Aug 02, 2023 Version 2

Whole-Genome Amplification of Respiratory Syncytial Virus (RSV) using Illumina CovidSeq reagents for Next-Generation Sequencing V.2

DOI
dx.doi.org/10.17504/protocols.io.eq2lyjzbrlx9/v2

Carlos Davina-Nunez[1,2], Sonia Perez-Castro[1,3], Montse Godoy-Diz[3], Benito Regueiro-Garcia[1]

[1]Microbiology and Infectology Department, IIS Galicia Sur; [2]Universidade de Vigo, Vigo, Spain; [3]Microbiology Department, Complexo Hospitalario Universitario de Vigo (CHUVI), Spain

Carlos Davina-Nunez
Microbiology and Infectology Department, IIS Galicia Sur, Un...

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Protocol Citation: Carlos Davina-Nunez, Sonia Perez-Castro, Montse Godoy-Diz, Benito Regueiro-Garcia 2023. Whole-Genome Amplification of Respiratory Syncytial Virus (RSV) using Illumina CovidSeq reagents for Next-Generation Sequencing. **protocols.io https://dx.doi.org/10.17504/protocols.io.eq2lyjzbrlx9/v2**Version created by **Carlos Davina-Nunez**

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

Created: August 02, 2023

Last Modified: August 02, 2023

Protocol Integer ID: 85833

Keywords: Respiratory Syncytial Virus, RSV, NGS, Illumina, CovidSeq, amplification, PCR, genome amplification of respiratory syncytial virus, respiratory syncytial virus, genome amplification, complete hrsv genome, numbered amplicon primer, amplicon primer, amplification of influenza rna, amplification of rsv, pcr reaction, using seegene allplex respiratory panel, seegene allplex respiratory panel, multiplex pcr, sequencing, pcr, influenza rna, positive nasopharyngeal swabs of ct value, illumina covidseq reagents for next, whole genome, positive nasopharyngeal swab, using illumina covidseq reagent, rna, rsv, primer, occurring mutation, amplicon

Abstract

This protocol has been tested for amplification of RSV-positive nasopharyngeal swabs of CT value up to 24 using Seegene Allplex Respiratory Panel (Seegene Inc, Seoul, South Korea). This protocol does not require prior subtyping as it covers RSV-A and RSV-B in the same reaction. Panel of primers is an optimisation of a previously published panel by Wang et al.

This panel was modified to optimise the 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 in the 22-23 season 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 into two pools of non-consecutive amplicons (odd-numbered amplicon primers in one pool, even-numbered amplicon primers in other). This allowed for Whole-genome amplification in two reactions.

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. The library preparation part of the protocol was performed according to the Illumina CovidSeq 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 uses 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 Prepare both primer mixes according to Table 1.

For a final concentration of 10uM: add 1017 ul of Nuclease-Free water to Pool 1 and 1035 ul to Pool 2.

	Primer	Volume (100uM)	Reference	Sequence	Base	Pool
	A1f	5	Wang	ACGSGAAAAAATGCGTACAAC	1	1
	A1r	5	Wang	GAAGATTGTGCTATACCAAAATGAACA	1779	1
	AB3f	10	Goya	GCYATGGCAAGACTYAGGAATG	2897	1
	A3r	5	Wang	GTTTGCYGAGGCTATGAATATGAT	4826	1
	A5f	5	Wang	GAACAACAGACTACTAGAGATTACCAG	6374	1
	A5r	10	This publication	AGGAGTTTRCTCATRGCAA	7929	1
	A7f	5	Wang	AGCTTAGGCTTAAGATGYGGA	9423	1
	A7r	5	Wang	TGAGTTTGACCTTCCATGAGT	10997	1
	A9f	7	Wang	GGGTTGGTTCATCTACACAAGAG	12316	1
	A9r	7	This publication	CGCAATAATAAATTCCCTGCTCC	14094	1
	B1f	5	Wang	ACGCGAAAAAATGCGTACTACA	1	1
	B1r	5	Wang	CATTGTTTGCCCTCCTAATTACTG	1661	1
	B3r	5	Wang	ATAGGGCCAAAATTTGCTTGTG	4309	1
	B5f	5	Wang	AGTGCAATCTTCCTAACTCTTGC	5700	1
	B5r	5	Wang	TGATTCCACTTAGTTGGTCTTTGC	7375	1

