May 05, 2025

Unbiased Metagenomic Protocol: From RNA to Sequencing

Emerging Infectious Diseases

DOI
https://dx.doi.org/10.17504/protocols.io.36wgq6545lk5/v1

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OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.36wgq6545lk5/v1

External link: https://doi.org/10.3201/eid3201.251224

Protocol Citation: Alexander Martínez, Claudia González, Shannon Whitmer, Oris Chavarría, Jessica Góndola, Ambar Moreno 2025. Unbiased Metagenomic Protocol: From RNA to Sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq6545lk5/v1

Manuscript citation:
Chen-Germán M, González C, Araúz D, Aguilar C, Vega M, Chavarria O, Moreno A, Santiago E, Franco D, Valdespino E, Gondola J, Pinedo M, Espino D, Salcedo T, Franco L, Obaldía N, Armien B, Martínez AA, Moreno B Detection of Oropouche and Punta Toro Virus Infections by Enhanced Surveillance, Panama, 2023–2024. Emerging Infectious Diseases 32(1). doi: 10.3201/eid3201.251224

Protocol status: Working
We use this protocol and have successfully sequenced viruses such as Oropouche, Respiratory Syncytial Virus, Equine Encephalitis Virus, Rhinovirus, Madariaga, and Influenza.

Created: March 25, 2025

Last Modified: May 05, 2025

Protocol Integer ID: 125642

Keywords: Metagenomics, DNA, RNA, Non-targeted sequencing, unbiased metagenomic protocol, microorganisms in clinical sample, clinical samples such as nasal swab, available rna to cdna, available rna, circulating dna, amplifying dsdna, clinical sample, sequencing process, rna, viral culture isolate, nasal swab, dsdna, kit nextera xt, novo, microorganism, library with kit nextera xt, dna, sequencing

Funders Acknowledgements:
Ministerio de Economía y Finanzas Panamá
Grant ID: 19911.031
This publication was in part supported by the Cooperative Agreement Number NU50CK000639 awarded to the Pan American Health Organization and funded by the Centers for Disease Control and Prevention. ts contents are solely the responsibility of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention or the Department of Health and Human Services.
Grant ID: NU50CK000639

Disclaimer

Abstract

To identify microorganisms in clinical samples with a diverse community through non-targeted sequencing, a de novo or shotgun sequencing process is proposed, starting from RNA obtained from clinical samples such as nasal swabs, serum, or viral culture isolates. Circulating DNA will be removed enzymatically, then converting the available RNA to cDNA → dsDNA → and then amplifying dsDNA. The obtained material can be sequenced by preparing a library with kit Nextera XT for Illumina platforms.

Guidelines

Use cooling blocks to maintain reagents during PCR steps.

Materials

Reagents

- Qubit 1X dsDNA High Sensitivity (HS) - Invitrogen™ Cat. Q3323
- Qubit RNA High Sensitivity (optional) - Invitrogen™ Cat. Q32852
- TURBO DNA-free™ Kit - Ambion Life Technologies Cat. AM1907
- Nuclease-free water Cat AM9937
- 10X Turbo DNase buffer.
- Turbo DNase, 2U/ µL.
- DNase Inactivation Reagent.
- 3M Sodium acetate CAT S7899-500ML
- 2-propanol
- Absolute ethanol molecular grade
- 70% Ethanol.
- 10nM dNTPs.
- Random Hexamers.
- Nuclease-free water.
- 0.1M DTT.
- RNase Inhibitor 40 U/ µL.
- Superscript IV RT CAT 18090010.
- Superscript IV First-Strand Synthesis System - Invitrogen Cat. 18091050
- NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - Cat. E6111S
- Agencourt AMPure XP - Cat. A63881
- GenomiPhi V2 DNA Amplification Kit - Cytiva Cat. 25660031
- Nextera XT sample prep Kit 96 Box 1 (-25°C to -15°C) CAT FC-131-1096
- Nextera XT library prep kit 96 sample Box 2 (4°C)
- Index adapters 96 samples: CAT FC-131-2001, CAT FC-131-2002, CAT FC-131-2003 and CAT FC-131-2004
- PhiX Control 10nM CAT FC-110-3001
- Qubit 1X dsDNA High Sensitivity (HS) - Invitrogen™ CAT. Q3323
- 1N NaOH CAT 06203-1KG

