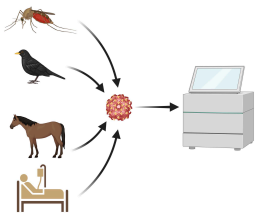
Feb 12, 2026 Version 2

Usutu virus (Orthoflavivirus usutuense) amplicon sequencing for Illumina (LowCost) V.2



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We use this protocol and it's working

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Abstract

Amplicon based targeted sequencing methods proved their importance in case of many human pathogenic viruses (e.g. Zika virus, Ebola virus, SARS-Cov 2). Application and development of this methods for other medically important viruses could be also beneficial as it ensure the possibility of molecular epidemiology.
Usutu virus (USUV, *Orthoflavivirus usutuense*) is widespread arbovirus which is mainly transmitted to humans via mosquito bites. In the natural circulation of USUV multiple hosts are involved which raise the need for sufficient sequencing method.
There are already available, established targeted sequencing methods for USUV, but we wanted to develop an additional one which uses shorter fragment length and optimised for Illumina iSeq 100. Shorter amplicon length works better on low quality samples and iSeq100 is the cheapest (most accessible) Illumina platform on the market.

Materials

	A	B	C
	Reagents		
	Component	Supplier	Catalog number
	QIAamp Viral RNA Mini Kit	Qiagen	52904
	RealStar® WNV RT-PCR Kit 2.0	altona Diagnostics GmbH	322013
	SuperScript IV Reverse Transcriptase	Invitrogen	18090010
	Random Hexamers (50 µM)	Invitrogen	N8080127
	RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen	10777019
	Q5® High-Fidelity DNA Polymerase	New England Biolabs	M0491S
	dNTP Mix (10 mM each)	Thermo Scientific™	R0192
	Individual primers	IDT or Eurofins etc.	
	NEBNext® Ultra™ II End Repair/dA-Tailing Module	New England Biolabs	E7546S
	QIAseq FX DNA Library UDI Kit (24)	Qiagen	180477
	Agencourt® AMPure® XP	Beckman Coulter	A63880
	Qubit™ 1X dsDNA High Sensitivity (HS)	Invitrogen	Q33230
	Absolute Ethanol (Molecular Biology Grade)	any	-
	Nuclease free water	any	-
	SeaKem® LE Agarose	Lonza	50004
	iSeq 100 i1 Reagent v2 (300-cycle)	Illumina	20031371
	Consumables		
	Qubit™ Assay Tubes	Invitrogen	Q32856
	PCR tubes 0,2 ml	any	-

	A	B	C
	1,5 ml tubes	any	-
	Pipette tips 0,1-1000 µl		
	Equipments		
	Magnetic stand or rack	any	-
	Pipettes 0,1-1000 µl	any	-
	Qubit™ 4 Fluorometer	Invitrogen	Q33238
	End-point PCR machines	any	-
	Gel electrophoresis system	any	-
	iSeq 100 System	Illumina	

Troubleshooting

Safety warnings

Multiplex PCR based amplification of a targeted pathogen genome could be highly effective, so it could serve as potential contamination source! This and other amplicon based method should be separated from diagnostic units to avoid cross-contamination. The implementation of negative controls are necessary to check the reagents purity.

Nucleic Acid Extraction

1 RNA extraction with QIAamp Viral RNA kit based on manufacturers recommendation. Other RNA extraction kits can be used as well e.g. *Quick*-RNA Viral Kit (Zymo Research).

Note

QIAamp® Viral RNA Mini Handbook

HB-0354-008_HB_QA_Viral_RNA_...

qRT-PCR

2 In house method, using the primers from Nikolay et. al. and optimised with OneStep RT-PCR Kit (Qiagen, Hilden, Germany).

Component	Volume
5x OneStep RT-PCR Buffer	5 µL
OneStep RT-PCR Enzyme Mix	1 µL
dNTP Mix	1 µL
Nuclease Free Water	11.4 µL
UsuFP	0.6 µL
UsuRP	0.6 µL
UsuP	0.4 µL
Sample	5 µL
Final volume	25 µL

Reaction conditions

Step	Temperature	Time	Cycles
Reverse transcription	50 °C	00:30:00	1

Denaturation	95 °C	00:15:00	1
	94 °C	00:00:30	45
Amplification	58 °C	00:00:45	45
	72 °C	00:01:00	45
Final extension	72 °C	00:10:00	1

Note

The publication for the primers:
Nikolay B, Weidmann M, Dupressoir A, Faye O, Boye CS, Diallo M, et al.
Development of a Usutu virus specific real-time reverse transcription PCR assay based on sequenced strains from Africa and Europe. J Virol Methods. 2014;197.
https://doi.org/10.1016/j.jviromet.2013.08.039

UsuFP: CAAAGCTGGACAGACATCCCTTAC

UsuRP: CGTAGATGTTTTCAGCCCACGT

UsuP: 6FAM-AAGACATATGGTGTGGAAGCCTGATAGGCA-TMR

OneStep RT-PCR Kit (Qiagen, Hilden, Germany) Handbook:

EN-QIAGEN-OneStep-RT-PCR-Kit-...

