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Sequence-Independent, Single-Primer Amplification of RNA viruses V.1

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

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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

MATERIALS

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

STEP MATERIALS

- Filter (0.22µm) **Costar** **Catalog #**8110
- Linear Acrylamide (5 mg/ml) (1 ml Tube) **Thermo Fisher** **Catalog #**AM9520
- QIAamp® Viral RNA Mini **Qiagen** **Catalog #**52906
- TURBO™ DNase (2 U/µL) **Thermo Fisher** **Catalog #**AM2238
- Zymo DNA Clean & Concentrator - 5 **Zymo Research** **Catalog #**D4014

Protocol materials

- RNA Clean & Concentrator™-5 **Zymo Research Catalog #**R1015
- SISPA-Primer A **IDT**
- Filter (0.22µm) **Costar Catalog #**8110
- AccuTaq LA DNA Polymerase **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8045-125UN
- Linear Acrylamide (5 mg/ml) (1 ml Tube) **Thermo Fisher Catalog #**AM9520
- QIAamp® Viral RNA Mini **Qiagen Catalog #**52906
- Linear acrylamide **Thermofisher Catalog #**AM9520
- TURBO™ DNase (2 U/µL) **Thermo Fisher Catalog #**AM2238
- Ampure XP beads **Beckman Coulter Catalog #**A63881
- SISPA-Primer B **IDT**
- Filter (0.22µm) **Costar Catalog #**8110
- Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #**D4014
- SuperScript™ IV First-Strand Synthesis System **Thermo Fisher Catalog #**18091200
- TURBO™ DNase (2 U/µL) **Thermo Fisher Catalog #**AM2238
- QIAamp® Viral RNA Mini **Qiagen Catalog #**52906
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- Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #**D4014
- TURBO™ DNase (2 U/µL) **Thermo Fisher Catalog #**AM2238

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) **Costar Catalog #**8110

2 Centrigue at 14,000 RPM for 5 minutes

Equipment

Centrifuge

NAME	Centrifuge
TYPE	Benchtop Centrifuge
BRAND	Eppendorf
SKU	5405000441
LINK	https://online-shop.eppendorf.us/US-en/Centrifugation-44533/Centrifuges-44534/Centrifuge-5425-PF-243560.html
SPECIFICATIONS	Any benchtop centrifuge will suffice

14000 rpm, 00:05:00

3 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) **Thermo Fisher Catalog #**AM9520

QIAamp® Viral RNA Mini **Qiagen Catalog #**52906

4 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.

5 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.

6 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

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

8 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.

9 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

10 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.

11 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 13, or to eliminate possible Buffer AW2 carryover, perform step 12 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

12 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

13 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

14 Centrifuge at 6000 x g (8000 rpm) for 1 min.
8000 rpm, 00:01:00

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

DNase Treatment

16 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) **Thermo Fisher Catalog #**AM2238

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

18 Incubate at 37°C for 30 minutes. 37 °C

00:30:00

Equipment

ThermoMixer

NAME

Benchtop Incubator

TYPE

Eppendorf

BRAND

5382000023

SKU

https://online-shop.eppendorf.us/US-en/Temperature-Control-and-Mixing-44518/Instruments-44519/Eppendorf-ThermoMixerC-PF-19703.html

LINK

Any heat block will suffice

SPECIFICATIONS

Zymo Clean-up and Concentrator

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

Zymo DNA Clean & Concentrator - 5 **Zymo Research Catalog #**D4014

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

21 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

Equipment

Centrifuge	NAME
Benchtop Centrifuge	TYPE
Eppendorf	BRAND
5405000441	SKU
https://online-shop.eppendorf.us/US-en/Centrifugation-44533/Centrifuges-44534/Centrifuge-5425-PF-243560.html	LINK
Any benchtop centrifuge will suffice	SPECIFICATIONS

22 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

23 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

24 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

25 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

26 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.

27 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.

Equipment

SimpliAmp Thermal Cycler	NAME
PCR	TYPE
Applied Biosystems	BRAND
A24811	SKU
https://www.thermofisher.com/order/catalog/product/A24811	LINK
Any standard PCR thermocycler will suffice	SPECIFICATIONS

28 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.

29 Add 5 µL master mix to reaction. Incubate at 50°C for 10 minutes.

50 °C 00:10:00

30 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.

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

32 Incubate at 37°C for 8 min.

37 °C 00:08:00

33 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.

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

35 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

36 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

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

38 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

Equipment

SimpliAmp Thermal Cycler	NAME
PCR	TYPE
Applied Biosystems	BRAND
A24811	SKU
https://www.thermofisher.com/order/catalog/product/A24811	LINK
Any standard PCR thermocycler will suffice	SPECIFICATIONS

Bead Clean-up

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

40 Incubate at RT for 10 minutes

Room temperature 00:10:00

41 Spin down briefly

Equipment

Mini-centrifuge	NAME
Centrifuge	TYPE
Fisher	BRAND
S67601B	SKU
https://www.fishersci.com/shop/products/fisherbrand-standard-mini-centrifuge-standard-mini-centrifuge/s67601b	LINK
Any standard mini centrifuge with adapters for different tube sizes will suffice	SPECIFICATIONS

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

Equipment

Magnetic Stand	NAME
Magnetic Stand	TYPE
Thermo Scientific	BRAND
MR02	SKU
https://www.thermofisher.com/order/catalog/product/MR02	LINK
Any magnetic rack that fits your tubes will suffice.	SPECIFICATIONS

43 Wash with 200µL of 70% EtOH.

44 Remove EtOH without disturbing beads.

45 Repeat wash. Remove EtOH without disturbing beads.

46 Remove samples from magnetic rack and spin down briefly.

47 Remove residual ethanol

48 Let air dry briefly - Do not overdry bead pellet

49 Resuspend sample in 50µL of H2O.

50 Incubate samples at RT for 5 minutes.
Room temperature 00:05:00

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

52 Continue onto library prep protocol of your choice.