Severe Acute Respiratory Syndrome (SARS)-like coronavirus RdRp sequencing

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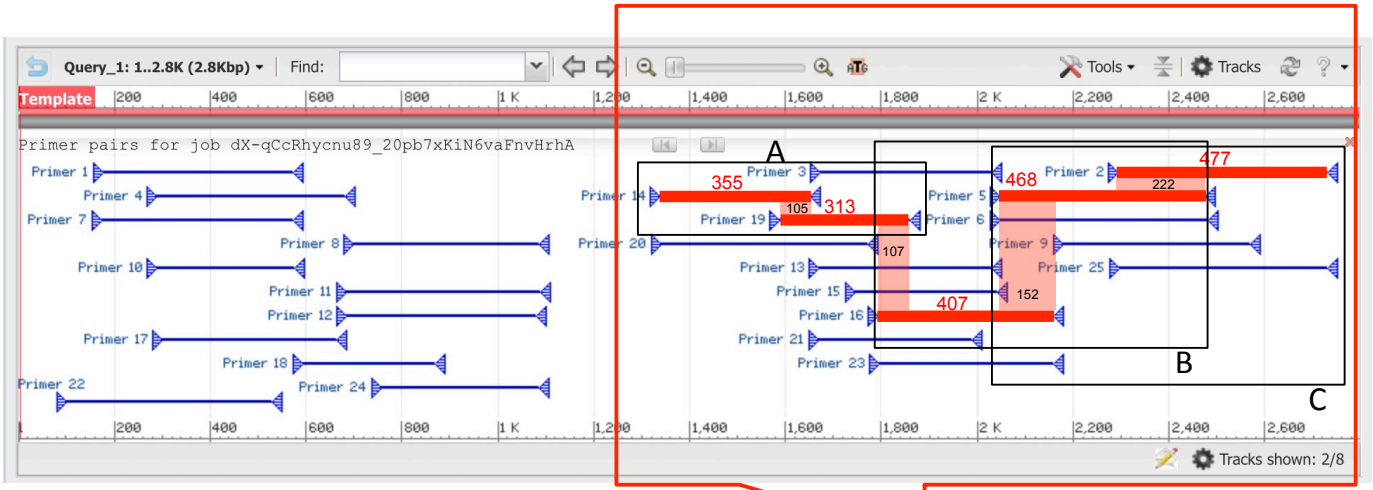
This protocol describes the semi-nested RT-PCR approach for the amplification and sequencing of the SARS-like Coronavirus RNA-dependent RNA-polymerase (RdRp) encoding gene for confirmatory sequence based typing and phylogenetic analysis. Co V are a group IV ((+)ssRNA) species of the Nidovirales order, Coronaviridae family, Coronavirinae subfamily and Betacoronavirus genus. Beta and alphacoronaviruses infect mammals only whereas gammacoronavirus infect birds and the host specificity of deltacoronaviruses remains unknown. Betacoronaviruses have 4 different lineages: lineage A represented by the human HCoV-Z29E, lineage B represented by human and bat SARS-CoV and bat ZB6V, lineage C represented by the human, camel and bat MERS-CoV, bat BtCoV, HKU4 & HKU5 and lineage D represented by the bat BtCoV-HKU9. The 29 to 33 kb CoV genome is non-segmented and contains 5′-methylated caps and 3′-polyadenylated tails. Their genome encodes for replicase, structural proteins like the spike glycoprotein (S), the envelope protein (E), membrane protein (M) and nucleocapsid protein (N) which are essential for virus-cell-receptor binding, virion assembly and immunomodulatory effects. The partially overlapping 5′-terminal ORF (ORF1a/b) encodes the large replicase polyprotein 1a (pp1a) and pp1ab which are cleaved by proteases to produce non-structural proteins including the RNA-dependent RNA polymerase (RdRp) and helicase (Hel), involved in the transcription and replication of CoVs.

