

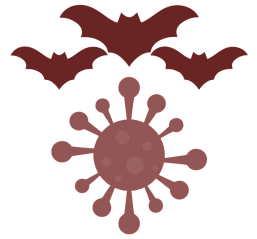


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🌐 RT-qPCR Detection of Nipah Virus (NiV) Targeting the Nucleocapsid (N) Gene

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We use this protocol and it's working

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Disclaimer

This protocol is provided for research, surveillance, preparedness, and laboratory capacity-building purposes. It describes a laboratory-developed RT-qPCR assay for detection of Nipah virus (NiV) RNA and has not been fully clinically validated across all specimen types, populations, or settings. Laboratories adopting this protocol are responsible for conducting appropriate local validation and verification in accordance with national regulations and institutional quality management systems.

The protocol has been optimized using specific reagents, extraction methods, and real-time PCR instruments. Use of alternative reagents, extraction methods, or instrumentation may be appropriate but requires local validation to ensure equivalent performance. All work involving specimens potentially containing NiV must comply with applicable biosafety, biosecurity, ethical, and regulatory requirements.

Results generated using this protocol should not be used as the sole basis for clinical diagnosis or public health decision-making and must be interpreted in conjunction with clinical, epidemiological, and laboratory information.

Abstract

The genus *Henipavirus* (family *Paramyxoviridae*) includes Hendra virus (HeV) and Nipah virus (NiV), highly pathogenic zoonotic viruses capable of causing severe respiratory and/or encephalitic disease in a broad range of mammalian species. Natural infections have been documented in humans, horses, pigs, dogs, and cats, while experimental infections have been studied in guinea pigs, golden hamsters, and ferrets. Old World fruit bats serve as the natural reservoir hosts, typically exhibiting subclinical infection. Among henipaviruses, NiV is a bat-borne zoonotic pathogen associated with particularly severe disease and high case-fatality rates in humans and animals. Here, we describe a one-step real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay for the detection and quantification of NiV RNA, targeting the nucleocapsid (N) gene.

This assay is a modified version of the method originally described by Feldman et al. (2009), optimized to improve analytical sensitivity and specificity. The RT-qPCR is suitable for detection of NiV RNA in a wide range of biological matrices, including clinical, animal, and environmental samples, when performed in laboratories with appropriate biosafety capacity for handling high-risk pathogens.

Viral RNA is extracted from a defined volume of sample using either the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) or the Quick-DNA/RNA Pathogen Miniprep Kit (Zymo Research, Irvine, CA, USA), according to the manufacturers' instructions. The assay combines reverse transcription and real-time PCR amplification in a single reaction using the SuperScript III Platinum One-Step Quantitative RT-PCR Kit (Invitrogen, Carlsbad, CA, USA) with primers and a modified probe adapted from Feldman et al. (2009). Each 25 μ L reaction contains the supplied reagents, primers, probe, and 5 μ L of extracted RNA template. Appropriate positive and negative controls are included in each run to ensure assay performance and result validity.

Guidelines

This protocol describes a one-step RT-qPCR assay for detection of Nipah virus (NiV) RNA, targeting the nucleocapsid (N) gene. It is intended for use by laboratories with appropriate molecular diagnostic expertise and biosafety authorization. Users should ensure that all reagents, equipment, and instruments are calibrated and validated according to institutional quality management systems prior to use.

Primer and probe sequences, reagents, and cycling conditions are based on a modified version of the assay described by Feldman et al. (2009). Laboratories implementing this protocol are encouraged to validate assay performance locally, including sensitivity, specificity, and control behavior, before routine diagnostic or surveillance use.

All work must be conducted in compliance with national regulations, institutional biosafety policies, and ethical approvals, as applicable. This protocol is suitable for qualitative detection of NiV RNA in clinical, animal, and environmental specimens when combined with appropriate controls and biosafety measures.



Troubleshooting

Problem

No amplification in positive control

Solution

Possible causes include degradation of reagents, improper storage of enzymes, incorrect master mix preparation, or incorrect cycling conditions. Verify reagent integrity and expiry dates, confirm that enzymes were kept cold during setup and returned promptly to $-20\text{ }^{\circ}\text{C}$, review master mix composition, and confirm that the correct cycling conditions were used. Repeat the run after corrections.

