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'VirION': Long-read, low-input, viral metagenomic sequencing; Library Preparation and MinION (Oxford Nanopore Technologies) Sequencing (lib. prep. kit SQK-LSK108; flow cell: R9.4) v4

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We use this protocol and it's working

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Abstract

This protocol describes a generalizable, long-read, low-input metagenomic sequencing approach ('VirION') for the survey of viral communities. A significant obstacle in adopting long-read technology for viral metagenomics lies in obtaining the amount of DNA required; e.g. viral DNA extraction from 20 L of seawater yields far less than the micrograms of DNA recommended for efficient long-read sequencing. To overcome this limitation, we developed a Long-Read Linker-Amplified Shotgun Library approach for long-read viral metagenomics. The VirION method has been demonstrated to be as relatively quantitative as short-read methods, and analyses that combined VirION long-read data with Illumina, short-read data, captured many abundant and ubiquitous viral genomes that were missed by short-read assemblies. This approach was also shown to have overcome issues of microdiversity, and to have captured more genomic islands than short-read assemblies. Thus, VirION provides a high throughput and cost-effective alternative to fosmid and single-virus genomic approaches.

Guidelines

Method contents

A: DNA fragmentation
B: End-prep and clean-up
C: PCR adapter ligation and clean-up **(possible stop stage)**
D: Amplification and clean-up **(possible stop stage)**
E: 2nd End prep and clean-up
F: Adapter ligation
G: Adapter bead binding
H: Elution of library
I: Preparation of host computer and performing Flow cell QC
J: Priming the flow cell
K: Loading the library
L: Completing the experiment

Note: Process sheared, diluted commercial lambda DNA as a postivie control, through stages A-E, in order to verify success of amplification stages.

Equipment and consumables

- Vortex mixer
- Pipettes P1000, P200, P100, P20, P10 and P2
- Tips: 1000 µl, 200 µl, 10 µl
- Magnet for bead separation (a decent make, e.g. DynaMag™ Spin Magnet)
- Micro centrifuge *(model: Eppendorf 5424, if possible)
- Covaris g-TUBE (1 per sample, plus one for a positive control)
- 1.5 ml Eppendorf LoBind centrifuge tubes (Important: some plastics inhibit library prep.)
- 2 ml thin-walled PCR tubes
- Thermal cycler set for:
- Program 1: 5 min at 20°C; 5 min at 65°C; 1 min 20°C; hold at 20°C
-

- Program 2: 3 mins at 95°C; 15 cycles of: 15 secs at 95°C, 15 secs at 62°C, 5 min at 72°C, followed by one cycle of 5 min at 72°C; and hold at 4°C
-

- Qubit fluorimeter
- Qubit reaction tubes and Qubit dsDNA HS and BR Assay Kits
- Heat block set to 55°C
- Timer
- Filled Ice box
- Bioanalyzer/TapeStation
- Hula mixer (rotator: optional)

- Desktop/laptop:
- Min. available memory: 50 GB
-

- Sleeptimer and update turned off
-

- For latest minimum computer specs: https://community.nanoporetech.com
-

- MinION Mk 1B

Reagents

- AMPure XP beads resuspended and at RT
- SQK-LSK108 sequencing kit (Oxford Nanopore)

Defrost following reagents on ice:

- ‘PCR Adapters’ (PCA),
- ‘Primers’ (PRM),
- ‘Running Buffer with Fuel Mix’ (RBF),
- 'Library Loading Beads' (LLB)‘
- Adapter Mix’ (AMX 1D) Note: leave in freezer until 10 min before needed

Defrost following reagents at RT:

-
- ‘Adapter Bead Binding’ (ABB)
-

- ‘Elusion Buffer’ (ELB)
-

- NEB Next Ultra II End-repair / dA-tailing Module (E7546S):
- ‘Ultra II End-Prep Buffer’ - defrost at RT; check for precipitate; vortex well to dissolve if necessary
-

- ‘Ultra II End-Prep Enzyme Mix’ - defrost on ice
-

- NEB Blunt / TA Ligase Master Mix (M0367S) – check for precipitate; take out of freezer 10 min before use and keep on ice
- NEB Next High-Fidelity 2X PCR Master Mix (Product number: M0541), take out of freezer ~15 min before use and put on ice
- Nuclease free water (NFW)
- Freshly prepared 80% EtOH (with NFW)
- Commercial lambda DNA sheared as detailed in stage ‘A’, and diluted to match sample concentration

Before start

Sample requirements

<1 µg of viral metagenomic DNA in 47 µl of elution buffer (EB) (e.g. 10 mM Tris-Cl, pH 8.5) or nuclease free water (NFW)

Important note: EDTA compromises flow cell pores, so do not use TE to re-suspend/elute viral DNA; If viruses were obtained by chemical flocculation and resuspension, further cleaning (post DNA extraction with Wizard DNA Clean-Up System) may be required (e.g. using DNeasy PowerClean Pro Kit (Cat No./ID: 12997-50).

