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Version 3

# Whole-Genome Amplification of Respiratory Syncytial Virus (RSV) using Illumina CovidSeq reagents for Next-Generation Sequencing V.3

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**We use this protocol and it's working**

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## Abstract

Protocol for amplification of RSV and for whole-genome sequencing. This protocol can amplify the whole genome in two parallel RT-PCR reactions in order to make RSV sequencing accessible and affordable. This protocol does not require prior subtyping as it covers RSV-A and RSV-B in the same reaction. The primer panel is an optimisation of a previously published panel by Wang et al.

Our optimisation from the original panel allows for multiplex PCR, so the whole genome can be amplified in just two PCR reactions. In addition to this, primers have been modified to account for commonly-occurring mutations that affect primer-binding areas and were causing suboptimal amplification. These primers were used to cover the complete hRSV genome (both A and B) by splitting them into two pools of non-consecutive amplicons (odd-numbered amplicon primers in one pool, even-numbered amplicon primers in other).

This protocol has been tested for RSV RNA amplification from RSV-positive nasopharyngeal swabs of CT value up to 26 using Seegene Allplex Respiratory Panel (Seegene Inc, Seoul, South Korea). However, it provides best results with CT values below 21. For samples of low viral load, an alternative primer panel is provided only with the amplicons sufficient to sequence the G and F genes. This alternative panel has successfully sequenced samples of CT values up to 30, albeit with higher amount of total reads required in the case of very high CT value.

Illumina CovidSeq (Illumina Inc, San Diego, USA) reagents were used for the RT-PCR, with a mix previously published for amplification of Influenza RNA and a thermocycling program optimised in our lab. Library preparation was performed according to the Illumina CovidSeq protocol.


### v3 (2024.02.22)

#### Modifications from v2:

1. Primer A10f has been replaced to solve the amplicon dropout for Nextclade variant A.D.5.2 (mutation G13793A).
2. The relative concentration of primers has been modified to improve amplicon homogeneity.
3. An alternative panel has been added to amplify the G and F genes suitable for low viral load samples.
4. A bioinformatic analysis section has been added with a link to a custom pipeline for this protocol.

## Materials

 QIASymphony DSP Virus/Pathogen Midi Kit **Qiagen Catalog #937055**

 Illumina CovidSeq Assay **Illumina, Inc.**

 Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q32851**

 BioAnalyzer High Sensitivity Chip **Agilent Technologies Catalog #5067-4626**

## Troubleshooting

### Before start

This protocol was tested using as input RNA extracted from nasopharyngeal swabs after confirmation of RSV infection via RT-PCR. Samples were extracted using the QIASymphony DSP Virus/Pathogen Midi Kit (Qiagen, Hilden, Germany).

## Primer pools preparation

### 1 Pools WG: for samples of high viral load, covering the whole RSV genome.

**For a final concentration of 10uM: add 607 ul of Nuclease-Free water to Pool 1 and 639 ul to Pool 2.**

	A	B	C	D	E	F
	Primer	Volume (100uM)	Source	Sequence	Base	Pool
	A1f	2,5	Wang	ACGSGAAAAAATGCGTACAAC	1	1
	A1r	2,5	Wang	GAAGATTGTGCTATACCAAATGAAC A	1779	1
	AB3f	10	Goya	GCYATGGCAAGACTYAGGAATG	2897	1
	A3r	3,5	Wang	GTTTGCYGAGGCTATGAATATGAT	482 6	1
	A5f	2,5	Wang	GAACAACAGACTACTAGAGATTACCA G	6374	1
	A5r	5	This Publication	AGGAGTTTRCTCATRGCAA	7929	1
	A7f	2,5	Wang	AGCTTAGGCTTAAGATGYGGA	942 3	1
	A7r	2,5	Wang	TGAGTTTGACCTTCCATGAGT	1099 7	1
	A9f	5	Wang	GGGTTGGTTCATCTACACAAGAG	1231 6	1
	A9r	5	This Publication	CGCAATAATAAATTCCTGCTCC	1409 4	1
	B1f	2	Wang	ACGCGAAAAAATGCGTACTACA	1	1
	B1r	2	Wang	CATTGTTTGCCCTCCTAATTACTG	1661	1
	B3r	5	Wang	ATAGGGCCAAAATTTGCTTGTG	430 9	1
	B5f	2,5	Wang	AGTGCAATCTTCCTAACTCTTGC	5700	1
	B5r	2,5	Wang	TGATTCCACTTAGTTGGTCTTTGC	7375	1