Equipments

- Thermal cycler
- Level II biosafety cabinet
- Vortex
- Centrifuge
- Microcentrifuge
- Plate centrifuge
- Plate shaker
- Magnetic rack for 1.5 ml microtubes CAT 12321D
- Fluorometer – Qubit

- Fragment analyzer

Qubit 1X dsDNA BR Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q33230

Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #**Q32852

TURBO DNA-free™ Kit **Thermo Scientific Catalog #**AM1907

Nuclease-Free Water (not DEPC-Treated) **Thermo Fisher Scientific Catalog #**AM9937

Sodium Acetate buffer solution 3 M pH 52 for molecular biology **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S7899-500ML

SuperScript™ IV Reverse Transcriptase **Thermo Fisher Catalog #**18090010

SuperScript™ IV First-Strand Synthesis System **Thermo Fisher Scientific Catalog #**18091050

NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 20 rxns **New England Biolabs Catalog #**E6111S

Agencourt AMPure XP beads **Beckman Coulter Catalog #**A63881

GenomiPhi V2 DNA Amplification Kit **Cytvia Catalog #**25660031

Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #**FC-131-1096

Nextera XT Index Kit v2 (set A B C D) **Illumina, Inc. Catalog #**FC-131-2001; FC-131-2002; FC-131 ,

phiX V3 control **Illumina, Inc. Catalog #**FC-110-3001

Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q33230

Invitrogen™ DynaMag™-2 Magnet **Thermo Fisher Scientific Catalog #**12321D

Safety warnings

Due to the unbiased amplification performed, follow a unidirectional workflow to avoid cross-contamination.

Ethics statement

This a research use only protocol, an approval by the users' Institutional Review Board (IRB) or equivalent ethics committee(s) must be obtained for the use on human or animal samples.

Before start

It is recommended to use freshly extracted nucleic acids or those stored for no longer than 3 days at -80 °C.

Consider the target (organisms, in case of a known suspect) you wish to obtain to determine the starting point of the process. If it is RNA, complete the entire process; if it is DNA, purification is recommended, followed by the GenomiPhi kit's amplification step.

Nucleic Acid Quantification (Optional)

1 Follow the nucleic acid quantification procedure (RNA) indicated by the manufacturer for the Qubit fluorometer or the method available in your laboratory for RNA quantification.

2 If identifying microorganisms whose genetic material is DNA is required, quantify the initial material in the sample under study, perform purification with beads at a 1:1 ratio, and then proceed with the Amplification step.

Removal of Contaminating DNA from RNA Samples

35m

3 Mix 5 µL of 10X Turbo DNase Buffer, 1 µL of TURBO DNase (2U), and 4 µL of nuclease-free water in a tube.

4 Dispense 10 µL of the mix into a microtube/8-tube strip or a plate, depending on the number of samples to be processed.

5 Add RNA (~ 40 µL) or 10 µg of RNA per reaction, with a total reaction volume of 50 µL , and mix gently.

6 Incubate at 37 °C for 00:30:00 .

30m

7 Mix the DNase Inactivation Reagent by pipetting or gently swirling the tube.

> Note
>
> This reagent has a milky appearance; ensure it is homogeneous before use.