Problem

Amplification is observed in the no-template control (NTC)

Solution

This is most commonly due to carry-over contamination or contaminated reagents or consumables. Discard reagents and consumables used for the run, decontaminate work surfaces and pipettes, prepare fresh master mix in a clean area, and repeat the assay using new reagents and filtered pipette tips.

Problem

Amplification is observed in the negative extraction control (NC)

Solution

This may result from cross-contamination during extraction or sample handling. Review the extraction workflow, ensure strict separation between samples and controls, and confirm that pipette tips are changed between all steps. Repeat RNA extraction and RT-qPCR.

Problem

Late amplification ($C_t > 40$) is observed in the first RT-qPCR run

Solution

Possible causes include low RNA concentration, RNA degradation, or non-specific amplification. Repeat the RT-qPCR from the same RNA extract. If late amplification persists, re-extract RNA from the original specimen and review RNA handling and storage conditions.

Problem

C_t values are inconsistent between replicate reactions

Solution

This may be due to pipetting variability, insufficient mixing of reagents, or the presence of air bubbles. Ensure accurate pipetting, mix reagents gently but thoroughly, and briefly centrifuge the plate before amplification



Problem

Amplification curves are weak or abnormal in shape

Solution

This may be caused by suboptimal baseline or threshold settings or instrument-specific analysis parameters. Review and adjust analysis settings according to instrument guidance and confirm that threshold settings (e.g., RFU = 50 for HEX in this protocol) are appropriate for the instrument used.

Problem

Standard controls fail to meet acceptance criteria during quantitative runs

Solution

Possible causes include incorrect preparation of serial dilutions, pipetting errors, or degradation of standard material. Prepare fresh dilutions, verify concentrations, and repeat the quantitative run.

Problem

No amplification is observed in samples while all controls perform as expected

Solution

This may indicate a true negative result or poor sample quality. Confirm sample integrity and consider repeat extraction if sample quality is uncertain.

Safety warnings

 ! Nipah virus is a high-consequence pathogen.

Improper handling of specimens suspected to contain NiV may pose a serious risk to laboratory personnel and public health.

 ! Do not perform this protocol without appropriate authorization.

Users must comply with all country-specific biosafety, biosecurity, and ethical requirements prior to handling specimens.

 ! Live virus work is not described here.

This protocol is limited to molecular detection following appropriate specimen inactivation. Virus isolation or propagation requires higher containment and is outside the scope of this protocol.

 ! Results should be interpreted cautiously.

False-negative results may occur due to improper specimen collection, transport, storage, or degradation of RNA. All results must be evaluated in conjunction with appropriate controls and clinical or epidemiological information.

Before start

Before starting this protocol, laboratories should conduct a documented, activity-specific biosafety risk assessment, in line with the WHO Laboratory Biosafety Manual, 4th Edition (2020). Nipah virus is generally considered a Risk Group 4 pathogen, and the appropriate containment, inactivation strategy, and workflow must be determined locally based on the nature of the specimens and procedures performed.

Ensure that:

- Personnel are trained and authorized to perform high-risk molecular diagnostics;
- Validated specimen inactivation and RNA extraction procedures are in place;
- Required reagents, primers, probes, and controls are available and within expiry;
- Appropriate positive, negative, and no-template controls are included in each run.

This protocol should only be implemented in laboratories with biosafety infrastructure and oversight and approval from relevant competent authorities.

Disclaimer and Limitations of Use

- 1 This protocol describes a laboratory-developed RT-qPCR assay for the detection of Nipah virus (NiV) RNA and is provided for research, surveillance, preparedness, and laboratory capacity-building purposes. While the assay has been optimized and routinely performed in our laboratory setting, **it has not been fully clinically validated** across diverse populations, specimen types, and epidemiological contexts.