Reverse transcription

3 Reverse transcription is conducted with SuperScript IV Reverse Transcriptase.

3.1 Mix the following components in a 0.2 mL tube.

Component	Volume
50µM random hexamers	1 µL
10mM dNTPs mix (10mM each)	1 µL
Template RNA	11 µL

Total volume	13 µL

3.2 Gently mix by pipetting, and spin down afterwards if needed. Incubate the reaction as follows: 6m

Temperature	Time
65 °C	00:05:00
Place on ice for	00:01:00

Note

The master mix should be prepared in a clean cabinet while the sample should be added in another one.

3.3 Prepare the following master mix:

Component	Volume
SSIV Buffer	4 µL
100mM DTT	1 µL
RNaseOUT RNase Inhibitor	1 µL
SSIV Reverse Transcriptase	1 µL
Total volume	7 µL

Add 7 µL of master mix to the 13 µL annealed template RNA. The final volume is 20 µL. Gently mix by pipetting, and spin down afterwards if needed.

3.4 Incubate the reaction as follows: 1h 15m

Temperature	Time
25 °C	00:05:00
50 °C	00:50:00
70 °C	00:20:00

Note

The master mix should be prepared in a clean cabinet while the sample should be added in another one.

Primer pool preparation

4 Primer pool preparation from preordered primers. The primers were designed with the usage of Primalscheme using the sequences from the list below:

Usuta_primer_set.ods

4.1 The list of designed primers and pool ID (1 or 2) are available in the attached table.

This table contains the necessary amount and ratios of individual primers what should be used for primer pool generation.

4.2 Primers should be sorted based on there pool ID and resuspend lyophilised oligos in nuclease free water to 100 µM concentration. We tried to optimise the primer concentrations and the current ratios are written in the table above. Use the mentioned amount (5 µL to 12.5 µL) from unique primers to generate primer stocks (Pool 1-2).

4.3 Dilute the primer stocks (100 µM cc) 1:10 in nuclease free water to get the reaction ready primer solution (10 µM).

Note

Primer pools should be prepared in a clean mastermix cabinet!

For one reaction 2.5 µL reaction ready primer solution (10 µM) is needed. It can be prepared and aliquoted in 100 µL to avoid contamination and degradation.

Multiplex-PCR

5 Set up the multiplex PCR reaction in 0.2 mL PCR tube.

Reactions with Pool 1 and 2 primers:

Component	Pool 1	Pool 2
5X Q5 Reaction Buffer	5 µL	5 µL
10 mM dNTPs	0.5 µL	0.5 µL
Q5 Hot Start DNA Polymerase	0.25 µL	0.25 µL
Primer Pool 1 or 2 (10µM)	2.5 µL	2.5 µL
Nuclease-free water	14.25 µL	14.25 µL
Total volume:	22.5 µL	22.5 µL

Note

The master mix should be prepared in a clean cabinet while the sample should be added in another one.

6 Add 2.5 µL cDNA directly to each tube and mix well by pipetting. The final volume is 25 µL .

Note

Inside the extraction and sample addition cabinet

7 Set-up the following program on the thermal cycler: 2m 5s

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:05	20-38

Annealing + Extension	60 °C	00:01:30	20-38
Hold	4 °C	Till further processes	

Note

The cycle number are determined based on the qPCR result. In our case the Ct number + ~3 cycles formula gave the best result (e.g. The Ct value is 26,54 use 30 cycles). In general, use higher number of cycles than the obtained Ct value.

This is a safe stopping point. The amplicons can be stored at 4°C till further processes.

Amplicon clean-up

8 Conduct clean-up with Ampure XP Beads.

Sample/Bead ratio = 1:1 (50 µL)
Washing: two times with 200 µL 80% EtOH.
Elution: 28 µL nuclease-free water.

