Feb 02, 2020

Sequence-Independent, Single-Primer Amplification of RNA viruses V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bb3miqk6

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ABSTRACT

This protocol outlines the methods to perform unbiased direct metagenomic sequencing of nucleic acid extracts from cell-free fluids. This protocol can be adapted to be run on Illumina and Nanopore sequencing platforms.
The protocol is based off of the work from Kafetzopoulou et al. (PMID: 30563591). Liana has provided the lab with detailed protocols, and has worked with us extensively on optimizing and getting protocols running efficiently. Please note that this protocol has been updated to use SuperScript IV with it's optimal temperature which has been reflected in Lewandowski et al. (DOI: https://doi.org/10.1128/JCM.00963-19)

Notes:
* This protocol has been used to sequence influenza direction from respiratory clinical samples (DOI: https://doi.org/10.1128/JCM.00963-19).
* A team from China published last week on a 2019-nCoV familial cluster using the SISPA protocol as for coronavirus whole genome sequencing (DOI: https://doi.org/10.1016/S0140-6736(20)30154-9)

SISPA-Primer A - 5'-GTT TCC CAC TGG AGG ATA-(N9)-3'
SISPA-Primer B - 5'-GTT TCC CAC TGG AGG ATA-3'

MATERIALS

NAME	CATALOG #	VENDOR
RNA Clean & Concentrator™-5	R1015	Zymo Research
Ampure XP beads	A63881	Beckman Coulter
QIAamp® Viral RNA Mini	52906	Qiagen
Linear acrylamide	AM9520	Thermofisher
Filter (0.22µm)	8110	Costar
SuperScript™ IV First-Strand Synthesis System	18091200	Thermo Fisher
Sequenase Version 2.0 DNA Polymerase	70775Y200UN	Thermo Fisher
TURBO™ DNase (2 U/µL)	AM2238	Thermo Fisher
dNTP Mix (10 mM each)	R0192	Thermo Fisher
AccuTaq LA DNA Polymerase	D8045-125UN	Sigma Aldrich
SISPA-Primer A		IDT
SISPA-Primer B		IDT

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Filter (0.22µm)	8110	Costar

NAME	CATALOG #	VENDOR
Linear Acrylamide (5 mg/ml) (1 ml Tube)	AM9520	Thermo Fisher
QIAamp® Viral RNA Mini	52906	Qiagen
TURBO™ DNase (2 U/µL)	AM2238	Thermo Fisher
Zymo DNA Clean & Concentrator - 5	D4014	Zymo Research

Nucleic Acid Extraction

1 Add 280µL cell-free liquid to 0.22µm centrifuge filter.

Note: (add in 2x what you want to get out)

Filter (0.22µm)
by Costar
Catalog #: 8110

2 Centrigue at 14,000 RPM for 5 minutes

Centrifuge
Benchtop Centrifuge
Eppendorf 5405000441
Any benchtop centrifuge will suffice

14000 rpm 00:05:00

3 Transfer 140µL of filtered sample to a new tube.

4 Prepare Buffer AVL and linear polyacrylamide mix.

* For one sample, 560µL Buffer AVL + 5.6µL linear polyacrylamide Reagent Volume (µL)

Linear Acrylamide (5 mg/ml) (1 ml Tube)
by Thermo Fisher
Catalog #: AM9520

QIAamp® Viral RNA Mini
by Qiagen
Catalog #: 52906

5 Pipet 560µL prepared Buffer AVL containing linear polyacrylamide into a 1.5 ml microcentrifuge tube.

* Note: If the sample volume is larger than 140 µl, increase the amount of Buffer AVL–linear polyacrylamide proportionally (e.g., a 280 µl sample will require 1120 µl Buffer AVL–linear polyacrylamide) and use a larger tube.

