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RSVAB WGS and GF protocols V.5

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Manuscript citation:
Generic novel system for genomic characterization of Respiratory Syncytial Virus obtaining whole genome sequencing and a full-length G and F sequences.

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

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Keywords: novel rsv amplicon, viral genome, cdna from rsv, rsvab wg, viral nucleic acid extract, rsv, specific sequences of the main antigen, systems for genomic characterization, generating cdna, producing amplicon, genomic characterization, main antigen

Abstract

This SOP describes the procedure for generating cDNA from RSV viral nucleic acid extracts and subsequently producing amplicons tiling the viral genome using. We propose two systems for genomic characterization of RSV. First, a novel RSV amplicon-based system for WGS, and second, a method focused on obtaining the specific sequences of the main antigens, G and F.

Sample management

1 No prior treatment is necessary. However, in samples with high Cts, the use of DNAseI in the sample as a pretreatment can improve coverage.

dsCDNA generation:

2

During this step three master mixes will be prepared: MMI, MMII and MMIII.

Materials: **Kit Superscript III First Strand (Invitrogen)**
100% DMSO
RNAseH (Invitrogen)
Klenow fragment 3' →5' exo (New England Biolabs)
Primer FR26RV-N : 5′GCC GGA GCT CTG CAG ATA TCNNNNNN 3′

Note

This step must be performed in a RNase free, pre-PCR environment in which post PCR RSV amplicons are not present, to minimise risk of sample contamination.

Citation

Díez-Fuertes F, Iglesias-Caballero M, García-Pérez J, Monzón S, Jiménez P, Varona S, Cuesta I, Zaballos Á, Jiménez M, Checa L, Pozo F, Pérez-Olmeda M, Thomson MM, Alcamí J, Casas I
(2021). A Founder Effect Led Early SARS-CoV-2 Transmission in Spain.

https://doi.org/10.1128/JVI.01583-20 LINK

3 **MMI Preparation:**

	A	B
	FR26RV-N (10uM)	2
	DMSO	0,5
	Total	2,5 ul

Mix thoroughly by vortexing.

4 **MMII Preparation:**

	A	B
	10x First Strand Buffer	2
	DTT 100 mM	2
	MgCl2 25mM	4
	dNTPs	1
	RNaseOUT	0,5
	SSIII RT	0,5
	Total	10 ul

Kit Superscript III First Strand (Invitrogen)

5 **MMIII Preparation:**

	A	B
	Klenow 5'-3'	1
	RNAseH	0,5
	Total	1,5 ul

6 Defrost extracted RNA.

Maintain **on ice** the MMI,MMII and MMIII mixes.

7 **MMI Amplification:**

Add 5 µL Sample in MMI mix

Place the tube on a thermocycler and run the following program:

	A	B
	65ºC	5 min
	4ºC	2 min

Briefly tube centrifugation

8 **MMII Amplification:**

Addition of 10 µL from MMII in the tube with the MMI and the viral extraction.

Place the tube on a thermocycler and run the following program:

	A	B
	25ºC	10 min
	50ºC	50 min
	85ºC	10 min
	4ºC	∞

Briefly tube centrifugation

9 **MMIII Amplification:**

Addition of 1.5 µL of MMIII into the tube with the previous mixes and the viral extraction

Place the tube on a thermocycler and run the following program:

	A	B
	37ºC	60 min

	A	B
	75ºC	15 min

Briefly tube centrifugation

10 **STOP POINT**: cDNA can be stored at 4°C (same day) or -20°C (up to a week).

RSVAB WGS protocol

11

Citation

Iglesias-Caballero M, Camarero-Serrano S, Varona S, Mas V, Calvo C, García ML, García-Costa J, Vázquez-Morón S, Monzón S, Campoy A, Cuesta I, Pozo F, Casas I (2023). Genomic characterisation of respiratory syncytial virus: a novel system for whole genome sequencing and full-length G and F gene sequences.

https://doi.org/10.2807/1560-7917.ES.2023.28.49.2300637 LINK

Materials:

2x MyTaqRed mix (Bioline)
Primers:

	A	B
	Primer ID	**Sequence (5′-3′)**
	Mix 1	
	RSVCombinitial	ACGCGAAAAAATGCGTACWACA
	RSVWGS1R.2	GCKATTGCAGATCCWACACC

