GENERATION OF TILED VIRUS AMPLICONS FOR RSV A SEQUENCING

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Adapted from: Quick, J. *et al*. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nature Protocols 12 (6), 1261-1276.* https://www.nature.com/nprot/journal/v12/n6/abs/nprot.2017.066.html

Notes: The panel has been optimized using RSV A positive samples circulating in Europe in 2025-2026. Processing samples from previous years or from regions outside of Europe might require further adjustments.

Reference sequence is A/England/397/2017, which is available at PP109421 and also deposited under accession number EPI_ISL_412866 in GISAID.

MATERIAL

- Nucleospin 8 Virus Core Kit (Macherey Nagel)
- Lunascript RT Supermix 5X kit (NEB)
- Q5 High-Fidelity PCR Kit (NEB) and dNTPs 10 mM
- Primers purchased at 200 µM from Sigma-Aldrich
- AMPure XP Bead-Based Reagent Protocol for PCR Purification (Beckman Coulter)

Equivalent kit and reagents can be used.

PROTOCOL

1.1. GENERATION OF TILED AMPLICONS

Reverse Transcription :

Composition	Reaction	Final Concentration
Lunascript Supermix 5X	2 µL	1X
RNA*	8 µL	-

*RNA was extracted from 100 µL of respiratory sample and eluted in 100 µL.

LUNA RT	
T°	**Time**
25°C	2 min
55°C	10 min
95°C	1 min
4°C	∞

Amplicon generation by PCR in 2 independent reactions:

Primer pools.
Primers were purchased at 200 µM from Sigma-Aldrich
Prepare two pre-mix (primers pools) with concentrations indicated in Annex 1

Composition	Reaction RSVA_pool 1	Reaction RSVA_pool 2	Final Concentration in 25 µL
5X Q5 reaction buffer	5 µL	5 µL	1X
10mM dNTP	0,5 µL	0,5 µL	200 µM
50X Primer pool 1 or primer pool 2 (see concentration in Annex 1)	0,5 µL	0,5 µL	See annex 1
cDNA	2,5 µL	2,5 µL	-
Q5 polymerase (2000 U/mL)	0,25 µL	0,25 µL	20 U
H2O RNase DNase free	For final volume 25 µL	For final volume 25 µL	

PCR (Q5)		
T°	**Time**	**Nb Cycles**
98°C	30 s	1
98°C	15 s	35
65°C	5 min	
4°C	∞	1

Check amplification on agarose gel:
Run 5 µL of each reaction on a 1% agarose gel. Each should produce a visible ~400 bp band and the negative control (no cDNA) should be clean.

1.2. PURIFICATION OF AMPLICONS USING MAGNETIC BEADS

Two purification techniques are described, one manual, and one automated using the King Fisher (Thermo Scientific).
Alternative purification techniques can be used but should be validated by the user.

1.2.A. MANUAL PURIFICATION OF AMPLICONS

1. Vortex Agencourt AMPureXP beads bottle (placed at room temperature) to resuspend magnetic particles
2. For each sample, combine the PCR products from pool 1 and pool 2 PCRs (~50 µL final volume)
3. Add 72 µL (1,44X) of RNAclean XP beads to PCR; mix by pipetting 10 times

4. Incubate samples 10 minutes at room temperature.
5. Place the tubes on an appropriate magnetic rack to separate beads from the supernatant.
6. When the solution is clear (about 5-10 minutes), discard the supernatant without disturbing the beads.
7. Add 200 µL of freshly prepared 70% ethanol to the tube while in the magnetic rack. Incubate at room temperature for 1 min, and then carefully remove and discard the supernatant
8. Repeat Step 7 once for a total of 2 washes.
9. Completely remove the residual ethanol, and air-dry beads on the magnetic rack (5-10 minutes).
10. Add 40 µL of RNase Dnase free H20 on the beads and remove the plate from the magnetic rack.
11. Mix well by pipetting up and down and put the tube in the magnetic rack until the solution is clear.
12. Transfer the 40 µL to a clean plate.
13. Quantify the DNA concentration using the Qubit High Sensitivity DNA kit (or equivalent) from 1 µL of each product. Expected range > 80 ng/µL DNA.
14. Proceed with NGS library construction or other downstream application. Alternatively, the samples can be stored at –20°C.

1.2.B. PURIFICATION OF AMPLICONS USING KING FISHER

1. Vortex Beckman Coulter AMPureXP beads bottle (placed at room temperature) to resuspend magnetic particles
2. For each sample, combine the PCR products from pool 1 and pool 2 PCRs (~50 µL final volume) and transfer the whole PCR products to a DeepWell plate
3. Add 108 µL of AMPureXP beads to the PCR products in a DeepWell plate
4. Fill a U-bottom plate with 150 µL of 70% ethanol for washing
5. Fill a U-bottom plate with 50 µL of Ambion water for elution
6. Start the protocol on the robot Kingfisher and follow the instructions.

