Whole genome sequencing of respiratory syncytial (RSV) virus from clinical samples with low viral load

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Abstract

Here is a description of a protocol for whole genome sequencing of RSV from clinical samples (nasopharyngeal aspirates -NPA-). The protocol was tested with samples with viral loads as low as 10^{+03} viral copies/ml NPA.

The RNA is amplified by RT-PCR in five overlapped fragments of around 2300-4500 nt in length by using specific primers which anneal in conserved regions of the genome. Briefly the protocol includes: viral RNA extraction from NPA done with silica membrane columns and fragment amplification performed in five independent reaction tubes with OneStep RT-PCR Kit (Qiagen). Each fragment size check performed in an agarose gel electrophoresis, clean-up step done with silica membrane columns and the quantification step performed by Qubit. Finally, amplicons pooled equimolarly and library prep done with Nextera XT Kit.

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Materials

- Agarose A5304 by Contributed by users
- DNA Clean & Concentrator™-5 D4003 by Zymo Research
- Qubit™ dsDNA HS Assay Kit Q32851 by Invitrogen - Thermo Fisher
- Ethidium bromide [EB, EtBr] EB0195.SIZE.25g by Bio Basic Inc.
- Agencourt Ampure XP A63880 by Beckman Coulter
- RiboLock RNase Inhibitor #EO0381 by Thermo

Fisher Scientific

Nextera XT DNA Library Preparation Kit FC-131-1096 by illumina

OneStep RT-PCR Kit 210210 by Qiagen

PureLink viral RNA/DNA mini kit 12280050 by Invitrogen - Thermo Fisher

QIAamp Viral RNA Mini Kit 52904 by Qiagen

Protocol

Viral RNA extraction

Step 1.

Centrifuge the nasopharyngeal aspirate (NPA) at 5,000 g for 5 min to remove cellular debris.

Viral RNA extraction

Step 2.

Extract viral RNA from 200 µl of NPA by following the protocol PureLink viral RNA/DNA mini kit (Thermo Fisher Scientific) according the manufacturer's instructions except for 2.8 µl of carrier RNA instead of 5.6 µl as recommended to reduce the presence of tRNA in the extracted RNA. Nevertheless, 140 µl of NPA should be used if QIAamp Viral RNA Mini Kit (Qiagen) is used instead of PureLink kit. In this case, no changes from the protocol should be done.

Viral RNA extraction

Step 3.

Elute viral-extracted RNA in 40 µl of DNase/RNase-free water. Add 1.25 µl of 40 U/µL RiboLock RNase Inhibitor (Thermo Fisher Scientific) to preserve the extracted RNA.

Viral RNA extraction

Step 4.

Extracted RNA can be storage at -80ºC.

RT-PCR

Step 5.

PRIMERS:

Fragment 1: 2384 bp

Forward 1f: 5'-ACGCGAAAAAATGCGTACwAC-3'
Reverse 2384r: 5'-GCrTCTTCTCCATGrAATTC-3'
Fragment 2: 2731 bp
Forward 2124f: 5'- GCwGGyCTAGGCATAATG-3'
Reverse 4875r: 5'- GTTGTTrGTGTrACTTTGT-3'
Fragment 3: 4351 bp
Forward 3314f: 5'-AyCCyGCATCACTwACAAT-3'
Reverse 7665r: 5'-CAGGAAACAGCTATGACCyAAGCA-3'
Fragment 4: 4114 bp
Forward 7094f: 5'-TGATGCATCAATATCTCAAGTC-3'
Reverse 11208r: 5'-CTCCTGTGTTAAGCTACCTATAG-3'
Fragment 5: 4331 bp
Forward 10438f: 5'-AGTyTkACAAGATATGGTGATCT-3'
Reverse 15180r: 5'-AAGTGTCAAAAACTAATATCTCGT-3'

All the procedure should be done in ice.
Amplify each fragment in an independent RT-PCR reaction with OneStep RT-PCR kit of Qiagen by mixing:
primer f (25 µM)--- 0.6 µl
primer r (25 µM)--- 0.6 µl
RNA--- 6 µl

Incubate at 65 °C for 5 min, then place 2 min in ice.

Add (master mix can be done):
H2O DNase/RNase free--- 5 µl
Buffer 5X--- 10.55 µl
RNAsa Inhibitor (40 U/µl)---0.25 µl
dNTP mix (10 mM)--- 1 µl
Enzime Mix--- 1 µl
(Final volume: 25 µl)

Incubate:
45 °C 30 min
95 °C 15 min
40 times: 94 °C 10 sec
55 °C 30 sec
68 °C 4.5 min
68 °C 10 min
4 °C hold

Fragment verification, quantification and pool

Step 6.
Run 4 µl of RT-PCR product on a 1.8% agarose gel stained with Ethidium Bromide.

Fragment verification, quantification and pool

Step 7.

Perform a clean-up of RT-PCR product with DNA Clean & Concentrator kit (Zymo Research) following the manufacturer's instructions. Elute with water PCR-grade, avoid elution with buffer containing EDTA.

Fragment verification, quantification and pool

Step 8.

Run 2 µl of each amplicon in a Qubit dsDNA HS Assay.

Fragment verification, quantification and pool

Step 9.

Pool equimolarly the 5 amplicons

NGS

Step 10.

Follow NexteraXT kit (Illumina) to perform library preparation.
After index addition by amplification and clean-up with Agencourt Ampure XP, quantify the libraries with the Qubit dsDNA HS Assay Kit.
Perform a standard normalization instead of beads normalization. Never normalize with
beads when libraries have a concentration lower than 15 nM
If you are not familiar with bioinformatic analysis in NGS, we recommend using UGENE or Geneious.

Warnings

Ethidium bromide is a potent mutagen. Ethidium bromide solution must be handled with extreme caution and decontaminated prior to disposal.