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Sequencing of Nipah Virus (NiV) Using Oxford Nanopore MinION

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Protocol status: Working

This protocol has been implemented in routine laboratory workflows and has consistently produced high-quality sequencing data.

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Disclaimer

This protocol is provided for research, surveillance, preparedness, and laboratory capacity-building purposes. It describes a laboratory-developed sequencing workflow for Nipah virus (NiV) and has not undergone full clinical validation. Performance may vary depending on sample type, laboratory conditions, reagents, instrumentation, and operator experience.

Laboratories implementing this protocol are responsible for conducting appropriate local verification and validation in accordance with national regulations, institutional policies, and quality management systems. This protocol is not intended to replace authorized diagnostic procedures or reference laboratory confirmation.

All work involving specimens potentially containing Nipah virus must be conducted in compliance with applicable biosafety, biosecurity, ethical, and regulatory requirements. Results generated using this protocol should be interpreted in conjunction with epidemiological, clinical, and other laboratory information.

Abstract

This protocol describes an amplicon-based whole-genome sequencing approach for Nipah virus (NiV) using the Oxford Nanopore MinION platform. It employs a tiled amplicon system, using overlapping primer sets designed to amplify conserved regions across the NiV genome. The protocol is designed to support genomic characterization of Nipah virus belonging to both the Bangladesh lineage and the Malaysia lineage and is applicable to RNA extracts derived from clinical, animal, or environmental specimens. The workflow enables generation of viral genome sequence data for use in molecular epidemiology, phylogenetic analysis, and surveillance activities. This protocol is intended for research, preparedness, and capacity-building purposes and assumes implementation within laboratories operating under appropriate biosafety oversight and local validation frameworks.

Guidelines

This protocol is intended for use by laboratories with experience in molecular virology, genomic sequencing, and the handling of high-consequence pathogens. Nipah virus is generally classified as a Risk Group 4 pathogen, and implementation of this protocol must be conducted under national and institutional biosafety and biosecurity frameworks, following a documented risk assessment consistent with the WHO Laboratory Biosafety Manual (4th Edition).

The protocol assumes that sequencing activities are performed on appropriately inactivated RNA or downstream nucleic acid products and does not describe work involving live virus. Laboratories must ensure that specimen collection, transport, inactivation, extraction, and downstream processing are conducted in accordance with local regulatory approvals, ethical requirements, and containment practices appropriate to the assessed risk.

This amplicon-based ARTIC-style sequencing approach is designed to detect and sequence Nipah virus belonging to both the Bangladesh and Malaysia lineages by targeting conserved genomic regions using overlapping primer sets. Laboratories adopting this protocol should verify primer performance against locally relevant strains and remain aware that ongoing viral evolution may necessitate primer review and updates.

All reagents, instruments, software, and analysis pipelines used with this protocol should be validated locally prior to routine implementation. Results generated using this protocol are intended to support research, surveillance, and preparedness activities and should be interpreted in conjunction with epidemiological, clinical, and other laboratory data.



Materials

Reagents

- Nuclease-free water, RNase/DNase-free (Ambion, cat. no. AM9935)
- Linear acrylamide (LA), 5 mg/mL (Ambion)
- 5× SuperScript IV Reaction Buffer
- Dithiothreitol (DTT), 0.1 M
- dNTP mix, 10 mM
- RNase inhibitor: SUPERase•In (Ambion, cat. no. AM2694) or RNaseOUT
- SuperScript IV Reverse Transcriptase (Invitrogen, cat. no. 1809050)
- Random primers (mostly hexamers) (Invitrogen, cat. no. 48190-011)
- Elution Buffer (10 mM Tris-HCl, pH 8.5; EB)
- Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, cat. no. M0493S)
- NEBNext Ultra II End Prep Module (New England Biolabs, cat. no. E7546S)
- NEBNext Quick Ligation Module (New England Biolabs, cat. no. E6056S)
- Native barcoding and ligation reagents (Oxford Nanopore Technologies, SQK-LSK114 or compatible native barcoding chemistry)
- AMX, LNB, SFB, EB, and SQB buffers (Oxford Nanopore Technologies, supplied with SQK-LSK114)
- Flow Cell Priming Kit (Oxford Nanopore Technologies, cat. no. EXP-FLP002)
- MinION R10.x Flow Cells (e.g. R10.4.1; Oxford Nanopore Technologies, cat. no. FLO-MIN114)

Consumables

- Low-bind microcentrifuge tubes (1.5 mL and 2.0 mL)
- Low-bind PCR tubes or 96-well PCR plates
- Filtered pipette tips (10 µL, 200 µL, 1000 µL)
- Agarose
- DNA gel stain
- DNA size ladder (100 bp)
- Magnetic bead-compatible tubes or plates
- Disposable gloves
- Laboratory wipes and decontamination wipes
- Freshly prepared ethanol (70% and 80%, molecular biology grade)

Equipment

- Class II biosafety cabinet (as required by institutional biosafety policy)
- Microcentrifuge
- PCR thermocycler with heated lid
- Agarose gel electrophoresis system
- Gel imaging system
- Magnetic separation rack (for tubes or plates)
- Fluorometric DNA quantification instrument (e.g. Qubit or equivalent)
- MinION sequencing device
- Computer workstation compatible with MinKNOW and downstream data analysis software



Note

This protocol has been validated using the reagents, consumables, and equipment listed above. Equivalent products may be used if validated to perform comparably, particularly when substituting sequencing kits or flow cell chemistries.



Troubleshooting

Problem

Low or no cDNA yield

Solution

Confirm RNA integrity and RT-qPCR Ct value prior to cDNA synthesis. Verify that RNA dilution was applied correctly for low Ct samples to reduce inhibition. Ensure reverse transcription reagents are not expired and were stored correctly. Minimize RNA handling time and avoid repeated freeze–thaw cycles.

Problem

PCR amplification failure in one or more primer pools

Solution

Check cDNA input quality and volume. Confirm correct primer pool preparation and labeling. Verify that PCR reagents were added correctly and mixed thoroughly. If amplification is weak, confirm that Ct values fall within the recommended range and that RNA dilution was applied where appropriate.

Problem

Uneven amplification across primer pools

Solution

Review primer pool preparation and confirm correct working concentrations. Ensure equal cDNA input was added to each pool. Uneven coverage may reflect sample quality or amplicon dropout rather than a procedural error.

Problem

No visible band on agarose gel

Solution

Do not proceed with library preparation for that sample. Confirm PCR setup and cycling conditions. For critical samples only, a repeat PCR with increased cycle number may be considered. Samples with no detectable amplification are unlikely to yield usable sequencing data.

Problem

Very faint bands on gel

Solution

Proceed with caution. Faint bands may still produce usable libraries but often result in reduced yield or incomplete coverage. Document this observation and consider re-amplification if the sample is critical.

Problem



Low DNA concentration after amplicon cleanup

Solution

Confirm bead ratios and ethanol wash steps were performed correctly. Ensure beads were fully resuspended during binding and elution. Very low yields may reflect weak upstream amplification rather than cleanup failure.

Problem

Beads difficult to resuspend or cracked after cleanup

Solution

Avoid over-drying beads during cleanup steps. Elute promptly once beads lose surface sheen. If beads appear cracked, resuspension efficiency and DNA recovery may be reduced.

Problem

Inaccurate or inconsistent DNA quantification

Solution

Ensure quantification reagents are equilibrated to room temperature and used within their linear range. Dilute highly concentrated samples prior to measurement. Avoid relying on spectrophotometric methods alone for low-concentration DNA.