	A	B	C	D	E	F
	B7f	2,5	Wang	GGTGAAGCTGAAATTAGAAGAACCAAC	8760	1
	B7r	2,5	Wang	CACCATATCTTGTCAAAGCTCTCAGG	1050 7	1
	B9f	5	Wang	GAACCAAGCTTACCCTCATGGATT	1186 0	1
	B9r	5	Wang	TTCTGGGGTTGGGTGATATAG	1365 0	1
	A2f	2,5	Wang	ACAGGCATGACTCTCCTGAT	1556	2
	A2r	2,5	Wang	TTGGGTGTGGATATTTGTTTCAC	340 0	2
	A4f	2,5	Wang	ACCTGGGACACTCTCAATCA	4697	2
	A4r	2,5	Wang	GACATGATAGAGTAACTTTGCTGTCT	654 0	2
	A6f	2,5	Wang	GTCACGAAGGAATCCTTGCA	7642	2
	A6r	2,5	Wang	CCCTCTACCTCTTTTATTATGTAGAA CC	9521	2
	A8f	5	Wang	GGTGTAACAATCTCTATTTTCCTGGT	1070 4	2
	A8r	5	Wang	CGATTAATAGGGCTAGTATCAAAGTG	1261 5	2
	A10f	3,5	This Publicatio n	CCCACACCAGAAACCCTAGAAA	1365 3	2
	A10r	3,5	Wang	ACGAGAAAAAAAGTGTCAAAAATA A	1522 5	2
	B2f	2	Wang	CAGRTTAGGAAGGGAAGACACTA	1316	2
	B2r	2	Wang	CAAGTCACTCAATTTTTTGGAGGTTG G	2982	2
	B4f	7,5	Wang	TGGAAGCAYACAGCTACACG	394 3	2
	B4r	7,5	Wang	CTACATGTYGATTGGTAAAGCTCC	5788	2
	B6f	2,5	Wang	CCTCTAGTGTTTCCTTCTGATGAG	7113	2
	B6r	2,5	Wang	GTTGTAGCAATTTGTTTCAGACGAG	883 4	2
	B8f	5	Wang	AAGTTCTCTGAAAGCGACAGATC	1023 1	2

	A	B	C	D	E	F
	B8r	5	Propio	TAATACTWGGTGATGTTACTCCTAC	1219 0	2
	B10f	2,5	Wang	TAGTCAATCAAGACACAAGTTTGC	1328 9	2
	B10r	2,5	Wang	ACGAGAAAAAAAGTGTCAAAAATA ATG	1522 2	2

**Table 1: mix of primers used for whole genome amplification.** Two mixes are required, one for pool 1 and another for pool 2. References for base number: hRSV/A/England/397/2017 and hRSV/B/Australia/VIC-RCH056/2019 for RSV-A and RSV-B respectively. Citation to the source papers for the primers can be found below.

