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WORKS FOR ME 1

RAPIDprep: A simple, fast protocol for RNA metagenomic sequencing of clinical samples

Forked from Nextera XT protocol for MiSeq HIVPR-RT sequencing

COMMENTS 0

This protocol is published without a DOI.

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DISCLAIMER

This assay has been used primarily in aresearch setting and is not necessarily validated from clinical use.

ABSTRACT

The protocol allows rapid RNA metagenome sequencing of pathogens of clinical respiratory samples. The protocol has been designed to be simple and quick to enable the utilization of existing sample extracts and data back within 24h from start to finish (i.e. library prep - sequencing - analysis). The method also uses commonly available reagents that pairs with amplicon based WGS assays, and works by essentially making rRNA depleted ds-cDNA and library amplification with enzyme based tagmentation using Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096** .

PROTOCOL CITATION

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FORK NOTE

FORK FROM

Forked from Nextera XT protocol for MiSeq HIVPR-RT sequencing, Paula Aulicino

KEYWORDS

metagenome, NGS, virome, rapid, transcriptome, Illumina, genome sequencing, cDNA synthesis, Nextera XT, Invitrogen

LICENSE

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MATERIALS TEXT

Quick-RNA Viral Kit **Zymo Research Catalog #R1034** Step 1
ezDNase™ Enzyme **Thermo Fisher Catalog #11766051**
QIAseq FastSelect -rRNA HMR Kit **Qiagen Catalog #334385**
QIAseq FastSelect –5S/16S/23S Kit **Qiagen Catalog #335921**
SuperScript™ IV VILO™ Master Mix **Invitrogen - Thermo Fisher Catalog #11756050**
Sequenase Version 2.0 DNA Polymerase **Thermo Fisher Catalog #70775Y200UN**
Mag-Bind® TotalPure NGS **Omega Biotek Catalog #M1378-01** In 2 steps
Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096**
Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit **Invitrogen - Thermo Fisher Catalog #Q33231** In 2 steps
Buffer EB **Qiagen Catalog #19086** In 2 steps
High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #5067-5584** Step 34
High Sensitivity D1000 Reagents **Agilent Technologies Catalog #5067-5585**

SAFETY WARNINGS

Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

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RNA extraction

1 Extract RNA with Quick-RNA Viral Kit **Zymo Research Catalog #R1034**, following manufacturer's instructions.

Note

DNase treatment

2 Setup the following reaction mix for each sample along with a no template control (NTC):

A	B
Reagent	Volume (µL)
10X ezDNase Buffer	1
ezDNase enzyme	1
DNA/RNA	8

total reaction volume - 10µL per sample

3 Mix well by gently pipetting up and down, and briefly centrifuge.

10m

4 Incubate the reaction as follows:

A	B
Temperature (°C)	Time (mm:ss)
37	10:00
4	∞

After incubation, spin down and place reaction on ice.

rRNA depletion

5 Add the following to the previous reaction (Setup reaction on ice).

A	B
Reagent	Volume (µL)
previous reaction	10
FastSelect Mix	1

Total reaction volume - 11µL per sample; FastSect Mix is a mixture of QIAseq FastSelect -rRNA HMR, QIAseq FastSelect -5S/16S/23S, and water, with the ratio of (QIAseq FastSelect -rRNA HMR):(QIAseq FastSelect -5S/16S/23S):water=1:1:1.

6 Mix well by pipetting up and down, and briefly centrifuge.

14m

7 Incubate the reaction as follows:

A	B
Temperature (°C)	Time (mm:ss)
75	02:00
70	02:00
65	02:00
60	02:00
55	02:00
37	02:00
25	02:00
4	∞

After incubation, spin down and place reaction on ice.

1st strand DNA synthesis

8 Add the following to the previous reaction (Setup reaction on ice).

A	B
Reagent	Volume (µL)
previous reaction	11
water	5
SuperScript IV VILO Master Mix (5X)	4

total reaction volume - 20µL per sample

9 Mix well by pipetting up and down, and briefly centrifuge.

35m

10 Incubate the reaction as follows:

A	B
Temperature (°C)	Time (mm:ss)
25	10:00
50	20:00
85	05:00
4	∞

After incubation, spin down and place reaction on ice.

