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Version 1

🌐 Discovery of RNA and DNA viruses using next-generation sequencing: Metagenomics V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.261ge34zol47/v1>



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CVR Genomics



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We use this protocol and it's working

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Abstract

Next-generation sequencing is a powerful tool for viral genomics. Viruses often constitute a very small proportion of any given sample meaning that methods that enable detection of viral nucleic acids are frequently needed for detection and characterisation. Improvement of sensitivity can be achieved by depletion of unwanted nucleic acid during sample pre-treatment or by enrichment such as PCR amplification with virus specific primers, or probe-based targeted enrichment. However, some methods for specific enrichment rely on prior knowledge of the viruses. Here we describe a method for next-generation sequencing to identify unknown viruses (see Figure 1).

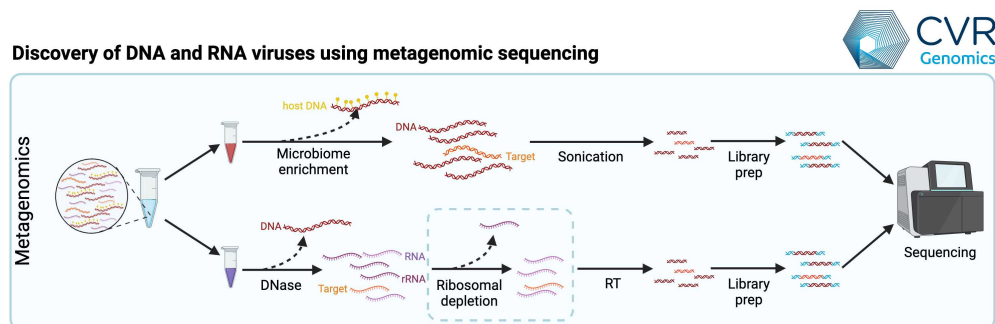


Figure 1: Diagram of workflow for discovery of DNA and RNA viruses using metagenomic sequencing. Optional ribosomal depletion step is shown in hashed box. Image prepared using BioRender.com.

Following simultaneous extraction of RNA and DNA samples are split into two and subjected to non-specific treatments that deplete host nucleic acids and improve chances of detecting RNA or DNA viruses, respectively. Illumina sequencing libraries are then prepared and these metagenomic libraries can directly sequenced. These sequencing libraries can then be subjected to targeted enrichment using a pan-viral probe set to achieve higher sensitivity (for details see accompanying protocol [Discovery of RNA and DNA viruses using next-generation sequencing: Targeted enrichment](#)).

We applied this approach to an outbreak of acute hepatitis of unknown aetiology in children, enabling the identification of adeno-associated virus 2 (AAV2) in all patients but not in samples from controls. This method also led to the identification of adenovirus and human herpesviruses.

This protocol describes the pre-treatment of nucleic acid extracts to improve detection of DNA and RNA viral reads followed by Illumina library preparation and metagenomic sequencing.



Materials

Reagents:

- ✕ Ambion® DNase I (RNase-free) **Thermo Fisher Catalog #AM2224**
- ✕ Agencourt RNAClean XP Magnetic Beads **Beckman Coulter Catalog #A63987**
- ✕ Illumina Ribo-Zero Plus rRNA Depletion Kit **Illumina, Inc. Catalog #20040526**
- ✕ Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog #N0447S**
- ✕ Random hexamers **Thermo Scientific Catalog #N8080127**
- ✕ SuperScript® III Reverse Transcriptase **Thermo Fisher Catalog #18080044**
- ✕ RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #10777019**
- ✕ NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**
- ✕ Agencourt AmPure XP beads **Catalog #A63880**
- ✕ NEBNext Microbiome DNA Enrichment Kit - 24 rxns **New England Biolabs Catalog #E2612L**
- ✕ KAPA LTP library prep kit **Roche Catalog #796188001**
- ✕ 40% Polyethylene Glycol MW 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1458**
- ✕ 5 M Sodium chloride (NaCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5150-1L**
- ✕ NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) **New England Biolabs Catalog #E6440-8**
- ✕ HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #KK2601**
- ✕ Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32851**
- ✕ Qubit RNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32852**
- ✕ Genomic DNA ScreenTape **Agilent Technologies Catalog #5067-5365**
- ✕ Genomic DNA Reagents **Agilent Technologies Catalog #5067-5366**