SARS-like CoV contig amplification assembly & sequencing approach (NC_004718.3),



Priority region (1434 bp, from position 1318 to 2751)

Note 1: The 3' half of the RdRp gene was given high priority for the purpose of confirmatory genotyping of SARS-like CoV sequences and to allow at least some phylogenetic analysis of nucleotide sequences. Other primer sequences have been omitted from this protocol. Initial RT-PCR considers fragment A-C amplification after which fragments 14, 19, 16, 5 and 2 are amplified in the semi-nested PCR. High-resolution image available from http://www.genomica.uaslp.mx/Research/Exotic_Pathogens/SARS_Seq.jpg.

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Oligonucleotide primers

Name	Sequence[1]	bp	Start	Stop	Tm	%GC	Hair	HomD	HetD	Amplicon
SARS-14F	TCTTCTTTGCTCAGGATGGCA	21	1318	1338	59.65	47.62	-0.63	-5.09	-6.31	355 bp
SARS-14R	GCTACGGTGCGAGCTCTATT	20	1672	1653	59.97	55	-0.61	-9.49		
SARS-19F	TCAAGATGCACTTTTCGCGT	20	1568	1587	58.77	45	-0.25	-10.36	-6.57	313 bp
SARS-19R	AGGCATGGCTCTGTCACATT	20	1880	1861	59.67	50	0.1	-5.38		
SARS-16F	GCAAGTTTTACGGTGGCTGG	20	1774	1793	60.04	55	0.38	-3.61	-7.81	407 bp
SARS-16R	GAGCCTGTGTTGTAGATTGCG	21	2180	2160	59.61	52.38	0.84	-3.61		
SARS-5F	CAGGTGGAACATCATCCGGT	20	2029	2048	59.75	55	-0.59	-9.75	-3.61	430 bp
SARS-5R	CTGGGTAAGGCAGGTACACG	20	2496	2477	60.11	60	-0.04	-3.65		
SARS-2F	CTGATGATGCCGTTGTGTGC	20	2275	2294	60.18	55	0.55	-3.61	-3.61	477 bp
SARS-2R	GTTCCCAGTACCGTGAGGTG	20	2751	2732	60.04	60	-3.06	-4.41		
14F+19R									-5.09	563 bp
16F+5R									-4.25	723 bp
5F+2R									-9.36	723 bp

Note 2: As produced by NCBI's Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Reverse oligonucleotide primer sequences given in this table are the reverse-complement of sequences present in alignments and as they should be ordered for synthesis; Hairpin (Hair), homodimer (homD) and heterodimer (HetD) DQ's given in kcal/mol.

The document http://www.genomica.uaslp.mx/Research/Exotic_Pathogens/SARS_CoV_RdRp_Aln_v4.pdf provides a detailed oligonucleotide map of RdRp-encoding region.

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One-step RT-PCR components and conditions

This One-step RT-PCR procedure uses Invitrogen's SuperScript™ One-Step RT-PCR System with Platinum™ *Taq* DNA Polymerase (Invitrogen Cat. No.: 10928-042). PCR is carried out using Vivantis Taq DNA Pol (Cat: PL1202).

RT-PCR		1x
dH_2O	cf	0 µL
2x Reaction buffer mix	1 x	6.25 µL
RT Enzyme mix	1 x	0.25 µL
10 µM Forward oligo	400 nM	0.25 µL
10 µM Reverse oligo	400 nM	0.25 µL
Template	< 1 µg	5.5 µL
		vf: 12.5 µl

↓

Run in "CoV" program in TC-1

→

Total time: 2:58 hrs		
50 ºC	30 min	
94 ºC	2 min	
94 ºC	20 sec	40 cycles
56 ºC	30 sec	
70 ºC	30 sec	
70 ºC	5 min	
4 ºC	5 min	
RT	∞	

IMPORTANT (Note 3): Dilute RT-PCR product 1:5 by adding 62.5 µL dH_2O to the 12.5 µL of RT-PCR product.