	Primer	Volume (100uM)	Reference	Sequence	Base	Pool
	B7f	5	Wang	GGTGAACTGAAATTAGAAGAACCAAC	8760	1
	B7r	5	Wang	CACCATATCTTGTCAAACTCTCAGG	10507	1
	B9f	7	Wang	GAACCAACTTACCCTCATGGATT	11860	1
	B9r	7	Wang	TTCTGGGGTTGGGTGATATAG	13650	1
	A2f	5	Wang	ACAGGCATGACTCTCCTGAT	1556	2
	A2r	5	Wang	TTGGGTGTGGATATTTGTTTCAC	3400	2
	A4f	5	Wang	ACCTGGGACACTCTCAATCA	4697	2
	A4r	5	Wang	GACATGATAGAGTAACTTTGCTGTCT	6540	2
	A6f	5	Wang	GTCACGAAGGAATCCTTGCA	7642	2
	A6r	5	Wang	CCCTCTACCTCTTTTATTATGTAGAACC	9521	2
	A8f	5	Wang	GGTGTACAATCTCTATTTTCCTGGT	10704	2
	A8r	5	Wang	CGATTAATAGGGCTAGTATCAAAGTG	12615	2
	A10f	10	This publication	CRTCTACAATGATTAGAACCAATTAC	13742	2
	A10r	10	Wang	ACGAGAAAAAAAGTGTCAAAAACTAA	15225	2
	B2f	5	Wang	CAGRTTAGGAAGGGAAGACACTA	1316	2
	B2r	5	Wang	CAAGTCACTCAATTTTTTGGAGGTTGG	2982	2
	B4f	10	Wang	TGGAAGCAYACAGCTACACG	3943	2
	B4r	10	Wang	CTACATGTYGATTGGTAAAACTCC	5788	2
	B6f	5	Wang	CCTCTAGTGTTTCCTTCTGATGAG	7113	2

	Primer	Volume (100uM)	Reference	Sequence	Base	Pool
	B6r	5	Wang	GTTGTAGCAATTTGTTC AGACGAG	8834	2
	B8f	5	Wang	AAGTTCTCTGAAAGCG ACAGATC	10231	2
	B8r	5	This publication	TAATACTWGGTGATGTT ACTCCTAC	12190	2
	B10f	5	Wang	TAGTCAATCAAGACACA AGTTTGC	13289	2
	B10r	5	Wang	ACGAGAAAAAAAGTGT CAAAAACTAATG	15222	2

Table 1: mix of primers used for 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 original papers for the primers can be found below.

RT-PCR

2 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.

	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 2: 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):

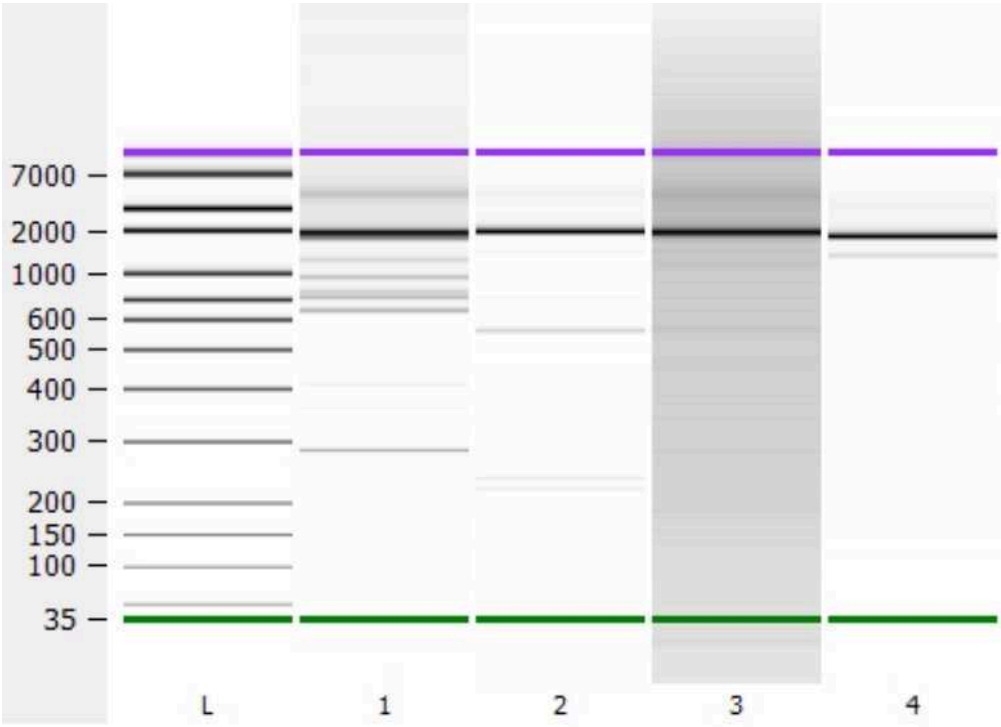
	A	B	C
	42º	60 min	
	98º	2 min	
	98º	15 s	35 cycles
	63º	7 min	
	4º	PAUSE	

