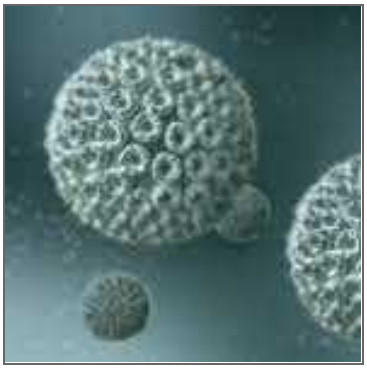

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Amplicon multiplex PCR sequencing of Rift Valley fever virus (RVFV) on Illumina MiSeq

John Juma[1,2], Samuel O. Oyola[1], Samson Konongoi[3], Isidore Nsengimana [Rwanda Inspectorate, Competition and Consumer Protection Authority][4], r.k.mwangi[1], James Akoko[1], Richard Nyamota[1], c.muli[1], e.kiritu[1], p.dobi[1], s.osiany[1], Amos Onwong'a[1], rwanja8[5], Rosemary Sang[3], Alan Christoffels[6], Kristina Roesel[6], Bernard Bett[6], Samuel O. Oyola[6]

[1]International Livestock Research Institute (ILRI);
[2]South Africa National Bioinformatics Institute (SANBI);
[3]Kenya Medical Research Institute (KEMRI);
[4]Rwanda Inspectorate, Competition and Consumer Protection Authority;
[5]Division of Immunology, Department of Human Pathology, University of Cape Town;
[6]South African National Bioinformatics Institute (SANBI), University of the Western Cape

RVFV

John Juma

ABSTRACT

Amplicon sequencing protocol for Rift Valley fever virus (RVFV)

Protocol status: Working
We use this protocol and it's working

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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

RT-qPCR

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

RVFV segment	*Primer name*	*Sequence 5'-3'*
L	RVFL-2912fwdGG	TGAAAATTCCTGAGACACATGG
L	RVFL-2981revAC	ACTTCCTTGCATCATCTGATG
L	RVFL-probe-2950	CAATGTAAGGGGCCTGTGTGGACTTGTG

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

Mix the following components in PCR strip-tubes/plate

	A	B
	Component	Volume (uL)
	KiCqStart™ One-Step Probe RT-qPCR ReadyMix™	7.5
	Nuclease-free water	4.75
	RVFV Oligos (2912fwdGG, 2981revAC, probe-2950)	0.75
	RNA	2.0
	Total	15

Note

Set up the reaction on ice.

Incubate the reaction on a Applied Biosystems machine as follows:

50 °C for 00:10:00
95 °C for 00:02:00
95 °C for 00:00:03 for 40 cycles
60 °C for 00:00:30

cDNA synthesis

30m

3

1. 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. 13m
2. Mix the following components in PCR strip-tubes/plate. Gently mix by pipetting and performing quick spin to collect the liquid.

	A	B
	Component	Volume
	LunaScript RT Supermix (5X)	2 uL
	Template RNA	8 uL
	Total	10 uL

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 should be cleaned with decontamination wipes and UV sterilised before and after use.

3. Incubate the reaction as follows:

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

Primer pool preparation

2h

4 If making up primer pools from individual oligos fully resuspend lyophilised 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.

Note

Primers should be diluted and pooled in the mastermix cabinet which should be cleaned with decontamination wipes and UV sterilised before and after use.

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 38 primers in pool 1 and 36 primers in pool 2, so the requirements is approx. 1.4 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

Multiplex PCR

4h

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 1	Reaction 2
	Q5 Hotstart Mastermix Buffer (5X)	12.5 uL	12.5 uL
	V1 Primer Pool 1	1.425 uL	0 uL
	V1 Primer Pool 2	0 uL	1.35 uL
	Nuclease-free water	6.575 uL	6.65 uL
	Mastermix Volume	20.5 uL	20.5 uL
	(cDNA)	4.5 uL	4.5 uL
	Total reaction Volume	25 uL	25 uL

> **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 should be cleaned with decontamination wipes and UV sterilised before and after use.

5.3 Set-up the following program on the thermal cycler: 5m 45s

Step	Temperature	Time	Cycles
Heat activation	98 °C	00:00:30	1
Denaturation	95 °C	00:00:15	35
Annealing	63 °C	00:05:00	35
Hold	4 °C	Indefinite	1

Amplicon clean-up

1h

6 Combine the two pools of amplicons:
Add 12.5 µL of each primer pool (Pool 1 and Pool 2, total of 25 µL) in new PCR strip-tubes/plate.

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

6.1 Add 20 µL (0.8X) of AMPure XP beads (thoroughly vortexed and at Room temperature) 5m 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).

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

6.3 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**

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

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

6.5 Repeat ethanol wash (step 6.3 and 6.4).
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.

6.6 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

> 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.**

6.7 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).

6.8 Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 00:02:0 2m (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.

