INSTITUT PASTEUR

CNR VIR

PROCEDURE

GENERATION OF TILED VIRUS AMPLICONS FOR RSV B SEQUENCING

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1. TOPIC

Initial design by Etienne Simon-Loriere (etienne.simon-loriere@pasteur.fr) available at https://github.com/SimonLoriereLab/RSV_amplicons_panels
G5 Génomique Evolutive des Virus à ARN for Centre National de Reference – Virus des infections respiratoires (dont la grippe et le SARS-CoV-2), Institut Pasteur, France.

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 B positive samples circulating in Europe in 2021-2022. Processing samples from previous years or from regions outside of Europe might require further adjustments.

2. STARTING MATERIAL

- RNA extracted with QiaAmp Viral RNA extraction kit (QIAGEN)
- 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.

3. PROTOCOL

3.1. 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 RSVB_pool 1	Reaction RSVB_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
Primers pool 1 or primers pool 2 (see concentration in Annex 1)	2,02 µL	1,95 µL	See annex 1
DNA	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	

Q5 PCR		
T°	**Time**	**Nb Cycle**
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.

3.2. PURIFICATION OF AMPLICONS ON BEADS

Can use alternative purification techniques.

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 (1,8X) 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 H20 RNase Dnase free 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 = 10-100 ng/µL DNA.
14. Proceed with NGS library construction or other downstream application. Alternatively, the samples can be stored at –20°C.

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.

4. ANNEX 1

Name	Seq	Pool	Conc. Pre-Mix (µM)	Conc. Final (nM)
RSVB_1_LEFT	CAGAAATGGGGTGCAATTCACTG	1	0,2	16
RSVB_1_RIGHT	TCATGAATGGAGATCAAGCCCAA	1	0,2	16
RSVB_3_LEFT	ACCATGAGCACTACAAAMGACAA	1	0,4	32
RSVB_3_RIGHT	GTTGGAAGTTACGGGTTGAGGT	1	0,4	32
RSVB_5_LEFT	TGACACTCCCAATTATGATGTGCA	1	0,4	32
RSVB_5_RIGHT	TTACAAGGGCAGCTATACACAGT	1	0,4	32
RSVB_7_LEFT	AGCACAATCATCCACAAGAGGG	1	3	242
RSVB_7_RIGHT	GAGTTGCTCTGCATATGCTTTGG	1	3	242
RSVB_9_LEFT	TCCAAAGATCCTAAGAAGAAAGATAGCA	1	0,15	12
RSVB_9_RIGHT	TATTCCGTCACGAGCCGAAGTR	1	0,15	12
RSVB_11_LEFT	TTGGAAGACAACGATAGCGACAA	1	0,2	16
RSVB_11_RIGHT	GCTTTACTAGTATGTTGATGCTTGCA	1	0,2	16
RSVB_13_LEFT	TGCAGTTTAACATGCTTRAAAGTAAAAAG	1	0,4	32
RSVB_13_RIGHT	GTGGTTTGATTGAAAAACGTGTAGC	1	0,4	32
RSVB_15_LEFT	CCTAGAGTGCGAATAGGTAAATAAAACAAG	1	2	162
RSVB_15_RIGHT	TGTGATGCCATGACTCTGTGAG	1	2	162
RSVB_17_LEFT	GAAAAGACCTGGGATACTCTTAATCATCT	1	1	81
RSVB_17_RIGHT	GTGCTTGGCTTGTTGTTCTGTG	1	1	81
RSVB_19_LEFT	AGAAACCACCATTAACCCAACAAAAA	1	0,2	16
RSVB_19_RIGHT	GCAAGAGTTAGGAAGATTGCACTTG	1	0,2	16
RSVB_21_LEFT	CAGAGGTTACTTGAGTGCTTTAAGAAC	1	2	162
RSVB_21_RIGHT	CTTTGTTCGTAAGCTGCAAAGCA	1	2	162
RSVB_23_LEFT	AGCACTTACATGTTAACAAACAGTGAG	1	0,4	32
RSVB_23_RIGHT	ACAAAGGCTGACTTCACTTGGT	1	0,4	32
RSVB_25_LEFT	GCTGTGATTATGTGTCAAACAAAGGA	1	0,4	32
RSVB_25_RIGHT	TGCAATATTATTGATTCCACTTAGTTGGTC	1	0,4	32
RSVB_27_LEFT	TACATCAACATCACADCACAGGC	1	2	162
RSVB_27_RIGHT	CTCTCTAGCACTCCAACTATACCAAG	1	2	162
RSVB_29_LEFT	CTTAAACGATTACCAGCAGACGTR	1	0,2	16
RSVB_29_RIGHT	GGTTGGATGATATAGAATCGTTATGATCAAG	1	0,2	16
RSVB_31_LEFT	ACGGCCCTTATCTTAAAAATGATTATACC	1	0,2	16
RSVB_31_RIGHT	ACCAGTGTATTAACCATGATGGAGG	1	0,2	16
RSVB_33_LEFT	AGATAATCAAACTTTGAGTGGTTTTCAGT	1	0,2	16
RSVB_33_RIGHT	ACTCTTGATAGTAGATCCTTTTGAGCC	1	0,2	16
RSVB_35_LEFT	ACTTGAGTGAGCTATATTTTCTCTTCAGA	1	0,2	16
RSVB_35_RIGHT	ACTAGTCCATATTAGATCTTTTGGAGGAG	1	0,2	16
RSVB_37_LEFT	AGAAAGAGAGCTCAGTGTGGGT	1	0,2	16
RSVB_37_RIGHT	TCCTTTATAAAAGGAGGTGCATGTCT	1	0,2	16
RSVB_39_LEFT	CACAGCTCTGATAAATGGTGATAATCAG	1	0,2	16
RSVB_39_RIGHT	TTGCAGAGCAATTTGATTGTATAACCA	1	0,2	16
RSVB_41_LEFT	GAGAACTCCAGACTTCCTTACAGAR	1	1	81
RSVB_41_RIGHT	GCGCACTTTTAGAAAATATTTTGTTTGGA	1	1	81