Problem

Poor barcode ligation or low pooled library yield

Solution

Confirm barcode reagents were added correctly and mixed thoroughly. Ensure the minimum recommended number of barcoded samples per library is met. Low input DNA may reduce ligation efficiency.

Problem

Excess adapter or barcode carryover

Solution

Confirm that Small Fragment Buffer (SFB) was used for cleanup steps where specified and that bead ratios were not increased. Do not substitute ethanol for SFB at adapter-cleanup stages.

Problem

Low final library concentration

Solution

Review cumulative losses across cleanup steps and confirm starting amplicon input was sufficient. Libraries with lower-than-expected yield may still be sequenced but often produce reduced coverage.



Problem

Low number of active pores on flow cell

Solution

Perform a flow cell check prior to loading. Ensure correct priming and avoid introducing air bubbles. If pore count is low, adjust expectations for data yield or consider using a different flow cell.

Problem

Poor sequencing yield or slow data generation

Solution

Confirm correct flow cell type and kit selection in MinKNOW. Ensure library was mixed with loading beads immediately prior to loading. Review pore activity and sequencing speed during the run.

Problem

Uneven genome coverage during run monitoring

Solution

Continue sequencing to increase depth if pores remain active. Review amplification performance of affected primer pools post-run. Uneven coverage is commonly linked to amplicon dropout rather than sequencing failure.

Problem

Insufficient genome coverage despite long run time

Solution

Stop the run once coverage gain plateaus. Flag affected samples for potential re-sequencing and review upstream steps (Ct value, PCR amplification, pooling accuracy).

Problem

Unexpected barcode cross-talk or misassignment

Solution

Verify correct barcode assignment during demultiplexing. Confirm barcode kit compatibility and cleanup performance. Low input DNA or excess free barcode may increase misassignment risk.

Problem

Failure to generate consensus sequence

Solution

Review coverage breadth and depth thresholds. Partial consensus sequences may be generated for samples with incomplete coverage. Document limitations and exclude samples that do not meet predefined acceptance criteria.

Safety warnings

 This protocol involves amplification-based sequencing and carries an inherent risk of amplicon contamination. Strict physical separation of pre-amplification and post-amplification areas must be maintained, and laboratory workflows should be designed to minimize the risk of cross-contamination.

Nipah virus is generally considered a high-risk pathogen. Although this protocol does not involve live virus, all work with specimens potentially containing Nipah virus or derived nucleic acids must comply with applicable national biosafety and biosecurity regulations and institutional risk assessments.

Failure to adhere to appropriate contamination control practices may result in false-positive results or compromised sequencing data. Users should follow good molecular biology practices, including appropriate use of controls, careful handling of amplified material, and routine decontamination of work surfaces and equipment.

Sequencing performance and genome recovery are highly dependent on sample quality, viral RNA concentration, and correct execution of the workflow. Poor-quality samples or deviations from recommended practices may result in incomplete genomes or failed sequencing runs.

This protocol has not been fully clinically validated and should not be used as the sole basis for clinical diagnosis or public health decision-making without appropriate authorization and confirmation through reference laboratory methods.

Ethics statement

This protocol may involve the use of human-derived specimens. Any work involving human subjects or human samples must be conducted in accordance with applicable local, national, and international regulations. Prior to implementation, informed consent must be obtained from participants where required, and the study must receive approval from the user's Institutional Review Board (IRB) or equivalent ethics committee(s). Users are responsible for ensuring that all ethical approvals and permissions are in place before applying this protocol.



Before start

Ensure that all required ethical, regulatory, and institutional approvals are in place prior to initiating this protocol. Laboratories should confirm that personnel involved in the work are appropriately trained in molecular biology techniques, genomic sequencing workflows, and biosafety practices relevant to high-consequence pathogens.

Verify that RNA extracts or downstream nucleic acid products have been prepared using approved methods and are suitable for sequencing. Sample quality, integrity, and viral RNA concentration will directly influence genome recovery and sequencing success. This protocol is best applied to samples with sufficient viral RNA to support amplicon-based sequencing.

Confirm availability of all required reagents, consumables, and equipment, and check that sequencing flow cells and library preparation reagents meet manufacturer quality specifications. Ensure that computational infrastructure for sequencing control, basecalling, and downstream analysis is available and functioning prior to starting the workflow.

Review laboratory workflow layout to maintain appropriate separation between pre-amplification and post-amplification areas, and implement measures to minimize the risk of cross-contamination. Familiarize all users with the overall workflow, stopping points, and quality control checkpoints before beginning the protocol.

Purpose and Scope

- 1 This protocol describes the sequencing of Nipah virus (NiV) RNA using Oxford Nanopore Technologies (ONT) MinION sequencing for the purpose of genomic characterization, lineage assignment, and molecular epidemiology.

The protocol is intended for use on RNA extracted from clinical or environmental specimens that have been confirmed positive for NiV by RT-qPCR. Sequencing is performed as a downstream application following molecular detection and is not intended for primary diagnostic use.

This method enables:

Generation of partial or near-complete NiV genome sequences

Assessment of viral genetic diversity

Support for outbreak investigation, surveillance, and research activities

All laboratory work described in this protocol must be conducted in accordance with institutional biosafety requirements and national regulations applicable to work with high-consequence pathogens.

Principle of the Method

- 2 This protocol is based on amplicon-based whole-genome sequencing of Nipah virus (NiV) using Oxford Nanopore Technologies (ONT) MinION sequencing.

Following confirmation of NiV RNA by RT-qPCR, extracted viral RNA is converted to complementary DNA (cDNA). The NiV genome is then amplified using a set of overlapping primers designed to collectively span the viral genome. This tiling amplicon approach increases sensitivity for samples with low viral RNA concentrations and supports genome recovery from a wide range of specimen types.

Amplified products are prepared for sequencing using ONT-compatible library preparation chemistries and sequenced on the MinION platform. The MinION device generates long-read sequence data in real time, allowing for rapid acquisition of viral genomic information.

Raw sequencing reads are processed using bioinformatic pipelines to:

Perform quality control and read filtering

Align reads to NiV reference genomes

Generate consensus sequences

Identify nucleotide substitutions and genomic variation

The resulting genome sequences can be used for phylogenetic analysis, lineage assignment, and molecular epidemiological investigations, including comparison with previously characterized NiV strains from human, animal, or environmental sources.

This sequencing approach is intended to complement RT-qPCR diagnostics by providing higher-resolution genetic information and is suitable for use in outbreak investigations, surveillance activities, and research studies. It is not intended to replace molecular diagnostic assays.

Safety and Responsibilities

- 3 All work involving clinical or environmental specimens suspected or confirmed to contain Nipah virus (NiV) must be conducted in accordance with institutional biosafety policies and national regulations. Laboratory activities may only be performed in facilities approved for work with high-consequence pathogens and after completion of an appropriate risk assessment.

This protocol describes sequencing as a downstream molecular application following RT-qPCR confirmation and does not replace institutional biosafety manuals or local standard operating procedures.

Only trained and authorized personnel may perform procedures described in this protocol. Staff must be familiar with molecular biology techniques, contamination control practices, and the operation of the MinION sequencing platform, and must have received training in incident reporting and emergency response.

Responsibilities include:

Ensuring that all required approvals and authorizations are in place prior to initiating work

Following established biosafety and contamination control procedures

Maintaining accurate documentation for samples, sequencing runs, and results

Reporting any incidents, deviations, or unexpected results according to institutional policy

Specimens, extracted nucleic acids, and sequencing outputs must be handled, labeled, stored, and shared in accordance with applicable ethics approvals and data governance requirements.