Performance characteristics such as analytical sensitivity, specificity, limit of detection, reproducibility, and robustness may vary depending on specimen quality, extraction methods, instrumentation, reagents, and laboratory workflows. Laboratories intending to implement this protocol are responsible for conducting appropriate local validation and verification studies in accordance with national regulations, institutional policies, and applicable quality management systems before use for diagnostic or clinical decision-making.

Results generated using this protocol should not be used as the sole basis for clinical diagnosis, patient management, or public health action without appropriate confirmation and interpretation in conjunction with clinical, epidemiological, and laboratory findings. Confirmatory testing using alternative methods, repeat sampling, or referral to reference laboratories may be required, particularly in settings involving suspected or confirmed Nipah virus infection.

The authors and contributors make no warranties regarding the performance or suitability of this protocol for any specific purpose. Use of this protocol implies acceptance that implementation occurs at the user's own responsibility, subject to local biosafety, biosecurity, ethical, and regulatory approvals. All work involving specimens potentially containing Nipah virus must comply with applicable national and international biosafety guidance.

This protocol may be updated as additional validation data, reference materials, or methodological improvements become available.

Note

This RT-qPCR assay detects Nipah virus RNA and does not distinguish between infectious and non-infectious virus, nor between genomic RNA and subgenomic transcripts. A negative result does not exclude infection if viral RNA levels are below the limit of detection or if sampling, transport, or storage conditions were suboptimal.

Background

- 2 Nipah virus (NiV; species *Henipavirus nipahense*) is an enveloped, negative-sense, single-stranded RNA virus belonging to the genus *Henipavirus* within the family *Paramyxoviridae*. NiV is a bat-borne zoonotic pathogen that causes sporadic outbreaks of severe disease in humans and animals, with reported case-fatality rates typically ranging from 40–75%, depending on outbreak context and healthcare capacity. Human infection may present as asymptomatic or mild illness, acute respiratory disease, or rapidly progressive and often fatal encephalitis. Limited human-to-human transmission has been documented, particularly among close contacts and in healthcare settings. Due to its high mortality, lack of licensed vaccines for routine use, and epidemic potential, Nipah virus is classified as a priority pathogen by the World Health Organization.

Reservoirs, transmission, and geographic distribution

Fruit bats of the genus *Pteropus* are the primary natural reservoirs of NiV. Spillover to humans has been associated with exposure to bat-contaminated food products, direct contact with infected animals (notably pigs during the initial Malaysia–Singapore outbreak), and subsequent human-to-human transmission. Human outbreaks have been reported primarily in South and Southeast Asia, with recurrent events in Bangladesh and India, including outbreaks involving healthcare workers. Beyond areas with confirmed human disease, NiV or NiV-like viruses have been detected in bat populations across a wider geographic range. In Cambodia, Nipah virus has been isolated from *Pteropus lylei*, and viral RNA has been detected in bat excreta, indicating that the ecological and virological prerequisites for spillover are present even in the absence of documented human infection. This highlights the importance of sensitive molecular diagnostics to support early detection and preparedness.

Virology and genome organization

The NiV genome is a non-segmented, negative-sense RNA genome of approximately 18 kb, making it substantially longer than those of many other paramyxoviruses. It encodes six major structural proteins: N (nucleocapsid), P (phosphoprotein), M (matrix), F (fusion), G (attachment glycoprotein), and L (RNA-dependent RNA polymerase). The P gene additionally encodes accessory proteins (C, V, and W) that contribute to immune evasion and pathogenicity.

Rationale for molecular detection targeting the N gene

The N gene protein plays a central role in the viral life cycle by encapsidating the genomic RNA and forming the ribonucleoprotein complex required for transcription and replication. As a result, N gene transcripts are produced at high levels in infected cells, particularly during the early stages of infection. This high transcriptional abundance, combined with strong sequence conservation across known NiV

lineages, makes the N gene a key molecular target for diagnostic detection. RT-PCR and real-time qRT-PCR assays targeting the N gene have demonstrated excellent analytical sensitivity, allowing detection of low viral RNA concentrations in a variety of specimen types, including whole blood or serum, cerebrospinal fluid (CSF), urine, and respiratory secretions.

Note

A recognized limitation of N-gene-based detection is that assays do not distinguish between genomic viral RNA and subgenomic N mRNA transcripts, which can complicate precise viral load quantification.

