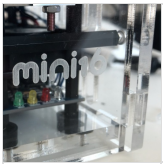

Version 1
Nov 17, 2020

Field-adapted PPRV whole genome MinION library build V.1

Frontiers in Veterinary Science

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

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ABSTRACT

Peste-des-petit-ruminants virus is currently the focus of a control and eradication program launched by the FAO and OIE. One of the important steps in the control strategy is strengthening the laboratory capacity and improve molecular methods to characterize field isolates. This protocol describes the preparation of samples for sequencing using the MinION sequencer, from cDNA synthesis, PCR amplification using multiplex primers to loading off the sample on the flow cell. The miniPCR is used as the thermo cycler, making this protocol fully field-adapted and ready to use in the field or in a less well-equipped lab.

EXTERNAL LINK

https://osf.io/9bfwe/

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Torsson E, Kgotlele T, Misinzo G, Wensman JJ, Berg M, Lindsjö OK, Field-Adapted Full Genome Sequencing of Peste-Des-Petits-Ruminants Virus Using Nanopore Sequencing. Frontiers in Veterinary Science doi: 10.3389/fvets.2020.542724

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PROTOCOL CITATION

Emeli Torsson, Emeli Torsson, Oskar Karlsson Lindsjö 2020. Field-adapted PPRV whole genome MinION library build. **protocols.io** https://dx.doi.org/10.17504/protocols.io.pnxdmfn

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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KEYWORDS

Peste des petits ruminants virus (PPRV), MinION, Whole Genome Sequencing, field-adapted

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OWNERSHIP HISTORY

Apr 23, 2018	Oskar Karlsson Lindsjö	Swedish University of Agricultural Sciences
Oct 25, 2019	Emeli Torsson	Swedish University of Agricultural Sciences

PROTOCOL INTEGER ID

11703

GUIDELINES

- For troubleshooting help, feel free leave your question in the comments section, or message us directly.
- The complete list of reagents with order numbers can be found in the 'Materials' section.
- Make sure to mix the magnetic beads thoroughly before use and not to disturb the pellet when removing liquid
- Hula mixer can be replaced by gently moving/shaking the sample by hand if needed

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MATERIALS TEXT

MATERIALS

Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0493L**

Blunt/TA Ligase Master Mix - 250 rxns **New England Biolabs Catalog #M0367L**

NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog #E7546L**

Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #Q32854**

Agencourt Ampure XP **Beckman Coulter Catalog #A63AA0**

Magnetic rack/stand **Contributed by users**

SuperScript First-Strand Synthesis System **Thermo Fisher Scientific Catalog #18091200**

Native Barcoding Expansion 1-12 (PCR-free) **Contributed by users Catalog #EXP-NBD104**

Ligation Sequencing Kit **Contributed by users Catalog #SQK-LSK109**

MinION Flow Cell R9.4.1 **Contributed by users Catalog #R9.4.1**

HighPrep™ PCR Clean-up System **MagBio Genomics Inc. Catalog #AC-60005**

ABSTRACT

Peste-des-petit-ruminants virus is currently the focus of a control and eradication program launched by the FAO and OIE. One of the important steps in the control strategy is strengthening the laboratory capacity and improve molecular methods to characterize field isolates. This protocol describes the preparation of samples for sequencing using the MinION sequencer, from cDNA synthesis, PCR amplification using multiplex primers to loading off the sample on the flow cell. The miniPCR is used as the thermo cycler, making this protocol fully field-adapted and ready to use in the field or in a less well-equipped lab.

RNA extraction

1 Extract RNA using prefered method, spin column or magnetic beads.

Amplicon preparation

2 Set up the following reaction

Random hexamers (50 ng/µl)	1 µl
dNTP mix (10 mM)	1 µl
RNA	1 - 11 µl
DEPC	to reach 13 µl

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Total	**13 µl**

Mix and centrifuge, then heat to anneal primers

65 °C

00:05:00

3 Put directly on ice for at least 1 minute

00:01:00

4 Prepare the following reaction

5x SuperScript IV buffer	4 µl
DTT (100 mM)	1 µl
Ribonuclease Inhibitor	1 µl
SuperScript IV Reverse Transcriptase	1 µl

Mix and centrifuge, then add to the annealed RNA (13 µl)

Set up the following thermocycling settings

23 °C	10 min
55 °C	15 min
80 °C	10 min

5 Add 1 µl RNase H to each tube to degarde left over RNA

37 °C

00:20:00

Primer design

6 Primers designed using the Zibra project: http://primal.zibraproject.org/

For PPRV we used an amplicon lenght of 800 bp and 100 bp overlap.