Specimen Types and Requirements

- 4 This protocol is intended for sequencing of Nipah virus (NiV) RNA extracted from specimens that have been confirmed positive by RT-qPCR. Sequencing should only

be performed on samples that meet minimum quality and documentation requirements.

Acceptable specimen types may include:

- Respiratory specimens (e.g., throat swab, nasopharyngeal swab, tracheal aspirate)
- Blood-derived specimens (e.g., serum, plasma, whole blood, where applicable)
- Cerebrospinal fluid (CSF)
- Other clinical or environmental specimens collected as part of approved surveillance or research activities

Specimen collection, transport, and primary processing must follow institutional and national guidelines appropriate for suspected or confirmed NiV infection. This protocol does not describe specimen collection procedures.

Specimens selected for sequencing should meet the following minimum criteria:

- Prior confirmation of NiV RNA by RT-qPCR
- Availability of sufficient extracted RNA for downstream sequencing
- Accompanying metadata sufficient for interpretation, including specimen type, collection date, and RT-qPCR result identifier

Specimens with evidence of severe degradation, insufficient volume, or incomplete documentation may be excluded from sequencing at the discretion of the laboratory or project lead.

Extracted RNA should be stored under conditions that preserve nucleic acid integrity and handled using contamination control practices appropriate for downstream amplification and sequencing workflows.

Sequencing results are intended for research, surveillance, and epidemiological purposes and must be interpreted in the context of specimen quality, viral RNA concentration, and assay limitations.

Controls and Quality Assurance

- 5 Appropriate controls must be included to monitor assay performance, detect contamination, and support interpretation of sequencing results. Controls are intended to assess workflow integrity and data quality rather than to provide diagnostic confirmation.

At minimum, the following controls should be included where applicable:

- A positive control consisting of NiV RNA or cDNA of known origin to verify amplification and sequencing performance
- A negative control processed alongside samples to monitor for contamination introduced during amplification or library preparation



- An extraction control, if included in the upstream workflow, to assess RNA extraction performance

Controls should be clearly labeled, tracked, and reviewed together with sample results for each sequencing run.

Quality assurance measures include:

- Verification that specimens selected for sequencing were previously confirmed positive by RT-qPCR
- Review of sequencing run metrics to ensure sufficient read yield and genome coverage
- Confirmation that control results are consistent with expected outcomes
- Assessment of consensus sequence quality prior to reporting or downstream analysis

Sequencing data that do not meet predefined acceptance criteria, or runs in which controls perform unexpectedly, should be reviewed and may be excluded from analysis or repeated according to laboratory policy.

All sequencing runs, control results, and quality assessments must be documented in accordance with institutional quality management practices.

Primer Reference Sequence and Amplicon Scheme

- 6 The design and genomic positioning of Nipah virus amplicon primers used in this protocol are based on the reference genome **JN808857.1 (Nipah virus isolate NIVBGD2008MANIKGONJ, complete genome)**.

Primer positions were selected to generate tiled, overlapping amplicons spanning the viral genome using this reference sequence as the coordinate framework. As a result, genome coverage and amplification performance may vary for samples that are genetically divergent from the reference, including strains with insertions, deletions, or nucleotide substitutions at primer binding sites.

Incomplete coverage or dropout of specific amplicons should be interpreted in the context of primer-template mismatch, sample quality, and viral RNA concentration. Where required, primer schemes may be updated or modified to improve coverage for divergent lineages, and any such changes should be documented alongside sequencing results.

Limitations of the Method

- 7 This sequencing protocol is intended for use on specimens that have been confirmed positive for Nipah virus (NiV) by RT-qPCR and is not suitable for primary diagnosis.

Successful genome recovery depends on specimen quality and viral RNA concentration. Samples with low viral RNA levels or degraded nucleic acid may yield partial genome coverage or fail to generate usable sequence data.

Amplicon-based sequencing may be affected by primer mismatches, which can result in uneven genome coverage or dropout of specific genomic regions, particularly in genetically divergent NiV strains.

Sequencing results may be influenced by contamination, amplification bias, or sequencing errors and must be interpreted in conjunction with control performance and quality metrics.

This method provides genomic information for research, surveillance, and epidemiological purposes and does not directly measure viral infectivity or clinical severity.

RNA Extraction and Preparation

- 8 Extract viral RNA using the QIAamp Viral RNA Mini Kit or equivalent validated method according to manufacturers' instructions

Note

RNA extraction kits other than the QIAamp Viral RNA Mini Kit may be used; however, any alternative extraction method must be validated prior to use with this sequencing protocol. Validation should demonstrate that the extracted RNA is of sufficient quality and free of inhibitors that could impact reverse transcription, amplification, or sequencing performance. Validation should include comparison with an established method using representative specimens across a range of RT-qPCR Ct values.

- 8.1 Run RT-qPCR Detection of Nipah Virus (NiV) Targeting the Nucleocapsid (N) Gene as per protocol: <https://www.protocols.io/view/rt-qpcr-detection-of-nipah-virus-niv-targeting-the-36wgg1mwkvk5/v1>

- 8.2 **Recommended RT-qPCR Ct range for sequencing input is Ct 18–29 for N gene.**

If Ct is between 12–15, dilute RNA 1:100 in nuclease-free water.

If Ct is between 15–18, dilute RNA 1:10 in nuclease-free water.

This dilution step reduces the risk of PCR inhibition.

- 8.3 Keep RNA on ice during handling and minimize time at room temperature. Proceed immediately to cDNA synthesis or store RNA at -80°C if processing is delayed. Handle all RNA using contamination control practices appropriate for pre-amplification workflows.

cDNA Synthesis

- 9 Primary method: SuperScript IV reverse transcription
- 9.1 Prepare an H_2O + linear acrylamide (LA) working solution by mixing 996 μL nuclease-free water with 4 μL linear acrylamide (5 mg/mL).
- 9.2 In a 0.2 mL PCR tube or plate well, add the following to 5 μL of extracted RNA (one tube per sample):
- 1 μL random hexamers
 - 1 μL dNTP mix (10 mM)
 - 6 μL H_2O + LA working solution
- Final volume: 13 μL
- 9.3 Heat-denature the RNA/primer mix at 70°C for 10 minutes in a thermocycler with a heated lid
- 9.4 Immediately place the tubes on ice for 1–2 minutes. Do not allow RNA to stand for extended periods prior to reverse transcription
- 9.5 Prepare the reverse transcription master mix as follows:
- 4 μL 5 $\times$ SuperScript IV reaction buffer
 - 1 μL DTT (100 mM)
 - 1 μL RNase inhibitor (RNaseOUT or SUPERase-In)
 - 1 μL SuperScript IV reverse transcriptase
- 9.6 Add 7 μL of the reverse transcription master mix to each tube (final volume 20 μL). Mix gently and spin down briefly if needed
- 9.7 Perform reverse transcription using the following thermocycler conditions:
- 23°C for 10 minutes
 - 55°C for 30 minutes
 - 80°C for 10 minutes
 - Hold at 4°C
- 9.8 Proceed directly to amplicon generation or store cDNA at 4°C short-term.



