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Preparation of viral sequencing library for Illumina using NEBNext ultra II

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

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We use this protocol and it's working

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Abstract

This method uses a metagenomic approach to analyze the genome sequence of RNA viruses. Nucleic acids outside the viral particles are reduced using nucleases and extracted to obtain template RNA. Templates are converted to double-stranded DNA, 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.

This protocol was folked from "Preparation of viral sequencing library for Illumina using WTA2 and QIAseq FX".

Guidelines

There are three advantages to using this method.
(1)No need to design virus-specific primers
(2)RNA viral genomes can be analyzed while reducing nucleotides of host and bacterial origin
(3)10 or more samples can be analyzed at a time on the iSeq100 (For viral genome less than 10,000 bases)

The method consists of two parts: pretreatment 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.

NEBNext Ultra II RNA Library Prep Kit for Illumina (E7770) is used for library preparation.
The following points are different from the method described in the attached manual.
(1) Use half the scale of the protocol in the manual.
(2) The number of cycles of PCR amplification is increased because the initial RNA input is small.
The amplified libraries are pooled into a tube for multiplex analysis. The size and molar concentration of the pooled libraries are adjusted to obtain a final library that can be applied to the flow cell.

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

(optional)

Qubit RNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32852

<Library preparation>

NEBNext Ultra II RNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs Catalog #**E7770S

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

NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns **New England Biolabs Catalog #**E7335S

NEBNext Multiplex Oligos for Illumina (Index Primers Set 2) - 24 rxns **New England Biolabs Catalog #**E7500S

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

NextSeq PhiX Control Kit **Illumina, Inc.** **Catalog #**FC-110-3002

Protocol materials

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

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

Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q33230 In Materials and 3 steps

Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852 Materials

NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns **New England Biolabs** **Catalog #**E7335S Materials

NEBNext Multiplex Oligos for Illumina (Index Primers Set 2) - 24 rxns **New England Biolabs** **Catalog #**E7500S Materials

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

NextSeq PhiX Control Kit **Illumina, Inc.** **Catalog #**FC-110-3002 Materials

NEBNext Ultra II RNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs** **Catalog #**E7770S Materials

Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880 In Materials and 4 steps

Reduction of nucleic acids derived from non-virus (pretreatment)

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 .

> **Note**
>
> If the concentration of RNA is measured here using the Qubit RNA HS Assay Kit or similar, it is not measurable due to low concentration. You can understand the large amount of viral genes are still included using real time PCR etc.

Fragmentaion of RNA and priming

8 0.2 mL PCR tubes are used to incubate mixtures.
For steps 9 to 47, refer to section 2 'Protocol for use with NEBNext rRNA Depletion Kit v2 (Human/Mouse/Rat)' in a manual of NEBNext Ultra II RNA Library Prep kit for Illumina (kit E7770). In our protocol, section 2.5 and beyond is referred.
Half volume of the reagent listed in the manual is used.

9 Mix the following components, keep On ice

Component	Volume / sample
RNA	2.5 µL
First Strand Synthesis Buffer (kit E7770)	2.0 µL
Random Primers (kit E7770)	0.5 µL

Total so far: 5 µL

10 Incubate in a thermal cycler set with the following program. 10m
Keep the heat-lid at 105 °C .

1. 94 °C for 00:10:00
2. Hold at 4 °C

Synthesis of 1st strand cDNA

11 Mix the following components, keep On ice

Component	Volume / sample
Product from step 10	5.0 µL
First Strand Sysnthesis Enzyme (kit E7770)	1.0 µL
Nuclease Free Water	4.0 µL

Total so far: 10 µL

12 Incubate in a thermal cycler set with the following program. 1h 15m

Keep the heat-lid at 80 °C .

1. 25 °C for 00:10:00
2. 42 °C for 00:50:00
3. 70 °C for 00:15:00
4. Hold at 4 °C

Synthesis of 2nd strand cDNA

13 Mix the following components, keep On ice

Component	Volume / sample
Product from step 13	10 µL
Second Strand Synthesis Buffer (kit E7770)	4.0 µL
Second Strand Synthesis Enzyme (kit E7770)	2.0 µL
Nuclease Free Water	24 µL

Total so far: 40 µL

14 Incubate in a thermal cycler set with the following program. 1h

Keep the heat-lid at 40 °C .

1. 16 °C for 01:00:00
2. Hold at 4 °C

Clean-up using magnetic beads

15 Clean-up products using Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

Add 72 µL (1.8x) of AMpure XP per sample.

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

17 Separate magnetic beads and remove supernatant.

18 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (1/2) 30s

19 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (2/2) 30s

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

21 Elute purified product in 26 µL of 0.1x TE (kit E7770).

22 Separate magnetic beads and transfer 25 µL of supernatant to a new 0.2 mL tube.

End Prep of cDNA Library

23 Mix the following components, keep On ice

Component	Volume / sample
Product from step 13	25 µL
End Prep Reaction Buffer (kit E7770)	3.5 µL
End Prep Reaction Enzyme (kit E7770)	1.5 µL

Total so far: 30 µL

24 Incubate in a thermal cycler set with the following program. 1h
Keep the heat-lid at 75 °C.

1. 20 °C for 00:30:00
2. 65 °C for 00:30:00
3. Hold at 4 °C

Adaptor ligation

25 Mix the following components in a 1.5 mL low-binding tube, keep On ice

Component	Volume
NEBNext Adaptor for Illumina (E7335 or E7500)	1.0 µL
Adaptor Dilution Buffer(kit E7770)	199 µL

26 Mix the following components as master mix in a 1.5 mL tube, keep On ice

Component	Volume / sample
Ligation Enhancer (kit E7770)	0.5 µL
Ligation Master Mix (kit E7770)	15 µL

27 Mix the following components, in the order given, keep On ice

Component	Volume / sample
Product from step 24	30 µL
Diluted Adaptor (step 25)	1.25 µL
Master mix (step 26)	15.5 µL

Total so far: 46.75 µL

28 Incubate in a thermal cycler set with the following program. 30m

Keep the heat-lid at 45 °C .