Stopping stages

If pausing (e.g. overnight) either after PCR adapter ligation and clean-up (Stage 'C') or after amplification and clean-up (Stage 'D'), some reagents will not be required immediately: check protocol to ensure reagents are not defrosted unnecessarily. Replace reagents taken from freezer to -20°C when they have been used and are no longer required (check later stages).

A: DNA fragmentation

1 Ensure DNA is not stuck to tube by gently flicking; then spin down; transfer <1 µg DNA in 47 µl NFW/EB into the top compartment of a Covaris g-TUBE; screw on lid

2 Shear DNA: Spin g-TUBE for 1 min at 6000 rpm (check all DNA has transferred into lower compartment of g-TUBE; if not, repeat 1 min spin); flip tube over (so base points upwards) and spin again for 1 min at 6000 rpm (check all DNA has transferred into lid of g-TUBE; if not, repeat 1 min spin);

Note

Keep processing time <15 min;

*if a microcentrifuge other than model specified is used, spin speed may have to be optimised to obtain 8 Kbp fragments

3 keep g-TUBE base upward, carefully unscrew tube and transfer DNA from lid to a fresh LoBind Eppendorf or (if proceeding directly to stage 'B') 0.2 ml PCR tube

Retain 1 µl of sheared DNA for later quantification via QuBit fluorimeter

Note

Assessment of fragment size Bioanalyzer/TapeStation not usually possible due to dilute nature of DNA

B: End-prep and clean-up

4 To 45 µl of fragmented and repaired DNA, add:
- 5 µl NFW
- 7 µl Ultra II End-Prep buffer
- 3 µl Ultra II End-Prep enzyme mix

(resulting in 60 µl total reaction volume)

5 Mix by gentle flicking, then spin down (transfer end-prep reaction to a 0.2 ml PCR tube, if needed)

6 Using thermocycler program 1, incubate for:
- 5 mins at 20 °C
- 5 min at 65 °C,
- 1 min 20 °C

Then transfer to fresh DNA LoBind tube

7 Resuspend AMPure beads (vortex); add 60 µl of beads to end-prep reaction; mix by gentle flicking

8 Incubate for 5 mins at RT, either on rotator, or gently flick every ~1 min

20 °C Room temp.

9 Spin down the sample and pellet on a magnet until the eluate is clear and colourless (~2 min)

10 Leaving the tube on the magnet, carefully pipette off and discard the supernatant

11 Keep tube on magnet, and wash beads with 200 µl of freshly prepared 80% ethanol (without disturbing the pellet; leave ~15 secs)

12 Remove the 80% ethanol using a pipette and discard

13 Repeat wash (steps 11 and 12)

14 Spin down briefly, replace on magnet, pipette off residual wash; briefly allow to dry (~1-2 min)

Note

It is important beads are allowed to dry to ensure all ethanol has been removed, however, over drying will result in some of the DNA not eluting efficiently leading to reduced recovery

15 Remove the tube from the magnetic rack and resuspend pellet in **31 µl** nuclease-free water by gentle flicking

16 Incubate for 2 minutes at 55 °C (heat block)

55 °C In heat block

17 Pellet beads on magnet until the eluate is clear and colourless (~2 min)

18 Transfer eluate to fresh DNA LoBind tube;

Retain 1 µl of fragmented and end-prepped DNA for later quantification (QuBit fluorimeter)

Note

Recovery aim: ≥ 20 ng (dependent on starting quantity).

C. PCR adapter ligation and clean-up

19 To 30 µl of end-prepped DNA, add:
- 20 µl of PCA
- 50 µl Blunt/TA Ligase Master Mix

(resulting in 100 µl total reaction volume)
Mix by gentle flicking, then spin down

Note

Note: mix between each addition by gentle flicking, and spin down

20 Incubate at RT for 10 minutes

20 °C Room temp.