10 Alternative method A: SuperScript III

- 10.1 Combine RNA, random hexamers, dNTPs, annealing buffer, and nuclease-free water according to the SuperScript III protocol, adjusting volumes based on RNA input.
- 10.2 Heat-denature the RNA/primer mix at 65 °C for 5 minutes, then place immediately on ice for 1–2 minutes.
- 10.3 Add the First-Strand Reaction Mix and SSIII/RNase inhibitor enzyme mix to reach a final reaction volume of 20–25 µL.
- 10.4 Incubate under the following conditions:
 - 25 °C for 10 minutes
 - 50 °C for 50 minutes
 - 85 °C for 5 minutes
 - Hold at 4 °C

11 Alternative method B: LunaScript RT SuperMix

- 11.1 If SuperScript enzymes are unavailable, LunaScript RT SuperMix may be used.
- 11.2 Add the following to a PCR tube:
 - 16 µL RNA
 - 4 µL LunaScript RT SuperMix (5×)Final volume: 20 µL.
- 11.3 Mix gently and incubate using the following program:
 - 25 °C for 2 minutes
 - 55 °C for 10 minutes
 - 95 °C for 1 minute
 - Hold at 4 °C

Generation of Tiled Amplicons

- 12 Prepare 100 µM working stocks of each primer then prepare each primer pool:
- 12.1 Pool 1



	A	B	C	D	E	F
	ONT_label	ID	Sequence	Start	End	Volume Needed (uL)
	Niv_1_LEFT	Niv_57F	GGAAMCAAGACAA ACACTTTTGG	56	79	5
	Niv_1_LEFT_alt	Niv_131F	GAGGCGGCTAGTT TTAGGAG	130	150	5
	Niv_1_RIGHT	Niv_735R	GCAAAGAACGGAT TGA CTCTC	714	735	7.5
	Niv_4_LEFT	NP1F	CTTGAGCCTATGTA TTTCAGAC	1174	1196	10
	Niv_4_RIGHT	Niv_2008R	GTGTAGTGTGTAAT TCATGTCC	1986	2008	10
	Niv_7_LEFT	Niv_2640F	TCCAGTACTTCTCC CACAGATGG	2639	2662	5
	Niv_7_RIGHT	Niv_3204R	GAGAAATGCCGAC TATYACTTCAC	3180	3204	5
	Niv_10_LEFT	Niv_3712F	CCTACCAAGAAGG CAAGAGTGTC	3711	3734	5
	Niv_10_RIGHT	Niv_4389R	CTCTGGACGAATC ATCTGCCA	4368	4389	5
	Niv_13_LEFT	Niv_5105F	GCAATGGAGCCRG ACATCAAGAG	5104	5127	7.5
	Niv_13_RIGHT	Niv_5711R	TGGCATTGTTTCTC CTGAACTC	5689	5711	5
	Niv_16_LEFT	Niv_6081F	CTGTGTTGCAGCC TTCTGTTCC	6080	6102	10
	Niv_16_RIGHT	Niv_6867R	GAGACATGTTYGAC ACATT CGGA	6844	6867	10
	Niv_19_LEFT	Niv_7671F	GTGATCTGCAACC AAGATTATGC	7670	7693	10
	Niv_19_RIGHT	Niv_8516R	CTCGTGTGAGAGA ATATGTT	8496	8516	10
	Niv_22_LEFT	Niv_9186F	CAGGCCRTGATCA AAGATGC	9185	9205	7.5
	Niv_22_RIGHT	Niv_9721R	CTGTCTAGTACCTC TCCA ACTC	9699	9721	5

	A	B	C	D	E	F
	Niv_25_ LEFT	NiV_1 0457 F	CTGGGAAGGRGTT TATAATGATGC	104 56	104 80	7.5
	Niv_25_ RIGHT	Niv_11 287R	GCACAATCAAGGA CCTGGGT	1126 7	1128 7	5
	Niv_28_ LEFT	Niv_11 575F	ACCTTCACGGGAA AAGAAAGAG	1157 4	1159 6	5
	Niv_28_ RIGHT	NiV_1 2322 R	TCCTTGAGTTGGAG TAGTGC	123 02	123 22	5
	Niv_31_ LEFT	NiV_1 2918 F	GAGCCAAGAAGAT TGGTTGAC	1291 7	129 38	5
	Niv_31_ RIGHT	Niv_1 3658 R	ACCGTATATCTCAT CYAGACG	136 37	136 58	5
	Niv_34_ LEFT	Niv_1 4155 F	GCTTTTGGTCAGA GACCCTGGT	1415 4	1417 6	5
	Niv_34_ RIGHT	Niv_1 4825 R	ACCAGCAATTCCT CTCTGGCA	148 03	148 25	5
	Niv_37_ LEFT	NiV- 1549 2F	GCAGGGAGTGTAC TCAACAG	154 91	1551 1	5
	Niv_37_ RIGHT	Niv_1 6261 R	AGGTAAGGACCCT GTGTAGG	162 41	162 61	5
	Niv_40_ LEFT	NiV_1 6977 F	CAGTCTACATTGGG GCAATCA	1697 6	169 97	5
	Niv_40_ RIGHT	Niv_1 7532 R	GCAAATYAGAGTG CTCAAGG	1751 2	1753 2	5

Total Final Volume of 100uM Stock = 185 uL

12.2 Pool 2

	A	B	C	D	E	F
	ONT_la bel	ID	Sequence	Star t	End	Volume Needed (uL)



	A	B	C	D	E	F
	Niv_2_ LEFT	Niv_4 87F	GGAAGGCTTGATGAG AATCCTC	486	508	5
	Niv_2_ RIGHT	Niv_1 026R	CCAATTTCTCTGTAG AGTAGCA	100 4	102 6	5
	Niv_5_ LEFT	Niv_1 598F	GCAACAGATGATCCA GCAATCAG	1597	162 0	5
	Niv_5_ RIGHT	Niv_2 329R	GGAGATCCTAGAGTA AATCTC	230 9	233 0	7.5
	Niv_8_ LEFT	Niv_3 019F	CTCCTGTGATTGCTG AACAC	301 8	303 8	5
	Niv_8_ RIGHT	Niv_3 663R	CGTTTTCCGTCCCAG CAGATGG	364 1	366 3	5
	Niv_11_ LEFT	Niv_4 180F	CTGAGCTTAAACCAG TGATAGGA	4179	420 2	5
	Niv_11_ RIGHT	Niv_4 759R	GACTAACTAAGAGCA AGATTAAGAGG	473 3	475 9	5
	Niv_14_ LEFT	Niv_5 597F	CAGCTCGACAAGCAT CAAGC	559 6	561 6	5
	Niv_14_ RIGHT	Niv_6 102R	GGAACAGAAGGCTGC AACACAG	608 0	610 2	5
	Niv_17_ LEFT	Niv_6 719F	CTCGGAGTGYAGTGT YGGGA	6718	673 8	7.5
	Niv_17_ RIGHT	Niv_7 529R	CTGTTGGATTTTCAGT CAGGATAGG	750 5	752 9	5
	Niv_20_ LEFT	Niv_8 291F	TGGGACRTAGTGTATT CAGA	829 0	831 0	10
	Niv_20_ RIGHT	Niv_9 047R	GTCCATGGTTCCGTA GTAGC	902 7	904 7	10
	Niv_23_ LEFT	Niv_9 394F	CACTGCCTCCCTTGA AAATCC	939 3	641 4	10
	Niv_23_ RIGHT	Niv_1 0155 R	GTCTAATCCCCATAG ATAGCCT	1013 3	1015 5	10
	Niv_26_ LEFT	Niv- 1069 4F	CACAGGAGACAATGT YATAAGACC	106 93	1071 7	10