8 Add 3 µL of DNase Inactivation Reagent to each well containing the samples at Room temperature .

9 Incubate for 00:05:00 at Room temperature , occasionally mixing by pipetting during the incubation.

5m

10 Individually transfer the contents of the wells into a 1.5 mL microtube labeled with the sample ID for the next step.

RNA Precipitation

39m

11 Complete the volume of your DNase-treated RNA solution to a total of 500 µL with nuclease-free water.

12 Add 50 µL of [M] 3 Mass Percent sodium acetate at Room temperature (pH 5.2-5.5).

13 Add 500 µL of 2-propanol at Room temperature and mix by inverting about 10 times to ensure homogeneity.

14 Let stand at Room temperature for 00:20:00 . 20m

15 Centrifuge at 12000 rpm, Room temperature, 00:15:00 to precipitate the RNA pellet. 15m

16 Be aware of which side of the tube the pellet is located.

17 Carefully remove the supernatant.

Note

- The sediment will appear transparent and glassy after the supernatant is removed.
- Be careful not to dislodge or remove the pellet with the pipette tip.

18 Add 500 µL of chilled 70% ethanol to wash the sediment (1/2).

19 Centrifuge at 12000 rpm, Room temperature, 00:02:00 . 2m

20 Remove the supernatant.

Note

Be very careful not to discard the pellet, which will appear white and more visible after the ethanol wash.

21 Wash with 500 µL of chilled 70% ethanol (2/2).

22 Centrifuge at 12000 rpm, Room temperature, 00:02:00 . 2m

23 Carefully remove all the remaining ethanol.

24 Leave the microtube open at Room temperature to allow all the ethanol to evaporate, and ensure the pellet appears matte and completely white.

Note

This step may take 5 to 30 minutes.

25 Resuspend the sediment in nuclease-free water in an appropriate volume (recommended: 30 µL).

Note

Note 1: Optionally, you may assess the RNA quantity and integrity using NanoDrop or the Fragment Analyzer.

First-Strand Synthesis 1h 41m

26

Note

Note 2: Consider processing each sample in duplicate. Consider serving reagents in a nucleic acid-free cabinet.

For the denaturation mix, add 1 µL of [M] 10 millimolar (mM) dNTPs, and 2 µL of random hexamers, and dispense 3 µL of the mix per reaction into a 96-well plate/tube according to the number of samples to be processed.

27 For reverse transcription, mix the following reagents:

	A	B
	water	2 µL
	SSIV buffer	4 µL
	0.1 M DTT	1 µL
	RNase inhibitor (40 U/µL)	1 µL
	SuperScript IV enzyme	1 µL

28 Gently mix the RNA and then perform a short centrifugation to remove droplets from the lid.
Dispense 12 µL of RNA into each well containing the denaturation mix, briefly centrifuge, and place in the thermal cycler at 65 °C for 00:05:00 .

5m

29 After the 00:05:00 at 65 °C , pause the RT-First Strand program to add the reverse transcription mix.

30 Remove the plate/microtube from the thermal cycler and place it in a cold rack or On ice for 00:01:00 .

1m

31 Add 9 µL of the reverse transcription mix to each tube, mix gently, briefly centrifuge, then place in the thermal cycler at 42 °C for 01:30:00 , 85 °C for 00:05:00 , and store at 4 °C .

1h 35m

32 Once the RT program has finished, proceed with the next step. Otherwise, store the cDNA at -20 °C .

Second Strand Synthesis

1h

33

Note

The second strand synthesis module is used to generate double-stranded cDNA from first-strand cDNA, as part of the NEBNext non-directional RNA library preparation workflow.

Note 3: **Consider processing each cDNA in duplicate**.

For the generation of the second strand, mix the following reagents:

	A	B
	NEBNext® Second Strand Synthesis Reaction Buffer	8 µL
	NEBNext® Second Strand Synthesis Enzyme Mix	4 µL

34 Dispense 12 µL of the mix per sample into the PCR plate or strip containing the cDNA sample, and mix by pipetting up to 10 times. The final reaction volume will be 36 µL .

35 Place in the thermal cycler for 01:00:00 at 16 °C, with the lid set to < 40 °C or turned off.

1h

AMPure Bead Purification

21m 30s

36

Note

This step uses beads for the purification of genomic DNA.

Prepare 80% ethanol according to the amount of DNA to be purified.