Additional reagents required:

Absolute ethanol
Nuclease-free water
10 mM Tris pH8

Equipment:

Equipment

LE220	NAME
High-throughput focused ultrasonicators	TYPE
Covaris	BRAND
500569	SKU
https://www.covaris.com/le220-plus-focused-ultrasonicator-500569 ^{LINK}	

Equipment

Qubit	NAME
Flurometer	TYPE
Invitrogen	BRAND
Q33228	SKU
https://www.thermofisher.com/order/catalog/product/Q33228 ^{LINK}	



Equipment

4200 TapeStation System	NAME
Electrophoresis tool for DNA and RNA sample quality control.	TYPE
TapeStation Instruments	BRAND
G2991AA	SKU
https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263	LIN K



Protocol materials

- ✕ Genomic DNA ScreenTape **Agilent Technologies Catalog #5067-5365**
- ✕ Illumina Ribo-Zero Plus rRNA Depletion Kit **Illumina, Inc. Catalog #20040526**
- ✕ RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #10777019**
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- ✕ 5 M Sodium chloride (NaCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5150-1L**
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- ✕ Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog #N0447S**
- ✕ NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**
- ✕ NEBNext Microbiome DNA Enrichment Kit - 24 rxns **New England Biolabs Catalog #E2612L**
- ✕ KAPA LTP library prep kit **Roche Catalog #796188001**
- ✕ NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) **New England Biolabs Catalog #E6440-8**
- ✕ Qubit RNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32852**

Before start

This protocol starts with nucleic acid extracts.



Initial sample preparation

- 1 Quantify the DNA and RNA concentration of your samples using Qubit HS reagents.

 Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32851**

 Qubit RNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32852**

Equipment

Qubit

NAME

Flurometer

TYPE

Invitrogen

BRAND

Q33228

SKU

<https://www.thermofisher.com/order/catalog/product/Q33228>^{LINK}



Note

Depending on the sample type the level of RNA/DNA may be undetectable and so quantification may not be required for all samples.

- 2 Split each nucleic acid extract into two subsamples for RNA and DNA virus detection.

2.1 If required, make up each sample to  50 µL with Nuclease-free water.

2.2 Prepare two 0.2 mL PCR tubes per sample labelled with **R** (for the RNA pre-treatment) or **D** (for DNA pre-treatment) along with the sample names.

Note

For multiple samples it is recommended that PCR strip tubes or plates are used, ensure that subsamples are grouped for RNA or DNA virus detection and able to be easily separated.

2.3 Add  25 µL sample to the tube **R** and  25 µL sample to tube **D**.

Subsample **R** → proceed to RNA virus detection - DNase I treatment (Step 3).

Subsample **D** → proceed to DNA virus detection - Microbiome enrichment (step 14).

Note

It is recommended to process the RNA samples first to minimise degradation.

RNA virus detection - DNase I treatment

27m

3 Prepare DNase I mix as follows (for multiple samples prepare a master mix with 10% excess):

A	B
Component	Volume (µl)
10X DNase I buffer	3
DNase I	2
Total	5

 Ambion®; DNase I (RNase-free) Thermo Fisher Catalog #AM2224

Note

For large amounts of DNA use the suppliers recommendations.



3.1 Add  5 μ L DNase I mix to  25 μ L RNA sample .

3.2 Incubate as follows:

15m

 37 °C for  00:15:00 then place  On ice .

4 Perform clean-up with 2X volume of RNAClean XP magnetic beads.

 Agencourt RNAClean XP Magnetic Beads **Beckman Coulter Catalog #A63987**

Note

Ensure RNAClean XP beads are equilibrated to room temperature for ~30 min and vortex well before use.