	A	B	C	D	E	F
	Niv_26 _RIGH T	NiV_11 475R	GACTATCCAAATGAC ATTCAGGGT	1145 1	1147 5	10
	Niv_29 _LEFT	NiV_1 2076 F	CAGACAAAACCTGGAA AGAAGTG	1207 5	120 97	5
	Niv_29 _RIGH T	Niv_1 2866 R	CCACTCGTCTTTGAT AGGAG	128 46	128 66	5
	Niv_32 _LEFT	Niv_1 3359 F	CTCAATAATCCGTGC CAAGC	133 58	1337 8	5
	Niv_32 _RIGH T	NiV_1 3913 R	GTCTCCTTGGACAAT TGCAG	138 93	1391 3	5
	Niv_35 _LEFT	NiV_1 4569 F	TGGGCCAGTGATCCA TACTC	1457 3	145 93	5
	Niv_35 _RIGH T	Niv_1 5226 R	GGTACTCTTATTGATG AGTGCTC	152 03	152 26	5
	Niv_38 _LEFT	Niv_1 5969 F	ACCTTGCAATAGACT CTGAC	159 69	159 89	10
	Niv_38 _RIGH T	Niv_1 6669 R	GTGGTATCTATAACTT CGGT	166 49	166 69	10
	Niv_41_ LEFT	Niv_1 7349 F	GCATACACCCCYGGA TTCCCA	173 48	1736 9	10
	Niv_41_ RIGHT	Niv_1 8134 R	TCCGATTATCTTCCA CCAGAT	1811 3	1813 4	7.5
	Niv_41_ RIGHT	NiV_1 8250 R	CGAACAAGGGTAAAG AAGAATCG	182 27	182 50	7.5

Total Final Volume of 100uM Stock = 200 uL

12.3 Pool 3

	A	B	C	D	E	F
	ONT_la bel	ID	Sequence	Star t	End	Volum e



	A	B	C	D	E	F
						Neede d (uL)
	Niv_3_ LEFT	NiV_8 08F	GTCAAGAAAGGAG GATCTGCT	823	844	10
	Niv_3_ RIGHT	NP1R	GCTTTTGCAGCCA GTCTTG	1553	1572	10
	Niv_6_ LEFT	Niv_2 052F	CCTATCCTTTCAAT GGTGCTTG	205 1	207 4	5
	Niv_6_ RIGHT	NiV_2 864R	AGGACAGCACCTT TGCATCAG	285 9	288 0	5
	Niv_9_ LEFT	Niv_3 181F	GTGAAGTRATAGTC GGCATTCTC	318 0	320 4	7.5
	Niv_9_ RIGHT	Niv_3 935R	GGTTTCGAGGTCA TAATCACC	391 4	393 5	5
	Niv_12_ LEFT	Niv_4 521F	GACGGTAACATTT GATCACTG	452 0	454 1	5
	Niv_12_ RIGHT	NiV_5 213R	CTGGYTCAACCTT ATCAAGATACC	518 9	521 3	5
	Niv_15_ LEFT	Niv_5 819F	CACTTGGGRAACT TTGTCCGT	581 8	583 9	7.5
	Niv_15_ RIGHT	Niv_6 441R	ACCAATCCTTTTG TTGATCC	642 1	644 1	5
	Niv_18_ LEFT	Niv_7 345F	AGGCTATATCTCAG GCATTCCGT	734 4	736 7	5
	Niv_18_ RIGHT	Niv_7 884R	GAGTTTGTCTCC TGACTGTGAG	7861	788 4	5
	Niv_21_ LEFT	Niv_8 719F	ACCCAGGTCCATA ACTCATTGG	8718	874 0	5
	Niv_21_ RIGHT	NiV_9 344R	GCTGATCTTTGAA CCTAACAG	932 3	934 4	5
	Niv_24_ LEFT	Niv_1 0009 F	CAGGCATCAAACA RGGTGACAC	100 08	100 30	7.5
	Niv_24_ RIGHT	NiV_1 0717 R	GGTCTTATRACATT GTCTCCTGTG	106 93	1071 7	7.5
	Niv_27_ LEFT	NiV_11 268F	ACCCAGGTCCTTG ATTGTGC	1126 7	1128 7	5



	A	B	C	D	E	F
	Niv_27_RIGHT	Niv_11 966R	CACTGCTCGCATC TCTGTTTTG	1194 4	1196 6	5
	Niv_30_LEFT	Niv_1 2513 F	CCTATTCTTGAGG CTAAAGTTGCT	1251 2	125 36	5
	Niv_30_RIGHT	Niv_1 3176 R	CCTTAACCATCCC GTTCTCC	1315 6	1317 6	5
	Niv_33_LEFT	Niv_1 3638 F	CGTCTRGATGAGA TATACGGT	136 37	136 58	7.5
	Niv_33_RIGHT	Niv_1 4399 R	TGCTGGGATTAAT GCTGCTG	143 79	143 99	5
	Niv_36_LEFT	Niv_1 5049 F	GAGTAATATACGGT CTTGAGGT	150 48	1507 0	5
	Niv_36_RIGHT	Niv_1 15727 R	CCTACATCAGCCA CTTCTTTGAC	1570 4	1572 7	5
	Niv_39_LEFT	Niv_1 6446 F	GCAAATCACCATA AACCTCCT	164 45	164 66	5
	Niv_39_RIGHT	Niv_1 7241 R	CCATGTCAGAATG GACAAGACC	1721 9	1724 1	5

Total Final Volume of 100uM Stock = 152.5 uL

- 13 Prepare 10 μ M working stocks for each primer pool by diluting primers 1:10 in nuclease-free water
- 14 For each sample, set up **three separate PCR reactions per sample** corresponding to primer Pool 1, Pool 2, and Pool 3
- 15 Prepare the following PCR reaction mix for each pool:
 - 12.5 μ L Q5 Hot Start High-Fidelity 2 $\times$ Master Mix
 - Primer pool (10 μ M) according to volumes below
 - q.s.p 25 μ L nuclease-free water
 - 5 μ L cDNA

Use the following primer pool volumes for Nipah virus:

- Pool 1: 4.6 μ L

- Pool 2: 5.0 μ L
- Pool 3: 3.8 μ L

Final reaction volume: 25 μ L per pool.

Note

This protocol has been validated using Q5 Hot Start High-Fidelity 2 $\times$ Master Mix. Use of alternative polymerases is possible but requires prior validation to confirm comparable amplification performance. Alternative enzymes may require different reaction setups and cycling conditions and should be used according to the manufacturer's recommendations.

- 16 Mix reactions gently, spin down briefly, and place in a thermocycler
- 17 Perform PCR using the following cycling conditions:
98 °C for 30 seconds
40–45 cycles of:
 - 95 °C for 15 seconds
 - 65 °C for 5 minutesHold at 4 °C
- 18 Upon completion, keep PCR products on ice or at 4 °C until downstream assessment

Amplicon Validation by Agarose Gel Electrophoresis

- 19 Prepare a 1% agarose gel using an appropriate nucleic acid stain and a 100 bp DNA ladder
- 20 Load 5 μ L of each PCR product from primer Pools 1, 2, and 3 into separate wells of the gel.
- 21 Run the gel under standard electrophoresis conditions until adequate separation of bands is achieved.
- 22 Visualize the gel and assess amplification success for each pool.



