Rabies LN34 PCR:
Frequently Asked Questions

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

Contents

Considerations for Implementation of Molecular Rabies Testing 1
CDC Support for LN34 2
Proficiency Testing 2
Assay Standardization 3
Assay Validation 3
Sample and Processing 3
Nucleic Acid Extraction and Virus Inactivation 4
LN34 Protocol 5
Resulting and Reporting 6
Quality Control and Environmental Monitoring 6
Staff Vaccination 7
Miscellaneous 7
Appendix A: Rabies RNA Control 8

Considerations for Implementation of Molecular Rabies Testing

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

A: Current APHL guidance is that 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 is negative or 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 for public health laboratory staff receiving member benefits. If you are unable to access these documents, ask your supervisor/director or email infectious.diseases@aphl.org.

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

A: LN34 primer and probe kits can be ordered from CDC by emailing a completed reagent order request form to referencereagents@cdc.gov. Available reagents include catalog numbers:

- KT0220 LN34 Pan-lyssavirus Assay Primer and Probe Set 2018
- KT0221 LN34 Positive Control RNA

These kits are provided for use in postmortem rabies diagnostic testing in animals and are provided for research use only 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 for technical assistance, comments or questions can be directed to 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. These are available under Catalog KT0220 LN34 Pan-lyssavirus Assay Primer and Probe Set 2018 and KT0221 LN34 Positive Control RNA and correspond to the protocol available on the APHL website. Interested groups can reach out to CDC if they are looking to validate the updated primers or multiplexed format in their laboratory.

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 and positive control RNA, subject to availability. CDC may also be able to provide blinded, inactivated RNA or sample panels (~approximately 5-12 samples; including positives and different variants) for assay validation or troubleshooting. CDC does not provide panels for proficiency testing, or reagents, consumables or equipment for performing routine PCR. Special requests, requests for technical assistance, questions or comments can be made 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.

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 to determine the limit of detection for the LN34 target; however, the panel provided by CDC is not ideal for this purpose because it includes host RNA matrix. Alternatively, a commercial RNA sequence matching the LN34 target sequence of a locally circulating rabies variant virus can be ordered (See Appendix A for an example quote). Another option is to order a DNA template for in vitro transcription which can be used to generate RNA in the laboratory. Custom RNA oligos can be purchased from several vendors. An example of a commercial RNA sequence matching the CDC-provided positive control sequence is attached for ease of use, however this is not an endorsement of that product by APHL, CDC or the US Government. 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, a negative DFA or PCR result should be reported as unable to rule out rabies infection. Positive results can be reported from suboptimal samples or samples where the required brain tissues are not available.

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. Laboratories should validate their NAAT extraction method against the LN34 protocol in their laboratory before implementation.

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 have not been sufficiently validated by the CDC using the currently distributed NAAT protocols. 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 a method that homogenizes tissues together, then extracting a representative subset of the required tissues. For livestock and other large brains, laboratories may consider separate extractions for brainstem and cerebellum.

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 for NAAT. However, the addition of RNA extraction buffer may render the tissue unsuitable for DFA. 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. Tissue homogenized in Zymo DNA/RNA shield or TRIzol reagent 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 TRIzol with the 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, the Zymo Quick RNA miniprep kit should be used. Successful extraction has also been demonstrated using automated extraction platforms and kits as shown below. Commercial names are provided for example purposes only and do not represent endorsement by APHL, CDC or the US Government. Validation and comparison data for some of these methods is not publicly available.

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 can share their inactivation data generated in the CDC rabies laboratory upon request via email. The performance characteristics as generated at CDC are provided by the Pox and Rabies Branch (PRB) Laboratory solely as an example for how the methods in the protocol performed within the PRB laboratory. The recipient laboratory is responsible for generating validation or verification data as applicable to establish performance characteristics as required by the testing laboratory's policies, applicable regulations and quality system standards.

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

A: Laboratories have reported successful virus inactivation using heat, TRIzol reagent, 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, contacting CDC 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 validation/verification. The CDC has validated the LN34 protocol on the ABI7500 fast and the ABI Viia7 and ABI QuantStudio 6 Flex. Other laboratories have validated the Thermo Fisher QuantStudio 5. While not formally 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, 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. The higher beta actin Ct value will mean additional testing may be required to confirm a percentage of negative samples 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, but they cannot detect all species of lyssavirus that cause rabies. The LN34 assay targets a highly conserved region of the lyssavirus genome and degenerate primers and three locked nucleotide probes to be able to detect all known lyssavirus species as described by Wadhwa et al. in 2017 and Gigante et al. in 2018. If a second target is desired, it should be run in a separate reaction from the LN34, or the entire assay will need to be repeated with diverse rabies virus variants and lyssavirus species.

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. Most commonly, this is due to a false positive DFA from non-specific staining. It may also be 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). If assistance is needed, contact your reference laboratory as stated in the Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing.

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

A: This decision is up to the discretion of the laboratory based on their in-house test validation requirements. 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.

© Copyright 2026, Association of Public Health Laboratories. All Rights Reserved.

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 Purification: Standard Desalting 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**

Place Order Now

IDT Terms and Conditions

IDT's Terms and Conditions are expressly incorporated by reference into and apply to any agreement between IDT and Customer. IDT's Terms and Conditions are the only terms and conditions upon which IDT will accept orders. Acceptance of any offer is limited to IDT's Terms and Conditions.
Any additional or different terms in Customer's form(s) are hereby deemed to be material alterations and notice of objection to them and rejection of them is hereby given. IDT's acceptance of any order is expressly conditional upon Customer's assent to IDT's Terms and Conditions, including any terms additional to or different from those contained in any form(s) from Customer.