4.1 Add  60 μ L RNAClean XP beads to the  30 μ L DNase I reaction and mix by pipetting.

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

5m

4.3 Place on a magnetic rack until beads and solution have fully separated.

4.4 Carefully remove supernatant being careful not to disturb the beads.

4.5 Wash 2X with  200 μ L 80% Ethanol .

Note

Ensure ethanol is freshly prepared.

4.6 Remove all traces of ethanol and air dry for up to  00:05:00 .

5m

**Note**

Take care not to over dry the beads.

- 4.7 To elute DNase I treated RNA add  12 μ L Nuclease-free water and incubate at  Room temperature for at least  00:02:00

2m

RNA virus detection - Ribosomal depletion

56m

- 5 Transfer  10 μ L DNase I treated RNA to fresh 0.2 mL tubes/plate for Ribo-zero probe hybridisation.

 Illumina Ribo-Zero Plus rRNA Depletion Kit **Illumina, Inc. Catalog #20040526**

Note

Ribo-depletion is recommended for sample that are likely to contain large levels of host or bacterial RNA (such as tissue biopsy, faecal, oral or nasal) but can be excluded from sample with lower levels of host RNA (such as plasma, serum or cerebrospinal fluid), or when the sample input is too low to enable library preparation.

- 5.1 Thaw DB1 and DP1 at  Room temperature , vortex to mix and centrifuge briefly.
- 5.2 Prepare the hybridisation probe mix on ice (for multiple samples prepare a master mix with 10% excess):

	A	B
	Component	Volume (μ l)
	DB1 (Depletion Probe Buffer)	3
	DP1 (Depletion Probe Pool)	1
	Total	4

5.3 Thoroughly pipette mix.

5.4 Add  4 µL hybridisation probe mix to each  10 µL sample and fully mix by pipetting 10 times.

5.5 Incubate samples as follows:

 95 °C for  00:02:00

Decrease  0.1 °C /sec until temperature reaches  37 °C then hold.

15m

Note

The program takes ~ 15 min to finish.

6 Prepare rRNA Depletion.

6.1 Thaw RDB and RDE at  Room temperature , vortex or flick (RDE) to mix and centrifuge briefly.

6.2 Prepare the rRNA Depletion mix (for multiple samples prepare a master mix with 10% excess):

A	B
Component	Volume (µl)
RDB (RNA Depletion Buffer)	4
RDE (RNA Depletion Enzyme)	1
Total	5

6.3 Thoroughly pipette mix.



6.4 Add  5 μ L rRNA Depletion mix to each  14 μ L and fully mix by pipetting 10 times.

6.5 Incubate samples as follows:

15m

 37 °C for  00:15:00

 4 °C hold

7 Prepare probe removal.

7.1 Thaw PRB and PRE at  Room temperature , vortex (PRB) or flick (PRE) to mix and centrifuge briefly.

7.2 Prepare the Probe Removal mix  On ice (for multiple samples prepare a mastermix with 10% excess):

A	B
Component	Volume (μ l)
PRB (Probe Removal Buffer)	7
PRE (Probe Removal Enzyme)	3
Total	10

7.3 Thoroughly pipette mix.

7.4 Add  10 μ L Probe Removal mix to each  19 μ L reaction and fully mix by pipetting 10 times.

7.5 Incubate samples as follows:

30m

 37 °C for  00:15:00

 70 °C for  00:15:00



🌡️ 4 °C hold

8 Perform clean-up with 2X volume of RNAClean XP magnetic beads.

⊗ Agencourt RNAClean XP Magnetic Beads **Beckman Coulter Catalog #A63987**

Note

Ensure RNAClean XP beads are equilibrated to room temperature for ~30 min and vortex well before use.