- 23 Expected amplicon size range is approximately 500–1700 bp.
- 24 Interpretation:
 - Clear bands within the expected size range indicate successful amplification.
 - Very faint bands may still be suitable for downstream processing, but success is not guaranteed.
 - If no visible band is present for a given pool, do not proceed with that sample.
- 25 For critical samples only, a repeat PCR with increased cycle number (up to 45 cycles total) may be considered if initial amplification is weak.
- 26 Samples that pass amplicon validation may proceed to PCR amplicon cleanup.

PCR Amplicon Cleanup

- 27 Perform all post-PCR cleanup steps in a designated post-PCR cabinet. Clean the workspace with appropriate decontamination wipes and UV-sterilize the cabinet before and after use.
- 28 Allow AMPure XP beads to equilibrate to room temperature and vortex thoroughly to resuspend magnetic particles.

Note

This protocol has been validated using AMPure XP beads. Equivalent SPRI-based magnetic bead cleanup reagents may be used; however, alternative beads must be validated prior to use to ensure comparable DNA recovery, size selection behavior, and compatibility with downstream library preparation. Bead-to-sample ratios and cleanup conditions may require adjustment when using alternative products.

- 29 Prepare fresh 80% ethanol using nuclease-free water.
- 30 To each 20 μ L PCR reaction, add 36 μ L (1.8 $\times$ volume) of AMPure XP beads. Mix by pipetting up and down at least 10 times.
- 31 Incubate samples at room temperature for 10 minutes to allow DNA binding to the beads.

- 32 Place tubes on a magnetic rack and allow the solution to clear (approximately 2–5 minutes). Carefully remove and discard the supernatant without disturbing the beads.
- 33 While tubes remain on the magnetic rack, add 200 μL of freshly prepared 80% ethanol to each tube. Incubate for 1 minute, then carefully remove and discard the ethanol.
- 34 Repeat the ethanol wash once for a total of two washes.
- 35 Briefly centrifuge tubes to collect residual liquid at the bottom, return to the magnetic rack, and remove any remaining ethanol.
- 36 Air-dry the beads on the magnetic rack for approximately 5 minutes, or until the bead pellet loses its surface sheen. Do not over-dry the beads.
- 37 Remove tubes from the magnetic rack and add 30 μL of Elution Buffer (EB) or 10 mM Tris-HCl (pH 8.0). Resuspend beads thoroughly by pipetting up and down until no clumps remain.
- 38 Place tubes back on the magnetic rack and allow the solution to clear (approximately 2 minutes).
- 39 Transfer 28 μL of the eluate to a new, clean tube, taking care not to carry over any beads.
- 40 Quantify DNA concentration using the Qubit High Sensitivity DNA kit with 1 μL of eluate from each pool.

Note

DNA quantification may be performed using alternative fluorometric or equivalent double-stranded DNA quantification methods if a Qubit instrument is not available. Any alternative method must be validated to provide accurate concentration estimates within the expected range and should be suitable for low-concentration amplicon DNA. Spectrophotometric methods alone are not recommended due to reduced accuracy at low DNA concentrations.

- 41 Expected DNA concentration after cleanup is approximately 10–100 ng/ μL . If DNA concentration exceeds the quantification range, dilute the sample 1:2 in EB prior to



measurement.

- 42 Proceed directly to downstream pooling and library preparation, or store purified amplicons at -20°C .

Quantification and Pooling of Amplicons

- 43 Review concentration values and confirm that each pool falls within an acceptable range for downstream library preparation

Note

Acceptable concentration range for downstream pooling and library preparation is typically 10–100 ng/ μL per pool (post-cleanup); dilute or repeat cleanup/quantification as needed to enable accurate normalization.

- 44 For each sample, calculate volumes required to pool amplicons from Pool 1, Pool 2, and Pool 3 at equimolar amounts.
- 45 Combine amplicons from the three primer pools for each sample to obtain a total of 100 ng DNA per pool, for a combined total of 300 ng.
- 46 Adjust the final pooled volume to 100 μL using nuclease-free water or 10 mM Tris-HCl (pH 8.0), resulting in a final concentration of approximately 3 ng/ μL .
- 47 Mix pooled amplicons thoroughly by pipetting up and down.
- 48 Use 10 μL of the pooled amplicon preparation (approximately 30 ng DNA) as input for library preparation.
- 49 If pooled amplicons are not used immediately, store at -20°C until library preparation.

Library Preparation – Native Barcoding

- 50 Preparation of pooled amplicon input



- 50.1 Ensure that purified amplicons from primer Pools 1, 2, and 3 have been quantified and pooled equimolarly as described in Section 6.6.
- 50.2 Prepare each pooled sample at the target concentration required for library preparation. Adjust volume using nuclease-free water or 10 mM Tris-HCl (pH 8.0) as needed.
- 50.3 Use the calculated input volume corresponding to the intended DNA mass for library preparation.
- 51 End-repair and dA-tailing
 - 51.1 For each pooled amplicon sample, add the following to a PCR tube:
 - 10 µL pooled amplicon DNA
 - 1.75 µL NEBNext Ultra II End Prep Reaction Buffer
 - 0.75 µL NEBNext Ultra II End Prep Enzyme Mix
 - 2.5 µL nuclease-free waterFinal volume: 15 µL
 - 51.2 Mix thoroughly by pipetting up and down at least 10 times and briefly spin down.
 - 51.3 Incubate in a thermocycler with heated lid using the following program:
 - 20 °C for 10 minutes
 - 65 °C for 5 minutes
 - Hold at 4 °C
 - 51.4 Keep samples on ice and proceed immediately to native barcode ligation.
- 52 Native barcode ligation
 - 52.1 Assign one unique native barcode to each sample using EXP-NBD104 (barcodes 1–12) or EXP-NBD114 (barcodes 13–24).
 - 52.2 To each end-prepped sample (15 µL), add:
 - 2.5 µL NBXX native barcode
 - 17.5 µL NEBNext Ultra II Ligation Master Mix
 - 0.5 µL Ligation EnhancerFinal volume: 35.5 µL.



52.3 Mix thoroughly by pipetting up and down at least 10 times. Briefly spin down.

52.4 Incubate in a thermocycler:

- 22.5 °C for 20 minutes
- 65 °C for 10 minutes

52.5 Place samples on ice for 1 minute.

53 Pooling of barcoded samples

53.1 Pool all barcoded samples into a single low-bind tube.

53.2 Mix gently by pipetting and record the total pooled volume.

53.3 Proceed immediately to library cleanup and quality control.

Cleanup and Quality Control of Barcoded Libraries

54 Preparation

54.1 Allow AMPure XP beads to equilibrate to room temperature for at least 30 minutes. Mix thoroughly to resuspend beads.

Note

This protocol has been validated using AMPure XP beads. Equivalent SPRI-based magnetic bead cleanup reagents may be used; however, alternative beads must be validated prior to use to ensure comparable DNA recovery, size selection behavior, and compatibility with downstream library preparation. Bead-to-sample ratios and cleanup conditions may require adjustment when using alternative products.

54.2 Prepare fresh 70% ethanol using nuclease-free water.



54.3 Thaw Small Fragment Buffer (SFB) and keep on ice until use.

55 Bead cleanup of pooled barcoded amplicons

55.1 Add 0.4× volume of AMPure XP beads to the pooled barcoded library (e.g., add 120 µL beads to a 300 µL pooled library).

Note

A 0.4× bead ratio is used to selectively retain amplicons ≥ 400 bp in the presence of ligation buffer. Do not increase bead ratio, as this may result in excess native barcode carryover.

