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Nanopore SISPA

Siu Fung Ho[1]

[1]University of Hong Kong

Siu Fung Ho
University of Hong Kong

ABSTRACT

Nanopore SISPA

Sample Preprocessing

1 Refer to sample preprocessing protocol for appropriate sample type.

2 Use 280 µl of the filtrate as starting material for the Qiagen Viral RNA Mini Kit.

RNA extraction with QIAamp Viral RNA Mini Kit

3 Add 1:500 linear acrylamide instead of carrier RNA to Buffer AVL.

4 Pipet 1120 µL prepared Buffer AVL into a 2 mL Eppendorf tube.

5 Add 280 µL of sample into the tube containing Buffer AVL.

6 Mix by pulse-vortexing for 15 s.

7 Incubate at room temperature for 10 min.

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

9 Add 1120 µL 100% ethanol to the sample, and mix by pulse-vortexing for 15 s.

10 After mixing, briefly centrifuge the tube to remove drops from inside the lid.

11 Carefully apply 630 µL of the lysate to the QIAamp Mini column without wetting the rim. Close the cap, and centrifuge at 6000 x g for 1 min.

Place the QIAamp Mini column into a clean 2 mL collection tube, and discard the tube containing the filtrate.

> **Note**
>
> If some lysate does not pass through the membrane after the initial spin, repeat the spin until it has completely flowed through.

12 Repeat the previous step until all of the lysate has been added to the column.

13 Carefully open the QIAamp Mini column, and add 500 µL Buffer AW1.

Close the cap, and centrifuge at 6000 x g for 1 min.

Place the QIAamp Mini column in a clean 2 mL collection tube, and discard the tube containing the filtrate.

14 Carefully open the QIAamp Mini column, and add 500 µL Buffer AW2.

Close the cap and centrifuge at 16,000 x g for 3 min.

15 Place the QIAamp Mini column in a new 2 mL collection tube, and discard the old collection tube with the filtrate.

Centrifuge at 16,000 x g for 1 min.

16 Place the QIAamp Mini column in a clean 1.5 mL microcentrifuge tube. Discard the old collection tube containing the filtrate.

Carefully open the QIAamp Mini column and add 50 µL Buffer AVE equilibrated to room temperature.

Close the cap, and incubate at room temperature for 1 min.

17 Centrifuge at 6000 x g (8000 rpm) for 1 min to collect eluate.

DNase treatment and RNA cocentration

18 Mix 50 µL eluate with 100 µL of RNA Binding Buffer.

19 Add and mix in 150 µL of 100% ethanol.

20 Transfer the sample to the Zymo-Spin IC Column3 in a CollectionTube and centrifuge.

Discard the flow-through

21 Add 400 µl RNA Wash Buffer to the column, centrifuge and discard the flow-through.

22 Make up a DNase I Reaction Mix for each sample to be treated:

Component	Volume

DNase I 5 µL

DNA Digestion Buffer 35 µL

23 Add 40 µl of DNase I Reaction Mix directly into column matrix and incubate at room temperature for 15 minutes.

24 Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flow-through.

25 Add 400 µl RNA Wash Buffer to the column and centrifuge for 1 minute ensure complete removal of the wash buffer.

Carefully, transfer the column into a RNase-free tube.

26 Add 10 µl DNase/RNase-Free Water directly to the column matrix and centrifuge.

Note

Pause point: store RNA at -20C

FastSelect rRNA removal

27 Bring FastSelect -rRNA HMR, FastSelect 5S/16S/23S, FastSelect FH Buffer, and Nuclease-free Water to room temperature.

28 For FastSelect 5S/16S/23S, incubate the tube at 37°C for 5 min, and then vortex to dissolve the precipitate.

29 Mix all other reagents vigorously by vortexing, and then briefly centrifuge.

30 Prepare depletion master mix on ice:

Component	Volume
FastSelect FH Buffer	1.5 µL
FastSelect -rRNA HMR	1 µL
FastSelect 5S/16S/23S	1 µL
Nuclease-free Water	1.5 µL

31 Add 5 ul of master mix to template RNA.

32 Incubation reactions as follows:

1. 2 min at 75 °C
2. 2 min at 70 °C
3. 2 min at 65 °C
4. 2 min at 60 °C
5. 2 min at 55 °C
6. 2 min at 37 °C
7. 2 min at 25 °C
8. Hold at 4°C

33 Add 19.5 µl QIAseq Beads (prewarmed to room temperature and mixed by vortexing) to the 15 µl reaction.

Mix thoroughly by vortexing, and then incubate for 5 min at room temperature.

34 Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).

35 Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

Note

Do not discard the beads, because they contain the RNA of interest.

36 Add 15 µl of Nuclease-free Water and 19.5 µl of QIAseq Bead Binding Buffer (prewarmed to room temperature and mixed by vortexing). Mix vigorously by vortexing, and then incubate for 5 min at room temperature.