8.1 Add 🧪 60 µL RNAClean XP beads to the 🧪 30 µL reaction , mix by pipetting.

8.2 Incubate at 🌡️ Room temperature for ⌚ 00:05:00

5m

8.3 Place on a magnetic rack until beads and solution have fully separated.

8.4 Remove and discard supernatant.

8.5 Wash 2X with 🧪 175 µL 80% Ethanol (freshly prepared) .

8.6 Remove all traces of ethanol and air dry for up to ⌚ 00:02:00

2m

Note

Do not over-dry the beads.

8.7 Elute in 🧪 12 µL ELB (Elution buffer) by incubating at 🌡️ Room temperature for ⌚ 00:02:00 .

2m



RNA virus detection - Low input reverse transcription

4h

- 9 Transfer  10 μL RNA sample to fresh 0.2 mL tubes/plates.

Note

This method for cDNA preparation has been tested for very low-input and undetectable RNA/DNA by Qubit HS reagents.

- 10 Prepare NTP/Hex mix (for multiple samples prepare a master mix with 10% excess):

A	B
Component	Volume (μL)
10 mM dNTP	1
Random Hexamers	1
Total	2

 Deoxynucleotide (dNTP) Solution Mix **New England Biolabs Catalog #N0447S**

 Random hexamers **Thermo Scientific Catalog #N8080127**

- 10.1 Add  2 μL NTP/Hex mix to each  10 μL sample .

- 10.2 Incubate as follows:

 65 °C for  00:05:00

immediately place  On ice

5m

- 11 Prepare the SSIII master mix (for multiple samples prepare a master mix with 10% excess):

A	B
Component	Volume (μL)



A	B
5X Reverse transcription buffer	4
SuperScript III	2
RNaseOUT	1
DTT	1
Total	8

 SuperScript[®] III Reverse Transcriptase **Thermo Fisher Catalog #18080044**

 RNaseOUT[™] Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #10777019**

11.1 Add  8 μ L SSIII mix to each  12 μ L reaction .

11.2 Incubate samples as follows:

1h 25m

 25 °C for  00:10:00

 55 °C for  01:00:00

 70 °C for  00:15:00

 4 °C hold

12 Prepare second strand mix (for multiple samples prepare a mastermix with 10% excess):

A	B
Component	Volume (μ l)
10X Second strand synthesis buffer	8
Second strand synthesis enzyme	4
Nuclease-free water	48
Total	60

 NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**



12.1 Add  60 μ L second strand mix to each  20 μ L reaction

12.2 Incubate as follows on PCR machine

2h 30m

 16 °C for  02:30:00

 4 °C hold

13 Perform clean up with 1X volume of AmPure XP beads.

 Agencourt AmPure XP beads **Catalog #A63880**

Note

Ensure AmPure XP beads are equilibrated to room temperature for 30 min and vortex well before use.

13.1 Add  80 μ L AmPure XP to each  80 μ L reaction (1:1 Ampure:sample ratio) and mix well.

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

5m

13.3 Place on a magnetic rack until beads and solution have fully separated.

13.4 Remove supernatant.

13.5 Wash 2X with  200 μ L 80% Ethanol (freshly prepared) .

13.6 Remove all traces of ethanol and air dry for  00:05:00 .

5m



13.7 Elute in  27 μ L 10 mM Tris pH8 by incubation at  Room temperature for  00:02:00 .

2m

Note

cDNA can be stored at 4°C overnight or -20°C longer term

cDNA → proceed to Section – Library prep (step 22) or store until DNA samples ready so can process together.

DNA virus detection - Microbiome enrichment

4h 43m

14 Pre-bind MBD2-Fc Protein to Magnetic Beads.



NEBNext Microbiome DNA Enrichment Kit - 24 rxns **New England Biolabs Catalog #E2612L**

See following attachment for reagent calculations:



Microbiome_calculations.xlsx

Note

Microbiome enrichment can be used to deplete CpG modified DNA.

14.1 Pipette  1 μ L Protein A magnetic beads (see column **B** of reagent calculation table) for every  6.25 ng input DNA into a 1.5 mL DNA LoBind tube.