RSVB_43_LEFT	TGTAAGAGAAAGATCTTGGTCATTATCCA	1	0,2	16
RSVB_43_RIGHT	ACTGTTAACCGGTGTAAATAATTKACAC	1	0,2	16
RSVB_45_LEFT	CTTAGCTTGATGTCAGTWGTGGAAC	1	0,2	16
RSVB_45_RIGHT	TGATCAGTTATATACCCYTCTCCCC	1	0,2	16
RSVB_47_LEFT	GCTACTGGAAATCTATGTCTAAAGTTTTCC	1	0,5	40
RSVB_47_RIGHT	GGGGTTGGGTGATATAGTTTGTTRTAATT	1	0,5	40
RSVB_49_LEFT	GTGATAGACAGGATTATAGATCATTCAGGT	1	0,2	16
RSVB_49_RIGHT	GTTGCATCTGTAGCAGGAATGGT	1	0,2	16
RSVB_51_LEFT	ACAGCCAATTGGAGTAAAATYATAATTGA	1	0,2	16
RSVB_51_RIGHT	TCTCCATTAACTACACTCTTCAATTTTGAC	1	0,2	16
RSVB_53_LEFT	AACAAGCTTATAAACCACAAGCATATGA	1	2	162
RSVB_53_RIGHT	TGTCTCGTTGTGTTGTAAATGCAC	1	2	162
RSVB_2_LEFT	AGTGAAATCTAACTTTACAACAATGCCA	2	1	78
RSVB_2_RIGHT	GGTGAGAGATGTTATTATTGAATCCATCG	2	1	78
RSVB_4_LEFT	TGGCGGGTTTCTAGAATGTATTGG	2	0,4	31
RSVB_4_RIGHT	TGATTTGCATCTTCAGTGATTAATAGCA	2	0,4	31
RSVB_6_LEFT	AAGCTTGACATCAGAAATACAAGTCAA	2	0,2	16
RSVB_6_RIGHT	CTTGTCCTGAACCATAGGCATTCA	2	0,2	16
RSVB_8_LEFT	AGCAGGTCTAGGCATAATGGGA	2	0,2	16
RSVB_8_RIGHT	TGTTGGTGCCAGATGTTATCGG	2	0,2	16
RSVB_10_LEFT	TGACCAAACAAATGATAACATTACAGCA	2	0,2	16
RSVB_10_RIGHT	GGTGTCTGTTTTGTTGATGYTGTT	2	0,2	16
RSVB_12_LEFT	CAGCAGCTGTCCAGTACAATGT	2	0,2	16
RSVB_12_RIGHT	TTCACATAGAGCAATGATCTCATGAGT	2	0,2	16
RSVB_14_LEFT	GGAGCATTCAAGTATATCAAGCCAC	2	0,4	31
RSVB_14_RIGHT	GCTTGTGAATTCTATTGTRATGGATGT	2	0,4	31
RSVB_16_LEFT	ACAGATGTATCAAATCGACACATAGTG	2	0,2	16
RSVB_16_RIGHT	GCAGAGATGATGAATATTATGGCTGC	2	0,2	16
RSVB_18_LEFT	ACCCAATACAAAATCAGAAACACACC	2	0,2	16
RSVB_18_RIGHT	ACAGTGGATTGTGGGGTGCT	2	0,2	16
RSVB_20_LEFT	GTGATCCCTCAAGCAAGAACKA	2	1	78
RSVB_20_RIGHT	TGATTGTATAGTTCATATACTGTGGTGCT	2	1	78
RSVB_22_LEFT	AGCAAGTGGTATAGCTGTATCCAAAG	2	2	156
RSVB_22_RIGHT	GCCTTACTATCTGAACATTGCTTGAC	2	2	156
RSVB_24_LEFT	TCCTTCTTTCCACAAGCTGACAC	2	0,2	16
RSVB_24_RIGHT	GAAGGAAACACTAGAGGGTCATAGT	2	0,2	16
RSVB_26_LEFT	CAAAGTTTAGCTTTTATTCGTAGATCYGA	2	0,2	16
RSVB_26_RIGHT	GTGTTGATTGCGATATTTGAGGCTT	2	0,2	16
RSVB_28_LEFT	TGTTAAACAAGATACTCAAGTCAATGGAC	2	0,2	16
RSVB_28_RIGHT	TGGTTGACTCTTTTGGGTTGCT	2	0,2	16
RSVB_30_LEFT	GCCACTCAACAATTTCTCCAACA	2	0,2	16
RSVB_30_RIGHT	AGTAATGACTGGAAATAAGTTGGTTCTTC	2	0,2	16
RSVB_32_LEFT	GTGATGAAAACTCAGTACTTACAACCA	2	0,4	31
RSVB_32_RIGHT	GCTGATGTCTTTCCATGTCAAAAATTG	2	0,4	31
RSVB_34_LEFT	CTATCACAATTATTCCTTTACGGAGATTGT	2	0,2	16
RSVB_34_RIGHT	TCATTGCAGTTAATTCTTACAGCATCC	2	0,2	16