8.1 Merge the two pools prior to adding the magnetic beads.

8.2 Add 50 µL AMPure XP beads to the sample and mix gently. The sample/magnetic bead ratio is 1:1.

8.3 Incubate for 00:05:00 at room temperature. 5m

8.4 Place the tube on a magnetic rack for 00:02:00 (until supernatant is completely clear). 2m

8.5 Remove supernatant without touching the bead pellet.

8.6 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

8.7 Remove supernatant and discard.

8.8 Repeat washing step with 80% ethanol (step 9.6-9.7).

8.9 Carefully remove the supernatant and dry the beads for around 00:02:00 by leaving the lid open. (Dull pellet indicates dryness) 2m

8.10 Remove tube from the magnetic rack and resuspend the pellets in 28 µL nuclease-free water and incubate at room temperature for 00:02:00 . 2m

8.11 Place the tube back to a magnetic rack for 00:02:00 until supernatant is completely clear and transfer eluate into clean tube. Make sure that no beads are transferred as well. 2m

Quantification and normalisation

9 Quantify 1 µL directly from the two different PCR reaction (Pool 1, Pool 2) with the 1x dsDNA HS Assaykit.

9.1 Aliquot 199 µL 1x Working solution to Qubit assay tube (equal to the number of your samples). Prepare two extra tubes for the standards with 190 µL 1x Working solution.

9.2 Add 1 µL sample for each test tube and 10 µL from the Standards (Standard 1, Standard 2). The final volume is 200 µL .

9.3 Mix each sample vigorously by vortexing and pulse centrifuge to collect the liquid.

9.4 Incubate at room temperature for 00:02:00 before measuring.

9.5 Calibrate Qubit fluorometer with the standards based on manufacturers recommendation.

9.6 Read your sample.

9.7 Normalization is not required if the concentrations of the two reactions (Pool 1, Pool 2) from the same sample differ by 20 % or less. If the difference exceeds this threshold, use the entire volume of the low concentration pool and add an equivalent concentration from the other pool.

Note

Alternative dsDNA quantification systems can be used as well (e.g.QuantiFluor® ONE dsDNA System).

End Repair and dA Tailing

10 Amplicon end preparation with NEBNext Ultra II End Repair/dA-Tailing Module.

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

Component	Volume
NEBNext Ultra II End Prep Reaction Buffer	1.75 µL
NEBNext Ultra II End Prep Enzyme Mix	0.75 µL
50 ng Amplicons	x µL
Nuclease-free water	12.5-x µL
Total volume	15 µL

Note

If the concentration does not reach the 4 ng / µl use the whole amount (25 µL) from the cleaned up amlicons.

10.2 Place sample in a thermal cycler, with the heated lid set to 75 °C, and run the following program: 45m

Temperature	Time
20 °C	00:30:00
70 °C	00:15:00

Adapter Ligation

11 Adapter ligation with QIAseq FX DNA Library Kit.

11.1

Component	Volume
DNA Ligase Buffer, 5x	10 µL
DNA Ligase	5 µL
UDI Adapter	5 µL
Nuclease-free water	17.5 µL
Previous reaction	15 µL
Final volume	50 µL

11.2 Place sample in a thermal cycler without heated lid, set and incubate: 15m

Temperature	Time
20 °C	00:15:00

Note

We used QIAseq FX DNA Library Kit as ligation module. Applied concentrations and reaction conditions are optimised for this protocol. Alternative ligase modules could be use after optimisation (e.g. from NEB).

Library clean up

12 Conduct clean-up with Ampure XP Beads.

Sample/Bead ratio = 1 : 0.6 (30 µL)
Washing: two times with 200 µL 80% EtOH.
Elution: 25 µL nuclease-free water.

12.1 Add 30 µL AMPure XP beads to the sample and mix gently. The sample/magnetic bead ratio is 1:0.6.

12.2 Incubate for 00:05:00 at room temperature.

12.3 Place the tube on a magnetic rack for 00:02:00 (until the supernatant is completely clear).

12.4 Remove supernatant without touching the bead pellet.

12.5 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

12.6 Remove supernatant and discard.

12.7 Repeat washing step with 80% ethanol (step 12.5-12.6).

12.8 Carefully remove the supernatant and dry the beads for around 00:02:00 by leaving the lid open. (Dull pellet indicates dryness)

12.9 Remove tube from the magnetic rack and resuspend the pellets in 25 µL nuclease-free water and incubate at room temperature for 00:02:00 .

12.10 Place the tube back to a magnetic rack for 00:02:00 until the supernatant is completely clear and transfer eluate into clean tube. Make sure that no beads are transferred as well.