**Pools GF: for samples of mid-to-low viral load, covering the G and F genes.**

**For a final concentration of 10uM: add 256 ul of Nuclease-Free water to Pool 1 and 270 ul to Pool 2.**

	A	B	C	D	E	F
	Prime r	Volum e (100u M)	Source	Sequence	Base	Pool
	AB3f	7,5	Goya	GCYATGGCAAGACTYAGGAATG	2897	1
	A3r	3,5	Wang	GTTTGCYGAGGCTATGAATATGAT	4826	1
	A5f	2,5	Wang	GAACAACAGACTACTAGAGATTACCA G	6374	1
	A5r	5	This Publicatio n	AGGAGTTTTRCTCATRGCAA	7929	1
	B5f	5	Wang	AGTGCAATCTTCCTAACTCTTGC	5700	1
	B5r	5	Wang	TGATTCCACTTAGTTGGTCTTTGC	7375	1
	A4f	5	Wang	ACCTGGGACACTCTCAATCA	4697	2
	A4r	5	Wang	GACATGATAGAGTAACTTTGCTGTCT	6540	2
	B4f	7,5	Wang	TGGAAGCAYACAGCTACACG	3943	2
	B4r	7,5	Wang	CTACATGTYGATTGGTAAACTCC	5788	2



	A	B	C	D	E	F
	B6f	2,5	Wang	CCTCTAGTGTTTCCTTCTGATGAG	7113	2
	B6r	2,5	Wang	GTTGTAGCAATTTGTTTCAGACGAG	8834	2

**Table 2: mix of primers used for G and F protein amplification.** Two mixes are required, one for pool 1 and another for pool 2. References for base number: hRSV/A/England/397/2017 and hRSV/B/Australia/VIC-RCH056/2019 for RSV-A and RSV-B respectively. Citation to the source papers for the primers can be found below.

## RT-PCR

- Two Master Mixes must be prepared per sample: one for Pool1 and one for Pool 2 (Table 2). Manipulate reagents according to the Illumina CovidSeq Reference Guide. The protocol is the same for primers WG or primers GF.

	Reagent	Amount (ul) Reaction 1	Amount (ul) Reaction 2
	IPM	15	15
	FSM	3.2	3.2
	RVT	1	1
	Nuclease-Free Water	3.6	3.6
	Primer pool 1 (10uM)	1.2	-
	Primer pool 2 (10uM)	-	1.2

**Table 3:** Master mixes required for amplification of the RSV genome. Reaction 1 targets odd-numbered amplicons while reaction 2 targets even-numbered amplicons.

In a PCR tube, mix 20 ul of MasterMix with 5 ul of extracted RNA.

Place all tubes (two per sample) in a thermocycler and run the following program (Table 3):





	A	B	C
	42°	60 min	
	98°	2 min	
	98°	15 s	35 cycles
	63°	7 min	
	4°	PAUSE	

**Table 4:** Thermocycler program for RT-PCR. Indicate 25 ul as volume and heat lid at 99°C.

### (OPTIONAL) Check RT-PCR result with Agilent Bioanalyzer

- 3 Use an Agilent Bioanalyzer to check for amplification peaks. Expect PCR peaks around ~2000 bps (Figure 1).

## Expected result



**Figure 1: RT-PCR result with peaks expected around 2000 bps.** Representative image of an Agilent bioanalyzer of the amplification products on the WG protocol. From left to right: ladder; RSV-A, pool 1; RSV-A, pool 2; RSV-B, pool 1; RSV-B, pool 2. Scale indicates size in base pairs.

## Library preparation

- 4 Mix 10ul of tube one and tube two on each sample for a final 20 ul of PCR product. Follow instructions of the Illumina CovidSeq Reference Guide to generate sequencing-ready libraries.

**Recommended:** To ensure optimal normalisation, perform the library Clean-up on each tube and normalise individually instead of pooling. This improves normalisation especially in the presence of low-concentration PCR products.

Quantify samples after Clean-up using Qubit Flex and normalise samples.

(OPTIONAL): Check library preparation on an Agilent Bioanalyzer. The pattern expected is the usual post-tagmentation pattern from Illumina libraries with the highest peak around ~330bps.

## Expected results

- 5 The following results were obtained after sequencing the samples on an Illumina NextSeq 1000 (Table 5).

Ct value for Whole-Genome amplification ranged from 15-21, while for G and F amplification was from 20 to 27. Average percentage of Illumina reads mapped to RSV was 35,52% for whole genome and 12,73% for GF amplification.