Note

For low or undetectable amounts of DNA (<30 ng) use 5 μ L of magnetic beads.



Note

Do not vortex the magnetic beads.

14.2 Add  0.1 V MBD2-Fc protein (see column **C** of reagent calculation table) to the Protein A magnetic beads.

14.3 Mix the bead-protein mixture by placing the tube in a rotating mixer for  00:10:00 .

10m

14.4 Prepare the 1X Bind/Wash buffer and keep it  On ice :

A	B
Component	Volume (μl)
5X NEBNext Bind/Wash buffer	800
Nuclease-free water	3200
Total	4000

14.5 After the incubation, briefly spin the tube and place on the magnetic rack for  00:05:00 until the beads have collected.

5m

14.6 Remove the supernatant with a pipette without disturbing the beads.

14.7 Add  1 mL 1X Bind/Wash buffer to the tube to wash the beads. Pipet up and down a few times to mix.

14.8 Mix the beads on a rotating mixer for  00:03:00 at  Room temperature .

3m

14.9 Briefly spin the tube and place on the magnetic rack for  00:05:00 until the beads have collected.

5m



- 14.10 Remove the supernatant with a pipette without disturbing the beads.
- 14.11 Repeat wash step (2 washes in total).
- 14.12 Resuspend the beads in the volume of 1X Bind/Wash buffer equal to the initial magnetic bead volume in step 13.1 (see sum of column **B** of reagent calculation table).
- 15 Capture Methylated Host DNA.
- 15.1 Add appropriate volume of 5X Bind/Wash buffer to fresh tubes for each sample to give a 1X solution (see column **D** of reagent calculation table).
- 15.2 Add the volume of sample to give up to  1 µg DNA (see column **A** of reagent calculation table).

Note

It is important that the buffer and DNA is combined before adding the beads!

Note

Use a minimum volume of 40 µl and make up with 10 mM Tris pH8 if necessary. Maximum volume is 200 µl.

- 15.3 Add appropriate volume of washed Fc-bead/protein mix (see column **B** of reagent calculation table) to the DNA/buffer mix.
- 15.4 Mix and incubate in a rotating mixer at RT for  00:15:00 to  04:00:00 depending on sample type .

4h 15m

**Note**

Although the manufacturer's protocol suggests 15 min we would not recommend less than 30 min, and in some cases much longer is required.

Note

Ensure that the liquid moves freely during the incubation to achieve efficient mixing.

16 Elute Microbiome DNA.

16.1 Briefly centrifuge and place on a magnet for at least  00:05:00 to separate the bead-bound host DNA.

5m

16.2 Remove the supernatant containing host depleted/microbiome enriched DNA to fresh tubes.

Note

The supernatant contains the host-depleted microbiome DNA, the beads contain the microbiome-depleted host DNA. If required the beads can be resuspended in 1X bind buffer and stored for analysis of host DNA see manufacturer's guidelines for details.

Note

The supernatant is used directly in the sonication but can be cleaned up with 2X Ampure (see column **E** of reagent calculation table for volume) for longer term storage.

 Agencourt AmPure XP beads **Catalog #A63880**

DNA virus detection - sonication

1h 5m

17 If required make sample up to  55 μ L with 10mM Tris pH8 .

18 Prepare sonicator for use.

Note

Here we describe sonication with the Covaris LE220 and 8microTUBE-50 AFA Fiber Strip V2 or 96 microTUBE AFA Fiber Plate Thin Foil. Be sure to follow the manufacturers recommendations for your own instruments. In addition, some companies offer alternatives to sonication such as the use of fragmentases, this would need to be optimised before use.

