integrated DNA Technologies, Inc.
1710 Commercial Park
Coralville, IA 52241, USA
Phone: 1 800 328 2661

Quote Ref Number: **QTE-381987Q**
Quote Date: 4/18/2024
Pricing is Valid for 30 days. It will expire on 5/18/2024
All Prices are quoted in USD
**Payment Terms**
Purchase Order Number or Credit Card Net 30 Days

**Customer Information**
BOL, PA Dept. of Health
Attention to: Venkata Vepachedu
110 Pickering Way
EXTON, PA 19341
United States

**Customer Billing Address**
Pennsylvania Department of Health B
110 Pickering Way
Exton, PA 19341-1310
United States

**Quotation Summary**

| Line Item # | Name | Product | Quantity | Guarantee | Est. Lead Time (Business Days) | Line Item Price |
|---|---|---|---|---|---|---|
| 1 | LN34 Pos Control | Ultramer™ RNA Oligo, 80 nmol | 1 | 80 nmol | 5-12 | $2,400.00 USD |
| | Quantity: 80 nmol<br>Purification: Standard Desalting<br>Sequence: rCrGrA rCrUrC rArCrU rArUrA rGrGrG rCrGrA rArCrG rCrUrU rArArC rArArC rCrArG rArUrC rArArA rGrArA rArGrA rUrCrC rArGrU rCrGrG rGrArA rArCrC rUrGrU rCrGrU rGrCrU rCrUrA rArCrA rCrCrU rCrUrA rCrArA rUrGrG rArUrU rCrArG rArUrC rArGrU rArUrG rArGrU rArCrA rArGrU rArCrC rCrUrG rUrGrC | | | | |
| | | | | **Quote Subtotal:** | | **$2,400.00 USD** |
| | | | | **Est. Shipping and Handling Charges:** | | **$22.00 USD** |
| | | | | ***Ship full order when complete (single shipment)** | | |
| | | | | **Quote Total (not including applicable sales tax):** | | **$2,422.00 USD** |

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