Table 3: Thermocycler program for RT-PCR. Indicate 25 ul as volume and heat lid at 99ºC.

(OPTIONAL) Check RT-PCR result with Agilent Bioanalyzer

3 Use an Agilent Bioanalyzer to check for amplification peaks. Expect PCR peaks around ~2000 bps.

Expected result



RT-PCR result: peaks expected around 2000 bps. Representative image of an Agilent bioanalyzer of the amplification products. 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.

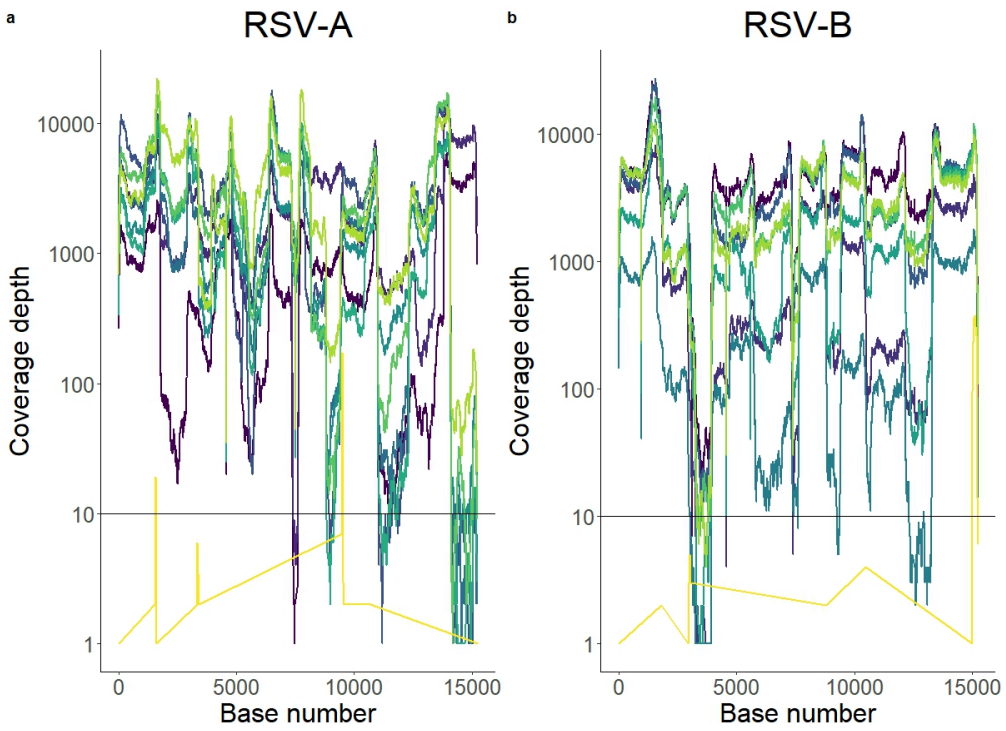
Quantify samples after Clean-up using Qubit Flex and normalise samples.

(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 Coverage obtained after an iSeq run with 16 samples: 8 from RSV-A, 7 from RSV-B and a negative control.
Average reads per sample (excluding negative control): 361k

Expected result



Expected result after sequencing: All samples had a depth above 10 for more than 85% of the genome. Aditionally depth over 10 was reached for the full G protein and 98% of the F protein in all samples.

The coverage of the negative control (Non-Template Control) appears in yellow after alignment to RSV-A reference (left) and RSV-B (right).

References

6 **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 (2021)
. 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
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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

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