	A	B
	RSVWGS2F	CACTWACAATATGGGTGCC
	RSVWGS2R.2	CRTTYCTTAARGTRGGCC
	RSVWGS4R	CATGWTGWYTTATTTGCCCC
	RSVWGS3.2F	ACATGGAAAGAYATYAGCC
	RSVWGS3.2R	TTGCATCTGTAGCAGGAATGG
	RSVWGS9F	GARCAACTCAAAGAAAATGG
	RSVWGS9R	AYTGRAACATRGGCACCC
	RSVCombending	ACGAGAAAAAAAGTGTCAAAAACTAA
	Mix 2	
	RSVCombinitial	ACGCGAAAAAATGCGTACWACA
	RSVWGS1R.2	AYTGRAACATRGGCACCC
	RSVWGS5F	CATAATTAYTTTGAATGGC
	RSVWGS5R	CAAACATTTAATCTRCTAAGGC
	RSVWGS6F	TTATAYAGATATCAYATGGGTGG
	RSVWGS6R	CCCTCTCCCCAATCTTTTTC
	RSVWGS13F	AAAAGATTGGGGAGAGGG
	RSV OG1_21	GGGGCAAATGCAACCATGTCC
	RSV GF_R	TTCGYGACATATTTGCCCC
	RSVCombending	ACGAGAAAAAAAGTGTCAAAAACTAA

	A	B	C	D	E	F	G
	1	EPI_ISL_18668201	-	13	RSVCombinitial	1	+
	2	EPI_ISL_18668201	2193	2212	RSVWGS9F	1	+
	3	EPI_ISL_18668201	2321	2340	RSVWGS4R	2	-
	4	EPI_ISL_18668201	3337	3355	RSVWGS_2F	1	+
	5	EPI_ISL_18668201	3349	3366	RSVWGS9R	2	-
	6	EPI_ISL_18668201	4656	4676	OG121	1	+
	7	EPI_ISL_18668201	6123	6142	RSVWGS_1R.2	2	-
	8	EPI_ISL_18668201	7647	7665	RSVGF-R	2	-
	9	EPI_ISL_18668201	7712	7730	RSVWGS_5F	1	+
	10	EPI_ISL_18668201	9374	9395	RSVWGS_5R	2	-
	11	EPI_ISL_18668201	9358	9376	RSVWGS_3.2F	1	+
	12	EPI_ISL_18668201	9986	10003	RSVWGS_2R.2	2	-
	13	EPI_ISL_18668201	10852	10874	RSVWGS_6F	1	+
	14	EPI_ISL_18668201	13076	13093	RSVWGS_13F	1	+
	15	EPI_ISL_18668201	13090	13109	RSVWGS_6R	2	-
	16	EPI_ISL_18668201	14267	14287	RSVWGS_3.2R	2	-
	17	EPI_ISL_18668201	15200	15225	RSVCombEnding	2	-
	1	EPI_ISL_1653999	1	22	RSVCombinitial	1	+
	2	EPI_ISL_1653999	2159	2178	RSVWGS9F	1	+
	3	EPI_ISL_1653999	2288	2307	RSVWGS4R	2	-

	A	B	C	D	E	F	G
	4	EPI_ISL_1653999	3311	3329	RSVWGS_2F	1	+
	5	EPI_ISL_1653999	3323	3340	RSVWGS9R	2	-
	6	EPI_ISL_1653999	4631	4651	OG121	1	+
	7	EPI_ISL_1653999	6102	6121	RSVWGS_1R.2	2	-
	8	EPI_ISL_1653999	7618	7636	RSVGF-R	2	-
	9	EPI_ISL_1653999	7699	7717	RSVWGS_5F	1	+
	10	EPI_ISL_1653999	9344	9365	RSVWGS_5R	2	-
	11	EPI_ISL_1653999	9328	9346	RSVWGS_3.2F	1	+
	12	EPI_ISL_1653999	9956	9973	RSVWGS_2R.2	2	-
	13	EPI_ISL_1653999	10822	10844	RSVWGS_6F	1	+
	14	EPI_ISL_1653999	13062	13079	RSVWGS_13F	1	+
	15	EPI_ISL_1653999	13060	13059	RSVWGS_6R	2	-
	16	EPI_ISL_1653999	14237	14257	RSVWGS_3.2R	2	-
	17	EPI_ISL_1653999	15209	15222	RSVCombEnding	2	-

Primer scheme with RSVA and RSVB RefSeq

Note

The protocol is based in the RSV genome amplification in two separate mixes with an unique amplification program. The mixes that will be mixed at the end of cycling.

