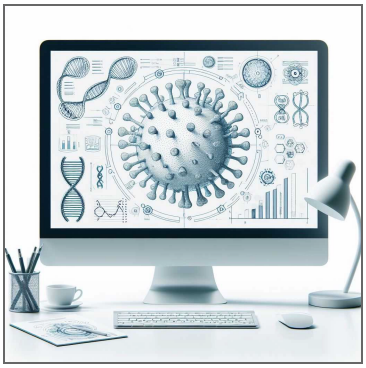

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Protocol status: Working
We use this protocol and it's working

Created: Feb 02, 2024

Preparation of viral sequencing library for Illumina using WTA2 and QIAseq FX

Forked from nCoV-2019 sequencing protocol for illumina

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DISCLAIMER

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ABSTRACT

This method uses a metagenomic approach to analyze the genome sequence of DNA and RNA viruses. Nucleic acids outside the viral particles are reduced using nucleases and extracted to obtain template DNA and RNA. Templates are converted to double-stranded DNA by random amplification, and library preparation is performed for analysis on Illumina sequencers.
Analysis data with reduced sequences of host and bacterial origin and abundant sequences of viral origin are obtained, allowing multiple samples to be analyzed even with the throughput of the iSeq100.

The library preparation protocol was originally folked from "nCoV-2019 sequencing protocol for illumina protocol V5" by Itokawa et al.

GUIDELINES

There are three advantages to using this method.
(1)No need to design virus-specific primers
(2)Applicable to both DNA and RNA viral genomes
(3)10 or more samples can be analyzed at a time on the iSeq100 (For genome analysis of coxsackievirus A6)

The method consists of three parts: pretreatment, random amplification, and library preparation.

The pretreatment is intended to increase the content of virus-derived nucleic acids in the sample and facilitate genome analysis. The main point of this method is to reduce host genome, ribosomal RNA, and nucleic acids derived from bacteria in advance, taking advantage of the fact that genomes in viral particles are not easily digested by Nuclease.

The random amplification using Merck millipore sigma's WTA2 kit can be used to obtain double-stranded DNA amplicon using DNA and RNA as templates. The following three points are different from the method described in the attached manual.
(1) This protocol is performed at one-fifth the scale of the protocol described in the manual.
(2) The initial denaturation temperature is changed so that DNA is also used as a template.
(3) The number of cycles of PCR amplification is increased due to the lower initial nucleic acid content.

The library preparation protocol was originally folked from "nCoV-2019 sequencing protocol for Illumina protocol V5" by Itokawa et al. Since the QIAseq FX DNA Library kit is used for library preparation in this method, multiplex analysis with the library of SARS-Cov-2 genome sequencing obtained using the protocol by Itokawa et al.

MATERIALS

<Pretreatment >

Equipment	
New Steradisc	NAME
0.45µm filter 50pcs	TYPE
Kurabo	BRAND
S-1304	SKU

Micrococcal Nuclease - 320,000 gel units **New England Biolabs Catalog #M0247S**

Benzonase® Nuclease 2.5ku **Contributed by users Catalog #70746-4CN**

High Pure Viral RNA Kit **Roche Catalog #11858882001**

Recipe for 100mL of homemade buffer (1M Tris, 100mM CaCl2, 30mM MgCl2, pH8)
1. Dissolve 15.06g of Trizma preset crystal pH7.5(M.W. 150.6) into 70mL of nuclease-free distilled water
2. Adjust to pH 8.0 by adding 4.92mL of NaOH (5N)
   pH is measured after the temperature drops to room temperature
3. Add 1.47 g of CaCl2-2H2O (M.W.* 147.01) and 0.813 g of MgCl2-6H2O (M.W. 203.30)
4. Dissolve, and meth up to 100mL
5. Filtrate through a 0.22-µm filter, dispense into tubes, and store.
*: molecular weight

<Random amplification>

TransPlex® Complete Whole Transcriptom Amplification Kit **Contributed by users Catalog #WTA2**

Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

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

<Library preparation>

QIAseq FX DNA Library CDI Kit (96) **Qiagen Catalog #180484**

or

QIAseq FX DNA Library UDI-A Kit (96) **Qiagen Catalog #180479**

PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001**

PROTOCOL MATERIALS

High Pure Viral RNA Kit **Roche Catalog #11858882001** Materials, Step 7

TransPlex® Complete Whole Transcriptom Amplification Kit **Catalog #WTA2** Materials, Step 9

Agencourt AMPure XP **Beckman Coulter Catalog #A63880** In Materials and 2 steps

QIAseq FX DNA Library CDI Kit (96) **Qiagen Catalog #180484** Materials, Step 24

QIAseq FX DNA Library UDI-A Kit (96) **Qiagen Catalog #180479** Materials, Step 24

PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001** Materials, Step 51

Micrococcal Nuclease - 320,000 gel units **New England Biolabs Catalog #M0247S** Materials, Step 4

Benzonase® Nuclease 2.5ku **Catalog #70746-4CN** Materials, Step 4

Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q33230** In Materials and 2 steps

Reduction of nucleic acids derived from non-virus

1 Collect 400 µL or more virus culture medium in a 1.5 mL tube.

Note

If the viral particles are broken, the genome could be digested in this later process.