Purification Protocol Steps for KingFisher Automation
Step 1 Pick up tips from position 1.
Step 2 – Binding (Position 2 - DW sample) • Pre-collect beads: ON • Release beads: ON • Mix for 6 minutes at medium speed • Collect beads: 10 cycles, 5 seconds each
Step 3 – Wash (Position 3 - 96-well plate with 150 µL of 70% ethanol) • Mix for 15 seconds at medium speed *(no bead release / pre-collect / collect)*
Step 4 – Dry (Position 3 - outside well) Dry outside the well for 2 minutes

Step 5 – Elution (Position 4 - 96-well plate with 50 µL NF-water) • Release beads: ON • Mix for 4 minutes at medium speed
Step 6 – Bead collection (Position 4) • Pre-collect beads: ON • Mix for 2 minutes at slow speed • Collect beads: 3 cycles, 30 seconds each
Step 7 Leave tips in wash plate at position 3
Step 8 - **Final step** Hold at **4°C indefinitely (∞)**

The panel is compatible with Oxford Nanopore Technologies and Illumina sequencing. If using a combined paired read length shorter than the amplicons size (~400nt) with DNA fragmentation or tagmentation, adapt the bioinformatic pipeline for primer trimming. 150 nt long reads are the minimum recommended.

2. ANNEX 1

Primers are ordered dry and resuspended at a concentration of 200 μM.
Two 50X concentrated primer pools are prepared (Pool 1 and Pool 2), aliquoted and frozen at -20°C until use (« pre-Mix » in tables below). Aliquots are freeze/thawed a maximum of 5 times to prepare PCR-Mixes.