21 Resuspend AMPure beads (vortex); add 40 µl of beads to PCR adapter reaction; mix by gentle flicking

Note

This 0.4x beadwash removes small fragments of template DNA before PCR step

22 Incubate for 5 mins at RT, either on rotator, or gently flick every ~1 min

20 °C Room temp.

23 Spin down the sample and pellet on a magnet until the eluate is clear and colourless (~3 min)

24 Leaving the tube on the magnet, carefully pipette off and discard the supernatant

25 Keep tube on magnet, and wash beads with 200 µl of freshly prepared 80% ethanol (without disturbing the pellet; leave ~15 secs)

26 Remove the 80% ethanol using a pipette and discard

27 repeat wash (steps 25 and 26)

28 Briefly spin down, replace on magnet, pipette of residual wash; briefly allow to dry (note importance of drying time, as above)

29 Remove the tube from the magnetic rack and resuspend pellet in **26 µl** nuclease-free water by gentle flicking

30 Incubate for 2 minutes at 55 °C (heat block)

55 °C Heat block

31 Pellet beads on magnet until the eluate is clear and colourless (~3 min)

32 Transfer eluate to fresh DNA LoBind tube

Conduct a High Sensitivity (HS) QuBit fluorimeter quantification on 1 µl of PCR adapted and ligated DNA (also quantify retained DNA from stages 'A' and 'B')

33 Calculate volume required to add ~20 ng of PCR-adapter ligated DNA into the PCR reaction below (e.g. 2.7 ng/µl x 8 = 21.6 ng; use 8 µl)

Note

If not proceeding directly to stage 'D', PCR-adapter ligated DNA should be stored post clean-up at -20°C.

D. Amplification and clean-up

34 Set up the following PCR reactions (total volume 100 µl) in 0.2 ml thin-walled tubes, on ice:

- *40 µl NFW
- 2 µl PRM (primers)
- *8 µl PCR adapted and ligated DNA (from stage 'C')
- 50 µl NEB Next High Fidelity 2X master mix

:Mix by gentle flicking and spin down briefly

Note

*Adjust volume of NFW and DNA as necessary for a 100µl reaction with ~20 ng of PCR-adapter ligated DNA, using the volumes of PRM and NEB Next master mix specified

Set up as many reactions as possible to increase yield of amplicons for sequencing; also set up the PCR-adapter ligated lambda as a positive control

35 Quickly transfer PCR reactions to the thermal cycler and run the following (Program 2):

- Initial denaturation: 3 mins at 95°C;
- 15 cycles of:
- Denaturation: 15 secs at 95°C;
-
- Annealing: 15 at 62°C;
-
- Extension: 5 min at 72°C;
-
- Final Extension: 5 min at 72°C;
- Hold at 4°C

36 Spin down and transfer each reaction to a fresh DNA LoBind tube

37 Resuspend AMPure beads (vortex); add 40 µl of beads to each PCR reaction; mix by gentle flicking

Note

This 0.4x beadwash removes small amplification products

38 Incubate for 5 mins at RT, either on rotator, or gently flick every ~1 min

20 °C Room temp.

39 Spin down the samples and pellet on a magnet until the eluates are clear and colourless (~3 min)

40 Leaving the tubes on the magnet, carefully pipette off and discard the supernatant

41 Keep tubes on magnet, and wash beads with 200 µl of freshly prepared 80% ethanol (without disturbing the pellets; leave ~15 secs)

42 Remove the 80% ethanol using a pipette and discard

43 Repeat wash (steps 41 & 42)

44 Briefly spin down, replace tubes on magnet, pipette of residual wash; briefly allow to dry (note importance of drying time, as above)

45 Remove the tubes from the magnetic rack and resuspend pellets in **26 µl** nuclease-free water by gentle flicking

46 Incubate for 2 minutes at 55°C (heat block)

55 °C Heat block

47 Pellet beads on magnet until the eluate is clear and colourless (~3 min)

48 Transfer eluate to fresh DNA LoBind tube

Conduct Broad Range (BR) QuBit fluorimeter quantification on 1 µl of each PCR product

Conduct Bionalyzer/Tapestation assay on 1 µl of each PCR product

Note

Check no high molecular weight product present in adapted and ligated PCR product via Tapestation/Bioanalyzer; very high MW product is associated with poor sequencing; if observed, repeat PCR with fewer cycles.