Equipment

LE220	NAME
High-throughput focused ultrasonicators	TYPE
Covaris	BRAND
500569	SKU
https://www.covaris.com/le220-plus-focused-ultrasonicator-500569	LINK

18.1 Fill the tank with water to FILL level -2.

18.2 Switch on the chiller and ensure set to  7 °C .

18.3 Switch on the water conditioning system, the Covaris and the computer.

18.4 Open the SonoLab software.

18.5 Select Home and the transducer will get submerged, the degas pump should start automatically.

18.6 Degas the water bath for ~  00:45:00 .

45m

19 Set up the sonication conditions as follows:

	A	B
	Peak power	450
	Duty factor	10
	Cycles/burst	1000
	Treatment time (s)	89
	Dithering	on

Example sonication conditions to achieve ~350 bp fragments.

Note

The precise conditions will need to be validated using a gDNA control prior to use with samples.

20 Sonicate samples.

20.1 Add  55 µL Sample to either the 8 microTUBE-50 AFA Fiber Strip V2 or 96 microTUBE AFA Fiber Plate Thin Foil.

20.2 Place the strip/plate in the appropriate holder and screw into place (ensure that it is set up the same as the program conditions).

20.3 Select Load position to move the support arm forward.

- 20.4 Press the green button and open the door to put the holder into the support arm, ensure in the correct orientation and close the door.
- 20.5 Press start position to submerge the rack and confirm the correct volume of water has been added (samples should NOT be fully submerged).
- 20.6 Press Run to start the sonication.
- 20.7 Once completed press Load Position to remove the plate.
- 20.8 Transfer  50 µL sheared DNA to PCR tubes.

Note

Option to check shearing by running 1 µL on TapeStation gDNA.

 Genomic DNA ScreenTape **Agilent Technologies Catalog #5067-5365**

 Genomic DNA Reagents **Agilent Technologies Catalog #5067-5366**

Equipment

4200 TapeStation System

NAME

Electrophoresis tool for DNA and RNA sample quality control.

TYPE

TapeStation Instruments

BRAND

G2991AA

SKU

<https://www.agilent.com/en/product/automated-electrophoresis/tapestation-systems/tapestation-instruments/4200-tapestation-system-228263>

LINK

- 21 1.4X Ampure clean up.

 Agencourt AmPure XP beads **Catalog #A63880**

21.1 Add  70 μ L Ampure XP to the samples (ratio 1.4:1).

21.2 Incubate at  Room temperature for  00:15:00 .

15m

21.3 Place on a magnetic rack until beads and solution have fully separated.

21.4 Remove supernatant.

21.5 Wash 2X with  200 μ L 80% Ethanol (freshly prepared) .

21.6 Remove all traces of Ethanol. Air dry for  00:05:00 .

5m

21.7 Elute samples in  25 μ L 10mM Tris pH8 .

21.8 Transfer  25 μ L sample to new tubes.

Note

Pause: DNA can be stored at 4°C overnight or -20°C for longer term.

Sheared DNA → proceed to Section – Library prep (step 22) can process alongside the prepared cDNA samples.

Illumina sequencing library preparation

1h 50m

22 Prepare the End Repair mix (for multiple samples prepare a master mix with 10% excess):

	A	B
	Component	Volume (μ l)



	A	B
	10X End repair buffer	3
	End repair enzyme	2
	Total	5

Note

This version uses the KAPA LTP kit that has been discontinued by the supplier. Reactions at at half the volume of the manufacturer's recommendations with the final PCR reaction being at full volume.

Note

Samples pre-treated for the detection of DNA and RNA viruses can be processed alongside each other. Treat as seperate samples and do not pool before library prep.

 KAPA LTP library prep kit **Roche Catalog #796188001**

22.1 Add  5 μ L End repair mix to each  25 μ L sample .

22.2 Incubate as follows (if using a PCR machine do not use hot lid):

 20 °C for  00:30:00

30m

23 1.4X Ampure XP clean up.

