Oct 17, 2025 Version 1

One Health metagenomic next-generation sequencing for virus discovery V.1

Forked from a deleted protocol

DOI
https://dx.doi.org/10.17504/protocols.io.8epv5koxnv1b/v1

Alexandru Tomazatos[1], Marike Petersen[1], Balázs orváth[1], Alexandra Bialonski[1], Heike Baum[1], Marc Lütgehetmann[2], Alexander Schlaphof[1], Jaime Costales[3], M. Alejandra Camacho[4], Santiago F. Burneo[4], Petra Emmerich[1], Ronald von Possel[1], Daniel Camprubí[5], Jose Muñoz[5], Stephanie Jansen[6], Renke Lühken[1], Jonas Schmidt-Chanasit[1], Gábor óth[1], Dániel adar[1]

[1]Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Hemorrhagic Fever Reference and Research, 20359 Hamburg, Germany;
[2]University Medical Center Hamburg-Eppendorf, 20359, Hamburg, Germany;
[3]Centro de Investigación Para la Salud en América Latina, Pontificia Universidad Católica del Ecuador, 170525, Quito, Ecuador;
[4]Museo de Zoología, Pontificia Universidad Católica del Ecuador,170525, Quito, Pichincha, , Ecuador;
[5]Barcelona Institute for Global Health (ISGlobal), Hospital Clínic, Universitat de Barcelona, Barcelona, Spain;
[6]Faculty of Mathematics, Informatics and Natural Sciences, Universität Hamburg, 20148 Hamburg, Germany

Dániel Cadar
BNITM

DOI: **https://dx.doi.org/10.17504/protocols.io.8epv5koxnv1b/v1**

Protocol Citation: Alexandru Tomazatos, Marike Petersen, Balázs orváth, Alexandra Bialonski, Heike Baum, Marc Lütgehetmann, Alexander Schlaphof, Jaime Costales, M. Alejandra Camacho, Santiago F. Burneo, Petra Emmerich, Ronald von Possel, Daniel Camprubí, Jose Muñoz, Stephanie Jansen, Renke Lühken, Jonas Schmidt-Chanasit, Gábor óth, Dániel adar 2025. One Health metagenomic next-generation sequencing for virus discovery. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.8epv5koxnv1b/v1**

Protocol status: Working
We use this protocol and it's working

Created: October 16, 2025

Last Modified: October 17, 2025

Protocol Integer ID: 229977

Keywords: Metagenomics, mNGS, Virus discovery, One Health, Sequencing protocol, Viral enrichment, health pathogen discovery, virus discovery emerging, west nile virus case, emerging viral disease, mosquito homogenate sample, genomic surveillance, free approach for viral detection, accessible tool for outbreak investigation, mosquito homogenate, including dengue, rna virus, sequencing platform, quality genomes for subsequent phylogenomic analysis, dna virus, prior pathogen knowledge, outbreaks of unknown origin, viral detection, most virus, outbreak investigation, viral disease, complete genome recovery, genome recovery, genomic characterization of rna, threat to global health security, traditional diagnostic assay, efficient mngs workflow, quality genome, traditional diagnostic assays such as pcr, microbial nucleic acid, genomic characterization, genome copy, global health security, outbreak

Funders Acknowledgements:
PREPMEDVET
Grant ID: 13N15449
NEED
Grant ID: 01KI2022
German national program for arbovirus surveillance
Grant ID: 2819107A22

Abstract

Emerging and re-emerging viral diseases pose an increasing threat to global health security. Traditional diagnostic assays such as PCR and serology depend on prior pathogen knowledge and therefore fail to identify novel or divergent agents, especially in outbreaks of unknown origin. Metagenomic next-generation sequencing (mNGS) has emerged as a transformative, hypothesis-free approach for viral detection and genome recovery, yet its implementation remains limited by cost, protocol complexity, and the need for specialized infrastructure. We developed and validated a universal, cost-efficient mNGS workflow optimized for both benchtop and portable Illumina sequencing platforms. The protocol integrates non-specific enzymatic depletion of host and microbial nucleic acids, random priming, and streamlined library preparation using QIAseq FX kit. Analytical sensitivity was evaluated in contrived serum and mosquito homogenate samples spiked with six RNA viruses of public-health importance. Diagnostic performance was further tested on clinical and surveillance specimens, including dengue, chikungunya, monkeypox, COVID-19, and West Nile virus cases. The protocol achieved a detection limit of 10 genome copies μl^{-1} in human serum and 10^2 copies μl^{-1} in mosquito homogenates, with near-complete genome recovery (≥ 90 %) for most viruses at 10^3 copies μl^{-1}. In true clinical and field samples, mNGS correctly identified all etiologic agents and generated high-quality genomes for subsequent phylogenomic analysis. Mean sequencing depth across all runs was $> 5 \times 10^5$ reads per sample on the Illumina iSeq100. This modular mNGS workflow delivers rapid, unbiased detection and genomic characterization of RNA and DNA viruses directly from diverse matrices. Its reduced cost, portability, and shorter turnaround time make it an accessible tool for outbreak investigation, genomic surveillance, and One Health pathogen discovery, particularly in resource-limited environments.

