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PCR-NGS for RNA virus - PMCV

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We use this protocol and it's working

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Abstract

This protocol describes a **high-throughput method for sequencing RNA viral genomes using the Oxford Nanopore MinION platform and multiplex PCR amplicon enrichment**. In this approach, overlapping primer pairs are used to generate amplicons that collectively tile the entire viral genome. The amplicons are then barcoded, pooled, and prepared for sequencing, allowing for cost-effective, flexible, and scalable genome sequencing from multiple samples in a single sequencing run.

Protocol materials

- SPRIselect reagent kit **Beckman Coulter Catalog #**B23317
- Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #**M0493L
- LunaScript RT SuperMix Kit **New England Biolabs Catalog #** E3010S
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S
- Native Barcoding Kit 24 V14 (SQK-NBD114.24) **Oxford Nanopore Technologies Catalog #**SQK-NBD114.24
- Blunt/TA Ligase Master Mix - 250 rxns **New England Biolabs Catalog #**M0367L
- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #**E6056S
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- SFB expansion **Oxford Nanopore Technologies Catalog #**EXP-SFB001
- UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #** AM2616

cDNA synthesis

1 In a PCR tube, thoroughly mix the following components:

LunaScript RT SuperMix Kit **New England Biolabs Catalog #** E3010S

	A	B
	Components	**Volume** (uL)
	LunaScript RT SuperMix	2
	Viral RNA	8
	Total	10

Incubate on a thermocycler as follows with heated lid at 105C:

	A	B
	Temperature (℃)	**Time**
	25	2 minutes
	55	20 minutes
	95	1 minute
	4	Hold

PCR amplification

2 Prepare PCRs with viral cDNA for pool A and pool B, with

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

	A	B	C
	Component	**Pool A (odd primers (uL)**	**Pool B (even primers) (uL)**
	Nuclease-free water	16.15	16.225
	5x Q5 reaction buffer	5	5
	10 mM dNTPs	0.5	0.5
	Q5 high fidelity hot start polymerase	0.25	0.25
	Primers	0.6	0.525
	cDNA	2.5	2.5
	Total	25	25

Note: Primer Required end concentration in PCR = 0.015uM per primer.

2.1 Run on a thermocycler with heated lid at 105°C:

	A	B	C	D
	Cycle numbers	**Step**	**Temperature (°C)**	**Time**
	1	Initial denature	98	1 minute
	37	Denature	98	30 seconds
	1	Anneal and extend	65	5 minutes
	1	Hold	4	Infinity

1XClean up and normalization

10m

3 WIth SPRIselect reagent kit **Beckman Coulter Catalog #**B23317

3.1 1) Prepare fresh 75% ethanol.

3.2 2) Transfer 21 µL contents of PCR tube to a 1.5 mL LoBind Eppendorf tube.

3.3 3) Vortex an aliquot of SPRI beads at room temperature to resuspend the beads.

3.4 4) Add 21ul of SPRI beads to the 1.5 mL Eppendorf tube.

3.5 5) Incubate on a rotator for 00:05:00 (or occasionally invert tubes to prevent beads settling). 5m

3.6 6) Spin down gently without pelleting the beads to the base, and then pellet on a magnet until clear.

3.7 7) Discard the supernatant (with pipette carefully).

3.8 8) Keep on magnet, wash twice with 200ul fresh 75% EtOH, discarding EtOH after each wash. Do not disturb pellet.

3.9 9) Briefly spin down, replace on magnet until clear.

3.10 10) Pipette off remaining wash. Briefly allow the pellet to dry.

3.11 11) Remove from magnet and resuspend the pellet in 21 µL of nuclease free water, incubate at Room temperature for 00:05:00 . 5m

3.12 12) Gently spin down without pelleting the beads to the bottom.

3.13 13) Pellet the beads on a magnet until clear.

3.14 14) Transfer the eluate to a fresh labelled LoBind tube without disturbing the beads.

3.15 15) Quantify DNA concentrations with a Qubit 3.0 fluorometer.

3.16 16) Normalise the cleaned PCR products from pool A and pool B with nuclease free water to a final concentration of 5 ng/µl.

End repair

31m

4 In a new PCR strip tube, set up the following reaction components, with

NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S

	A	B
	Component	**Amount (µL)**
	Normalized amplicons	8.3
	Ultra II End Prep Reaction Buffer	1.2
	Ultra II End Prep Enzyme Mix	0.5
	Total	10

4.1 Incubate on thermocycler with heated lead >= 75 °C , at following temperatures: 31m

20 °C for 00:15:00

65 °C for 00:15:00

4 °C for 00:01:00

Native barcode ligation

31m

5 In a new PCR strip tube, set up the following with

Native Barcoding Kit 24 V14 (SQK-NBD114.24) **Oxford Nanopore Technologies Catalog #**SQK-NBD114.24

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

	A	B
	Component	**Amount (µL)**
	dA-tailed amplicons	0.75
	Native barcode (NB01-NB24)	1.25
	Blunt/TA Ligase master mix	5
	Nuclease free water	3
	Total	10

5.1 Incubate on thermocycler at following temperatures:

31m

25 °C for 00:20:00

65 °C for 00:10:00

4 °C for 00:01:00

0.4X Cleanup

11m

6 Cleanup is performed with

SPRIselect reagent kit **Beckman Coulter Catalog #**B23317

SFB expansion **Oxford Nanopore Technologies Catalog #**EXP-SFB001

6.1 1) Transfer all required volume to a 1.5 mL LoBind Eppendorf tube to pool samples.

6.2 2) Vortex an aliquot of SPRI beads at Room temperature to resuspend the beads.

