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Title: Development protocol for Nanotrap® particle enrichment of virus for sequencing applications.		
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Objective: This protocol outlines the Nanotrap® Particle method to capture and concentrate viruses in Transport Media prior to next generation sequencing performed on an Oxford Nanopore minION sequencer.

Materials and equipment:

1. Nanotrap® Magnetic Virus Particles (Ceres Nanosciences, SKU# 44202)
2. Rapid PCR barcoding kit (Oxford Nanopore, Cat # SQK-RPB004)
3. RNeasy MinElute Cleanup Kit (Qaigen , Cat # 74204)
4. Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Fisher, Cat # K2561)
5. AMPure XP Beads (Beckman Coulter, Cat # A63880)
6. Taq 2X Master Mix (New England Biolabs, Cat # M0270L)
7. 10x Tris Buffered Saline (Polysciences, Cat # 24082-500)
8. PCR plate
9. Transport Media
10. RNase/DNase free water
11. Centrifuge and other standard lab equipment
12. Heat/Cold Block
13. Shaker
14. Vortex
15. 75% Ethanol
16. 80% Ethanol
17. 2 mL lo bind tubes
18. Thermocycler

Procedure:

This protocol contains seven sections (A, B, C, D, E, F, G), **Nanotrap® particle processing only occurs in section A (30 mins)**, all other sections are sequencing-specific preparation steps and are open to user customization (B, C, D, E , F , and G).

Current Start to Finish Processing Time: 6-7 hrs

A. Capture and Concentrate Virus using Nanotrap® Particles (Nanotrap Virus Capture kit)

Processing Time: 30 min

1. Add upto 1.5 mL of virus sample (e.g. viral sample in viral transport media) to 2 mL lo-bind tube.
2. Add 1 unit of Nanotrap® Magnetic Virus particles (NT) to sample. Resuspend thoroughly.
3. Incubate at room temperature with rotation/mixing for 30 minutes. Alternatively, you can gently mix every five minutes to ensure NT particles stay in suspension.
4. Separate NT sample on magnetic rack for 1-2 minutes.
5. Discard the supernatant carefully without disturbing the NT pellet.

B. Virus Extraction (Qaigen RNeasy MinElute kit , ***Nanotrap particles are compatible with most RNA/DNA extraction kits - current protocol optimized using RNeasy MinElute kit**)*

Processing Time: 30 min

1. Add 100 µL of RNase/DNase free water to NT pellet .
2. Add 350 µL of Buffer RLT to NT pellet, resuspend completely.
3. Incubate NT sample at room temperature, with rotation/mixing for 10 minutes.
4. Separate NT sample on magnetic rack for 1-2 minutes.
5. Move/save supernatant in new 2mL lobind tube, discard NT pellet.
6. Add 250 µL of 96-100% Ethanol to water/RLT sample, mix well by pipetting.
7. Transfer the sample (700 µL) to an RNeasy MinElute spin column placed in a 2 mL collection tube (supplied).
8. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through. For samples >700 µL, transfer the remaining sample and repeat the centrifugation. Discard the flow-through. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied).
9. Add 500 µL Buffer RPE to the spin column. Centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
10. Add 500 µL of 80% ethanol to the RNeasy MinElute spin column. Centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Centrifuge at full speed for 5 min to dry the membrane. Discard the flow-through and collection tube.
11. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied).
12. Add 20 µL RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

C. cDNA synthesis (Maxima H Minus Double-Stranded cDNA Synthesis Kit)

Processing Time: 2 hrs

First DNA Strand Reaction

1. Add .5 µL of Random Hexamer to 20 µl RNA sample in 2 mL lo bind tube.
2. Gently mix, incubate on heat block at 65C for 5 min.
3. Briefly cool down on ice.
4. Add 5 µL of 4x First Strand mix to sample.
5. Add 1 µL of First Enzyme Mix to sample.
6. Gently mix sample, incubate at room temperature for 10 min.
7. Incubate on heat block at 50C for 30 min.
8. Incubate on heat block at 85C for 5 min (stops first strand DNA reaction) .

Second DNA Strand Reaction

9. Add 34 µL of RNAse/DNAse free water to sample.
10. Add 16 µL of 5x Second Strand Mix to sample.
11. Add 4 µL of Second Strand Enzyme Mix to sample.
12. Gently mix sample, incubate at 16C for 60 min.
13. Incubate on heat block at 85C for 5 min (stops second strand DNA reaction).

D. DNA cleanup (AMPure XP beads**)**

Processing Time: 15 min

1. Add 50 µL of AMPure XP beads to cDNA sample, mix completely.
2. Incubate at room temperature for 5 mins.
3. Place sample on magnetic rack, allow to sit for several minutes until solution is clear.
4. Discard supernatant.
5. Add 200 µL of 75% EtOH to AMPure XP beads , resuspend completely.
6. Incubate at room temperature for 1 min.
7. Place sample on magnetic rack, allow to sit for several minutes until solution is clear.
8. Discard supernatant.
9. Add 24 µL of RNase/DNAse free water AMPure XP beads , resuspend completely.
10. Incubate at room temperature for 5 min.
11. Place sample on magnetic rack, allow to sit for several minutes until solution is clear.
12. Carefully extract DNA containing water, move to fresh 2 mL lo bind tube.
13. Discard AMPure XP beads.

E. Library Prep/PCR Amplification (Nanopore Rapid PCR barcoding Kit)

Processing Time: 2.5 hrs

1. Add 1 µL of FRM to 24 µl DNA sample.
2. Incubate on heat block at 30C for 2 min, directly add to heat block at 80C for 1 minute.
3. Briefly place sample on ice.
4. Add 1 µL of Barcode to sample (12 barcodes for 12 samples).
5. Add 25 µL of Taq 2X Master Mix.
6. Gently mix sample.
7. Add sample to PCR plate and run PCR procedure below.
    a. 95C 3 min
    b.
        i. 95C -15 sec
        ii. 56C -15 sec
        iii. 65C -2.5 min
        iv. Repeat b.- 40 cycles

F. DNA cleanup -sample concentration (AMPure XP beads)

Processing Time: 15 min

1. Combine all pcr samples into 1 - 2 mL lobind tube (protocol assumes 1 sample, can combine upto 12 samples).
2. Add 40 µL of AMPure XP Beads to PCR amplified DNA samples, mix completely (Add 0.8 vol of AMPure XP beads to combined sample ex: 5 samples 50 µL per sample = 250 µL total; load 200 µL of AMPure XP beads).
3. Incubate at room temperature for 5 mins.
4. Place sample on magnetic rack, allow sample to sit for several minutes until solution is clear.
5. Discard supernatant.
6. Add 200 µL of 75% EtOH to AMPure XP beads , resuspend completely.
7. Incubate at room temperature for 1 min.
8. Place sample on magnetic rack, allow sample to sit for several minutes until solution is clear.

9. Discard supernatant.
10. Add 15 µL of **1x** Tris Buffered Saline to AMPure XP beads , resuspend completely.
11. Incubate at room temperature for 5 min.
12. Place sample on magnetic rack, allow to sit for several minutes until solution is clear
13. Carefully extract DNA containing TBS, move to new 2 mL lobind tube.
14. Discard AMPure XP beads.

G. Flow Cell Loading (Nanopore Minion)

Processing Time: 15 min

1. Add 1 µL of RAP to DNA-TBS sample.
2. Incubate at room temperature for 5 min.
3. Add 34 µL of SQB to sample.
4. Add 25 µL of LB to sample.
5. Sample vol total = 75 µL.
6. Follow Nanopore Flow Cell Loading Procedure (available online at Oxford Nanopore Website).