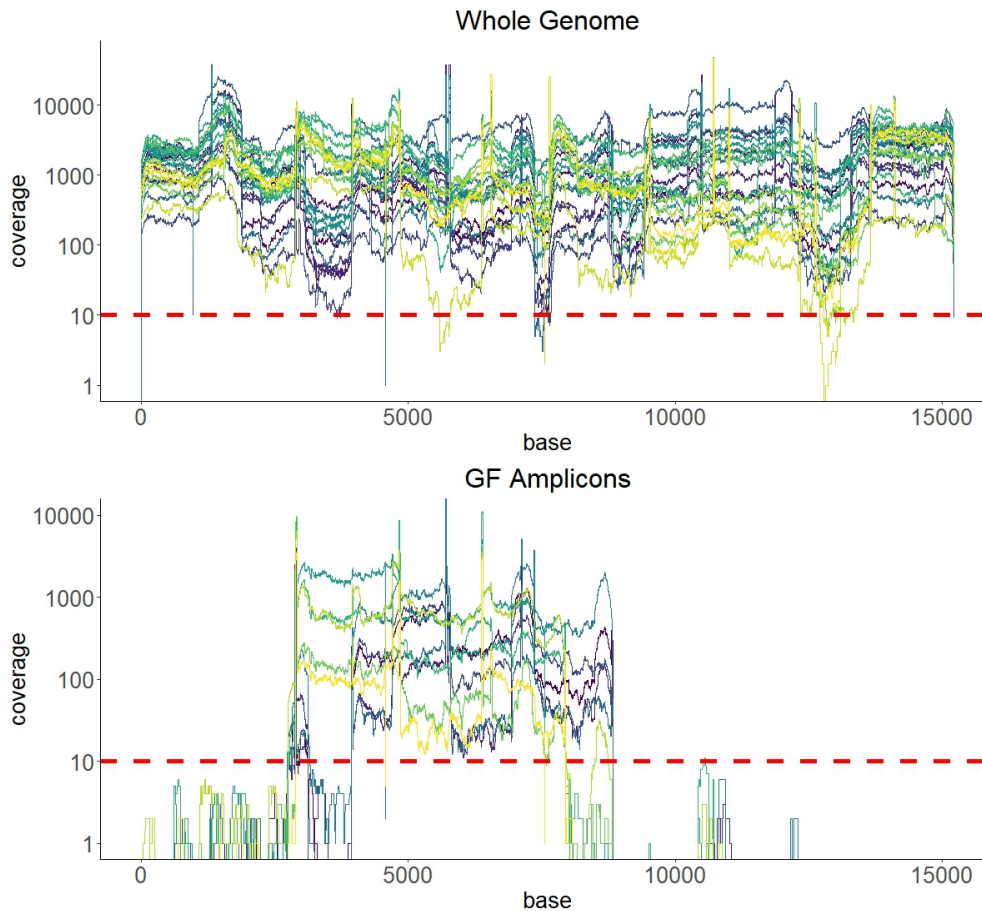
Average number of total reads was 195k for whole genome and 56k for GF, meaning that 41 and 143 samples could be sequenced in a single iSeq 100 run respectively (expecting 8M reads per run, as specified by the manufacturer).