6.3 3) Add correct volume of SPRI beads to the 1.5 mL Eppendorf tube (0.4x).

6.4 4) Incubate on rotator for 00:05:00 (or occasionally invert tubes to prevent beads settling). 5m

6.5 5) Spin down gently without pelleting the beads to the base, and then pellet on a magnet until clear.

6.6 6) Discard the supernatant.

6.7 7) Remove from magnet, and add 250 µL of SFB. Pipette to mix.

6.8 8) Briefly spin down, replace on magnet until clear.

6.9 9) Pipette off remaining wash. No need to allow to dry.

6.10 10) Remove from magnet, and add 250 µL of SFB. Pipette to mix.

6.11 11) Briefly spin down, replace on magnet until clear.

6.12 12) Pipette off remaining wash. No need to allow to dry.

6.13 13) Perform a single ethanol wash: Keep on magnet, wash with 200 µL fresh 75% EtOH discarding EtOH after wash. Do not disturb pellet.

6.14 14) Briefly spin down, replace on magnet until clear.

6.15 15) Pipette off remaining wash. Briefly allow to dry for about 00:01:00 until loses shine. 1m

6.16 16) Remove from magnet and resuspend the pellet in 31 µL of Qiagen EB ([M] 10 millimolar (mM) Tris-Cl pH 8.5), incubate at Room temperature for 00:05:00 . 5m

6.17 17) Gently spin down without pelleting the beads to the bottom.

6.18 18) Pellet the beads on a magnet until clear. Transfer the eluate to a fresh labeled LoBind tube without disturbing the beads.

Native adapter ligation 20m

7 In a 1.5mL Eppendorf tube, set up the following reaction; Mix gently by flicking. This step is performed with

NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #**E6056S

Native Barcoding Kit 24 V14 (SQK-NBD114.24) **Oxford Nanopore Technologies Catalog #**SQK-NBD114.24

	A	B
	Component	**Amount (µL)**
	Pooled barcoded sample	30
	NEBNext Quick Ligation Reaction Buffer (5x)	10
	Native adapter	5
	Quick T4 DNA Ligase	5

	A	B
	Total volume	50

Incubate on a thermocycler for 20 minutes at 20°C with heated lid set to 30°C (or off)).

7.1 Incubate on a thermocycler for 00:20:00 at 20 °C with heated lid set to 30 °C (or off)). 20m

1X Cleanup 10m

8 Cleanup is performed with
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
SFB expansion **Oxford Nanopore Technologies Catalog #**EXP-SFB001

8.1 1) Add 50 µL of AMPure beads previously homogenized.

8.2 2) Mix by inversion for 00:05:00 to promote the binding of the library to the beads. 5m

8.3 3) Spin down and place on the magnetic rack.

8.4 4) Once the beads have pelleted and the liquid is completely clear, remove the supernatant.

8.5 5) With the tube still on the magnetic rack, wash the pellet with 250 µL of SFB. Close tube lid, resuspend beads by flicking.

8.6 6) Spin down, beads on magnet and remove supernatant- discard the supernatant and wash again with 250 µL of SFB. After discarding the second time, close the tubes, spin down and place again on magnetic rack.

8.7 7) Remove the residual SFB then elute the pellet by adding 15 µL of EB (Elution Buffer) and resuspend the beads by flicking or pipetting, make sure all beads have been eluted from the wall tube.

8.8 8) Incubate for 00:05:00 at room temperature. 5m

8.9 9) Spin down, place on the magnetic rack, let the pellet beads settle on magnet, transfer the eluted solution to a new tube.

Priming and loading the SpotON flow cell 5m

9 This step is performed with

Native Barcoding Kit 24 V14 (SQK-NBD114.24) **Oxford Nanopore Technologies Catalog #**SQK-NBD114.24

UltraPure™ BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #** AM2616

9.1 1) Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flush Buffer (FCF) at room temperature before placing the tubes on ice as soon as thawing is completeMix the Sequencing Buffer (SB) and Flow Cell Flush Buffer (FCF) tubes by vortexing, spin down and return to ice.

9.2 2) Spin down the Flow Cell Tether (FCT) tube, mix by pipetting, and return to ice.

9.3 3) Priming mix: add 30 µL of thawed and mixed Flow Cell Tether (FCT) directly to the tube of thawed and mixed 1170 µL Flow Cell Flush Buffer (FCF), and mix by vortexing. Then add 5 µL Bovine Serum Albumin (BSA) at [M] 50 mg/mL .

9.4 4) Take out a flow cell from the fridge, perform quality check of the flow cell.

9.5 5) Open the priming port. Draw back with the P1000 draw back 20-30 ul of buffer to make sure there is continuous buffer flow from the priming port across the sensor array and that there are no bubbles in the flow cell.

9.6 6) Load 800 µL of the FCF+FCT priming mix slowly through the priming loading port using a P1000 pipette. It is extremely important not to introduce or push any air 5m

bubbles into the flow cell. Wait 00:05:00

9.7 7) Gently lift the SpotON port cover to make the sample port accessible.

9.8 8) Load 200 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles.

9.9 9) Prepare the library for loading sample:

	A	B
	Component	**Amount (µL)**
	Sequencing Buffer (SB)	37.5
	Library Beads (LIB), mixed immediately before use	25.5
	DNA library (20 ng – can dilute if needed with EB)	12
	Total	75

9.10 10) Mix gently by pipetting and briefly spin down. Using a P200 pipette, carefully add 75 µL of the diluted library to the flow cell's sample port by allowing droplets to fall onto the SpotON port, ensuring the pipette tip does not touch the port.

9.11 11) Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.

9.12 12) Start the sequencing.