 Agencourt AmPure XP beads **Catalog #A63880**

23.1 Add  20 μ L 10 mM Tris pH8 increase volume of each sample to a total of  50 μ L .

23.2 Add  70 μ L Ampure XP (1.4:1 Ampure:sample ratio). Pipette up and down to mix.

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

5m



23.4 Place samples on a magnetic rack until beads and solution have fully separated.

23.5 Remove supernatant.

23.6 Wash 2X with  200 μ L 80% Ethanol (freshly prepared) .

23.7 Remove all traces of ethanol and air dry for  00:05:00 .

5m

23.8 Elute in  21 μ L 10mM Tris pH8 leaving beads in solution.

24 13. Prepare the A-Tail mix (for multiple samples prepare a master mix with 10% excess):

A	B
Component	Volume (μ l)
10X A-Tail buffer	2.5
A-Tail enzyme	1.5
Total	4

 KAPA LTP library prep kit **Roche Catalog #796188001**

24.1 Add  4 μ L A-Tail mix to each  21 μ L reaction .

24.2 Incubate as follows:

 30 °C for  01:00:00

1h

25 1.4X SPRI clean up.

**Note**

SPRI is a solution containing 20% PEG 8000 and 2.5 M NaCl and allows for Ampure clean up using the beads contained within the reaction. SPRI is included as part of the KAPA LTP library prep kit but if required additional SPRI can be made by mixing equal quantities of 40% PEG 8000 and 5 M NaCl.

⚗ 40% Polyethylene Glycol MW 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P1458**

⚗ 5 M Sodium chloride (NaCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5150-1L**

25.1 Add  25 µL 10 mM Tris pH8 to increase volume of each sample to a total of  50 µL .

25.2 Add  70 µL SPRI (1.4:1 SPRI:sample ratio). Pipette up and down to mix.

Note

The magnetic beads should remain in solution from the previous step, alternatively Ampure XP can be used instead of SPRI reagent.

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

5m

25.4 Place the samples on a magnetic rack until beads and solution have fully separated.

25.5 Remove supernatant.

25.6 Wash 2X with  200 µL 80% Ethanol (freshly prepped) .

25.7 Remove all traces of ethanol and air dry for  00:05:00 .

5m

25.8 Elute in  15 µL 10 mM Tris pH8 leaving beads in solution.

26 Quantify  1 µL using Qubit high sensitivity dsDNA.

 Qubit™ dsDNA HS Assay Kit Invitrogen - Thermo Fisher Catalog #Q33228

Equipment

Qubit NAME

Fluorometer TYPE

Invitrogen BRAND

Q33228 SKU

<https://www.thermofisher.com/order/catalog/product/Q33228> LINK



26.1 Calculate the amount of pmol per in each  14 µL A-tail reaction as follows (alternatively use the calculation in the attached sheet):

$$Sample(pmol) = \frac{reaction(ng)}{size(bp) * 660} * 1000$$

Note

If the fragment size is not known estimate it to be 350 bp.



LTP_adapterCalculation.xlsx

26.2 Calculate the amount of adapter required (aim for 20:1 ratio adapter to sample - see calculation sheet).

Note

The adapter is contained in the NEBNext multiplex oligo kit.



NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) **New**
England Biolabs Catalog #E6440-8

- 26.3 Dilute adapter in water to achieve appropriate concentration in a total volume of 5 μL per reaction.

Note

If DNA is undetectable by Qubit HS reagents use 5 μL of 0.15 μM adapter.

- 27 Prepare the Ligation mix (for multiple samples prepare a master mix with 10% excess):

	A	B
	Component	Volume (μL)
	5X ligation buffer	5
	DNA ligase	2.5
	Total	7.5



KAPA LTP library prep kit **Roche Catalog #796188001**

- 27.1 Add 7.5 μL Ligation mix to 14 μL reaction (ensure remains on the beads).

- 27.2 Add 5 μL diluted NEBNext adapter .

- 27.3 Incubate as follows (if using a PCR machine ensure the hot lid is not turned on):
 20 $^{\circ}\text{C}$ for 01:00:00 .

1h



28 Add  0.75 μ L USER enzyme to each tube.

Note

USER enzyme is included as part of the NEBNext multiplex oligo kit.



NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) **New**
England Biolabs Catalog #E6440-8

28.1 Step 15 Incubate as follows:

 37 °C for  00:15:00

 4 °C hold

15m

29 0.9X SPRI clean up.