37 Vortex the bottle containing the magnetic beads for 00:00:30 to ensure they are fully resuspended.

30s

38 Place the sample DNA into a 1.5 mL LoBind tube.

Note

Note 4: If each sample was processed in duplicate, combine both DNAs into a 1.5 mL LoBind
microtube.

39 Add a 1.8X ratio of beads according to the total volume obtained from the second strand synthesis.

40 Vortex or mix by pipetting at least 10 times.

41 Incubate for 00:05:00 at Room temperature . 5m

42 Briefly centrifuge to remove droplets from the walls of the tube.

43 Place on the magnetic stand until the supernatant becomes clear, approximately 00:05:00 . 5m

44 Remove and discard the supernatant.

Note

Be careful not to disturb or discard the bead pellet.

45 Wash with 200 µL of 80% ethanol and incubate for 00:00:30 (1/2). 30s

Note

If using 1.5 mL microtubes for purification, perform the washes with 300 µL of ethanol to cover the bead pellet.

46 Remove and discard the supernatant.

47 Wash with 200 µL of 80% ethanol and incubate for 00:00:30 (2/2). 30s

48 Remove all ethanol residues with a 10 µL pipette tip.

49 Briefly centrifuge to concentrate all remaining ethanol at the bottom, place on the magnetic stand for 00:01:00, and remove the ethanol with a 10 µL pipette tip. 1m

> Note
>
> Make sure all ethanol is removed.

50 Keep the microtube on the magnetic stand with the lid open to dry the beads for up to 00:05:00. 5m

> Note
>
> Avoid over-drying or fragmenting the beads.

51 Remove the tube from the magnetic stand and resuspend it with 35 µL of nuclease-free water.

52 Vortex and centrifuge the tube. Verify that the mixture is resuspended.

53 Incubate for 00:02:00 at Room temperature. 2m

54 Place on the magnetic stand for 00:02:00. 2m

55 Transfer 30 µL of the supernatant into a new 1.5 mL LoBind tube.

Amplification

1h 44m

56

Note

The GenomiPhi V2 DNA Amplification Kit The Illustra™ GenomiPhi™ V2 DNA amplification kit enables whole-genome amplification at a mini scale through isothermal displacement amplification of multiple strands. The amplification is highly uniform across the genome, ensuring the representation is as close as possible to the original DNA sample. The DNA polymerase enzyme used is Phi29, which has proofreading activity.

Note 5: Consider processing each DNA sample in duplicate.

Add 9 µL of sample buffer in a tube/plate for each sample to be processed.

57

- In a new tube, mix 9 µL of reaction buffer and 1 µL of enzyme for each sample to be processed.
- Mix gently and briefly centrifuge the 'Mix' tube to remove droplets from the lid.
- Keep it On ice or in a cooler.

58

Add 5 µL of DNA into each well containing the sample buffer and place in the thermal cycler at 95 °C for 00:03:00 .

3m

59

- Then, pause the program and place it On ice or a cold block for at least 00:01:00 .
- After the pause, dispense 10 µL of the amplification mix into each tube of denatured DNA, using a cold block.
- Mix gently.

1m

60

Place in the thermal cycler and continue the incubation at 30 °C for 01:30:00 , inactivate at 65 °C for 00:10:00 , and then store at 4 °C .

1h 40m

Note

Note 6: Perform product purification (optional).

Sequencing

7m 30s

61 Quantify the samples according to the Qubit DNA High Sensitivity kit procedure and dilute them to 0.2 µL .

62
- In a new 96-well plate, add 10 µL of tagmentation buffer (TB), 5 µL of the amplification mix (ATM), and 5 µL of the PCR product diluted to 0.2 µL .
- Gently mix by pipetting and briefly centrifuge.

63
- Place the 96-well plate in the thermocycler at 55 °C for 00:02:30 , followed by storage temperature at 10 °C .
- Once the thermocycler reaches 10 °C , stop the program and proceed to add 5 µL of NTB.
- Mix by pipetting and briefly centrifuge.