2 Centrifuge 00:03:00 at 17,000 x g and aspirate the supernatant with a 1 mL tuberculin syringe. 3m

3

Equipment	
New Steradisc	NAME
0.45µm filter 50pcs	TYPE
Kurabo	BRAND
S-1304	SKU

Filter the medium through a 0.45µm filter into a 1.5 mL tube.

4 Micrococcal Nuclease - 320,000 gel units **New England Biolabs Catalog #M0247S**
Benzonase® Nuclease 2.5ku **Contributed by users Catalog #70746-4CN**

Mix the following reagents in a new 1.5mL tube.

Component	Volume / sample
Micrococcal nuclease	1 µL
Benzonase	2 µL
Homemade buffer*	7 µL

*see MATERIALS

5 Add 200 µL of filtrate into the tube, then mix by pipetting.

6 Incubate at 37 °C for 02:00:00 . 2h

7 High Pure Viral RNA Kit **Roche Catalog #11858882001**

Extract RNA from total volume (210 µL) and elute to 50 µL .

Whole transcriptome amplification independent of 3' end sequence

8 Prepare 2.5 µL of template nucleic acid in an 0.2mL 8-strip tube on ice.

9

TransPlex® Complete Whole Transcriptom Amplification Kit **Contributed by users Catalog #WTA2**

> **Note**
>
> This protocol uses 1/5 reagents per sample compared to the original WTA2 kit.

Add the following components in the tube.

Component	Volume / sample	
Nuclease-free water	0.32 µL	(possible to be replaced by template nucleic acid)
Synthesis solution (WTA2)	0.5 µL	

Total so far: 3.32 µL

10 Mix and incubate the reaction as follows: 5m

1. 95 °C for 00:05:00
2. Hold at 18 °C

11 Set the thermal cycler with a program below and start. 1h 20m

1. 18 °C pose
2. 18 °C for 00:10:00
3. 25 °C for 00:10:00
4. 37 °C for 00:30:00
5. 42 °C for 00:10:00
6. 70 °C for 00:20:00

7. Hold at 4 °C

12 Mix the following components, keep at 18°C, and add to the template from step 10.

Component	Volume / sample
Library Synthesis Buffer (WTA2)	0.5 µL
Nuclease-free water	0.78 µL
Library Synthesis Enzyme (WTA2)	0.4 µL

Total so far: 5 µL

13 Transfer the reaction tubes on the thermal cycler kept at 18 °C, and immediately skip to the next step (10m 18 °C for 00:10:00).

14 Mix the following components as master mix.

Component	Volume / sample
Nuclease-free water	60.2 µL
Amplification Mix (WTA2)	7.5 µL
WTA dNTP Mix (WTA2)	1.5 µL
Amplification Enzyme (WTA2)	0.75 µL

Add the master mix to the Library Synthesis reaction from step 13.

Total so far: approximately 75 µL

15 Transfer the reaction tubes on the thermal cycler. 7m 30s

Set the thermal cycler with a program below and start.

1. 94 °C for 00:02:00
2. 20 cycles x (94 °C for 00:00:30 , 70 °C for 00:05:00)
3. Hold at 4 °C

PCR clean-up and quantification

16 Clean-up the amplicons using Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

Add 90 µL of AMpure XP per sample.(Mixing ratio that removes below 100 bp)

17 Incubate at Room temperature for 00:05:00 5m

18 Separate magnetic beads and remove supernatant.

19 To wash beads, add 150 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (1/2) 30s

20 To wash beads, add 150 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (2/2) 30s

21 Allow the beads to dry for 00:02:00. 2m

22 Elute purified amplicon in 37.5 µL of Nuclease-free water.

23 Quantify the purified amplicon using fluorescent based method using Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q33230** .

Concentrations in the range of 10-100 ng/µL of purified amplicon are sufficient for the next section.

Fragmentation, End-prep & Adapter ligation

24 The use of 38m

QIAseq FX DNA Library CDI Kit (96) **Qiagen Catalog #180484** or
QIAseq FX DNA Library UDI-A Kit (96) **Qiagen Catalog #180479**
is assumed in this protocol.