Guidelines

1. Preventing RNA Degradation

- **Work quickly and keep samples cold.**
  Always keep RNA and reagents on **ice** during processing. Minimize freeze–thaw cycles.

- **Use RNase-free materials.**
  Use certified **RNase-free tubes, pipette tips, and reagents**. Autoclaving alone does not inactivate RNases.

- **Wear gloves at all times.**
  Skin carries RNases; change gloves frequently and avoid touching surfaces.

- **Use RNase inhibitors when applicable.**
  Add RNase inhibitor enzymes to reactions involving sensitive RNA samples.

2. Avoiding RNase Contamination

- **Clean the workspace before use.**
  Wipe benches and pipettes with **RNase-decontamination solutions** (e.g., RNaseZap or 70% ethanol).

- **Designate RNA-only areas and equipment.**
  Use separate pipettes and racks for RNA work; label them clearly.

- **Use sterile, single-use consumables.**
  Never reuse tubes or tips that have contacted RNA or potential contaminants.

3. Sample Handling and Storage

- **Use appropriate buffers.**
  Store RNA in **RNase-free water** or **TE buffer (pH 7.0–8.0)**.

- **Snap-freeze samples in liquid nitrogen** and store at **−80 °C** for long-term preservation. **Avoid repeated thawing.**
  Aliquot RNA to minimize freeze–thaw cycles.

4. General Laboratory Practice

- **Plan workflow in advance.**
  Prepare reagents, label tubes, and organize materials before beginning.

- **Use filtered pipette tips** to prevent aerosol contamination.

- **Handle reagents gently.**
  Avoid vortexing RNA; mix by pipetting or gentle flicking.

- **Document everything.**
  Record RNA concentrations, integrity (e.g., RIN values), and storage conditions.

Materials

Reagents

	A	B	C	D
	Reagent	**Provider**	**Cat. no.**	**Note**
	QIAamp Viral RNA Mini Kit	Qiagen	52906	
	Stainless Steel Beads	-	-	For homogenisation
	Aluminum Beads	-	-	For homogenisation -80°C
	Ultrafree-MC, HV 0,45 µm	Merck Millipore	UFC30HV00	
	Proteinase K	Invitrogen	AM2548	
	Turbo DNAse	Invitrogen	AM2239	
	Baseline Zero DNAse	Epicenter	DB0715K	
	Benzonase Nuclease	Merck Millipore	70664-3	
	RNAse A	Thermo Scientific	EN 0531	
	EDTA 0,5M	Thermo Scientific	AM9260G	
	RNAsecure	Invitrogen	AM7006	
	RNAseOUT	Invitrogen	10777019	
	dNTP Mix	Qiagen	201901	
	SuperScript IV	Thermo Scientific	180 90 200	
	Klenow Fragment	NEB	M0212L	
	D3N (CAT GCT GAT AGT CGT ACC GCN NNN NNNN)	IDT	-	Primer for RT, used with various concentration!
	D3 (CAT GCT GAT AGT CGT ACC GC)	IDT	-	Primer for SISPA
	HotStar Taq plus PCR-Kit	Qiagen	203605	
	QIAseq FX DNA Library UDI Kit	Qiagen	180477	with UDI indexes

	A	B	C	D
	AMPure XP Beads	Beckman Coulter	A63881	
	Qubit dsDNA HS Assay Kit	Thermo Scientific	Q32854	
	Qubit Assay Tubes	Thermo Scientific	Q32856	
	EB buffer (10 mM Tris-Cl, pH 8.5)	Qiagen	19086	
	Nuclease free Water	Qiagen	129114	
	Ethanol	Thermo Scientific	BP28184	
	iSeq 100 i1 Reagent v2 (300-cyc)	Illumina	20031374	
	Other Illumina reagent for different platforms	Illumina	-	

Necessary reagents for the protocol.