Diagnostic and Biosafety Considerations

- 3 Work involving Nipah virus (NiV) or potentially NiV-containing specimens requires careful consideration of biosafety risks to protect laboratory personnel, the community, and the environment. NiV is a high-consequence zoonotic pathogen with documented laboratory exposure risks; therefore, all activities must be conducted in accordance with national regulations, institutional biosafety policies, and international guidance.**



Laboratories considering implementation of this protocol are strongly advised to conduct a documented, activity-specific biosafety risk assessment prior to use. In line with the WHO Laboratory Biosafety Manual, 4th Edition (LBM4, 2020), the appropriate biosafety measures should be determined based on the pathogen, the nature of the procedures performed, the type of specimens handled, and the competency and training of personnel, rather than by default assignment of a fixed biosafety level.

Nipah virus is generally classified as a Risk Group 4 pathogen, and work involving live virus or virus propagation typically requires maximum containment. However, LBM4 recognizes that certain activities such as molecular detection performed on appropriately inactivated specimens may be managed using risk-appropriate heightened control measures, provided that inactivation procedures are validated and effective. *Users should consult and comply with national biosafety and biosecurity regulations, institutional biosafety committees, and relevant competent authorities to determine the acceptable containment and control measures for their specific setting.*



Preparation of Sample for Extraction

- 4 Samples are prepared for RNA extraction using the **QIAamp Viral RNA Mini Kit (Qiagen)** or the **Quick-DNA/RNA Pathogen Miniprep Kit (Zymo Research)**, following the manufacturers' instructions. Extracted RNA (or total nucleic acid, as applicable) is used directly for downstream RT-qPCR detection of NiV.

Note

Extracted RNA should be processed as soon as possible. If RNA has been previously frozen, it should be thawed on ice and analysed immediately to minimize degradation.

Note

Alternative RNA extraction methods or kits may also be suitable for downstream RT-qPCR detection of Nipah virus (NiV); however, any alternative method must be validated locally by the implementing laboratory prior to use. Validation should confirm that the extraction method provides RNA of sufficient quality and yield, does not introduce PCR inhibition, and produces consistent assay performance with respect to sensitivity, specificity, and control behavior. The choice of extraction method should also be compatible with institutional biosafety requirements and approved through local risk assessment and quality management processes.

Preparation and Equipment Setup

- 5 Prepare a clean work area dedicated to RNA handling and RT-qPCR setup, following laboratory contamination-control practices.
- 6 Ensure that all required equipment (pipettes, tube racks, centrifuge, vortex, and real-time PCR instrument) is available, functional, and calibrated according to institutional quality procedures.
- 7 Complete the RT-qPCR plate worksheet and determine the total number of reactions (N), including samples, positive controls, and no-template controls.
- 8 Calculate the total volume of RT-qPCR master mix required based on the number of reactions (N), including an appropriate excess to account for pipetting error, according to laboratory practice.



- 9 Remove required RT-qPCR reagents from refrigerated or frozen storage and allow them to thaw on ice or at 2–8 °C.

Note

Enzyme components must be removed from the –20 °C freezer only immediately before use, kept on ice at all times, and returned promptly to –20 °C after use.

- 10 Defrost positive control and any required samples and maintain them on ice.

Prepare Primers and Probe

11

	A	B
	Name	Sequence
	NFWD	GATATITTT GAMGAGGC GGCTAGTT
	NREV2	TCCCATCT GAGCTCTG GACTATTAG T
	NPRNiV- HEX	HEX- GCAACTGC TACTTTGA CAACC- BHQ2

Table 1: Primer and probe sequences used for RT-qPCR detection of Nipah virus (NiV). Primer and probe sequences target the nucleocapsid (N) gene of NiV and are shown in the 5'–3' orientation. All oligonucleotides are used at a working concentration of 10 µM.