29.1

Add  25 μ L 10 mM Tris pH8 to make volume up to  50 μ L .

29.2 Add  45 μ L SPRI to the samples (ratio 0.9:1).

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

5m

29.4 Place on a magnetic rack until beads and solution have fully separated.

29.5 Remove supernatant.

29.6 Wash 2X with  200 μ L 80% Ethanol (freshly prepared) .

29.7 Remove all traces of ethanol and air dry for up to  00:05:00 .

5m

**Note**

Take care not to over-dry the beads.

29.8 Elute samples in  22 μ L 10 mM Tris pH8 .

29.9 Transfer  20 μ L to new fresh 0.2 mL PCR tubes.

30 Add  5 μ L NEBNext multiplex oligos each sample.

Note

It is essential the UDI primers are used to prevent index hopping on the sequencer. It is recommended that the primer kits are rotated between batches, there are currently 5 NEBNext multiplex oligo kits.



NEBNext Multiplex Oligos for Illumina (96 Unique Dual Index Primer Pairs) **New**
England Biolabs **Catalog #E6440-8**

30.1 Add  25 μ L KAPA HotStart Ready mix to each sample.

Note

KAPA HotStart Ready mix is contained within the KAPA LTP kit but may need to be topped up with additional reagent.



KAPA LTP library prep kit **Roche Catalog #796188001**



HotStart ReadyMix (KAPA HiFi PCR kit) **Kapa Biosystems Catalog #KK2601**

30.2 Incubate as follows:

 98 $^{\circ}$ C for  00:00:45

4-20* cycles of

 98 $^{\circ}$ C for  00:00:15

3m

🌡️ 65 °C ** for ⌚ 00:00:30

🌡️ 72 °C for ⌚ 00:00:30

Final cycle of

🌡️ 72 °C for ⌚ 00:01:00

🌡️ 4 °C hold 40C hold

Note

*The number of cycles depends on the input DNA concentration, too few cycles and not enough library is produced, too many results in a high proportion of PCR duplicates and daisy chains.

	A	B	C
	Input DNA (into ER and AT)	Number of cycles required to generate:	
		100 ng library	1 µg library
	1 µg	3	3
	500 ng	3	3–4
	250 ng	3	4–6
	100 ng	3	6–7
	50 ng	3–5	7–8
	25 ng	5–7	8 – 10
	10 ng	7–9	11 – 13
	5 ng	9 – 11	13 – 14
	2.5 ng	11 – 13	14 – 16
	1 ng	13 – 15	17 – 19

KAPA recommendations for number of PCR cycles required for different amounts of input DNA. As the PCR primers contain the full Illumina adapter/index sequences at least 3 cycles are required.

**Note**

******The annealing temperature of 65°C is optimal for the NEBNext primers, if other primers are used this may need to be altered.

Note

It is recommended that after this step (i.e. post-PCR when samples are more concentrated and contain common adapters) work continues in a different area to prevent contamination.

- 31 Clean up and QC the libraries as in protocol [Library clean up and quality control for Illumina sequencing](#).

Metagenomic sequencing

- 32 Metagenomic sequencing can speed up identification of any viruses in the samples and, depending on the virus and viral load, may be sufficient to generate full genomes.
- 33 Using the bp size and ng/μl concentration calculate the nM concentration for each library as follows:

$$Conc(nM) = \frac{sample(ng/\mu l)}{size(bp) * 660(g/mol)} * 1000000$$

- 34 Pool the libraries by equal molarity and QC the pools as described in the protocol [Library pooling and quality control for Illumina sequencing](#).

Note

Ensure that enough unpooled library remains to perform targeted enrichment .

- 35 Sequence the pools on an Illumina sequencer following the manufacturer's guidelines.



Note

For metagenomic viral discovery sequencing we recommend sequencing at 40 million reads per sample (20 million for the RNA viral detection and 20 million for the DNA viral detection).