Consumables

	A	B	C	D
	Consumable	**Provider**	**Ca. no.**	**Note**
	Low binding Tubes 1,5ml	Sarstedt	72706700	
	Safe Seal SurPhob Tips 10µl	Biozym	VT0200	
	Safe Seal SurPhob Tips 300µl	Biozym	VT0250	
	Biosphere Filter Tips 100µl	Sarstedt	70.3030.25	
	Biosphere Filter Tips 1250µl	Sarstedt	701186210	
	Multiply Strip 0,2ml	Sarstedt	72985002	
	Multichannel reagent reservoirs 25 mL	Integra	4316	

Necessary consumables for the protocol.

Equipments

	A	B	C	D
	Equipment	**Provider**	**Ca. no.**	**Note**
	Eppendorf Research® plus pipette set	Eppendorf	3123000900	
	DynaMag™-PCR Magnet	Thermo Scientific	492025	
	BioSan Mini-Zentrifuge/Vortex KombiSpin	PROFILAB BIOSAN	22004	
	miniPCR® thermal cycler	miniPCR Bio	QP-1016-01	
	Qubit 4 Fluorometer	Thermo Scientific	Q33238	
	PCR Workstation	StarLabs	E2396-9693	
	iSeq100 Instrument	Illumina	20021532	
	Other Illumina platform	Illumina	-	

Necessary equipments for the protocol.

Troubleshooting

Safety warnings

!CAUTION.
Any potentially infectious clinical or environmental samples should be handled and made safe in accordance with biosafety regulations.

!CAUTION.
Please follow local institutional review board guidelines covering the collection and storage of clinical samples for research/diagnostic purposes.

!CAUTION.
RNA is highly susceptible to degradation and contamination; therefore, special care must be taken during all steps of RNA handling, extraction, and downstream processing.

!CAUTION.
To avoid stock contamination master mix preparation and reagents handling should be conducted in a dedicated clean cabinet!

Before start

Read the manufacturer`s instruction and be familiar with the reagents and equipments are involved in this protocol!

Sample preparation

1 **Plasma/serum/CSF/culture supernatants or other cell free fluids**

1.1 Remove 250 µL of biological material and clarify by centrifugation at 12,000 rpm for 00:05:00 . (if available volume is less than 250 µL , use 1xPBS to bring to 250 µL)

1.2 Transfer the debris free supernatant on a Ultrafree-MC HV 0.45µm sterile filter (Millipore).

1.3 Centrifuge at 8,000 rpm for 00:05:00 , then transfer 200 µL filtrate to a 1.5 ml tube. Proceed to step 5 (Viral Enrichment).

2 **Feces sample**

2.1 Take ~ 200 mg fecal sample and add 800 µL 1x PBS (Phosphate-Buffered Saline).

2.2 Vortex vigorously for ~ 00:05:00 until the fecal mass is fully homogenized.

> Note
>
> Homogenisation with beads is optional.

2.3 Clarify the supernatant by centrifugation at 12,000 rpm for 00:05:00 .

2.4 Remove 300 µL supernatant into fresh tube. Keep original tube at -80 °C for future extractions.

2.5 Clarify the new tube by centrifugation at 12,000 rpm for 00:05:00 .

2.6 Transfer 250 µL the debris free supernatant on a Ultrafree-MC HV 0.45µm sterile filter (Millipore).

2.7 Centrifuge at 8,000 rpm for 00:05:00 , then transfer 200 µL filtrate to a 1.5 ml tube. Proceed to step 5 (Viral Enrichment).

3 Tick sample

Place 1 tick into 2ml Tube.

3.1 Put the Tube in liquid nitrogen and incubate for 00:05:00

3.2 Add stainless steel beads to the tube and homogenize at 30hz for 00:03:00

3.3 Add 500 µL cooled (4 °C) 1x PBS and homogenize at 30hz for 00:03:00 .

3.4 Centrifuge the sample at 8,000 rpm for 00:02:00 .

3.5 Transfer 250 µL homogenate supernatant to a new 1,5ml Tube and add 2.5 µL Proteinase K. Incubate at 50 °C for 00:30:00 .

3.6 Centrifuge at 12,000 rpm for 00:05:00 .

3.7 Transfer 250 µL the debris free supernatant on a Ultrafree-MC HV 0.45µm sterile filter (Millipore).

3.8 Centrifuge at 8,000 rpm for 00:05:00 , then transfer 200 µL filtrate to a 1.5 ml tube. Proceed to step 5 (Viral Enrichment).

4 Tissue samples

4.1 Take ~ 200 mg tissue sample and add 500 µL 1x PBS (Phosphate-Buffered Saline).

4.2 Homogenize at 30hz for 00:03:00 .

4.3 Centrifuge the sample at 8000rpm for 00:02:00 .

4.4 Transfer 250 µL homogenate supernatant to a new 1,5ml Tube and add 2.5 µL Proteinase K. Incubate at 50 °C for 00:30:00 .