Note

Primer and probe sequences, reagents, and cycling conditions are based on a modified version of the assay described by Feldman et al. (2009):

<https://www.sciencedirect.com/science/article/pii/S0166093409002481>



- 12 Primers and probe should be reconstituted and diluted to a working concentration of 10 μM using RNase/DNase-free water or buffer, according to manufacturer recommendations. Aliquots should be prepared to minimize freeze–thaw cycles and stored at temperatures appropriate for oligonucleotides.
- 13 Before use, primers and probe should be thawed on ice, mixed gently, and briefly centrifuged to collect contents at the bottom of the tube.

Master Mix Preparation (Clean Room)

- 14 Perform master mix preparation in a clean area designated for RT-qPCR setup, following laboratory practices to prevent contamination.
- 15 Thaw all working solutions of reagents and maintain them on ice, except **Super Script III RT/Platinum Taq DNA polymerase** (Enzyme) which should be taken out of the -20°C freezer just before use.
- 16 Mix and Spin down the reagents using the micro-centrifuge before opening the tubes. (DO NOT vortex the enzyme).
- 17 Prepare the RT-qPCR master mix according to the volumes listed below, based on the total number of reactions (N) calculated previously. Include an appropriate excess volume to account for pipetting variability.

	A	B	C
	Reagent	Working Solution	Volume (μL)
	2X Reaction Mix	2x	12.5
	MgSO ₄ 50mM	-	1
	NFWD	10 μM	1
	NREV2	10 μM	1
	NPRNiV-HEX	10 μM	0.5
	RNAasin 20-40U/ μl	-	0.3
	SuperScript III/Platinum Taq DNA polymerase	-	0.5
	Nuclease Free-water	-	3.2
	Total		20

Table 2: Composition of the RT-qPCR master mix for detection of Nipah virus (NiV). Volumes are given per reaction for a final reaction volume of 25 μ L (including RNA template) and are based on a one-step RT-qPCR assay targeting the NiV nucleocapsid (N) gene.

- 18 Mix the master mix gently by pipetting up and down or brief vortexing, then centrifuge briefly to remove air bubbles.

Preparation of RNA and Control Samples (Extraction Room)

- 19 Retrieve extracted RNA samples from refrigerated or frozen storage and keep them on ice throughout setup.
- 20 If RNA samples are frozen, thaw them on ice and briefly centrifuge to collect contents at the bottom of the tube.
- 21 Gently mix RNA samples by pipetting or brief vortexing, followed by a short centrifugation to remove bubbles.
- 22 Arrange RNA samples, positive controls, and no-template controls in clearly labeled tubes or wells according to the completed plate layout.
- 23 Minimize RNA exposure time at room temperature and proceed directly to RT-qPCR reaction setup once samples and reagents are ready.

Addition of RNA (Extraction Room)

- 24 Perform RNA addition in a laboratory area designated for handling extracted nucleic acids, physically separated from reagent preparation and post-amplification areas, in accordance with contamination-control practices.
- 25 Ensure that the RT-qPCR reaction plate or tubes containing aliquoted master mix are clearly labeled and arranged according to the completed plate worksheet.
- 26 Keep extracted RNA samples on ice throughout the procedure.
- 27 Briefly centrifuge RNA samples prior to opening to collect contents at the bottom of the tube.



- 28 Using filtered pipette tips, dispense **20 µL of RT-qPCR master mix** into each designated reaction well or tube corresponding to the total number of planned reactions.
- 29 Using a new filtered pipette tip, add **5 µl** of RNA from sample (S), negative extraction control (NC) and distilled water for template control (NTC), into its designated well. Discard the pipette tip.

Note

Avoid touching the pipette tip to the sides of wells when dispensing RNA or control material.

- 30 For viral RNA qualitative detection, add **5 µl** of RNA NiV positive control (PC).