2m 30s

64 Incubate for 00:05:00 at Room temperature . Final volume per well: 25 µL .

5m

Note

Note 7: Preparing libraries for metagenomic applications requires diluting the indexes to avoid excess adapter dimers when working with low sample concentrations. Index dilution: perform a 1:10 dilution of the indexes, calculating based on the amount of sample you are working with.

65 For the addition of indexes, add 5 µL of each diluted index (i5 and i7) to the 96-well plate according to the set available in your laboratory.
Mix by pipetting 5 to 10 times.

66 Add 15 µL of NPM to each well and mix by pipetting 5 to 10 times. Final volume per well: 50 µL .

67 Seal the plate with Microseal B or plate strips, briefly centrifuge, and place it in the thermocycler with the following conditions:

	A	B	C
	Step	**Temperature (°C)**	**Time**
	Enzyme tagmentation inactivation	72	3 minutes
	Denaturalization and activation	95	30 seconds
	PCR cycles (16)	95	10 seconds
		55	30 seconds
		72	30 seconds
	Final extension	72	5 minutes
	Hold	4	∞

68 At the end of the program, the procedure can be stopped if necessary. The 96-well plate can be stored at 2-8 °C for up to 2 days; otherwise, proceed with the next step.

Library Purification

12m

69 Prepare 80% ethanol according to the number of samples to be processed.

Note

Note 8: The AMPure beads used during library purification should be brought to Room temperature 00:30:00 before use. Homogenize in the tube rotator during this time.

70 Mix the beads well in the vortex before use and ensure they are homogeneous. Serve a 1:1 ratio of beads/samples in this step.

71 Cover the plate with adhesive seal B/strip caps and mix using the plate shaker at 1800 rpm, 00:02:00 .

72 Incubate at Room temperature for 00:05:00 .

5m

73 Place the plate on the magnetic stand and let it sit for about 00:02:00 .

2m

74 Maintain the plate on the magnetic stand, and carefully remove and discard the supernatant.

75 With the plate still on the magnetic stand, add 200 µL of 80% ethanol into each well without mixing and incubate for 00:00:30 (1/2). 30s

76 Remove and discard the supernatant.

77 Add 200 µL of 80% ethanol into each well without mixing and incubate for 00:00:30 (2/2). 30s

78 Remove and discard the supernatant.

> Note
>
> It is important to ensure that most of the supernatant has been removed from the wells.

79 With the plate still on the magnetic stand, allow air to dry the plate for 10 to 12 minutes at Room temperature .

> Note
>
> Avoid fragmenting the bead pellet.

80 Carefully remove the plate from the magnetic stand and add 35 µL of RSB to each well of the plate. Mix up and down 10 times.

81 Seal the plate and mix at 1800 rpm, 00:02:00 .

82 Incubate the plate at Room temperature for about 00:02:00 . 2m

83 Place the plate on the magnetic stand for 00:02:00 and allow the beads to form a pellet. 2m

84 Remove 30 µL of supernatant from the Index PCR Clean-up plate and transfer it to the newly labeled CIA plate.

Note

Note 9: The protocol can be safely stopped at this point. The CIA plate can be stored in the refrigerator at 2-8 °C °C or Overnight at 25 °C to 15 °C for 7 days.

Library Quantification

85 Follow the nucleic acid quantification procedure (Qubit DNA High Sensitivity kit) indicated by the manufacturer for the Qubit fluorometer or the method available in your laboratory for DNA quantification.

Library Pooling

86 Label a 1.5 mL LoBind microtube as 'PAL' (Pool amplification library).

87 Add the appropriate number of samples to the previously labeled microtube, as indicated in the 'Pooling library spreadsheet'. The volume of samples will be calculated using the following formula.