Library amplification

13 Conduct library amplification with the usage of HiFi PCR Master Mix.

13.1 Mix the following components in an 0.2 mL tube.

Component	Volume
HiFi PCR Master Mix, 2x	12.5 µL
Primer Mix Illumina Library Amp	0.75 µL
Adapter ligated amplicons	11.75 µL
Final volume	25 µL

13.2

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	00:02:00	1
Denaturation	98 °C	00:00:20	4-8
Annealing	60 °C	00:01:30	4-8
Extension	72 °C	00:00:30	4-8
Final extension	72 °C	00:01:00	1
Hold	4 °C	Till further processes	

5m 20s

Note

Under 0,5 ng the applied cycle number should be 8 while above 4 ng 4 cycles is suitable. Reduced number of PCR cycles can result balanced amplicon distribution. Concentration measurment is recommended at this point.

Final Library clean up

14 Conduct clean-up with Ampure XP Beads.

Sample/Bead ratio = 1 : 0.7 (17.5 µL)
Washing: two times with 200 µL 80% EtOH.
Elution: 25 µL Elution Buffer.

14.1 Add 17.5 µL AMPure XP beads to the sample and mix it gently. The sample/magnetic bead ratio is 1:0.7.

14.2 Incubate for 00:05:00 at room temperature.

14.3 Place the tube on magnetic rack for 00:02:00 (until the supernatant is completely clear).

14.4 Remove supernatant without touching the bead pellet.

14.5 Add 200 µL freshly prepared 80% ethanol to the tube. Float the beads through ethanol either by replacing to another magnetic rack or mix via pipetting.

14.6 Remove supernatant and discard.

14.7 Repeat washing step with 80% ethanol (step 14.5-14.6).

14.8 Carefully remove the supernatant and dry the beads for around 00:02:00 by leaving the lid open. (Dull pellet indicates dryness)

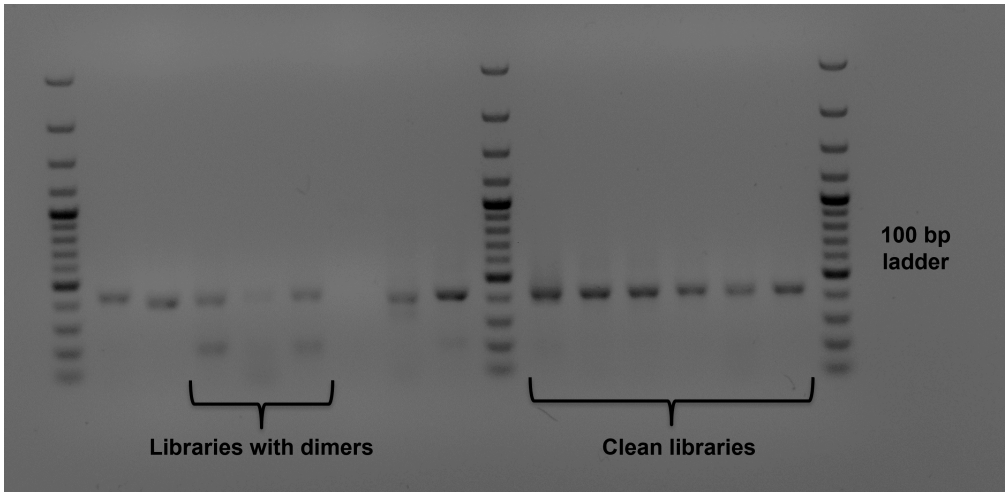
14.9 Remove tube from the magnetic rack and resuspend the pellets in 25 µL elution buffer and incubate at room temperature for 00:02:00 .

14.10 Place the tube back to the magnetic rack for 00:02:00 until the supernatant is completely clear and transfer the eluate into clean tube. Make sure that no beads are transferred as well.

Library Size check

15 Run 5 µL from the cleaned up final library on 1,5% agarose gel. Low concentration of WNV genome in the sample could affect the multiplex PCR effectivity. Missing template genome results high amount of primer dimers. The expected final library size is 400bp to 420 bp. The dimers are appearing around 200 bp.

Note



Examples of clean and primer dimer containing libraries.

Fragment size should be considered in the case of pooling to be sure about the equal sequencing depth of individual samples.Fragment size should be considered in the case

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Pooling and loading final libraries

16 Fragment size should be considered in the case of pooling to be sure about the equal sequencing depth of individual samples.

16.1 Pool all samples together in a 1.5 mL Eppendorf Low Binding tube in equimolar concentration.

16.2 Dilute the pooled libraries in RSB to 100 pM final concentration. Add PhiX control.

16.3 Load 20 µL onto an iSeq 100 2×150 bp flow cell.

16.4 Start the sequencing.

Protocol references

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Usutu virus specific real-time reverse transcription PCR assay

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