Note

Positive controls are essential to verify assay performance, reagent integrity, and amplification efficiency. For this RT-qPCR assay targeting the Nipah virus (NiV) nucleocapsid (N) gene, several types of positive control materials may be used, provided they have been appropriately validated and are handled in accordance with institutional biosafety requirements. Acceptable positive controls include extracted RNA from previously confirmed NiV-positive clinical or animal specimens, RNA extracted from inactivated NiV cell culture supernatants, and nucleic acid derived from plasmids or synthetic constructs containing the NiV N-gene target sequence. RNA-based controls most closely reflect clinical samples and are therefore preferred for qualitative detection workflows, while plasmid or synthetic DNA controls provide stable, quantifiable reference material and are commonly used for assay standardization and quantitative applications. When RNA derived from cell culture supernatants is used, it should be prepared at a validated working dilution (e.g., 10^{-3}) and fully inactivated prior to downstream processing. The choice of positive control should be guided by the intended application (qualitative versus quantitative detection), availability of reference material, and biosafety considerations, and should be validated locally to ensure consistent Ct values and reproducible assay performance.

- 31 Once all RNA templates and controls have been added, seal the plate immediately using appropriate optical sealing film or caps.
- 32 Briefly centrifuge the sealed plate to collect liquid at the bottom of the wells and remove air bubbles at 1500 rpm for 2 minutes.

- 33 Proceed directly to RT-qPCR amplification. If a delay is unavoidable, keep the sealed plate on ice or at 2–8 °C until loading into the real-time PCR instrument.

RT-qPCR Amplification and Cycling Conditions (PCR Room)

- 34 Transfer the sealed RT-qPCR plate or reaction tubes to the PCR room or designated amplification area, following laboratory workflows that maintain separation between pre- and post-amplification activities.
- 35 Switch on the real-time PCR instrument and allow it to complete any required startup or calibration checks according to the manufacturer's instructions.

Note

This protocol has been optimized and routinely performed using **Bio-Rad CFX and Opus real-time PCR instruments** with detection settings compatible with the probe reporter dye specified in this assay. While the assay is expected to be broadly transferable to other real-time PCR platforms, laboratories using alternative instruments should perform full local validation prior to implementation. This includes verification of cycling conditions, fluorescence detection channels, baseline and threshold settings, and overall assay performance (e.g., sensitivity, specificity, and control behavior). Differences in instrument optics, thermal uniformity, and data analysis algorithms may affect assay performance, and appropriate optimization and validation are required to ensure reliable results.

- 36 If required, switch on the associated computer and launch the instrument control software. Allow the system to complete startup checks and establish communication between the instrument and software.
- 37 Confirm that the instrument has completed initialization and any required calibration or self-checks before proceeding.
- 38 Confirm that the correct detection channel is selected for the probe reporter dye (HEX) and that passive reference settings, if applicable, are configured according to instrument requirements.
- 39 Define the plate layout by assigning sample identifiers and control types (samples, negative extraction control, no-template control, positive control, and standards if applicable) to the corresponding wells, according to the completed RT-qPCR plate worksheet.

40 Verify that all sample and control positions in the software match the physical layout of the PCR plate before starting the run.

41 Program or confirm the RT-qPCR cycling conditions as follows:

Step	Temperature (°C)	Time	Cycles	Data acquisition
Reverse transcription	50	30 min	1	No
Initial denaturation	95	2 min	1	No
Denaturation	95	15 s	45	No
Annealing/extension	60	30 s	45	Yes

Table 3. RT-qPCR cycling conditions for detection of Nipah virus (NiV) targeting the nucleocapsid (N) gene. Fluorescence data are acquired during the annealing/extension step using the HEX reporter dye.

42 Confirm that the total number of amplification cycles is set to 45 and that fluorescence data acquisition is enabled during the annealing/extension step.

43 Place the sealed PCR plate or tubes into the real-time PCR instrument, ensuring correct orientation and secure placement.

44 Review all run parameters, including plate layout, cycling conditions, detection channels, and sample identifiers, before starting the run.

45 Start the RT-qPCR run and monitor the instrument during initiation for any error messages.

46 Upon completion of the run, save the raw data, amplification plots, and run files according to laboratory data management practices.

47 Do not open reaction plates or tubes in the PCR room after amplification. Dispose of amplified material according to institutional procedures for post-amplification waste.

Interpretation of Results

48 **For NiV RNA qualitative detection**

48.1 The PCR is **positive** if:

- A clear amplification curve is observed with a Cq value below 40 cycles.
- The fluorescence threshold has been set appropriately.
- All positive controls amplify as expected within the laboratory-defined acceptable Cq range.
- All negative controls, including the negative extraction control and the no-template control, show no amplification (no Cq or undetermined).

