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Targeted sequencing of Rift Valley fever virus (RVFV) on Oxford Nanopore MinION platform

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We use this protocol and it's working

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Abstract

This protocol is adapted for
Rift Valley fever (RVF) samples and is based on the methods for the NEBNext ® both for Illumina and Oxford Nanopore Technologies (ONT). The protocol utilizes amplicon sequences to generate consensus genomes of the virus. The targeted sequencing approach offers a viable alternative to generating RVF virus (RVFV) genomes without the need to culture, thereby reducing turnaround time for generating genomes. The protocol utilizes primer sets of overlapping sequences for Illumina (n = 78) and Oxford Nanopore Technologies (n = 258) sequencing.

1. RNA extraction

1 Extract viral RNA from serum or cell-culture supernatants using QIAamp Viral RNA kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Begin with a volume of 140 µL .

2. RT-qPCR

25m 6s

2 Determine cycle threshold (Ct) values on RNA samples using probe-based reverse transcription quantitative real-time PCR against the highly conserved domain on the L-segment of the virus (using 5' Fam reporter dye and 3' BHQ1 quencher dye). 12m 33s

2.1

	A	B	C
	RVFV segment	Primer name	Sequence 5′-3′
	L	RVFL-2912fwdGG	TGAAAATTCCTGAGACACATGG
	L	RVFL-2981revAC	ACTTCCTTGCATCATCTGATG
	L	RVFL-probe 2950	CAATGTAAGGGGCCTGTGTGG ACTTGTG

Table 1. Primers and probe set used for RT-qPCR assay **(Bird et al., 2007)**.

2.2 Mix the following components in PCR strip-tubes/plate

2.3

	A	B
	Component	Volume (µL)
	KiCqStart One-Step Probe RT qPCR ReadyMix	7.5

	A	B
	Nuclease-free water	4.75
	RVFV Oligos (2912fwdGG, 2981revAC, probe-2950)	0.75
	RNA	2.0
	Total	15

Table 2: Reaction components for RT-qPCR

Note

Set up the reaction on ice.

2.4 Incubate the reaction on an Applied Biosystems machine as follows: 12m 33s

50 °C for 00:10:00
95 °C for 00:02:00
95 °C for 00:00:03 for 40 cycles
61 °C for 00:00:30

3. cDNA synthesis 13m

3 Prepare RNA samples and include a negative control (nuclease-free water) per library. If previously frozen, mix by vortexing briefly and quick spin to collect the liquid. At all times, keep the samples on ice.

3.1 Mix the following components in PCR strip-tubes/plate. Gently mix by pipetting and performing a quick spin to collect the liquid.

3.2 13m

	A	B
	Component	Volume

	A	B
	LunaScript RT Supermix (5X)	2 µL
	Template RNA 8 µL	
	Total	10 µL

Table 3: cDNA synthesis reaction components

Note

To prevent pre-PCR contamination the mastermix should be added to the PCR strip tubes/plate in the mastermix cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use. RNA samples should be added in the extraction/sample addition cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

25 °C for 00:02:00
55 °C for 00:10:00
95 °C for 00:01:00
Hold at 4 °C

4. Primer pool preparation

4 If making up primer pools from individual oligos fully resuspend lyophilized oligos in 1xTE to a concentration of [M] 100 micromolar (µM) , vortex thoroughly and spin down.

4.1 Sort all odd regions primers into one or more tube racks. Add 5 µL of each odd region primer to a 1.5 mL Eppendorf tube labelled “Pool 1 ([M] 100 micromolar (µM)) ”. Repeat the process for all even region primers for Pool 2. These are your [M] 100 micromolar (µM) stocks of each primer pool.

4.2 Dilute [M] 100 micromolar (µM) pools 1:10 in molecular grade water, to generate [M] 10 micromolar (µM) primer stocks.

Note

Primers are used at a final concentration of [M] 15 nanomolar (nM) per primer. In this case, V1 pools have 130 primers in pool 1 and 128 primers in pool 2, so the requirement is approx. 4.8 µL primer pool ([M] 100 micromolar (µM)) per 25 µL reaction.

Note

Make up several 100 µL aliquots of [M] 10 micromolar (µM) primer dilutions and freeze them in case of degradation and/or contamination.

5. Multiplex PCR

5

5.1 Set up the two PCR reactions per sample as follows in strip-tubes or plates. Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

	A	B	C
	Component	Reaction volume (Pool 1)	Reaction volume (Pool 2)
	Q5 Hotstart Mastermix Buffer (5X)	12.5 µL	12.5 µL

	A	B	C
	V1 Primer Pool 1	4.8 µL	-
	V2 Primer Pool 2	-	4.8 µL
	Nuclease-free water	3.2 µL	3.2 µL
	(cDNA)	4.5 µL	4.5 µL
	Total reaction Volume	25 µL	25 µL

Table 4. Reaction components for the multiplex tiling PCR using primer pools 1 and 2

Note

To prevent pre-PCR contamination the mastermix for each pool should be made up in the mastermix cabinet which should should be cleaned with decontamination wipes and UV sterilised before and after use and aliquoted into PCR strip-tubes/plate.