4.5 Centrifuge at 12,000 rpm for 00:05:00 .

4.6 Transfer 250 µL the debris free supernatant on a Ultrafree-MC HV 0.45µm sterile filter (Millipore).

4.7 Centrifuge at 8,000 rpm for 00:05:00 , then transfer 200 µL filtrate to a 1.5 ml tube. Proceed to step 5 (Viral Enrichment).

Viral Enrichment

5 The nuclease treatment should be prepared on ice/coolrack. Prepare the following Nuclease Mix:

Reagent	**Amount**
Turbobuffer 10x	24 µL
Turbo DNAse	7 µL
Baseline Zero DNAse	3 µL
Benzonase	3 µL
RNAse A	2 µL
Final volume:	39 µL

5.1 Add to the 200 µL sample and incubate at 37 °C for 01:30:00 .

5.2 Inactivate the nucleases by adding 25 µL of [M] 30 millimolar (mM) EDTA and 10.5 µL RNAsecure to each sample and incubate for 00:10:00 at 65 °C in a heating block.

RNA extraction

6 Extract RNA from 140 µL sample matrix using the QIAamp Viral RNA Mini Kit according to the manufacturer’s instructions **without adding the carrier RNA**, elution should be conducted with 60 µL of elution buffer (EB).

Note

The manual for QIAamp Viral RNA Mini Kit:
HB-0354-008_HB_QA_Viral_RNA_...

6.1 After elution add 1 µL l RNase OUT to the extracted RNA and mix it gently.

Reverse Transcription

7 Prepare everything on ice/coolrack. Add 1 µL D3N 100µM primer to each well/tube then add 10 µL freshly extracted RNA and mix carefully.

Note

The sequence of D3N primer: 5`-CAT GCT GAT AGT CGT ACC GCN NNN NNNN-3`

7.1 Incubate in a PCR machine for 00:10:00 at 75 °C .

7.2 Prepare the following reaction mix on ice/coolrack:

Component	**Amount**

5xSSIV Buffer	4 µL
dNTP Mix	1.5 µL
Superscript IV RT	1 µL
RNAse OUT, 40 U/µl	1 µL
Final volume:	8.5 µL

7.3 Add 8.5 µL to each reaction and mix by pipetting. Incubate with the following conditions.

Temperature	Time
25 °C	00:05:00
50 °C	01:00:00
70 °C	00:15:00
4 °C	Till further processes

Second strand synthesis

8 Prepare the following master mix on ice/coolingrack.

Component	Amount
D3N 20µM	1 µL
NE Buffer 2 10x	2.5 µL
dNTP-Mix 10µM	1 µL
Final volume:	4.5 µL

8.1 Add 4.5 µL master mix to each tube and mix it by pipetting. Incubate the tubes with the following conditions.

Temperature	Time
95 °C	00:02:00
4 °C	00:05:00

8.2 Add 1 µL Klenow-Fragment (5000U/ml, NEB) to each reaction and mix it by pipetting. Incubate the tubes with the following conditions.

Temperature	Time
37 °C	01:00:00
75 °C	00:20:00
4 °C	Till further processes

Clean-up with AMPure XP Beads

9 **Sample/Bead ratio** = 1:1.8 (25 µL sample/ 45 µL bead)

Washing: 3 times with 200 µL 80% EtOH

Elution: 25 µL elution buffer (EB)

Note

Before usage, beads should be at room temperature and mix properly.

9.1 Add 45 µL AMPure XP beads to the sample and mix gently.

9.2 Incubate for 00:05:00 at room temperature.

9.3 Place the tube on a magnetic rack for 00:02:00 (until supernatant is completely clear).

9.4 Remove supernatant without touching the bead pellet.

9.5 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

9.6 Remove supernatant and discard.

9.7 Repeat washing step with 80% ethanol additional two times (Step 22-23).

9.8 Carefully remove the supernatant and dry the beads approximately for 00:05:00 by leaving the lid open (Dull pellet indicates dryness).

9.9 Remove tube from the magnetic rack and resuspend the pellets in 25 µL elution buffer (EB) and incubate at room temperature for 00:02:00

9.10 Place the tube back to a magnetic rack for 00:02:00 until supernatant is completely clear and transfer 23 µL eluate into clean tube. Make sure that no beads are transferred alongside the supernatant.

Note

5 µL of this dsDNA is used for the next reaction, the rest is stored at -20 °C .