If not proceeding directly to stage 'E', PCR products should be stored post clean-up at -20°C.

E. 2nd End prep and clean-up

49 Concentrate (e.g. via SpeedVac) 3-3.5 µg of PCR product into 45 µl of NFW

50 To 45 µl of PCR product in NFW add:

- 5 µl NFW
- 7 µl Ultra II End-Prep buffer
- 3 µl Ultra II End-Prep enzyme mix

(resulting in 60 µl total reaction volume)

51 Mix by gentle flicking, then spin down; transfer end-prep reaction to a 0.2 ml PCR tube

52 Using thermocycler program 1, incubate for:
- 5 mins at 20 °C
- 5 min at 65 °C,
- 1 min 20 °C

Then transfer to fresh DNA LoBind tube

53 Resuspend AMPure beads (vortex); add 60 µl of beads to end-prep reaction; mix by gentle flicking

54 Incubate for 5 mins at RT, either on rotator, or gently flick every ~1 min

20 °C Room temp.

55 Spin down the sample and pellet on a magnet until the eluate is clear and colourless (~2 min)

56 Leaving the tube on the magnet, carefully pipette off and discard the supernatant

57 Keep tube on magnet, and wash beads with 200 µl of freshly prepared 80% ethanol (without disturbing the pellet; leave ~15 secs)

58 Remove the 80% ethanol using a pipette and discard

59 Repeat wash (steps 57 & 58)

60 Spin down, replace on magnet, pipette of residual wash; briefly allow to dry (note importance of drying time, as above)

61 Remove the tube from the magnetic rack and resuspend pellet in **31 µl** nuclease-free water by gentle flicking

62 Incubate for 2 minutes at 55 °C (heat block)

55 °C Heat block

63 Pellet beads on magnet until the eluate is clear and colourless (~2 min)

64 Transfer eluate to fresh DNA LoBind tube

Retain 1 µl of fragmented and repaired DNA for (later) quantification using a QuBit fluorimeter;

Note

Recovery aim: >2500 ng.

F. Adapter ligation

65 To 30 µl of end-prepped amplified DNA, add:
- 20 µl AMX
- 50 µl NEB Blunt / TA Master Mix

(resulting in a final volume of 100 µl)
Mix by gentle flicking, then spin down

Note

Mix between each addition by gentle flicking; spin down

66 Incubate at RT for 10 minutes

20 °C Room temp.

G. Adapter bead binding

67 Resuspend AMPure beads (vortex); add 40 µl of beads to the adapter ligation reaction; mix by gentle flicking

68 Incubate for 5 mins at RT, either on rotator, or gently flick every ~1 min

20 °C Room temp.

69 Spin down the sample and pellet on a magnet until the eluate is clear and colourless (~3 min)

70 Leaving the tube on the magnet, carefully pipette off and discard the supernatant

71 Add 140 µl of **ABB** to the beads; remove from magnet and resuspend by gentle flicking

72 Pellet beads on magnet until the eluate is clear and colourless (~5 min)

73 Leaving the tube on the magnet, **very** carefully remove supernatant using a pipette and discard

74 Repeat addition and removal of 140 µl of ABB (steps 71 -73)

H. Elution of library

75 Resuspend pelleted beads in **15 µl ELB** by gentle but tenacious flicking; incubate at RT for 10 minutes

20 °C Room temp.

76 Pellet beads on magnet until the eluate is clear and colourless (~5 min)

77 Remove and **retain 15 µl of eluate** into a clean 1.5 ml Eppendorf DNA LoBind tube

Place the tube of (adapted and tethered) library on ice until required for library loading

Note

This elution will form the basis of the library loading mix

78 Conduct a second elution on the beads: add 15 µl ELB to the pellet; resuspend; incubate at RT for 10 min; pellet on magnet; remove and retain second eluate into clean 1.5. ml tube; place this on ice

Note

If the first elution is somewhat low on DNA, this second one may form the basis of a second library loading mix. It may be worth loading the second library mix onto a flow cell at a point in the run where there does not appear to be enough DNA for the 'single pores' still available.

79 Quantify 1 µl of DNA library via QuBit fluorimeter (both elutions); also quantify DNA retained from previous stages at this time.

