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Technique: Nested RT-PCR and Sequencing

Methodology: The protocol is designed to target two genes: *ORF1a* and *S*. The results are evaluated through agarose gel electrophoresis followed by sequencing to determine the match to WH-human1 sequence (MN908947).

Material:

- QIAamp Viral RNA Mini Kit (Qiagen)
- SuperScript IV Reverse Transciptase (ThermoFisher)
- Random Primer (ThermoFisher)
- OligodT Primer (ThermoFisher)
- Quick Taq® HS DyeMix (TOYOBO)
- AMpure XP for PCR Purification

- <u>Primers sequences</u>

  ORF1a gene

  A) NIID_WH-1_F501: 5'-TTCGGATGCTCGAACTGCACC-3'
  B) NIID_WH-1_R913: 5'-CTTTACCAGCACGTGCTAGAAGG-3'
  C) NIID_WH-1_F509: 5'-CTCGAACTGCACCTCATGG-3'
  D) NIID_WH-1_R854: 5'-CAGAAGTTGTTATCGACATAGC-3'
  E) NIID_WH-1_Seq_F519: 5'-ACCTCATGGTCATGTTATGG-3'
  F) NIID_WH-1_Seq_R840: 5'-GACATAGCGAGTGTATGCC-3'

  S gene

  G) WuhanCoV-spk1-f: 5'-TTGGCAAAATTCAAGACTCACTTT-3'
  H) WuhanCoV-spk2-r: 5'-TGTGGTTCATAAAAATTCCTTTGTG-3'
  I) NIID_WH-1_F24381: 5'-TCAAGACTCACTTTCTTCCAC-3
  J) NIID_WH-1_R24873: 5'-ATTTGAAACAAAGACACCTTCAC-3
  K) NIID_WH-1_Seq_F24383: 5'-AAGACTCACTTTCTTCCACAG-3
  L) NIID_WH-1_Seq_R24865: 5'-CAAAGACACCTTCACGAGG-3

Protocol:

1. Viral RNA extraction, performed according to QIAamp viral RNA mini kit manufacturer's instructions

2. Synthesis of first strand cDNA using SuperScript IV Reverse Transciptase (ThermoFisher), Random Primer (ThermoFisher) and OligodT Primer (ThermoFisher), according to manufacturer's instructions.

3. Preparation of 1st PCR reactions with Quick Taq® HS DyeMix (TOYOBO) according to manufacturer's instructions. Primers used in each PCR and PCR conditions are presented below. DDW should be used as negative control.

Primers used in each PCR reaction				
	***ORF1a* gene**		***S* gene**	
	Primers*	**Expected size (bp)**	**Primers***	**Expected size (bp)**
1st PCR	A and B	413	G and H	547
2nd PCR	C and D	346	I and J	493
Sequencing	E and F	-	K and L	-

* Primers concentration in all reactions is 400nM.

Temperature (°C)	Time	No of Cycles
94	1 min	1
94 56 68	30 sec 30 sec 1 min	40

4. Preparation of nested PCR (2nd PCR) with the according primers and 1µL of the product of 1st PCR. The PCR is performed under the same conditions as 1st PCR. DDW should be used as negative control.

5. Visualization of each PCR product (1st and 2nd) by 2% agarose gel electrophoresis.

6. Purification of PCR products with AMpure XP.

7. Sequencing of PCR products with the appropriate primers (indicated above). The results are compared with the sequence of WH-human1 (MN908947).