RSVA_1_LEFT	CTTGCGTAAACCAAAAAAATGGGG	1	30	1,50
RSVA_1_RIGHT	TGGTCATTGTTGAATCACTTAGTTTTTTG	1	30	1,50
RSVA_3_LEFT	TAGAGACATCATAACACATAAATTTATATACTTG	1	15	0,75
RSVA_3_RIGHT	CAACTTGACTTTGCTAAGAGCCAT	1	15	0,75
RSVA_5_LEFT	AGGTATGTTATATGCTATGTCTAGATTAGGA	1	75	3,76
RSVA_5_RIGHT	TGGGTAATAAACCTTTATAGCGTTTCA	1	75	3,76
RSVA_7_LEFT	AAGTGATGTTACGGTGGGGG	1	15	0,75
RSVA_7_RIGHT	ACTCAAAGCTCTACATCATTATCTTTTGG	1	15	0,75
RSVA_9_LEFT	AAACCCAATAAATGAGACAGATGATACT	1	15	0,75
RSVA_9_RIGHT	TCCTGAGTCTTGCCATAGCTTC	1	15	0,75
RSVA_11_LEFT	GCTACCAAACTGTACATCAAAACACA	1	8	0,38
RSVA_11_LEFT_bis	GCTACCAAACTGTATATCAAAACACA	1	8	0,38
RSVA_11_RIGHT	TCTTAATGAGGGTCCCTTGGGT	1	15	0,75
RSVA_13_LEFT	ACACTCAACCCAACACATGACA	1	113	5,64
RSVA_13_LEFT_bis	ACACTCAACCCCACACATGACA	1	38	1,88
RSVA_13_RIGHT	TTGTATGAATCTACTCATTGATGTAGAGG	1	113	5,64
RSVA_13_RIGHT_bis	TTGTGTGAATCTGCTCATTGATGTAGAGG	1	38	1,88
RSVA_15_LEFT	ACACGCTAGATAAAATCAACCAATGG	1	15	0,75
RSVA_15_RIGHT	CATTTGCCCCAGCGTTRTTYTT	1	15	0,75
RSVA_17_LEFT	CCACAAAGTCACACTAACAACTGC	1	15	0,75
RSVA_17_RIGHT	TGGTGGTGGTTTTCTTTCCAGG	1	15	0,75
RSVA_19_LEFT	AAACCCTCCACTCAACCACCTC	1	15	0,75
RSVA_19_RIGHT	TCTGTACCATTACACTTATTTTCCTTGAT	1	15	0,75
RSVA_21_LEFT	GGCATTGCCGTATCCAAGGT	1	15	0,75
RSVA_21_RIGHT	TGCTGTCTAACTATTTGAACATTGCT	1	15	0,75
RSVA_23_LEFT	GTGACAATGCAGGATCAGTATC	1	15	0,75
RSVA_23_RIGHT	CAGAGGGGAACACTAATGGATC	1	15	0,75
RSVA_25_LEFT	TATACTGCAAGGCCAGAAGCAC	1	15	0,75
RSVA_25_RIGHT	CATGGGGTGGCCATTCAAAATAA	1	15	0,75
RSVA_27_LEFT	ACTGAACTCAACAGCGATGACA	1	75	3,76
RSVA_27_RIGHT	CATTGAATGGTTGATCCGGTGG	1	75	3,76
RSVAb_29_LEFT	CTCAGAATGTAACGCTTTAGGAAG	1	150	7,52
RSVAb_29_RIGHT	GTAGTAATGACTGAGTTGTCTTCATC	1	150	7,52
RSVAb_31_LEFT	GCAACATCCTCCATCATGGTTAATAC	1	75	3,76
RSVAb_31_RIGHT	GTAGAACCCCTCATTGTGGAATAG	1	75	3,76
RSVA_33_LEFT	TGCAGGTGACAATAACCTCAACA	1	30	1,50
RSVA_33_LEFT_bis	TGCAGGTGACAACAACCTCAACA	1	30	1,50
RSVA_33_RIGHT	AAATTTTTAGGAGGTGATATAGCCTTATCA	1	30	1,50
RSVA_33_RIGHT_bis	AAATTTTTAGGAGGTGATATAGCCTTGTCA	1	30	1,50
RSVA_35_LEFT	AGGCAAAGAAAGAGAACTCAGTGT	1	15	0,75
RSVA_35_RIGHT	GGGGGTGCATGCCTATATGT	1	15	0,75
RSVA_37_LEFT	TCACTATTAGATCTAATATCTCTCAAAGGGA	1	150	7,52
RSVA_37_RIGHT	ACTGCATAATAGACTTTCACCTCTATATTC	1	150	7,52
RSVA_39_LEFT	TCGAAGTTTCTATAGAAGAACTCCTGA	1	15	0,75
RSVA_39_RIGHT	TCATAAACAACTCTTAGCCCGTGA	1	15	0,75
RSVA_41_LEFT	GAGATAAAAGAGAAATATTGAGTATGGAAAACC	1	75	3,76
RSVA_41_RIGHT	TGGCCTTCTCATATGTTAACCCA	1	75	3,76
RSVA_43_LEFT	TGGCCTTAGCTTAATGTCTGTAGT	1	15	0,75
RSVA_43_RIGHT	ACATATGATCAGTTATATATCCCTCTCCC	1	15	0,75
RSVA_45_LEFT	AGCTTCAAACTATGGTTTCTTAAACG	1	150	7,52
RSVA_45_RIGHT	CCTATACAATAGTCACTCAGTGTC	1	150	7,52
RSVA_47_LEFT	CCTTAGTGCACAATAGCACATCAC	1	15	0,75
RSVA_47_RIGHT	CAGGCAATTCAGCATCACAGAC	1	15	0,75
RSVA_49_LEFT	GGTCCTGCAAATGTGTTCCCA	1	75	3,76
RSVA_49_RIGHT	AGCTGTTTAACAATTCACTTAGATGAGG	1	75	3,76