PCR Amplification (SISPA)

10 Prepare the following master mix on ice/coolingrack.

Component	**Amount**
Buffer 10x	5 µL
dNTP Mix	1 µL
$MgCl_2$	4 µL
Primer D3, 100µM	1 µL
Hot Start Taq Polymerase	0.5 µL
Nuclease free water	33.5 µL
Final volume:	45 µL

10.1 Add 45 µL PCR Mix to a new PCR Tube or 8 Strip.

10.2 Add 5 µL dsDNA (Sample) to each reaction and mix well by pipetting. Incubate the reaction with the following PCR conditions

Temperature	Time	Cycles
95 °C	00:15:00	1
94 °C	00:00:30	30
55 °C	00:00:45	30
72 °C	00:01:30	30
72 °C	00:10:00	1
4 °C	Till further processes	

10.3 Spin down the tubes briefly.

Clean-up with AMPure XP Beads

11 **Sample/Bead ratio** = 1:0.9 (50 µL sample/ 45 µL bead)

Washing: 3 times with 200 µL 80% EtOH

Elution: 25 µL elution buffer (EB)

Note

Before usage, beads should be at room temperature and mix properly.

11.1 Add 45 µL AMPure XP beads to the sample and mix gently.

11.2 Incubate for 00:05:00 at room temperature.

11.3 Place the tube on a magnetic rack for 00:02:00 (until supernatant is completely clear).

11.4 Remove supernatant without touching the bead pellet.

11.5 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

11.6 Remove supernatant and discard.

11.7 Repeat washing step with 80% ethanol additional two times (Step 22-23).

11.8 Carefully remove the supernatant and dry the beads approximately for 00:05:00 by leaving the lid open (Dull pellet indicates dryness).

11.9 Remove tube from the magnetic rack and resuspend the pellets in 25 µL elution buffer (EB) and incubate at room temperature for 00:02:00

11.10 Place the tube back to a magnetic rack for 00:02:00 until supernatant is completely clear and transfer 23 µL eluate into clean tube. Make sure that no beads are transferred alongside the supernatant.

Quantification with Qubit Fluorometer

12 Quantify 1 µL cleaned PCR product with the Qubit 1x dsDNA HS Assay kit.

12.1 Aliquot 199 µL 1x Working solution to Qubit assay tube (equal to the number of your samples). Prepare two extra tubes for the standards with 190 µL 1x Working solution.

12.2 Add 1 µL sample for each test tube and 10 µL from the Standards (Standard 1-2). The final volume is 200 µL .

12.3 Mix each sample vigorously by vortexing and pulse centrifuge to collect the liquid.

12.4 Incubate at room temperature for 00:02:00 before measuring.

12.5 Calibrate Qubit fluorometer with the standards based on manufacturers recommendation.

12.6 Read your sample.

Note

The manual for Qubit 1x dsDNA HS Assay kit.
Qubit_1X_dsDNA_HS_Assay_Kit_UG...

Note

Alternative dsDNA quantification systems can be used as well (e.g.QuantiFluor ONE dsDNA System).

Normalisation and dilution

13 The NGS library preparation is conducted with the QIAseq FX DNA Library Kit from Qiagen. The input amount should be 100 ng in 35 µL . Dilute the sample with nuclease free water to reach the suitable concentration.

Component	**Amount**
100 ng amplicon	x µL
Nuclease Free water	35-x µL

Note

The leftover from amplicons could be stored at -20 °C .

NGS Library Preparation

14 **Fragmentation and end repair**

Note

The manual for QIAseq FX DNA Library Kit.
HB_QIAseq_FX_DNA_Library_0624_...

14.1

Prepare the following master mix on ice/coolingrack.

Component	**Amount**
FX Buffer 10x	5 µL
FX Enzym Mix	10 µL
Final volume:	15 µL

14.2 Add 15 µL master mix to 35 µL (previously diluted to 100 ng / 35 µL) sample and mix it with pipetting. Final volume is 50 µL .

14.3 Incubate with the following thermal profile:

Temperature	**Time**
4 °C	00:01:00
32 °C	00:08:00
65 °C	00:30:00

4 °C	Till further processes

15 **Adapter Ligation**

Note

Important!
-Track the barcodes from each adapter well used for each sample.
-Only 1 adapter should be used per ligation reaction.
-If adapters are used from another supplier, follow the manufacturer`s instructions.
-Do not use adapter wells, if the foil seal has been pierced.