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

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

Gel electrophoresis or Tapestation 20m

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

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

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

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

7.4 Run at 240V for 00:20:00. Visualize PCR products, confirm bands of approximately 400bp s 20m

Run pooled cDNA amplicons on a TapeStation® without cleanup. To run on a TapeStation, dilute an

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

Amplicon quantification

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

Library preparation

1h 30m

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

9.1 End-Prep 1h

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

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

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

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

Temperature	Time
20 °C	00:30:00

65 °C
4 °C

00:30:00
Indefinite

9.2 Adaptor-ligation

Add the following components directly to the End Prep Reaction Mixture

	A	B
	Component	Volume
	End Prep Reaction Mixture (step 9.1)	30 uL
	NEBNext Adaptor for Illumina	1.25 uL
	NEBNext Ultra II Ligation Master Mix	15 uL
	Total volume	46.25

Note

1. Mix the NEBNext Ultra II Ligation Master Mix by pipetting up and down several times prior to adding to the reaction
2. The NEBNext adaptor is provided in NEBNext Oligo kits. NEB has several oligo options which are supplied separately from the library prep kit. Please see www.neb.com/oligos for additional information

Do not premix adaptor with the Ligation Master Mix.

9.3 Set a 100 µL or 200 µL 2pipette to 40 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

Note

Caution: The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance)

9.4 Incubate at 20 °C for 00:15:00 (15 minutes) in a thermal cycler with the **heated lid off**. 15m

9.5 Add 1.5 µL of USER® Enzyme to the ligation mixture from Step 9.4.

Note

Steps 9.5. and 9.6. are only required for use with NEBNext Adaptors. USER enzyme can be found in the NEBNext Multiplex Oligos (www.neb.com/oligos).

9.6 Mix well and incubate at 37 °C for 00:15:00 (15 minutes) with the heated lid set to ≥ 47 °C. 15m

Note

Samples can be stored overnight at −20°C.
Note: Only a portion of the ligation reaction (7.5 µl) will move forward to PCR enrichment.

PCR Enrichment of Adaptor-ligated DNA

6m 55s

10 Follow Section 10.1. if you are using the following oligos: Use option A for any NEBNext Oligo kit where index primers are supplied in tubes. These kits have the forward and reverse primers supplied in separate tubes. Primers are supplied at [M] 10 micromolar (µM).

Follow Section 10.2. if you are using the following oligos: Use Option B for any NEBNext Oligo kit where index primers are supplied in a 96-well plate format. These kits have the forward and reverse (i7 and i5) primers combined. Primers are supplied at [M] 10 micromolar (µM).

10.1 Add the following components to a sterile strip tube:

Separate Forward and Reverse Primers

	A	B
	Component	Volume
	Adaptor Ligated DNA Fragments (step 9.4 or 9.6)	7.5 uL
	NEBNext Library PCR Master Mix	12.5 uL
	Universal PCR Primer/i5 Primer	2.5 uL
	Index (X) /i7 Primer	2.5 uL
	Total volume	25 uL

10.2 Add the following components to a sterile strip tube:

Premixed Forward and Reverse Primers

	A	B
	Component	Volume
	Adaptor Ligated DNA Fragments (step 9.4 or 9.6)	7.5 uL
	Adaptor Ligated DNA Fragments (step 9.4 or 9.6)	12.5 uL
	Index Primer Mix	5 uL
	Total volume	25 uL

10.3 Set a 100 µL pipette to 20 µL and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect all liquid from the sides of the tube.

10.4 Run the PCR program to amplify the libraries: 6m 55s

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:10	7
Annealing	65 °C	00:01:15	7
Extension	65 °C	00:05:00	1
Hold	4 °C	Indefinite	

Library Clean-up

11 Clean Up Libraries
Repeat the same clean up process as step 6 using 20 µL of AMPure beads or NEBNext Sample Purification Beads and 28 µL of Elution Buffer (EB)/ 0.1X TE.

Library quantification and normalization

12

12.1 Analyze 2 µL library using a Qubit dsDNA HS Assay kit

12.2 Calculate the molarity value using the following formula. Use the band size from gel electrophoresis or Tapestation readings (step 7).

Library concentration ([M] 0 µg/µL) / (660 g/mol * average library size (bp)) * 10^6

12.3 Normalize each library by dilution with nuclease free water.

12.4 Pool equal volume (e.g. 5 µL) from each of the normalized libraries into a single 1.5 mL Eppendorf tube.

Sequencing

5m

13 Denature and load pooled libraries as follows:

13.1 Denature the pooled libraries by mixing 5 µL of pooled libraries and 5 µL of 0.2N NaOH solution.

13.2 Incubate for 00:05:00 (5 minutes) 5m

13.3 Add 990 µL of HT1 buffer and mix well with denatured pooled library by pipetting up and down 10 times with P1000.

13.4 Load 600 µL of the denatured, diluted pooled library into the loading position of the Illumina reagent cartridge (V2, 300 cycle kit). Load reagent cartridge, flow cell, and PR2 buffer into Miseq instrument, confirm the metrics and start the run.