37 Centrifuge in a table-top centrifuge until the beads are completely pelleted (~2 min).

38 Place the tubes/plate on a magnetic rack for 2 min. Once the solution has cleared, with the beads still on the magnetic stand, carefully remove and discard the supernatant.

39 With the beads still on the magnetic stand, add 200 µl of 80% ethanol. Wait for 30 s.

Carefully remove and discard the wash.

Note

Do not discard the beads, because they contain the RNA of interest.

40 Repeat the ethanol wash.

Note

Completely remove all traces of ethanol after this second wash. Remove the ethanol with a 200 µl pipette tip first, and then use a 10 µl pipette tip to remove any residual ethanol that will settle.

41 With the beads still on the magnetic stand, air-dry at room temperature for 6–10 min until all liquid has evaporated but without overdrying the beads.

Note

Visually inspect the pellet to confirm that it is completely dry.

42 Remove the beads from the magnetic stand, and elute the RNA from the beads by adding 12 µl Nuclease-free Water. Mix well by pipetting or vortexing, and then allow the tubes to sit at room temperature for 2 min to appropriately hydrate the beads.

43 Return the tubes/plate to the magnetic rack until the solution has cleared.

44 Transfer 10 µl of the supernatant to nuclease-free tube.

Note

Pause point: Samples can be store at -80C

Reverse Transcription

45 Prepare RT reaction master mix. Per sample volumes:

Component	Volume
DNase I treated RNA	6 µL
dNTP 10mM	0.5 µL
SISPA-A primer 40uM	1 µL
Nuclease-free water	2.5 µL

46 Mix and briefly centrifuge.

47 Incubate at 65C for 5min and then on ice for 1 min

48 Pre-warm the 5x SSIV buffer to RT. Vortex and briefly centrifuge.

49 Prepare RT mastermix:

Component	Volume
5x SSIV Buffer	2 µL
DTT 100 mM	0.5 µL
RNase Inhibitor	0.5 µL
Reverse Transcriptase	0.5 µL

50 Add 3.5ul of RT master mix to annealed RNA.

51 Incubate at 42C for 10 min and then at 80C for 10 min.

Second Strand cDNA Synthesis (Klenow fragment)

52 Add 1ul of 10x NEB buffer 2 to the RT product.

53 Incubate at 95C for 3 min and then one ice for 1min.

54 Add 0.5ul of Klenow fragment to the mixture.

55 Incubate at 37C for 1 h and then at 75C for 15 min.

AMPure cleanup

56 Prepare the AMPure XP beads for use; resuspend by vortexing.

57 Transfer the cDNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

58 Add 11 µl of resuspended AMPure XP beads to the cDNA samples and mix by pipetting up and down.

59 Incubate for 5 minutes at room temperature.

60 Prepare 500 µl of fresh 70% ethanol in nuclease-free water.

61 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

62 Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard.

63 Repeat the previous step.

64 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 3 min, but do not dry the pellet to the point of cracking.

65 Remove the tube from the magnetic rack and resuspend pellet in 10 µl nuclease-free water. Incubate for 2 minutes at room temperature.

66 Pellet the beads on a magnet until the eluate is clear and colourless.

67 Remove and retain 10 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Single Primer PCR amplification

68 Setup PCR master mix:

Component	Volume
2x LongAmp PCR buffer	12.5 µL
SISPA-B primer 10 uM	5 µL
Nuclease-free water	5 µL

Note

Mastermix should be made up and aliquoted into PCR tubes in a mastermix cabinet. Cabinet and tubes should be cleaned with decontamination wipes/70% ethanol and UV sterilised before and after use.

69 Program the following PCR cycles into the thermal cycler.

Step	Temperature	Time	Cycles
Inital Denaturation	95C	3 min	1
Denaturation	95C	15 s	20
Annealing	50C	30 s	20
Extension	65C	50 s	20
Final Extension	65C	5 min	1

AMPure cleanup 2

70 Prepare the AMPure XP beads for use; resuspend by vortexing.

71 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

72 Add 25 µl of resuspended AMPure XP beads to the PCR product and mix by pipetting up and down.

73 Incubate for 5 minutes at room temperature.

74 Prepare 500 µl of fresh 70% ethanol in nuclease-free water.

75 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

76 Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard.

77 Repeat the previous step.

78 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 3 min, but do not dry the pellet to the point of cracking.

79 Remove the tube from the magnetic rack and resuspend pellet in 24 µl nuclease-free water. Incubate for 2 minutes at room temperature

80 Pellet the beads on a magnet until the eluate is clear and colourless.

81 Remove and retain 24 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Note

Pause point: Store samples at -20C

Native barcoding and sequencing by ligation

82 Refer to the nanopore protocol.