RSVB_36_LEFT	TTGCGGTTCTATCGTGAGTTTCA	2	0,4	31
RSVB_36_RIGHT	CAGGGAAGAATTGTAAAATATTTTCGGC	2	0,3	23
RSVB_36_RIGHT_bis	CAGGGAAGAATTGTAAAATATTTTCAGC	2	0,1	8
RSVB_38_LEFT	ACGAACTGCATGGRGTACAATC	2	0,2	16
RSVB_38_RIGHT	TCCCTTAAGCTTATGGCCTATGC	2	0,2	16
RSVB_40_LEFT	AGGTAGCTTAACACAGGARTTAGAATAC	2	1,5	117
RSVB_40_RIGHT	GACACAAGTCAAGAATTTGTTCAGTCT	2	1,5	117
RSVB_42_LEFT	ATCCACAGGCTTTAGGGTCTGA	2	0,2	16
RSVB_42_RIGHT	GGCTATAGTGCTAGTTGTATATTTAATRTCC	2	0,2	16
RSVB_44_LEFT	CAAAGATGAATTCATGGAAGAACTGAGT	2	0,2	16
RSVB_44_RIGHT	GCTTCAACTTGATGATATCRACATCTCC	2	0,2	16
RSVB_46_LEFT	TTAGCTGGACATTGGATTCTGA	2	1	78
RSVB_46_RIGHT	CAACCCAAGGGCATACGGT	2	0,75	59
RSVB_46_RIGHT_bis	CAACCCAAGGGCATACAGT	2	0,25	20
RSVB_48_LEFT	CTTTTCAGACAACACTCATCTGCT	2	1	78
RSVB_48_RIGHT	CTGATCTTGCATCCTGTGGAACT	2	1	78
RSVB_50_LEFT	CCCAGTTGTATAGCATTCATAGGTGA	2	0,4	31
RSVB_50_RIGHT	TTTGAAATCAATATCATCTTGAGCATGGT	2	0,4	31
RSVB_52_LEFT	GATGTTGTGCAAAATGCTAAATTGATTCT	2	0,2	16
RSVB_52_RIGHT	CACTACCTGTTATTTTAATCAGCTTCTTGA	2	0,2	16