15.1 Prepare the following master mix on ice/coolingrack.

Component	**Amount**
DNA Ligase Buffer 5x	20 µL
DNA Ligase	10 µL
Nuclease free water	15 µL
Final volume:	45 µL

15.2 Add 5 µL UDI indexes to each reaction. The master mix volume is 50 µL .

Note

UDI indexes
-Unique dual indexing is a sequencing strategy that has distinct, unrelated index sequences for each of the i5 and i7 index reads.
-Unique dual indexing is a known mitigation for filtering index-hopped reads seen in downstream analyses. Misassigned reads will be flagged as "undetermined reads" and can be excluded from analysis.

PDF index-hopping-white-paper.pdf

15.3 Add the whole ligation master mix (50 µL) to the whole amount of sample (50 µL) and mix it via pipetting. The total volume is 100 µL .

Incubate the samples at 20 °C for 00:15:00 .

16 **Clean-up with AMPure XP Beads**

Sample/Bead ratio = 1:0.8 (100 µL sample/ 80 µL bead)

Washing: 2 times with 200 µL 80% EtOH

Elution: 52.5 µL elution buffer (EB)

> Note
>
> Before usage, beads should be at room temperature and mix properly.

16.1 Add 45 µL AMPure XP beads to the sample and mix gently.

16.2 Incubate for 00:05:00 at room temperature.

16.3 Place the tube on a magnetic rack for 00:02:00 (until supernatant is completely clear).

16.4 Remove supernatant without touching the bead pellet.

16.5 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

16.6 Remove supernatant and discard.

16.7 Repeat washing step with 80% ethanol (Step 22-23).

16.8 Carefully remove the supernatant and dry the beads approximately for 00:05:00 by leaving the lid open (Dull pellet indicates dryness).

16.9 Remove tube from the magnetic rack and resuspend the pellets in 52.5 µL elution buffer (EB) and incubate at room temperature for 00:02:00

16.10 Place the tube back to a magnetic rack for 00:02:00 until supernatant is completely clear and transfer 50 µL eluate into clean tube. Make sure that no beads are transferred alongside the supernatant.

17 Clean-up with AMPure XP Beads

Sample/Bead ratio = 1:1 (50 µL sample/ 50 µL bead)
Washing: 2 times with 200 µL 80% EtOH
Elution: 26 µL elution buffer (EB)

Note

Before usage, beads should be at room temperature and mix properly.

17.1 Add 50 µL AMPure XP beads to the sample and mix gently.

17.2 Incubate for 00:05:00 at room temperature.

17.3 Place the tube on a magnetic rack for 00:02:00 (until supernatant is completely clear).

17.4 Remove supernatant without touching the bead pellet.

17.5 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

17.6 Remove supernatant and discard.

17.7 Repeat washing step with 80% ethanol (Step 22-23).

17.8 Carefully remove the supernatant and dry the beads approximately for 00:05:00 by leaving the lid open (Dull pellet indicates dryness).

17.9 Remove tube from the magnetic rack and resuspend the pellets in 26 µL elution buffer (EB) and incubate at room temperature for 00:02:00

17.10 Place the tube back to a magnetic rack for 00:02:00 until supernatant is completely clear and transfer 23.5 µL eluate into a clean PCR tube. Make sure that no beads are transferred alongside the supernatant.

18 **Library amplification**

Prepare the following master mix on ice/coolingrack.

Component	Amount
HIFI PCR Master Mix 2x	25 µL
Universal Primer Mix	1.5 µL
Final volume:	26.5 µL

18.1 Add 26.5 µL master mix to 23.5 µL sample. The total volume is 50 µL . Incubate with the following cycling conditions.

Temperature	Time	Cycles
98 °C	00:02:00	1

98 °C	00:00:20	8
60 °C	00:00:30	8
72 °C	00:00:30	8
72 °C	00:01:00	1
4 °C	Till further processes	

19 **Clean-up with AMPure XP Beads**

Sample/Bead ratio = 1:1 (50 µL sample/ 50 µL bead)

Washing: 2 times with 200 µL 80% EtOH

Elution: 52.5 µL elution buffer (EB)

Note

Before usage, beads should be at room temperature and mix properly.

19.1 Add 50 µL AMPure XP beads to the sample and mix gently.

19.2 Incubate for 00:05:00 at room temperature.

19.3 Place the tube on a magnetic rack for 00:02:00 (until supernatant is completely clear).

19.4 Remove supernatant without touching the bead pellet.

19.5 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

19.6 Remove supernatant and discard.

19.7 Repeat washing step with 80% ethanol (Step 22-23).

19.8 Carefully remove the supernatant and dry the beads approximately for 00:05:00 by leaving the lid open (Dull pellet indicates dryness).