Sample volume to add = (Fv* Fc) / (# * Ic)

Fv= Final Volume

Fc = Final Concentration

= Total number of samples to pool

Ic = Initial Concentration

88 PAL can be brought to a final concentration of [M] 4 nanomolar (nM) , [M] 2 nanomolar (nM) , or [M] 1 nanomolar (nM) in a final volume of approximately 25 µL . The final volume of the PAL and the number of samples can be modified.

89 Use the resuspension buffer to complete the final volume to which the library will be brought, as indicated in the spreadsheet.

90 The final concentration of the libraries in the microtube labeled as PAL can be confirmed by quantifying the library again.

Library Purification and Quantification

11m

91 Prepare 3 mL of 80% ethanol.

92 Add a 1:1 ratio of AMPure XP beads to the microtube containing the pooled library.

93 Mix and incubate for 00:05:00 at Room temperature . 5m

94 Place on the magnetic rack until the supernatant is clear (00:05:00).

95 Remove and discard the supernatant.

> Note
>
> Be careful not to disturb the bead pellet or discard the beads.

96 Wash the beads with 80% ethanol.

97 Keep the microtube on the magnetic rack, wash with 1000 µL of 80% ethanol, and incubate for 00:00:30 (1/2). 30s

98 Remove and discard the supernatant.

99 Wash the beads with 1000 µL of 80% ethanol and incubate for 00:00:30 (2/2). 30s

100
- After the second wash, remove all ethanol with a 10 µL pipette tip.
- Then centrifuge to ensure all ethanol is at the bottom, place the tube on the magnetic rack for 00:01:00, and remove with a 10 µL pipette tip.

1m

101 Remove the tube from the magnetic rack and resuspend it with 35 µL of RSB.

102 Vortex and centrifuge. Ensure the mixture is resuspended.

103 Incubate for 00:02:00. 2m

104 Place on the magnetic rack for 00:02:00. 2m

105 Transfer 30 µL of the supernatant into a new 1.5 mL microtube labeled 'Purified PAL'.

106 Quantify the library again to start the dilution and denaturation process.

Note

Note 10: Depending on the final concentration of your library, proceed to the next step by diluting the sample starting from [M] 4 nanomolar (nM), [M] 2 nanomolar (nM), or [M] 1 nanomolar (nM). If, when quantifying the library, a concentration lower than [M] 1 nanomolar (nM) is obtained, convert the obtained concentration to picomolar and gradually dilute the library to the indicated loading concentration.

Dilution and Denaturation

107 Preparation of Fresh NaOH and Dilution

107.1 Prepare [M] 1.0 Mass Percent NaOH.

107.2 Dilute [M] 1 Mass Percent NaOH to [M] 0.2 Mass Percent. Add the following reagents:

	A	B
	molecular-grade water	800 µL
	1.0 N NaOH	200 µL

107.3 Invert the microtube several times to mix. The result is 1 mL of [M] 0.2 Mass Percent NaOH.

Denaturation and Dilution of the Library

5m

108 Combine the following volumes in a 1.5 mL LoBind, depending on your library concentration:

	A	B	C	D
	Library concentration	**1 nM**	**2 nM**	**4 nM**
	Purified PAL (µL)	20	10	5
	NaOH 0.2 N (µL)	20	10	5
	Hyb (µL)	960	980	990
	The total volume of the library at 20 pM (µL)	1000		

109 Mix by vortexing and perform a short centrifugation to concentrate the contents at the bottom of the tube.

110 Incubate at Room temperature for 00:05:00.

5m

111 Add Hyb to the tube containing the denatured library. The result is 1 mL of denatured library at [M] 20 picomolar (pM) .

112 Depending on the sequencing chemistry of the kit, perform the corresponding loading dilution. For MiSeq v2, the final loading concentration is up to [M] 10 picomolar (pM) .

113
- Prepare PhiX at [M] 12.5 picomolar (pM) . Discard 60 µL of the library and add 60 µL of the PhiX control at [M] 12.5 picomolar (pM) to achieve a 10% PhiX concentration in the library.
- Mix the solution thoroughly and briefly centrifuge.

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