Recovery aim for RT eluate: >1000 ng (~75ng/µl); ;

Remove flow cell from 4°C to RT 30 min before performing QC and loading library (check for bubbles on 'chip'; if bubbles noted, use alternative flow cell)

I. Preparation of host computer and performing Flow cell QC

80 On host computer ensure that:
- The internet is working;
- **>150 GB of memory is free on the C drive**;
- All sleep modes (including screensavers and log-offs) are disabled

81 Connect MinION to host computer (light and fan operation indicates that the device is successfully plugged in); open MinKNOW software; Check for MinKNOW software updates

> Note
>
> Check that the MinION configuration has been run prior to loading flow cell

82 Insert flow cell into MinION (orientated with flow chip adjacent to hinge); close cover; check that MinKNOW is reporting a 'Flowcell connection' number and flowcell ID (if not, close MinKNOW, eject MinION, and repeat)

83 Select running parameters:
- Label Experiment' and 'Sample ID' (as required)
- Select the appropriate operation (i.e. Platform QC)

84 'Start' QC; to assist MinION gain correct temperature (approximately 34 °C), place MinION on insulating or conductive surface and modify airflow (as needed)

Allow script to run to completion (this will QC the flow cell; total time ~10 min); note number of pore channels available (if <1000, consider using a different flow cell)

Proceed with preparation of priming buffer

J. Priming the flow cell

85 Mix RBF thoroughly by vortexing/pipetting; briefly spin down; prepare priming buffer by adding **576 µl** of RBF to **624 µl** NFW (total: 1200); mix by vortexing, spin down.

86 Open the Minion lid and flow cell 'Priming Port' (turn clockwise)

87 To ensure liquid-liquid contact between priming buffer flow cell buffer, and a continuous buffer flow from the Priming Port port across the sensor array:

- Place pipette tip of a Gilson 1000 µl pipette at flow cell Priming Port; hold vertically and slowly wind pipette (anticlockwise) to draw back a small volume of the (yellow) QC buffer from the flow cell (a few µls)
- Visually check that there is continuous buffer from the Priming Port across the sensor array; continue to wind pipette if necessary to remove any small bubbles, **but do not draw bubbles from post-sensor channel into the array**
-

88 Avoiding the introduction of air bubbles, **load 800 µl of the priming mix** via the Priming Port: wind a vertical Gilson pipette (clockwise), use pipette plunger (with care) under 200-100 µl volume, and remove pipette with a few µls left in tip

89 Proceed with preparing library for loading while flow cells 'primes' for 5 min

Note

Secondary priming of flow cell with **200 µl priming buffer** (see below) should take place immediately before loading the library

K. Loading the library

90 To a fresh low bind tube, add the following (in order):

- 35 µl RBF;
- 2.5 µl NFW;
- 25.5 µl LLB;
- 12 µl of adapted and tethered DNA library (i.e.: 'RT eluate')

(Total library loading mix: 75 µl)

Mix by gentle flicking (gently spin down if needed)

Note

LLB will settle out very quickly; resupend by pipetting prior to transfter

91 Open the Spot-ON sample port cover with care

Very carefully and slowly, add 200 µl of **priming buffer** to the flow cell via the **Priming Port** (NOT the Spot-ON Sample Port), avoiding bubbles and maintaining liquid-liquid

contact as before (including removal of a few µls beforehand if needed); proceed immediately to next step

92 Resuspend beads in the library loading mix via pipette, and immediately **load into the Spot-ON Sample Port in a dropwise fashion**; ensure each drop flows into the port before adding the next

93 Gently replace the Spot-ON Sample Port cover ensuring that the bung enters the port; close the Priming Port cover and replace the MinION lid

94 **Wait for 1 hour** prior to starting sequencing run

L. Starting the sequencing run

95 Complete experiment parameters:
- Choose correct library prep. kit (SQK-LSK108)
- Select correct flow cell (FLO-MIN106; R9.4)
- Switch **base calling to 'Off'**

(select other parameters as required; 48 hour run recommended)

96 Click on 'Begin Experiment' and:

- Note new number of pores reported in pre-sequencing QC ('Mux')
- Check pore occupancy figures (i.e. high 'in-stand pore' to 'single pore' ratio)
- Monitor messages in the Message panel in the MinKNOW GUI
- Allow the protocol to proceed in MinKNOW until it reports that the run is complete, or use 'Stop' in MinKNOW control panel to finish protocol

Note

The read files are stored in :\data\reads (? 'C\MinION_data\reads)

Base call reads after sequencing has been completed.