5.2 Add 4.5 µL cDNA to each of the PCR reactions, gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

Note

cDNA should be added in the extraction and sample addition cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

5.3 Set-up the following program on the thermal cycler:

	A	B	C	D
	Step	Temperature	Time	Cycles
	Heat activation	98°C	30 seconds	1
	Denaturation	95°C	15 seconds	35
	Annealing	63°C	5 minutes	35
	Hold	4°C	Indefinite	1

Table 5. Reaction conditions for the multiplex tiling PCR assay

6. Amplicon clean-up

22m 30s

6 Combine the two pools of amplicons:

6.1 Add 12.5 µL of each primer pool (Pool 1 and Pool 2, total of 25 µL) in new PCR strip-tubes/plate.

6.2 Perform NEBNext Sample Purification Beads/AMPure XP bead cleanup as follows: 5m

Add 20 µL (0.8X) of AMPure XP beads (thoroughly vortexed and at Room temperature)
to the combined amplicons plate.

Cover the plate with seal, gently mix on a plate mixer and pulse spin to bring down the components at the bottom of the tube.

Incubate at Room temperature for 00:05:00 (5 minutes).

6.3 Place the tube/plate on a magnetic stand for 00:05:00 or until the beads have pelleted and the supernatant is completely clear. 5m

6.4 Remove and discard the liquid from each well with a multichannel pippette, being careful not to touch
the bead pellet.

Note

Caution: do not discard the beads

6.5 Add 200 µL of freshly prepared, Room temperature 80% ethanol to each well/tube, incubate for 00:00:30 at Room temperature and then carefully remove and discard the supernatant. 30s

Note

Be careful not to disturb the beads that contain DNA targets.

6.6 Repeat ethanol wash (step 6.4 and 6.5)

6.7 Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

6.8 Air dry the beads for up to (00:05:00 5 minutes) while the tube/plate is on the magnetic stand with the lid open. 5m

Note

**Caution: Do not over-dry the beads. This may result in lower recovery of DNA. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has
evaporated. When the beads turn lighter brown and start to crack, they are too dry.**

6.9 Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 28 µL 0.1X TE or Elution Buffer (EB).

6.10 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:00 (2 minutes) at Room temperature . If necessary, quickly spin the sample to collect the liquid from
the sides of the tube or plate wells before placing back on the magnetic stand. 2m

6.11 Place the tube/plate on the magnetic stand. After 00:05:00 (5 minutes) (or when the solution is clear). 5m

6.12 Transfer 25 µL to a new PCR tube, ensuring no beads are transferred.

7. Gel electrophoresis or Tapestation 2m

7

7.1 Use remaining volumes from Pool 1 and Pool 2 to confirm amplification (step 5.3)

7.2 Make 1% agarose gels with enough wells for all samples.

7.3 Load 2 µL of the 100 bp ladder into gel on either side of each row of wells.

7.4 Dispense 2 µL of 6X loading dye into each sample with a multichannel pipette, mix and load 2 µL of this mix into the gel.

7.5 Run at 240V for 00:02:00 (2 minutes) . Visualize PCR products, confirm bands of approximately 400bp size. 2m

7.6 Run pooled cDNA amplicons on a TapeStation® without cleanup.

To run on a TapeStation, dilute an aliquot of the pooled amplicons 10-fold with 0.1X TE Buffer and run 2 µL on a DNA High Sensitivity ScreenTape.

8. Amplicon quantification

8

8.1 Quantify amplicons using Qubit dsDNA High Sensitivity kit and plate reader according to directions.

9. Library preparation

1h 9m

9 Prepare sequencing libraries with NEBNext Ultra II RNA Library Prep kit at half volume, as follows:

9.1 End-Prep

Add the following components to a sterile nuclease-free tube:

	A	B
	Component	Volume
	NEBNext Ultra II End Prep Enzyme Mix	1.5 µL
	NEBNext Ultra II Reaction Buffer	3.5 µL
	Target cDNA amplicon	25 µL
	Total volume	30 µL

Table 6. Library end-prep reaction components

9.2 Set a 100 µL or 200 µL pipette to 25 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. 20m

Perform a quick spin to collect all liquid from the sides of the tube.

In a thermal cycler with lid heated to 75 °C and run the following program:

20 °C for 00:10:00
65 °C for 00:10:00
4 °C Indefinite

9.3 Barcode-ligation using SQK-NBD112-96 31m

Add the following components directly to the End Prep Reaction Mixture and incubate

	A	B
	Component	Volume
	End Prep Reaction Mixture (step 9.1)	30 µL
	Oxford Nanopore native barcodes (SQK-NBD112-96)	5 µL
	Blunt/TA ligase Master Mix	15 µL
	Total volume	50

Table 7. Barcode-ligation reaction components

In a thermal cycler with lid heated to 75°C , run the following program:

25 °C for 00:20:00

65 °C for 00:10:00
On Ice for 00:01:00

9.4 Pool the barcoded-adaptor ligated samples into one 1.5 mL DNA LoBind tube.

9.5 Clean up the pooled sample using 0.4X Agencourt AMPure XP beads and mix gently by pipetting.

9.6 Incubate for 00:10:00 (10 minutes) at Room temperature. 10m

9.7 Place on a magnetic rack and incubate for or until the beads have pelleted and the supernatant is
completely clear.

9.8 Carefully remove and discard the supernatant without touching the bead pellet.

9.9 Wash the beads using 250 µL of ONT's Short Fragment Buffer (SFB). Flick the tube or pipet up and down 10 times to mix to resuspend pellet.