6 Add 140 µl plasma, serum, urine, cell-culture supernatant or cell-free body fluid to the Buffer AVL–LPA in the microcentrifuge tubes. Mix by pulse-vortexing for 15 s. **00:00:15**

* Note: To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution. Frozen samples that have only been thawed once can also be used.

7 Incubate at room temperature (15–25°C) for 10 min.

* Note: Viral particle lysis is complete after lysis for 10 min at room temperature. Longer incubation times have no effect on the yield or quality of the purified RNA.
00:10:00

8 Briefly centrifuge the tube to remove drops from the inside of the lid.

9 Add 560 µl ethanol (96–100%) to each sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid. **00:00:15**

* Note: Use only ethanol, since other alcohols may result in reduced RNA yield and purity. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone. If the sample volume is greater than 140 µl, increase the amount of ethanol proportionally (e.g., a 280 µl sample will require 1120 µl ethanol). To ensure efficient binding, it is essential that the sample is mixed thoroughly with the ethanol to yield a homogeneous solution.

10 Carefully apply 630 µl of the solution from step 8 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

* Note: Close each spin column to avoid cross-contamination during centrifugation.

* Note: Centrifugation is performed at 6000 x g (8000 rpm) to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of the viral RNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all of the solution has passed through.

8000 rpm 00:01:00

11 Carefully open the QIAamp Mini column, and repeat step 9. If the sample volume was greater than 140 µl, repeat this step until all of the lysate has been loaded onto the spin column.

12 Carefully open the QIAamp Mini column, and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 14, or to eliminate possible Buffer AW2 carryover, perform step 13 and then continue.

* Note: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in flow- through, containing Buffer AW2, contacting the QIAamp Mini column.

14000 rpm 00:03:00

13 Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min. **14000 rpm 00:01:00**

14 Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 30 µl DNase/RNase Free Water equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.800

15 Centrifuge at 6000 x g (8000 rpm) for 1 min.

8000 rpm 00:01:00

16 Transfer 30µL of eluted nucleic acid to a new microcentrifuge tube.

DNase Treatment

17 Create a master mix of TURBO DNase Mix:

Add 1 µL TURBO DNase (2 U) (for up to 10 µg RNA in a 50 µL reaction), 5µL TURBO DNase Buffer, and 14µL of H2O per sample.

TURBO™ DNase (2 U/µL)
by Thermo Fisher
Catalog #: AM2238

18 Add 20µL of TURBO DNase Mix to 30µL of sample.

19 Incubate at 37°C for 30 minutes. **37 °C**

00:30:00

ThermoMixer
Benchtop Incubator
Eppendorf 5382000023
Any heat block will suffice

Zymo Clean-up and Concentrator

20 Add 2 volumes RNA Binding buffer to each sample (ex. 100µL buffer + 50 µL sample)

Zymo DNA Clean & Concentrator - 5
by Zymo Research
Catalog #: D4014

21 Add an equal amount of 100% ethanol and mix. (ex. 150µL ethanol)

22 Transfer the sample to the Zymo-Spin IC Column in a Collection Tube and centrifuge for 30 seconds. Discard the flow-through

* For samples >800 µL, Zymo-Spin columns can be reloaded

14000 rpm 00:00:30

Centrifuge
Benchtop Centrifuge
Eppendorf 5405000441
Any benchtop centrifuge will suffice

23 Add 400 µL RNA Prep Buffer to the column and centrifuge at 10,000g– 16,000g for 30 seconds. Discard flow-through.

14000 rpm 00:00:30

24 Add 700 µL RNA Wash Buffer to the column and centrifuge at 10,000g– 16,000g for 30 seconds. Discard flow-through.
14000 rpm 00:00:30

25 Centrifuge at 10,000g– 16,000g for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase free tube (Not provided in RNA Clean & Concentrator-5 kit).
14000 rpm 00:02:00

26 Add 8-10 µL Nuclease-Free water directly to the column matrix and centrifuge at 10,000g– 16,000g for 30 seconds. The eluted RNA can be used immediately or stored at -70°C.