Primer	Sequence	Pool	Conc. Final (nM)	Conc. Pre-Mix (µM) (50 X)
RSVA_2_LEFT	TGGCCTAATAGATGACAATTGTGAAA	2	30	1,50
RSVA_2_RIGHT	TGACCAGAAATGTAAATGTGGCCT	2	30	1,50
RSVA_4_LEFT	CAAACAATCCGAAATAACAACTTTATGCA	2	15	0,75
RSVA_4_LEFT_bis	CAAACAATCCAAAATAACAGCTTTATGCA	2	15	0,75
RSVA_4_RIGHT	TCCACTCCATTTGCCTTAACATGA	2	15	0,75
RSVA_4_RIGHT_bis	TCCACTCCATTTGCTTTGACATGA	2	15	0,75
RSVA_6_LEFT	AGCAGCAGGAGATAGATCAGGT	2	15	0,75
RSVA_6_RIGHT	GGTTGTTCAATATATGGTAGAATCCTGC	2	15	0,75
RSVA_8_LEFT	AAATGGTGTGATTAACTACAGTGTATTAGA	2	11	0,56
RSVA_8_RIGHT	GGCGTAGGGTCTTCTTTGAAACT	2	11	0,56
RSVA_10_LEFT	AGGATTGATGAGAAATTAAGTGAAATACTAGG	2	15	0,75
RSVA_10_RIGHT	TCAGCAGATAGATGTTTGGTTGGA	2	15	0,75
RSVA_12_LEFT	TCCATGCCAGCAGATCTACTCA	2	15	0,75
RSVA_12_RIGHT	TCAAGTGTGTTCAGATCTTTATTTCTGA	2	15	0,75
RSVA_14_LEFT	CAACAAATTGGAAGCACACAGCT	2	15	0,75
RSVA_14_RIGHT	TGTTATCATGTGTATTAGTGTAAAGTAAGGC	2	15	0,75
RSVA_16_LEFT	TCTCACCATGCAAGCCATYATC	2	15	0,75
RSVA_16_RIGHT	TGGGTGAGGTATGTTGGGGTT	2	15	0,75
RSVA_18_LEFT	ACCCAACAATGATTTTCACTTTGAAGT	2	15	0,75
RSVA_18_RIGHT	GTGTTGGATGAAGATRGAGRTTGTGAT	2	15	0,75
RSVA_20_LEFT	GCAAAGGCTATCTTAGTGCTCTAAG	2	15	0,75
RSVA_20_RIGHT	AGCCTTGTTTGTGGATAGTAGAGC	2	15	0,75
RSVAb_22_LEFT	GTGTAACTACACCTGTAAGCAC	2	75	3,76
RSVAb_22_RIGHT	CAGAGATTTACCTCACTTGGTAATG	2	75	3,76
RSVA_24_LEFT	GGGTGGATACTGTGTCTGTAGG	2	15	0,75
RSVA_24_RIGHT	GGTGCTATTTTTATTCAGTTACTAAATGCA	2	15	0,75
RSVA_26_LEFT	GGGGCAAATATGTCACGAAGGA	2	15	0,75
RSVA_26_RIGHT	GCTTTCAATATATGATATGACAGTATTGTACAC	2	15	0,75
RSVA_28_LEFT	CAAGTAATTGTAGAGTCACTATGTATAACCA	2	15	0,75
RSVA_28_LEFT_bis	CAAGTAATTGTAGAGTCACTATGTATAATCA	2	15	0,75
RSVA_28_RIGHT	AGTTGGTATAATCATTTTTGAGATAAGGACC	2	30	1,50
RSVAb_30_LEFT	CTTATGACATACAAGAGTATGACCTC	2	15	0,75
RSVAb_30_RIGHT	CTCACTAGATCGATATTGTGTTAATATGC	2	15	0,75
RSVA_32_LEFT	TGTGGATTCAATAATGTTATCTTGACACA	2	15	0,75
RSVA_32_RIGHT	AGCATCCATGGCTTGTCTTTCA	2	15	0,75
RSVA_34_LEFT	TCAGGACTACGTTTCTATCGAGAGT	2	15	0,75
RSVA_34_RIGHT	TGTTTTCTGCTATCATTTTCTCTGCT	2	15	0,75
RSVA_36_LEFT	TGGATGAACTGCATGGTGTACA	2	30	1,50
RSVA_36_RIGHT	TGTGGCCTATTCCTGCATACTC	2	30	1,50
RSVA_38_LEFT	GGACCGTGGATAAACACTATACTTGA	2	56	2,82
RSVA_38_LEFT_bis	GGACCGTGGATAAACACCATACTTGA	2	19	0,94
RSVA_38_RIGHT	CTTGAAGTTTGTCTTTTAAATCATGGTTTG	2	75	3,76
RSVA_40_LEFT	GTGCACAACACTATACCACCAC	2	15	0,75
RSVA_40_RIGHT	TGTCCATTGTATACATGATACTGGGT	2	15	0,75
RSVA_42_LEFT	AGCAAAATTGGATTGGGTGTATGC	2	15	0,75
RSVA_42_RIGHT	ACATCACCTGTGAATATGGGAGG	2	15	0,75
RSVA_44_LEFT	ACTAATTTAGCTGGACATTGGATTCTT	2	30	1,50
RSVA_44_RIGHT	TGTTAACAACCCAAGGGCAAAC	2	30	1,50
RSVA_46_LEFT	ACAACAAATTATATCATCCYACACCAG	2	150	7,52
RSVA_46_RIGHT	TATGATGCCAAGGAAGCATGC	2	150	7,52
RSVA_48_LEFT	GACCATTCCTGCTACAGATGCA	2	30	1,50
RSVA_48_RIGHT	GGCATGATGAAATTTTTGGTTCTTGA	2	30	1,50
RSVA_50_LEFT	CGATAAAGAGTCTATTGATGCAAATATTAAGAG	2	75	3,76
RSVA_50_RIGHT	TGGATTTAAACTTATTCTTCCTAGATCAAAA	2	75	3,76