## Expected result

	A	B	C	D	E	F	G
	ID	Protocol	Type	Ct	Reads	Mapped	% Mapped
	1	WG	RSVA	18,78	257281	95837	37,25
	2	WG	RSVA	16,03	217155	99218	45,69
	3	WG	RSVA	21,02	166115	63323	38,12
	4	WG	RSVA	18,3	252610	151591	60,01
	5	WG	RSVA	16,15	180537	108683	60,20
	6	WG	RSVA	18,72	151806	39181	25,81
	7	WG	RSVA	16,56	163103	54346	33,32
	8	WG	RSVA	17,57	198450	75927	38,26
	9	WG	RSVA	19,79	101188	13114	12,96
	10	WG	RSVA	19,83	185337	56954	30,73
	11	WG	RSVA	19,54	160465	59019	36,78
	12	WG	RSVB	20,68	195553	65041	33,26
	13	WG	RSVB	20,53	164314	20950	12,75
	14	WG	RSVB	19,33	273521	129813	47,46
	15	WG	RSVB	20,1	216850	38274	17,65
	16	WG	RSVB	19,77	190689	31540	16,54
	17	WG	RSVB	19,32	40702	7127	17,51
	18	WG	RSVB	15,78	352487	248221	70,42
	19	WG	RSVB	19,11	150137	57172	38,08
	20	WG	RSVB	18,83	152340	14259	9,36
	21	WG	RSVB	17,78	237441	128883	54,28
	22	WG	RSVB	19,16	231418	106105	45,85
	23	WG	RSVB	18,98	194417	59997	30,86
	24	WG	RSVB	19,57	236938	93306	39,38

	A	B	C	D	E	F	G
	25	GF	RSVA	24,5	64596	19831	30,70
	26	GF	RSVA	26,6	49369	7899	16,00
	27	GF	RSVA	26,9	59690	1731	2,90
	28	GF	RSVA	20,5	47327	13242	27,98
	29	GF	RSVA	20,8	33785	1223	3,62
	30	GF	RSVB	21,6	56690	5584	9,85
	31	GF	RSVB	24,3	63607	1781	2,80
	32	GF	RSVB	22,9	61577	5739	9,32
	33	GF	RSVB	26,5	60855	1424	2,34
	34	GF	RSVB	22,6	59949	13057	21,78

**Table 5: Results of sequenced samples in a NextSeq1000 run.** 24 samples sequenced full genome and 10 sequences GF in the same run.



**Figure 2: Coverage per base of all samples described in Table 3.**

## Bioinformatic Analysis

- 6 A pipeline for RSV sequencing analysis is provided in <https://github.com/OMIC-G/RSV>.

This pipeline uses Illumina reads as input and includes the BED file with the primers in this version for analysis, as well as the reference genomes recommended for the current season. The pipeline does not require prior categorization in RSV-A and B as it sorts reads in RSV-A and RSV-B.



Output provided from the pipeline includes:

1. RSV typing in RSV-A and RSV-B.
2. Two fasta files with all RSV-A and RSV-B consensus sequences.
3. A report including quality data and clade designation.
4. CLI-based upload to epiRSV GISAID database.

## References

### 7 The illumina CovidSeq protocol can be found in:

#### Illumina CovidSeq Reference Guide

**The primers found in Table 1 were obtained from:**

##### Citation

Wang L, Ng TFF, Castro CJ, Marine RL, Magaña LC, Esona M, Peret TCT, Thornburg NJ (2022)  
. Next-generation sequencing of human respiratory syncytial virus subgroups A and B genomes..

<https://doi.org/10.1016/j.jviromet.2021.114335>

LINK

##### Citation

Stephanie Goya, Gabriel L. Rojo, Mercedes S. Nabaes Jordar, Laura E. Valinotto, Alicia S Mistchenko, Mariana Viegas  
. Whole genome sequencing of respiratory syncytial (RSV) virus from clinical samples with low viral load.  
protocols.io.

<https://protocols.io/view/whole-genome-sequencing-of-respiratory-syncytial-r-bmhak32e>

LINK



## The Master Mix used for RT-PCR with Illumina CovidSeq was first published in:

### Citation

Ying Lin, Jeffrey Koble, Priyanka Prashar, Anita Pottekat, Christina Middle, Scott Kuersten, Michael Oberholzer, Robert Brazas, Darcy Whitlock, Robert Schlaberg, Gary P. Schroth

. A sequencing and subtyping protocol for Influenza A and B viruses using Illumina® COVIDSeq™ Assay Kit.

protocols.io.

<https://protocols.io/view/a-sequencing-and-subtyping-protocol-for-influenza-crv3v68n>

LINK