9.10 Place tube on a magnetic stand for 00:03:00 (3 minutes) or until the solution is clear to separate the beads from the supernatant. Remove the supernatant. 3m

9.11 Repeat the steps 9.8 and 9.9.

9.12 Perform a final wash with 500 µL of 80% freshly prepared ethanol and incubate at Room temperature for 00:03:00 (3 minutes). Carefully remove and discard the supernatant without disturbing the beads that contain DNA targets. 3m

9.13 Repeat the step above (9.12)

9.14 Briefly centrifuge the tube in pulses to ensure that all residual ethanol flows to the bottom. Use a P10 pipette to carefully extract the residual ethanol without disturbing the pellet.

9.15 Allow the tube to dry while the lid is open.

> Note
>
> **Do not let the pellet to completely dry. Over-drying may render resuspension difficult.**

9.16 Remove the tube from the magnetic stand and elute the pellet in 33 µL of Nuclease free water (NFW) and gently mix by pipetting followed by incubation at room temperature for 00:02:00 (2 minutes). 2m

9.17 Place on a magnetic stand and transfer the sample to a clean 1.5 mL Eppendorf DNA LoBind tube without transferring any beads.

9.18 Measure the concentration of the samples using Qubit.

10. Adaptor ligation using ONT adapter mix II 50m 3s

10 Use the Qubit measurements from the previous step to dilute purified Native barcoded DNA pool
in nuclease-free water. Add the following reaction components in a 1.5 mL Eppendorf DNA LoBind tube.

	A	B
	Component	Volume
	Native barcoded and purified DNA (step 9.17)	30 µL
	NEBNext Quick Ligation Reaction Buffer	10 µL
	Adapter Mix II (AMII)	5 µL

	A	B
	NEBNext Quick T4 Ligase	5 µL
	Total Volume	50 µL

Table 8. Reaction components for adaptor ligation

Note

Mix the NEBNext Quick Ligation Reaction Buffer by pipetting prior to use in the reaction

10.1 Flick the reaction tube to mix thoroughly. Perform a quick spin for 00:01:00 (1 minute) to collect all the liquid from the sides. 1m

Note

NEBNext Quick Ligation Reaction Buffer is viscous. Ensure adequate mixing of the ligation reaction, since incomplete mixing will lead to reduced ligation efficiency.

10.2 Incubate at 25 °C for 00:20:00 (20 minutes). 20m

10.3 Vortex NEBNext Sample Purification Beads to resuspend.

10.4 Add 50 µL (1X) resuspended beads to the ligation reaction mix. Mix thoroughly by flicking the tube followed by a quick spin for 00:00:03 (3 seconds) 3s

10.5 Incubate the samples for 00:10:00 (10 minutes) at Room temperature 10m

10.6 Place the tube on a magnetic stand to separate the beads from the supernatant.

10.7 After 00:03:00 (3 minutes) or when solution is clear, carefully remove and discard the supernatant without disturbing the beads. 3m

> Note
>
> **Caution: Do not discard the beads.**

10.8 Wash the beads with 250 µL Short Fragment Buffer (SFB). Flick the tube to resuspend the pellet.

10.9 Wait for 00:03:00 (3 minutes) or until the solution is clear to separate the beads from the supernatant. 3m
Carefully remove the supernatant.

10.10 Repeat step 10.8 and 10.9.

10.11 Remove the tube from the magnetic stand and elute the DNA with 15 µL Elution Buffer (EB) provided in the SQK-NBD112-96 kit.

10.12 Resuspend the pellet in EB by flicking.

10.13 Incubate for 00:10:00 (10 minutes) at Room temperature 10m

10.14 Place the tube on a magnetic stand for 00:03:00 (3 minutes) or until the solution is clear, then transfer 15 µL to a new Eppendorf LoBind DNA tube. 3m

10.15 Determine the concentration of the DNA by Qubit.

11. Sequencing

11

11.1 Follow Oxford Nanopore Protocol SQK-LSK109 to prepare the MinION flowcell and DNA library sequencing
mix using up to 20 ng adapter-ligated DNA sample (previous step).

11.2 Mix thoroughly 117 µL of Flush Buffer and 3 µL Flush Tether in a LoBind tube.

11.3 Prime the flowcell by slowly pipetting much of the previous mix, while avoiding pushing too fast or
bubble formation.

11.4 In a separate LoBind tube, add 15 µL of the Sequencing Buffer II, 10 µL of freshly vortexed
Loading Beads II and 5 µL (approximately 20fmol) DNA library.

11.5 Load 30 mL on the flow cell to initiate the sequencing.