Prepare two primers pools (10µM), one with even numbered primer pairs and on with odd number primer pairs (https://www.nature.com/articles/nprot.2017.066)

Amplicon preparation

7 Prepare the following reaction

Q5 reaction buffer (5x)	5 µl
dNTP mix (10 mM)	0.5 µl
Q5 DNA polymerase	0.25 µl
Primer pool 1 or 2 (10 µM)	variable*
DEPC	Up to 22.5 µl
Total	**22.5 µl**

* final concentration of each primer: 0.015 µM per primer https://www.nature.com/articles/nprot.2017.066

Add 2.5 µl of cDNA from step 4

8 Set up the following thermocycling settings

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1	98 °C	30 sec
2-40	98 °C	15 sec
2-40	65 °C	5 min

9 Pool each sample, so both primer pools per sample are in the same tube

Optional: Gel eletrophoresis

10 (Optional step to check PCR reaction)

Run 3-5 µl of sample on a 1 % agarose gel, 6-7 V/cm, 50-60 min.

Expected outcome: A clear band at amplicon size (e.g. 800 bp) and possibly a second band at x3 amplicon size (e.g. ~2400 bp)

Amplicon purification

11 Add AMPure/HighPrep magnetic beads to PCR reaction tube according to the following equation

Volume of beads per reaction = 1.8 x Reaction Volume

For example:

PCR reaction volume 50 µl + 90 µl AMPure beads

12 Mix the beads and PCR reaction thoroghly by pipetting up and down 10 times.

Incubate in RT for 3-5 minutes

00:03:00 Incubate at RT

13 Place tube in magnetic rack for 5-10 minutes to seperate beads

00:10:00

Aspirate the now clear solution from the reaction and discard

14 Add 200 µl newly prepared 70% ethanol to reaction tube when still on magnetic rack. Incubate for 30 sec at room temperature (RT).

00:00:30 Incubate at RT

Aspirate the etahnol and discard. Repeat wash once more.

00:00:30 Incubate at RT

15 Allow reaction tubes to air-dry in RT until completly dry and total evaporation of residual ethanol. 10-20 min

00:20:00

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16 Add 40 µl of elution buffer (TE buffer, 10 mM Tris-Acetate pH 8.0, or reagent grade water) to each reaction tube. Mix thoroghly by vortex or pipetting up and down.

Replace tube back to magnetic rack. When solution is cleared, remove and save for downstream reactions.

Quantification

17 Quantify 1 µl purified DNA using Qubit flourometer.

End Repair / dA-tailing

18 Dilute 0.12 pmol PCR products in nuclease free water to 25 µl

For our amplicons of 800 bp this means 60 ng of PCR product in 25 µl water.

To convert mass/moles: https://nebiocalculator.neb.com/#!/dsdnaamt

19 For each sample, set up the following end-repair /dA-tailing reaction

Ultra II End Prep Reaction Buffer	3.5 µl
Ultra II End Prep Enzyme Mix	1.5 µl
DNA from previus step	25 µl
Total	**30 µl**

Gently mix reaction by pipetting up and down at least 10 times, then quickly centrifuge to collect all liquid.

20 In thermocycler (heated lid at >75 °C) sett up the following protocol

20 °C	5 minutes
65 °C (inactivate enzyme)	5 minutes

21 Put directly on ice for at least 30 seconds

00:00:30

Magnetic bead purification

22 Add 30 µl of AMPure/HighPrep beads to reaction and mix by flicking tube

23 Incubate on Hula mixer for 5 min at RT

00:05:00

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24 Centrifuge tube and place on magnetic rack to pellet the magnetic beads. When solution is clear, pipet of supernatant and discard

25 While on magnatic rack, add 200 µl of freshly prepared 70 % ethanol to the tube.
Remove ethanol without disturbing the bead pellet. Repeat wash one more time.

26 Spin down beads and then place again on magnetic rack. Pipette of any residual ethanol. Allow to air dry for 3-5 min at RT

00:05:00

27 Remove tube from magnetic rack and resuspend pellet in 15 µl nuclease-free water and mix by flicking the tube.

Incubate for 2 min at RT.