19.9 Remove tube from the magnetic rack and resuspend the pellets in 52.5 µL elution buffer (EB) and incubate at room temperature for 00:02:00

19.10 Place the tube back to a magnetic rack for 00:02:00 until supernatant is completely clear and transfer 50 µL eluate into clean tube. Make sure that no beads are transferred alongside the supernatant.

Library quantification with Qubit and Agarose Gel

20 **Gel Electrophoresis**

Running the Gel is optional. If the parameters in this protocol have been followed as stated the samples should range around 450bp and you can use this for the further calculations.

20.1 Load 5 µL librariy on a 2% Agarose Gel and let it run at 100 Volts for 45-60min.

20.2 The library should show a distribution centered around the size of the fragmented DNA plus 120 bp (450 bp). The increase in library length reflects the addition of sequencing adapters to the DNA fragments or not sufficient fragmentation.

21 **Qubit Measurment**

Quantify 1 µL cleaned Final Library with the Qubit 1x dsDNA HS Assay kit.

21.1 Aliquot 199 µL 1x Working solution to Qubit assay tube (equal to the number of your samples). Prepare two extra tubes for the standards with 190 µL 1x Working solution.

21.2 Add 1 µL sample for each test tube and 10 µL from the Standards (Standard 1-2). The final volume is 200 µL .

21.3 Mix each sample vigorously by vortexing and pulse centrifuge to collect the liquid.

21.4 Incubate at room temperature for 00:02:00 before measuring.

21.5 Calibrate Qubit fluorometer with the standards based on manufacturers recommendation.

21.6 Read your sample.

Note

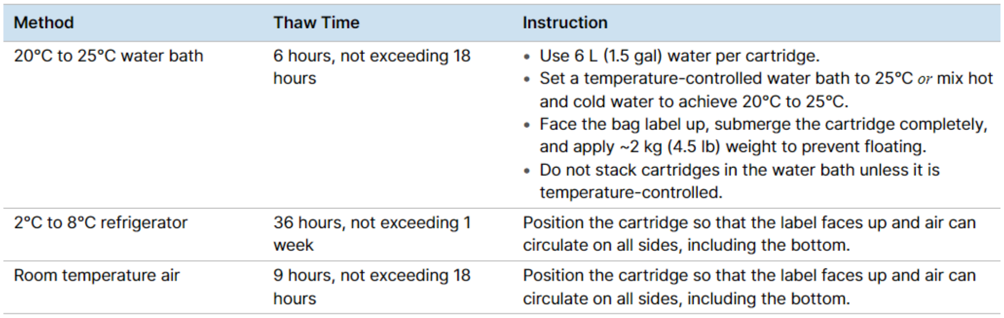
The manual for Qubit 1x dsDNA HS Assay kit.
Qubit_1X_dsDNA_HS_Assay_Kit_UG...

Note

Alternative dsDNA quantification systems can be used as well (e.g.QuantiFluor ONE dsDNA System).

Preparing the sequencing run (iSeq)

22 Thaw the cartridges according to the manufacturer`s instruction.



Method	Thaw Time	Instruction
20°C to 25°C water bath	6 hours, not exceeding 18 hours	• Use 6 L (1.5 gal) water per cartridge. • Set a temperature-controlled water bath to 25°C *or* mix hot and cold water to achieve 20°C to 25°C. • Face the bag label up, submerge the cartridge completely, and apply ~2 kg (4.5 lb) weight to prevent floating. • Do not stack cartridges in the water bath unless it is temperature-controlled.
2°C to 8°C refrigerator	36 hours, not exceeding 1 week	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.
Room temperature air	9 hours, not exceeding 18 hours	Position the cartridge so that the label faces up and air can circulate on all sides, including the bottom.

Summary of Thaw Methods for iSeq cartridge

22.1 The samples are entered directly on the device or a sample sheet in CSV format. If you are using sample sheet prepare it in advance.

> Note
>
> Using the QIAseq FX DNA Library Prep with UDIs Template for the Local Run Manager (LRM) v2 Excel File from Illumina
>
> XLSX PROM-16094-002_TF_QIAseqFX_U...

22.2 Mix the samples in equimolar concentration. Mix it properly!

22.3 Dilute the mixed libraries in 100 µL volume using EB to [M] 1 nanomolar (nM) concentration.

22.4 From previous dilution, make a dilution in 100 µL volume using EB to [M] 50 picomolar (pM) concentration. This means take 95 µL EB and 5 µL diluted library.

22.5 Dilute your PhiX Control to [M] 50 picomolar (pM) . The concentration of the Original Tube is [M] 10 nanomolar (nM) .