12 **..2Preparation of RSV Amplification Mix 1:**

	A	B
	MyTaq Red 2x	15
	H20	8,4

	A	B
	RSV Combinitial (5 uM)	0,2
	RSVWGS1R2.2 (5uM)	0,2
	RSVWGS2F (5 uM)	0,2
	RSVWGSW2R.2 (5 uM)	0,2
	RSVWGS4R (5 uM)	0,2
	RSVWGS3.2F (5 uM)	0,2
	RSVWGS3.2R (5 uM)	0,2
	RSVWGS9F (5uM)	0,2
	RSVWGS9R (5 uM)	0,2
	RSV Combending (5uM)	0,2
	Total	25

13 **Preparation of RSV Amplification Mix 2:**

	A	B
	2x My Taq Red	15
	H2O	7,6
	RSV Combinitial (5uM)	0,2
	RSVWGS1R.2 (5 uM)	0,2
	RSVWGS5F (5 uM)	0,2
	RSVWGS5R (5 uM)	0,2
	RSVWGS6F (5 uM)	0,2
	RSVWGS6R (5 uM)	0,2
	RSVWGS9F (5 uM)	0,2
	RSVWGS9R (5 uM)	0,2
	RSVWGS13F (5 uM)	0,2
	RSV OG1_21 (5 uM)	0,2
	RSV GF_R (5 uM)	0,2

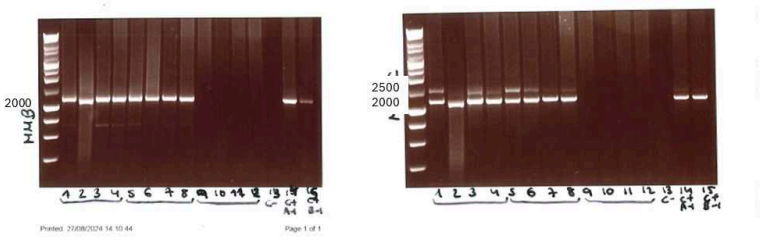
	A	B
	RSV Combending (5 uM)	0,2
	Total	25 ul

14 Addition of 5 µL of the previous prepared double stranded cDNA on each mix.

15 **Amplification protocol:**

	A	B	C
	95ºC	1 min	
	95ºC	30 seg	x45
	55ºC	8 min	
	72ºC	2 min	
	72ºC	5 min	
	12ºC	∞	

16 To assess PCR performance, the amplicons can be loaded onto a 1% agarose gel for electrophoresis.



1-4: <22
5-8: 22-28
9-12: >30

17 Finally, mix in one single tube both mixes and proceed to purification and library preparation.

RSVAB GF protocol starting from ds cDNA

18 Due to the significance of achieving accurate RSV genomic characterization, it was developed the RSVAB-GF PCR to complement the genomic coverage of both antigenic major proteins in cases where WGS encounters difficulties, and to provide a simpler and more cost-effective method of obtaining the sequences of both antigens.

19 Materials:
2x MyTaqRed mix (Bioline)
Primers:

	A	B
	RSVWGS_2F	CACTWACAATATGGGTGCC
	RSVGF-R	TTCGYGACATATTTGCCCC

20 **Preparation of cDNA GF amplification mix:**

	A	B
	H20	5,5
	2X MyTaqRed	12,5
	RSVWGS_2F	1
	RSVGF-R (10 uM)	1
	Total	20 ul

21 Addition of 5 µL of the previous prepared double stranded cDNA on the mix.

22 **Amplification protocol cDNA GF:**

	A	B	C
	95ºC	1 min	
	95ºC	30 seg	x35
	60ºC	3 min	
	72ºC	2 min	
	72ºC	5 min	
	12 ºC	∞	

RSVAB GF protocol starting from viral extraction

23 Materials:
Qiagen OneStep RT-PCR kit.

24 **Preparation of GF amplification mix:**

	A	B
	H20	10
	5xQ PCR MM	6
	dNTPs	1
	RSVWGS_2F (10uM)	1
	RSVGF-R (10 uM)	1
	RT-PCR mix	1
	Total	20 ul

25 Addition of 10 µL of the viral extraction

26 **Amplification protocol GF:**

	A	B	C
	48ºC	60 min	
	95ºC	15 min	
	95ºC	30 seg	x 45
	55ºC	2 min	
	72ºC	1 min	
	72ºC	5 min	
	12ºC	∞	

Citations

Step 11

Iglesias-Caballero M, Camarero-Serrano S, Varona S, Mas V, Calvo C, García ML, García-Costa J, Vázquez-Morón S, Monzón S, Campoy A, Cuesta I, Pozo F, Casas I. Genomic characterisation of respiratory syncytial virus: a novel system for whole genome sequencing and full-length G and F gene sequences.
https://doi.org/10.2807/1560-7917.ES.2023.28.49.2300637

Step 2

Díez-Fuertes F, Iglesias-Caballero M, García-Pérez J, Monzón S, Jiménez P, Varona S, Cuesta I, Zaballos Á, Jiménez M, Checa L, Pozo F, Pérez-Olmeda M, Thomson MM, Alcamí J, Casas I. A Founder Effect Led Early SARS-CoV-2 Transmission in Spain.
https://doi.org/10.1128/JVI.01583-20