Note

The fluorescence threshold has been set appropriately. In our laboratory and on our instruments, the threshold is set at RFU = 50 for the HEX reporter dye. Laboratories using different instruments or analysis software should establish and validate their own threshold settings.

If any positive control fails to amplify or if any negative control shows amplification, the run must be considered invalid and results must not be reported. Samples with late or borderline amplification near the cutoff should be interpreted with caution and may require repeat testing or re-extraction according to laboratory policy.

48.2 The PCR is **negative** if:

- No amplification is observed, or the Ct value exceeds 40 cycles after a second repeat RT-qPCR run.
- The fluorescence threshold has been set appropriately.
- All positive controls amplify as expected within the laboratory-defined acceptable Ct range.
- All negative controls, including the negative extraction control and the no-template control, show no amplification.

If control performance does not meet acceptance criteria, the run must be considered invalid and results must not be reported.

48.3 The PCR **cannot be interpreted** and has to be repeated, if:

- Amplification is observed with a Ct value exceeding 40 cycles in the first RT-qPCR run.
- The fluorescence threshold has been set appropriately.
- The positive control fails to amplify.
- Any negative control, including the negative extraction control or the no-template control, shows amplification, which is indicative of potential carry-over contamination.

In these situations, **results must not be reported**. The assay should be repeated after identifying and correcting the underlying cause, such as reagent issues, contamination, or technical error.

49 For NiV RNA quantification

A standard graph of the cycle threshold (Cq) values obtained can be obtained using a serial dilution of a constructed plasmid. The Cq values from unknown samples can be plotted on the respective standard curve, and the copy number of NiV RNA calculated.

When using the BioRad CFX 96/Opus, the detailed interpretation of results is provided below:

49.1 Quantitative results can only be obtained if:

Ct value of samples is within the range of control standards.

All negative controls are negative.

Efficiency of the assay should be 90–105%,

R^2 value (correlation coefficient) ≥ 0.98

Slope value is within the range between -3.1 and -3.6.

49.2 Repeat the assay if:

Cq value of sample is outside the range of control standards.

Negative controls are positive.

R^2 value (correlation coefficient) < 0.98

Slope value is outside the range between -3.1 and -3.6.

49.3 Copy number calculation:

The NiV viral load in sample is expressed as NiV copies per reaction or milliliter (ml).

Formula: number of NiV copies per ml = number of copies calculated by the machine x 12 (multiplicative factor from 5 μ l of extracted RNA to 60 μ l elution volume, equal 140 μ l of sample) x 7.14 (multiplicative factor from 140 μ l of sample to 1ml)

49.4 Method limitation:

Limit of detection: ranging from **5-50 copies per reaction**

Protocol references

Adapted from: K.S. Feldman, A. Foord, H.G. Heine, I.L. Smith, V. Boyd, G.A. Marsh, J.L.N. Wood, A.A. Cunningham, L.-F. Wang,

Design and evaluation of consensus PCR assays for henipaviruses,

Journal of Virological Methods,

Volume 161, Issue 1,

2009,

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(<https://www.sciencedirect.com/science/article/pii/S0166093409002481>)

Abstract: Henipaviruses were first discovered in the 1990s, and their potential threat to public health is of increasing concern with increasing knowledge. Old-world fruit bats are the reservoir hosts for these viruses, and spill-over events cause lethal infections in a wide range of mammalian species, including humans. In anticipation of these spill-over events, and to investigate further the geographical range of these genetically diverse viruses, assays for detection of known and potentially novel strains of henipaviruses are required. The development of multiple consensus PCR assays for the detection of henipaviruses, including both SYBR Green and TaqMan real-time PCRs and a conventional heminested PCR is described. The assays are highly sensitive and have defined specificity. In addition to being useful tools for detection of known and novel henipaviruses, evaluation of assay efficiency and sensitivity across both biological and synthetic templates has provided valuable insight into consensus PCR design and use.

Keywords: Henipavirus; PCR; SYBR Green; Consensus PCR

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