2nd strand DNA synthesis

11 Add the following to the previous reaction (Setup reaction on ice).

A	B
Reagent	Volume (µL)
previous reaction	20
water	11
Sequenase reaction buffer (5X)	8
Sequenase Dilution	1

total reaction volume - 40µL per sample; Sequenase Dilution is a mixture of Sequenase Dilution Buffer and Sequenase Version 2.0 DNA Polymerase, with the ratio of (Sequenase Dilution Buffer):(Sequenase Version 2.0 DNA Polymerase)=2:1.

12 Mix well by pipetting up and down, and briefly centrifuge.

17m 30s

13 Incubate the reaction as follows:

A	B

A	B
Temperature (°C)	Time (mm:ss)
4	∞
37	10:00
95	02:00
4	∞

Slow ramp (0.1°C/second) from 4°C to 37°C; After incubation, spin down and place reaction on ice.

14 Add the following to the previous reaction (Setup reaction on ice).

A	B
Reagent	Volume (µL)
previous reaction	40
Sequenase Dilution	1

total reaction volume - 41µL per sample

15 Mix well by pipetting up and down, and briefly centrifuge.

30m

16 Incubate the reaction as follows:

A	B
Temperature (°C)	Time (mm:ss)
37	30:00
4	∞

After incubation, spin down and place reaction on ice.

ds-cDNA purification

17 Perform purification of the previous reaction with magnetic beads (Mag-Bind® TotalPure NGS **Omega Biotek Catalog #M1378-01**).

Note

5m

DNA libarary preparation and sequencing

18

Note

19 Combine 10 µL Tagment DNA Buffer (TD) and 5 µL neat ds-cDNA from previous reaction for each sample into a PCR plate or strip.

20 Mix well by pipetting up and down 10 times.

21 Add 5 µL Amplicon Tagment Mix (ATM) to each well on top of the TD/DNA mix.

22 Mix well by pipetting up and down 10 times. Seal and briefly centrifuge.

7m

23 Incubate the reaction mix as follows:

A	B
Temperature (°C)	Time (mm:ss)
55	07:00
10	∞

Following tagmentation, immediately remove the reaction from the thermocycler.

24 Add 2.5 µL Neutralize Tagment Buffer (NT) to stop the reaction.

25 Mix well by pipetting up and down 10 times. Seal and briefly centrifuge.

5m

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

27 Add indexes and Nextera PCR Master Mix (NPM) to the neutralised tagmentation reaction for each sample as below:

A	B
Reagent	Volume (µL)
previous reaction	25
IDT® for Illumina Nextera DNA Unique Dual Indexes	10
Nextera PCR Master Mix (NPM)	15

total reaction volume - 50µL per sample

28 Mix well by pipetting up and down 10 times. Seal and briefly centrifuge.

25m 10s

29 Incubate the reaction as follows:

A	B	C
Temperature (°C)	Time (mm:ss)	cycles
72	03:00	1X

A	B	C
95	00:30	1X
95	00:10	16X
55	00:30	
72	00:30	
72	05:00	1X
4	∞	

After incubation, spin down and place reaction on ice.

30 Perform purification of the previous reaction with magnetic beads (Mag-Bind® TotalPure NGS **Omega Biotek Catalog #M1378-01**).

Note

31 Quantify all purified DNA using Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit **Invitrogen - Thermo Fisher Catalog #Q33231** .

32 Pool the individual libraries equally in DNA amount based on the Qubit values. Here, we assume the libraries will have similar fragment lengths and distributions.

33 Quantify the final pool of libraries using Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit **Invitrogen - Thermo Fisher Catalog #Q33231** .

34 Analyse 2 µL final pool of libraries with High Sensitivity D1000 ScreenTape **Agilent Technologies Catalog #5067-5584** ensuring the whole fragment peak is captured.

35 Scale the calculated molarity from the Tapestation to the Qubit DNA concentration using the following formula: Final molarity = (Tapestation DNA molarity) x (Qubit DNA concentration) / (Tapestation DNA concentration)

36 Dilute the final pooled libraries down to [M] 1 nanomolar (nM) (at least 50µL) using Buffer EB **Qiagen Catalog #19086**, and add PhiX sequencing control if required.

37 Combine 10 µL 1nM libary pool and 90 µL Buffer EB **Qiagen Catalog #19086** to dilute the final pool of libraries to [M] 0.1 nanomolar (nM) for loading.

38 Load 20 µL 0.1nM library pool into a defrosted Illumina iSeq cartridge with flow cell.

39 Run in Illumina iSeq.

Equipment	
iSeq	NAME
sequencer	TYPE
Illumina	BRAND
ILM20021532	SKU
https://www.illumina.com/systems/sequencing-platforms/iseq.html	LINK