PCR		1x
dH_2O	cf	5.275 µL
2x Reaction buffer mix	1 x	1.25 µL
$MgCl_2$	2.5 mM	0.625 µL
10 mM dNTPs	200 µM	0.25 µL
10 µM Fwd Oligo	400 nM	0.5 µL
10 µM Rev Oligo	400 nM	0.5 µL
Taq DNA Polymerase	400 nM	0.13 µL
Template	< 1 µg	4 µL
		vf: 12.53 µl

↓

Run in "CoV" program in TC-1 or TC-2

→

Total time: 2:28 hrs		
94 ºC	3 min	
94 ºC	20 sec	40 cycles
56 ºC	30 sec	
70 ºC	30 sec	
72 ºC	5 min	
4 ºC	5 min	
RT	∞	

Note 4: Samples with low RNA or viral load might benefit from using 6 - 9 µL of 1:5 diluted RT-PCR product (decreasing dH_2O volume to 3.275 or 0.275 µL, correspondingly).

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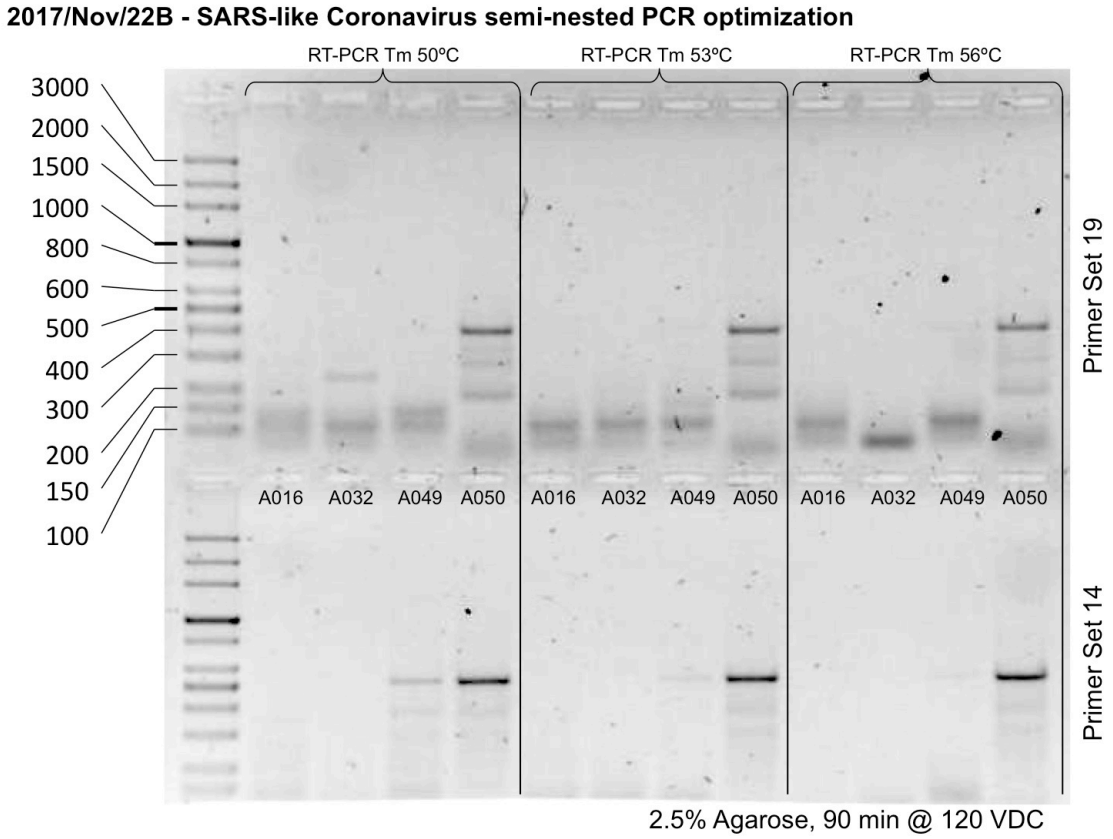
Agarose gel electrophoresis

Prepare 2.5% agarose gel using 6.25 grs agarose in 250 mL TBE buffer. Run at 120 VDC for 90 minutes.

Add 5 µL of orange G loading buffer to 12.53 µL of PCR product and load 15 µL into each well (see http://www.genomica.uaslp.mx/Protocolos/Mol_Load_Buffers.pdf for loading buffer preparation).



References

1. Invitrogen SuperScript® One-Step RT-PCR with Platinum® Taq insert. Document part number 10928.pps, Revision 3.0, Publication number: MAN0000919.

Revision history

1.0 Original document.

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