SISPA A: Reverse Transcription and 2nd strand cDNA synthesis Primer A Addition

27 Make a working stock of your primer A. Stock: 100pmol/1µL. Add 4µL of stock + 6µL H2O. You now have a 10µL of a 40pmol/µL working stock.

28 Add 1 µL Primer A working stock to 4 µL extracted RNA. Heat to 65°C for 5 minutes in a thermocycler and let cool at 4°C for 5 minutes.

SimpliAmp Thermal Cycler
PCR
Applied Biosystems A24811
Any standard PCR thermocycler will suffice

29 While reaction is on the thermocycler, make a master mix for 1 reaction (scale up as needed) consisting of 2 µL 5X RT buffer, 1 µL 10 mM dNTP, 1 µL water, 0.5 µL 0.1M DTT, and 0.5 µL SSIV RT.

30 Add 5 µL master mix to reaction. Incubate at 50°C for 10 minutes.
50 °C **00:10:00**

31 While reaction is on the thermocycler, make a master mix for 1 reaction (scale up as needed) consisting of 1 µL 5X Sequenase buffer, 3.8 µL water, and 0.15 µL Sequenase.

32 After 10 minute incubation, Add 5 µL of Sequenase Mix #1 to the reaction.

33 Incubate at 37°C for 8 min.
37 °C **00:08:00**

34 While reaction is on the thermocycler, make a master mix for 1 reaction (scale up as needed) consisting of 0.45 µL Sequenase dilution buffer, and 0.15 µL Sequenase.

35 After 8 min incubation, add 0.6µL of Sequenase Mix #2 to the reaction.

36 Incubate at 37°C for 8 min.

37 °C 00:08:00

* Round A is now complete and samples can be stored at -20°C

SISPA B: PCR Amplification of Randomly Primed cDNA cDNA Amplification

37 Make a master mix for 1 reaction (scale up as needed) consisting of 5 µL AccuTaq LA 10x Buffer, 2.5 µL dNTP mix, 1µL DMSO, 0.5 µL AccuTaq LA DNA Polymerase, 35 µL nuclease free water, and 1 µL Primer B.

*Do not dilute Primer B

38 Add 5 µL of product from SISPA Round A to 45 µL master mix.

39 Run PCR with the following conditions 98°C for 30s, followed by 30 cycles of 94°C for 15 s, 50°C for 20 s, and 68°C for 2 min, a final step of 68°C for 10 min, and then a 4°C hold until you're ready for the cleanup.

* Can freeze in the -20°C until ready

SimpliAmp Thermal Cycler
PCR
Applied Biosystems A24811
Any standard PCR thermocycler will suffice

Bead Clean-up

40 Amplified cDNA was purified using a 1:1 ratio of AMPure XP beads. Add 50µL of resupsended AMPure XP beads to 50µL SISPA product.

41 Incubate at RT for 10 minutes

Room temperature 00:10:00

42 Spin down briefly

Mini-centrifuge
Centrifuge
Fisher S67601B
Any standard mini centrifuge with adapters for different tube sizes will suffice

43 Place on magnet. Remove and discard supernatant once solution turns clear. Be sure not to disturb beads.

Magnetic Stand
Magnetic Stand

Thermo Scientific MR02

Any magnetic rack that fits your tubes will suffice.

44 Wash with 200µL of 70% EtOH.

45 Remove EtOH without disturbing beads.

46 Repeat wash. Remove EtOH without disturbing beads.

47 Remove samples from magnetic rack and spin down briefly.

48 Remove residual ethanol

49 Let air dry briefly - Do not overdry bead pellet

50 Resuspend sample in 50µL of H2O.

51 Incubate samples at RT for 5 minutes.

Room temperature **00:05:00**

52 Transfer tubes to magnetic rack. Transfer 48µL of eluted product to a new tube.

53 Continue onto library prep protocol of your choice.