55.2 Mix by pipetting up and down at least 10 times and incubate at room temperature for 10 minutes.

55.3 Place the tube on a magnetic rack and allow the solution to clear (approximately 5 minutes).

If the total volume exceeds 500 µL, split the sample into two tubes prior to placing on the magnetic rack.

55.4 Carefully remove and discard the supernatant without disturbing the bead pellet.

55.5 Add 250 µL Small Fragment Buffer (SFB) to the beads and resuspend by gentle pipette mixing.

If the cleanup was split across two tubes, resuspend beads in the first tube with 250 µL SFB and use this suspension to resuspend beads in the second tube.

55.6 Place tubes on the magnetic rack and allow the solution to clear (approximately 5 minutes). Remove and discard the supernatant.

55.7 Repeat the SFB wash once for a total of two SFB washes.



- 55.8 Wash beads once with 200 μ L of 70% ethanol while on the magnetic rack. Incubate for 1 minute, then remove and discard ethanol.
- 55.9 Briefly centrifuge the tube, return it to the magnetic rack, and remove any residual ethanol.
- 55.10 Air-dry the beads on the magnetic rack for approximately 5 minutes.
- 56 Elution and quantification
- 56.1 Remove the tube from the magnetic rack and add 33 μ L Elution Buffer (EB) or 10 mM Tris-HCl (pH 8.0).
- 56.2 Resuspend beads thoroughly by pipetting up and down until fully homogeneous.
- 56.3 Place the tube back on the magnetic rack and allow the solution to clear.
- 56.4 Transfer 31 μ L of the eluate to a new low-bind tube.
- 56.5 Quantify the DNA concentration using a fluorometric double-stranded DNA assay
- 56.6 The expected total amount of pooled barcoded library at this stage is approximately:
- 40 ng when using 8 barcodes
 - Up to 160 ng when using 24 barcodes
- 56.7 Store cleaned barcoded libraries at -20°C if not proceeding immediately.

Native Barcode Adapter Ligation

- 57 For each pooled and cleaned barcoded library, add the following components to 30 μ L of barcoded amplicon pool:
- 10 μ L NEBNext Quick Ligation Reaction Buffer (5 $\times$)
 - 5 μ L Native Adapter (NA) adapter mix



- 5 μ L Quick T4 DNA Ligase

Final volume: 50 μ L.

58 Mix the reaction gently but thoroughly by pipetting up and down.

59 Incubate the ligation reaction at room temperature for 15 minutes.

60 Proceed immediately to post-ligation cleanup and quality control.

Cleanup and Quality Control After Adapter Ligation

61 Preparation

61.1 Allow AMPure XP beads to equilibrate to room temperature for at least 30 minutes. Mix thoroughly to resuspend beads.

Note

This protocol has been validated using AMPure XP beads. Equivalent SPRI-based magnetic bead cleanup reagents may be used; however, alternative beads must be validated prior to use to ensure comparable DNA recovery, size selection behavior, and compatibility with downstream library preparation. Bead-to-sample ratios and cleanup conditions may require adjustment when using alternative products.

61.2 Thaw Small Fragment Buffer (SFB) and keep on ice.

62 Bead cleanup of adapter-ligated library

62.1 Add 1 $\times$ volume of AMPure XP beads (50 μ L) to the 50 μ L adapter-ligated library.

62.2 Mix by pipetting up and down at least 10 times and incubate at room temperature for 10 minutes.

- 62.3 Place the tube on a magnetic rack and allow the solution to clear (approximately 5–10 minutes).
- 62.4 Carefully remove and discard the supernatant without disturbing the bead pellet.
- 62.5 Add 250 μ L Small Fragment Buffer (SFB) to the beads and resuspend by gentle pipette mixing.

Note

Do not substitute SFB with ethanol at this step, as SFB is required to remove excess adapters without disrupting adapter–protein complexes.

- 62.6 Place the tube on the magnetic rack and allow the solution to clear. Remove and discard the supernatant.
- 62.7 Repeat the SFB wash once for a total of two SFB washes.
- 62.8 Briefly centrifuge the tube and remove any residual SFB. Air drying is not required after SFB washes.

63 Elution and final quantification

- 63.1 Remove the tube from the magnetic rack and add 15 μ L Elution Buffer (EB) or 10 mM Tris-HCl (pH 8.0).
- 63.2 Resuspend beads thoroughly by pipetting up and down.
- 63.3 Place the tube back on the magnetic rack and allow the solution to clear.
- 63.4 Transfer 15 μ L of the eluate to a new low-bind tube.



- 63.5 Quantify the final library using a fluorometric double-stranded DNA quantification method.

Note

The expected DNA amount at this stage is approximately 50% of the input amount prior to adapter ligation.

- 63.6 If not proceeding immediately to sequencing, store the final library in 10 mM Tris-HCl (pH 8.0) at 4 °C for up to one week or at -20 °C for longer-term storage.

Priming and Loading the MinION Flow Cell (R10.x)

64 Preparation

- 64.1 Thaw the following reagents at room temperature and then place on ice:
- Sequencing Buffer (SQB)
 - Loading Beads (LB)
 - Flush Buffer (FB/FLB)
 - Flush Tether (FLT)
- 64.2 Mix all reagents by gentle inversion and briefly spin down before use.

65 Flow cell priming

- 65.1 Prepare the priming mix by adding 30 µL of Flush Tether (FLT) to the Flush Buffer (FB/FLB). Mix well by vortexing and briefly spin down.
- 65.2 Insert an R10.x MinION flow cell (e.g., R10.4.1, FLO-MIN114) into the MinION device and close the lid securely.

Note

This protocol has been validated using R10.x MinION flow cells. If alternative MinION flow cell versions are used, run configuration and sequencing performance should be verified according to the manufacturer's recommendations.

- 65.3 Perform a flow cell check in MinKNOW to assess the number of active pores.
- 65.4 Rotate the inlet port cover clockwise by 90° to expose the priming port.
- 65.5 Using a P1000 pipette set to approximately 770 µL, gently remove any air from the inlet port by slowly turning the volume dial anticlockwise until a small volume enters the pipette tip.
- Take care not to remove excessive volume or introduce air into the flow cell.
- 65.6 Slowly load 800 µL of the Flush Buffer + Flush Tether mix into the flow cell via the inlet port. Dispense smoothly and avoid introducing air bubbles.
- 65.7 Incubate the flow cell at room temperature for 5 minutes.
- 66 Preparation of the sequencing library for loading
- 66.1 In a new tube, prepare the sequencing library dilution as follows:
- 37.5 µL Sequencing Buffer (SQB)
 - 25.5 µL Loading Beads (LB)
 - 12 µL final sequencing library
- Final volume: 75 µL
- 66.2 Mix Loading Beads immediately before use, as beads settle rapidly.
- 66.3 If less than 12 µL of library is available, adjust the volume with Elution Buffer or 10 mM Tris-HCl (pH 8.0).

Note

The recommended amount of final library loaded onto a flow cell is **20–40 ng**. If the final library concentration exceeds this range, dilute the library to a total volume of 12 µL such that the total input mass falls within 20–40 ng.

67 Loading the library onto the flow cell

67.1 Gently lift the SpotON cover to expose the SpotON sample port.

67.2 Load 200 µL of the Flush Buffer + Flush Tether mix into the inlet port. This initiates siphoning at the SpotON port.

67.3 Immediately before loading, gently mix the prepared library dilution by pipetting up and down.

67.4 Load the 75 µL library dilution dropwise into the SpotON sample port, ensuring that each drop is fully drawn into the port before adding the next.