> **Note**
>
> This protocol uses 1/8 reagents per sample compared to the original QIAseq FX DNA library kit.

32 °C

Set the thermal cycler with a program below and start.

Keep the heat-lid at 80 °C.

1. 32 °C pose
2. 32 °C 00:08:00
3. 65 °C 00:30:00

25 Place new 8-strip tubes at 96 well aluminum block On ice.

26 Prepare a reaction mix per one sample as below.

Component	Volume / sample
FX Buffer, 10x	0.625 µL
FX Enzyme Mix	1.25 µL
Purified amplicon	Use liquid volume equivalent to between 20 to 100 ng.
Nuclease-free water	up to 4.375 µL
Total	6.25 µL

27 Transfer the tubes from the ice to the thermal cycler, and immediately skip to the next step (32 °C).

28 Add 0.5 µL adapter solution to each end-prepped DNA mixture.

29 Prepare a master mix per sample below On ice .

Component	Volume / sample
DNA Ligase Buffer, 5x	2.5 µL
DNA Ligase	1.25 µL
Nuclease-free water	2 µL
Total	5.75 µL

Add 5.75 µL of above master mix to each end-prepped DNA mixture mixed with adapter On ice .

Total so far: 12.5 µL

30 Set a thermal cycler with the following program with heat lid at 80 °C . 35m

1. 20 °C 00:15:00
2. 65 °C 00:20:00

Place the tubes, and start the thermal program immediately.

Library pooling & purfication 7m

31

> **Note**
>
> Ideally, library pooling should result in the collection of 200 ng or more in order to obtain a visible agarose gel electrophoresis in the next section.

Take the ligated mixture from each well and pool them into the 1.5 mL low-binding tube.

Adjust the volume to be pooled to average the amount of DNA in each sample.

Note

For example, if 20 ng is measured from each sample, 10 µL of the 25 ng/12.5µL sample and 5 µL of the 50 ng/12.5µL sample should be aliquoted.

Briefly measure the volume of pooled mixture using pipette.

32 Clean-up the pooled library using Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

Add AMpure XP to library using x0.8 volume of the libary (Mixing ratio that removes below 150 bp)

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

34 Separate magnetic beads and remove supernatant.

35 To wash beads, add 500 µL of 80% ethanol, and mix.
Separate magnetic beads and remove supernatant.(1/2)

36 To wash beads, add 500 µL of 80% ethanol, and mix.
Separate magnetic beads and remove supernatant.(2/2)

37 Allow the beads to dry for 00:02:00. 2m

38 Elute DNA in 50 µL of nuclease-free water.

39 Transfer the eluted DNA to a new 1.5 mL low-binding tube.

40 Purify again by adding 60 µL of AMpure XP (x1.2 volume of the elution which allow to remove below 100 bp).

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

42 Separate magnetic beads and remove supernatant.

43 To wash beads, add 500 µL of 80% ethanol, and mix.
Separate magnetic beads and remove supernatant.(1/2)

44 To wash beads, add 500 µL of 80% ethanol, and mix.
Separate magnetic beads and remove supernatant.(2/2)

45 Allow the beads to dry for 00:02:00 . 2m

46 Finally, elute DNA in 30 µL low-TE (10mM Tris-HCl pH8.0, 0.1mM EDTA).

Transfer the eluted DNA to a new 1.5 mL low-binding tube.

Preparation of 50pM library for Illumina iSeq100

47 Quantify the purified library using
Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q33230**

48 Mix 5 µL of the library with loading dye and electrophoresis on a 2% agarose gel alongside molecular markers.

Obtain a smear image of the library.

49 Estimate approximate average library size (base pairs) on the smear image.

The size of the most concentrated region can be read and used as an estimate.

Note

Image J is helpful to recognize distribution of the library size. You can obtain a densitogram of the gel image.
https://imagej.net/ij/

50 Calculate molar concentration of the library using the formula below.

$Y\ (nM) = X\ (ng/\mu L) \div Z\ (base\ pairs) \div 660\ (g/mol) \times 10^6$

Y: molar concentration of the library
X: mass concentration of the library
Z: average library size

Note

See the Illumina website.
'Converting ng/µl to nM when calculating dsDNA library concentration'
https://knowledge.illumina.com/library-preparation/dna-library-prep/library-preparation-dna-library-prep-reference_material-list/000001240

51 PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001**

Dilute the library to 1 nM using resuspension buffer of PhiX Control.

Prepare final library mixture as below.

Components	**volume**
Resuspension buffer	93 µL
PhiX control (50 pM)	2 µL
Library (1 nM)	5 µL