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Discovery of RNA and DNA viruses using next-generation sequencing: Metagenomics

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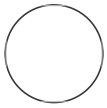
CVR Genomics



Katherine Smollett

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Protocol status: Working
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).

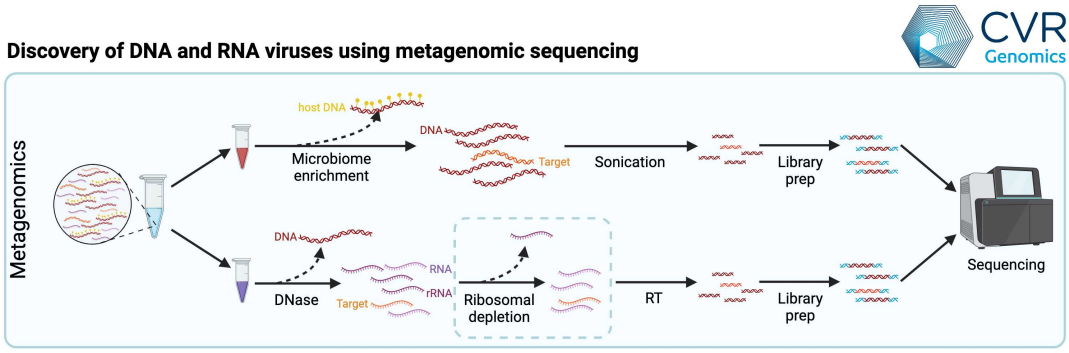

Figure 1: Diagram of workflow for discovery of DNA and RNA viruses using metagenomic sequencing. Optional ribosomal depletion step is shown in hashed box. Image prepared using BioRender.com.

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.

PROTOCOL integer ID:
71354

Keywords: Viral genomics, Next-generation sequencing, viral discovery, Illumina, Metagenomics, RNA, DNA, Untargeted detection of viruses

MATERIALS

Reagents:

- Ambion™ DNase I (RNase-free) **Thermo Fisher Catalog #AM2224**
- Agencourt RNAClean XP Magnetic Beads **Beckman Coulter Catalog #A6398**
- Illumina Ribo-Zero Plus rRNA Depletion Kit **illumina 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 **Contributed by users 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 **Sigma – Aldrich Catalog #P1458**
- 5 M Sodium chloride (NaCl) **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 LINK

BEFORE START INSTRUCTIONS

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 µL DNase I mix to 25 µL 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 µL RNAclean XP beads to the 30 µL 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 µL 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 µL Nuclease-free water and incubate at Room temperature for at least 00:02:00 2m

RNA virus detection - Ribosomal depletion 56m

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

Illumina Ribo-Zero Plus rRNA Depletion Kit **illumina 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 (µl)
	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: 15m

95 °C for 00:02:00
Decrease 0.1 °C /sec until temperature reaches 37 °C then hold.

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 µL rRNA Depletion mix to each 14 µL 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 (µl)
PRB (Probe Removal Buffer)	7
PRE (Probe Removal Enzyme)	3
Total	10

7.3 Thoroughly pipette mix.

7.4 Add 10 µL Probe Removal mix to each 19 µL 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: 5m

65 °C for 00:05:00

immediately place On ice

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

	A	B
	Component	Volume (µl)
	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 µL SSIII mix to each 12 µL 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

	A	B
	Component	Volume (µl)
	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 µL second strand mix to each 20 µL 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 **Contributed by users Catalog #A63880**

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

13.1 Add 80 µL AmPure XP to each 80 µL 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 µL 80% Ethanol (freshly prepared) .

13.6 Remove all traces of ethanol and air dry for 00:05:00 . 5m

13.7 Elute in 27 µL 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 µL 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 µL 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)

	A	B
	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 **Contributed by users Catalog #A63880**

17 If required make sample up to 55 µL 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 confrim 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 **Contributed by users Catalog #A63880**

21.1 Add 70 µL 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 µL 80% Ethanol (freshly prepared) .

21.6 Remove all traces of Ethanol. Air dry for 00:05:00 . 5m

21.7 Elute samples in 25 µL 10mM Tris pH8 .

21.8 Transfer 25 µL 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 (µl)
	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 µL End repair mix to each 25 µL sample.

22.2 Incubate as follows (if using a PCR machine do not use hot lid): 30m
20 °C for 00:30:00

23 1.4X Ampure XP clean up.
Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

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

23.2 Add 70 µL 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 µL 80% Ethanol (freshly prepared).

23.7 Remove all traces of ethanol and air dry for 00:05:00. 5m

23.8 Elute in 21 µL 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 (µl)
	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 µL A-Tail mix to each 21 µL reaction.

24.2 Incubate as follows: 1h
30 °C for 01:00:00

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 art 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 **Sigma – Aldrich Catalog #P1458**
> 5 M Sodium chloride (NaCl) **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 form 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 prepred).

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 #Q32851**

Equipment

Qubit	NAME
Flurometer	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_adapterCalcultion.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): 1h

20 °C for 01:00:00.

28 Add 0.75 µL 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: 15m

37 °C for 00:15:00

4 °C hold

29 0.9X SPRI clean up.

29.1 Add 25 µL 10 mM Tris pH8 to make volume up to 50 µL.

29.2 Add 45 µL 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 µL 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 µL 10 mM Tris pH8 .

29.9 Transfer 20 µL to new freah 0.2 mL PCR tubes.

30 Add 5 µL 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 µL 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: 3m

98 °C for 00:00:45

4-20* cycles of

98 °C for 00:00:15

65 °C ** for 00:00:30

72 °C for 00:00:30

Final cycle of

72 °C for 00:01:00

4 °C hold 4oC 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 suficient 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/µl)}{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).