1. 20 °C for 00:15:00
2. 20 °C pose *
3. 37 °C for 00:15:00
4. Hold at 15 °C

* Add USER Enzyme (kit E7770) 1.5 µL / sample and mix

Total so far: 48.25 µL

Clean-up using magnetic beads

29 Clean-up products using Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

Add 43.5 µL (0.9x) of AMpure XP per sample.

30 Incubate at Room temperature for 00:10:00 10m

31 Separate magnetic beads and remove supernatant.

32 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (1/2)

33 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (2/2)

34 Allow the beads to dry for 00:02:00.

35 Elute purified product in 8.0 µL of 0.1x TE (kit E7770).

36 Separate magnetic beads and transfer 7.5 µL of supernatant to a new 0.2 mL tube.

PCR Enrichment of Adaptor Ligated DNA

37 Mix the following components, keep On ice

Component	Volume / sample
Adaptor Ligated DNA from step 36	7.5 µL
Q5 Master Mix (kit E7770)	12.5 µL
Index (X) Primer (E7335 or E7500)	2.5 µL
Universal PCR Primer (E7335 or E7500)	2.5 µL

For multiplex analysis of specimens fewer than seven, use 'Index oligo selector' to verify that the index combination is acceptable.
NEBNext Index oligo selector

38 Incubate in a thermal cycler set with the following program. 1m 55s

1. 98 °C for 00:00:30
2. 20 cycles x (98 °C for 00:00:10, 65 °C for 00:01:15)

3. Hold at 4 °C

Clean-up of PCR product using magnetic beads and quantification of DNA

39 Clean-up products using Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

Add 22.5 µL (0.9x) of AMpure XP per sample.

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

41 Separate magnetic beads and remove supernatant.

42 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (1/2)

43 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (2/2)

44 Allow the beads to dry for 00:02:00.

45 Elute purified product in 11.5 µL of 0.1x TE (kit E7770).

46 Separate magnetic beads and transfer 11 µL of supernatant to a new 0.2 mL tube.

47 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.

Library pooling

48 Take the purified PCR product from each tube 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 you want to obtain 100 ng from each sample, 2 µL of the 50 ng/µL sample and 4 µL of the 25 ng/µL sample should be aliquoted.

In the next section, purification with 0.6x AMPureXP to remove DNA of less than 300 bp resulted in the loss of much DNA (reduced to about one-eighth of the amount). Therefore, if the total amount of DNA obtained in this section is small (approximately 500 ng / 50 µL or less), it will be difficult to obtain a visible agarose gel electrophoresis in the section on library size estimation.

If the total amount of DNA from library pooling is small, removal of less than 300 bp of DNA should not be performed.

Briefly measure the volume of pooled mixture using pipette.

Add 0.1x TE (kit E7770) up to 50 µL of total volume.

Purification of the library for size selection

49 Clean-up products using Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

Add 30 µL (0.6x) of AMpure XP per sample.

50 Incubate at Room temperature for 00:05:00

51 Separate magnetic beads and remove supernatant.

52 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (1/2)

53 To wash beads, add 200 µL of 80% ethanol, incubate for 00:00:30, and remove supernatant (2/2)

54 Allow the beads to dry for 00:02:00.

55 Elute purified product in 30 µL of 0.1x TE (kit E7770).

56 Separate magnetic beads and transfer supernatant to a new 0.2 mL tube.

57 Quantify the purified amplicon using fluorescent based method using
Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q33230 .
Concentrations 1.5 ng/µL or more of purified library is sufficient for the next section.

Estimation of library size

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

Note

More accurate molar concentrations can be determined using commercially available library quantification kits instead of the methods shown in this section.

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

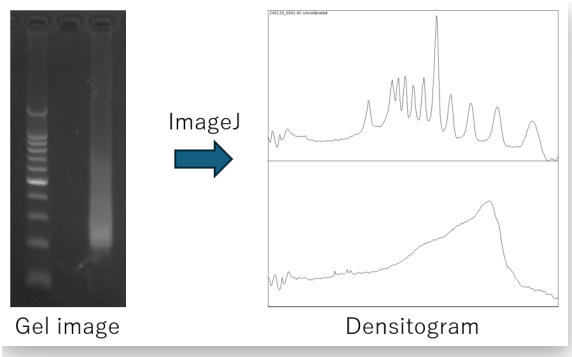
Obtain a smear image of the library.

60 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 estimation.

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/



Preparation of 50pM library for Illumina iSeq100

61 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

Setting up the local run manager in iSeq100

62 To analyze libraries using NEBNext® multiplex oligos for Illumina, you need to load the index and other information into a local run manager.

Refer to the manuals on Illumina site
How to use a custom library prep kit in Local Run Manager v2
How to use custom library prep and index kits with Local Run Manager v3 and v4

Obtain a .tsv file for configuration from the NEBNext® multiplex oligo page on NEB.

Change DefaultReadLength1, 2 in the .tsv file from 251 to 151.

Start the Local Run Manager on iseq100 and open "Tools" on the dashboard.

In the drop-down menu, select "Index & Library Prep Kits", "Index Kit", and "Add Index Kit".

Select and load the modified .tsv file.

Note

If libraries are created using both E7335 and E7500, a .tsv file that has both index information can be used.

You can consolidate the information under [Indices] in the two .tsv files into one file, and change the "Name" and "Description" under [kit] as desired.

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