22.6 To your final 100µl of 50pM library add 2µl of the 50pM PhiX. That equals to a spike in of ~2%.
Keep going your library is ready to load! :)

Start the sequencing run (iSeq)

23 On the iSeq open the “Illumina iSeq Control Software”.

23.1 Press the “Sequence” button on the center of the screen. The device will open and you can watch a video explaining the next steps.

23.2 Remove the thawed cartridge and the room temperature flow cell from the foil bags.

23.3 Invert the cartridge 5 times and lightly tap it on your work surface to help the reagents settle to the bottom.

23.4 Pierce the silver foil on the cartridge with a pipette tip.

23.5 With a new pipette tip load 20 µL of the finished [M] 50 picomolar (pM) pool to the bottom of the cartridge.

23.6 Insert the flowcell into the cartridge.

23.7 Place the Cartridge into the iSeq and select “close door”.

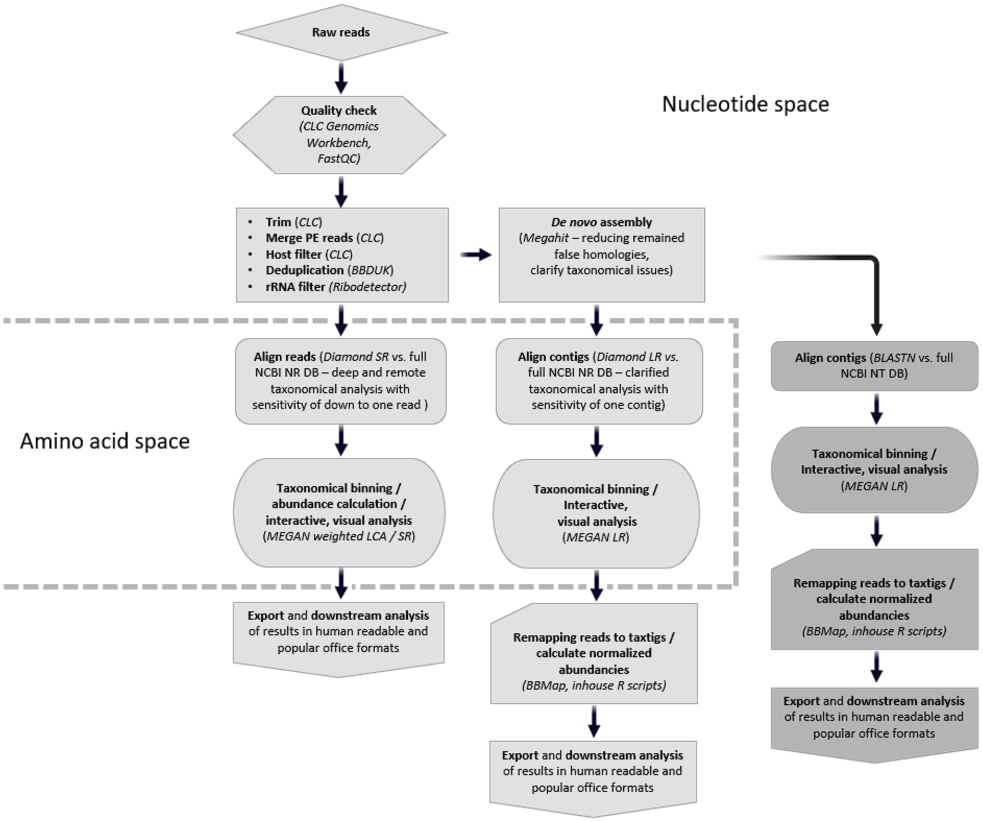
23.8 In the “Run Setup” your run should be automatically selected (if you already added the sample sheet).

23.9 Once the device has checked the cartridge and flow cell and you have double checked the details you can press “start run”.

Bioinformatic analysis

24 The generated FASTQ files are processed using MegaVir.

MegaVir is a bioinformatic pipeline for virome characterization and genome assembly.



The workflow of the MegaVir pipeline.

Note

The link for the pipeline:
https://github.com/balazshorvathreal/MegaVir?tab=readme-ov-file

Acknowledgements

This research was financed by the German Federal Ministry of Education and Research within project PREPMEDVET no. 13N15449. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript/protocol.

RL is funded by the Federal Ministry of Education and Research of Germany (BMBF) under the project NEED (01KI2022). Mosquitoes and dead birds were collected as part of the German national program for arbovirus surveillance funded by the German Federal Ministry of Food and Agriculture (BMEL) through the Federal Office for Agriculture and Food (BLE) with the grant number 2819107A22.