67.5 Gently replace the SpotON cover, ensuring the bung is properly seated, then close the inlet port and MinION lid.

68 Starting the sequencing run

68.1 Open MinKNOW and select **New Experiment**.

68.2 Configure the run using the following settings:

- Flow cell: R10.x MinION flow cell (e.g., R10.4.1, FLO-MIN114)
- Kit: Native Barcoding Kit 24 (v14 or compatible)
- Experiment name: enter a unique run identifier (e.g., YYYYMMDD_Project_RunX)
- Run duration: 6 hours (or until sufficient data are generated)
- Basecalling: High-accuracy
- Output settings: adjust file output parameters as required for real-time monitoring

68.3 Click **Start run** to initiate sequencing.

- 68.4 Monitor sequencing performance and data yield in real time using the MinKNOW interface.

Run Monitoring and Stopping Criteria

- 69 Monitor sequencing progress in real time using the MinKNOW interface to assess:
- Active pore count
 - Read yield
 - Sequencing speed
 - Basecalling status
- 70 Where available, use RAMPART (Read Assignment, Mapping, and Phylogenetic Analysis in Real Time) or an equivalent real-time monitoring tool to track:
- Genome coverage across the Nipah virus reference
 - Coverage uniformity across amplicons and primer pools
 - Sample demultiplexing and barcode performance
- 71 Continue sequencing until sufficient data have been generated to meet project objectives. Typical stopping criteria may include one or more of the following:
- Near-complete genome coverage achieved for most or all samples
 - Adequate depth of coverage across the genome for downstream consensus generation
 - Diminishing returns in coverage gain despite continued sequencing
- 72 Sequencing runs may be stopped manually once coverage targets are reached, rather than running for the full planned duration.
- 73 If coverage is uneven or incomplete for specific samples or amplicons, consider:
- Allowing the run to continue to increase depth
 - Flagging affected samples for potential re-sequencing
 - Reviewing upstream amplification performance for the affected primer pools
- 74 Record the following information at the end of the run:
- Total run duration
 - Total reads generated
 - Per-sample read counts
 - Coverage metrics used to justify stopping the run
- 75 After stopping the run, proceed with downstream data processing, consensus generation, and analysis according to the project's bioinformatic workflow.

Anticipated Results

76 This protocol is expected to generate amplicon-based Nanopore sequencing data suitable for downstream genomic analysis of Nipah virus (NiV)-positive specimens.

For samples with RT-qPCR Ct values within the recommended input range and successful amplification across primer pools, sequencing typically yields partial to near-complete NiV genome coverage. Genome completeness and depth of coverage may vary between samples and across genomic regions, reflecting differences in sample quality, viral RNA concentration, and amplification efficiency.

- Following data processing and consensus generation, anticipated outputs include:
- Demultiplexed Nanopore sequencing reads assigned to individual samples
 - Per-sample coverage profiles across the NiV genome
 - Consensus genome sequences suitable for phylogenetic analysis and lineage comparison
 - Partial consensus sequences for samples with incomplete coverage, with low-confidence regions masked or documented

Samples with strong amplification and balanced primer pool performance are expected to produce higher genome breadth and more uniform coverage. Samples with low viral RNA concentration, uneven amplification, or primer dropout may yield fragmented or incomplete genomes.

Sequencing results generated using this protocol are intended for research, surveillance, and molecular epidemiology applications, including outbreak investigation and comparative genomic analysis. Results are not intended for primary clinical diagnosis and should be interpreted in conjunction with RT-qPCR findings and quality control metrics.

Timing and Safe Stopping Points

77

Workflow stage	Approximate duration	Safe stopping point
RNA preparation and setup	15–20 min	Yes – extracted RNA can be stored at –80 °C
cDNA synthesis (reverse transcription)	~1 hour	Yes – cDNA can be stored at 4 °C (short term) or –20 °C
Subtotal: RNA → cDNA	~1–1.5 hours	
Amplicon PCR setup (3 primer pools)	~30 min	No – proceed directly to PCR
Amplicon PCR cycling	~3.5–4 hours	Yes – PCR products can be stored at 4 °C (overnight) or –20



Workflow stage	Approximate duration	Safe stopping point
		°C
Subtotal: Amplicon generation	~4–4.5 hours	
Agarose gel electrophoresis	30–45 min	No – proceed to cleanup once validated
PCR amplicon cleanup and quantification	~1–1.5 hours	Yes – cleaned amplicons can be stored at –20 °C
Subtotal: Validation & cleanup	~1.5–2 hours	
Amplicon pooling and normalization	~30 min	Yes – pooled amplicons can be stored at –20 °C
End-repair and native barcode ligation	~1–1.5 hours	No – proceed directly to cleanup
Barcoded library cleanup and QC	~1.5–2 hours	Yes – cleaned barcoded library can be stored at –20 °C
Adapter ligation and final cleanup	~1–1.5 hours	Yes – final library can be stored at 4 °C (≤1 week) or –20 °C
Subtotal: Library preparation	~4–5 hours	
Flow cell check and priming	30–45 min	No – load library immediately after priming
Library loading and run setup	20–30 min	No
Subtotal: Sequencing setup	~1–1.25 hours	
MinION sequencing run	~4–6 hours	Yes – run may be stopped once sufficient coverage is achieved
Basecalling and demultiplexing	~1–2 hours	Yes – data can be processed later
Mapping and consensus generation	~1–2 hours	Yes
Subtotal: Data processing	~2–4 hours	
Estimated hands-on time	~8–10 hours	
Total elapsed time	~18–24 hours	

Notes on this Protocol

78 This protocol is intended for sequencing of samples previously confirmed positive for Nipah virus by RT-qPCR and is not suitable for primary diagnostic use.

- **RT-qPCR Ct range:**

Optimal sequencing performance is typically obtained with samples having Ct values between **18 and 29**.

For samples with Ct **12–15**, dilute extracted RNA **1:100** in nuclease-free water prior to cDNA synthesis.

For samples with Ct **15–18**, dilute extracted RNA **1:10** prior to cDNA synthesis.

These dilutions reduce the risk of PCR inhibition during downstream amplification.

- **RNA quality:**

RNA integrity is critical for successful amplification and sequencing. Minimize freeze–thaw cycles, limit time at room temperature, and keep RNA on ice during handling.

- **Primer pools:**

This protocol uses **three tiled amplicon primer pools (Pools 1, 2, and 3)**. Uneven amplification or dropout of individual pools can result in uneven genome coverage and is a common source of incomplete genomes.

- **Amplicon validation:**

Expected amplicon sizes are approximately **500–1700 bp**. Samples with no visible amplification for one or more pools are unlikely to yield usable sequencing data and should not proceed to library preparation.

- **DNA concentration after cleanup:**

Following PCR amplicon cleanup, expected DNA concentrations are typically in the range of **10–100 ng/μL per pool**. Concentrations outside this range may require dilution or indicate suboptimal amplification.

- **Pooling strategy:**



Amplicons from all three primer pools should be combined at equal mass for each sample. Calculations for final concentration and input volume must be internally consistent and adjusted based on the intended DNA mass for library preparation.

▪ **Reagents and flow cells:**

This protocol has been validated using **Q5 Hot Start High-Fidelity 2× Master Mix**, **AMPure XP beads**, and **R10.x MinION flow cells**. Use of alternative reagents or flow cell versions requires prior validation and may require modification of conditions.

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