00:02:00

28 Place tube on magnatic rack and allow beads to pellet.

Quantification

29 Quantify 1 µl of end-prepped DNA using Qubit flourometer.

Recovery aim: 50-70 % of input

Barcoding

30 Thaw Native Barcodes at RT, individually mix by pipetting, and then place on ice.

31 Pepare the following barcoding reaction.

Native barcode	1 µl
Blunt/TA Ligase master mix	10 µl
End-prepped DNA*	9 µl
Total	20 µl

* at least 0.04 pmol, but as much as possible https://nebiocalculator.neb.com/#!/dsdnaamt

32 Mix gently by flicking the tube, spin down.

Incubate the reaction at RT for 10 min.

00:10:00

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Magnetic bead purification

33 Add 20 µl of AMPure/HighPrep beads to reaction and mix by flicking tube

34 Incubate on Hula mixer for 5 min at RT

00:05:00

35 Centrifuge tube and place on magnetic rack to pellet the magnetic beads. When solution is clear, pipet of supernatant and discard

36 While on magnatic rack, add 200 µl of freshly prepared 70 % ethanol to the tube.
Remove ethanol without disturbing the bead pellet. Repeat wash one more time.

37 Spin down beads and then place again on magnetic rack. Pipette of any residual ethanol. Allow to air dry for 3-5 min at RT

00:05:00

38 Remove tube from magnetic rack and resuspend pellet in 10 µl nuclease-free water and mix by flicking the tube.

Incubate for 2 min at RT.

00:02:00

39 Place tube on magnatic rack and allow beads to pellet.

Quantification

40 Quantify 1 µl of end-prepped DNA using Qubit flourometer.

Recovery aim: > 0.011 pmol /sample (equal to ~5.5 ng per sample for 800 bp amplicons).

Adaptor ligation

41 Pool samples in equimolar concentration in DNA LoBind 1,5 ml Eppendorf tubes to get a total of 0.15 pmol.

Dilute pooled sample to 20 µl (or as much as needed if volume needed exceeds 20 µl) in Nuclease-free water.

42

Barcoded and pooled DNA from previous step	20 µl

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Barcode Adapter Mix II (AMII)	5 µl
Blunt/TA Ligase master mix	25
Total	50 µl

If DNA excceds 20 µl, keep AMII at 5µl and increase Ligase master mix so that it is 50 % of total volume.

43 Incubate for 10 min at RT

00:10:00

Purification and wash

44 Add 20 µl of AMPure/HighPrep beads to reaction and mix by flicking tube.

If you needed to increase smaple volume in prevoius step, calculate 0.4X beads per reaction volume.

45 Incubate on Hula mixer for 5 min at RT

00:05:00

46 Centrifuge tube and place on magnetic rack to pellet the magnetic beads. When solution is clear, pipet of supernatant and discard

47 Add 250 µl of S Fragment Buffer (SFB). Flick the tube to resuspend beads, then return tube to magnetic rack to pellet the beads. Pipet of supernatant. Repeat this step once more. Make sure to get all of the SFB out. Spin down and pipet out again if needed.

48 Remove tube from magnetic rack and resuspend pellet in 15 µl nuclease-free water and mix by flicking the tube.

Incubate for 10 min at RT.

00:10:00

49 Place tube on magnatic rack and allow beads to pellet. Remove the now clear solution and add to new 1.5 ml DNA LoBind eppendorf tube. Store on ice.

Loading samples to flow cell

50 Thaw Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT), and one tube of Flush Buffer (FLB) at RT, then place on ice as soon as thawed.

51 Take out flow cell. Open lid and slide the priming port cover clockwise to make the priming port visiable.

52 Check for small bubbles under the cover. Draw back a small volume of buffer to remove possible bubbles (Set P1000 pipette to 200 µl, insert tip in port, turn until pipette set to 220-230 µl)

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53 Prepare the flow cell priming mix.

Add 46 µl of thawed FLT directly to tube of thawed and mixed FLB. Mix by pipetting up and down.

54 Load 800 µl of priming mix into flow cell via priming port, avoid introducing air. Wait for 5 minutes.

00:05:00

55 Prepare the following reaction

Sequencing Buffer (SQB)	37.5 µl	
Loading Beads (LB)	25.5 µl	
DNA Library	12 µl	
Total	75 µl	

56 Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

Load 200 µl of priming mix into the flow cell via the **priming port (**not SpotOn port), do not introduce air.

57 Mix the prepared library gently by pipetting up and down just before loading.

Add 75 µl of sample to the flow cell via the SpotON sample port dropwise, making sure that each drop flows into the port before adding the next.

Gently replace the SpotON sample port cover, close priming port and replace the MinION lid.

58 Start sequence run

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