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Supplemental Information

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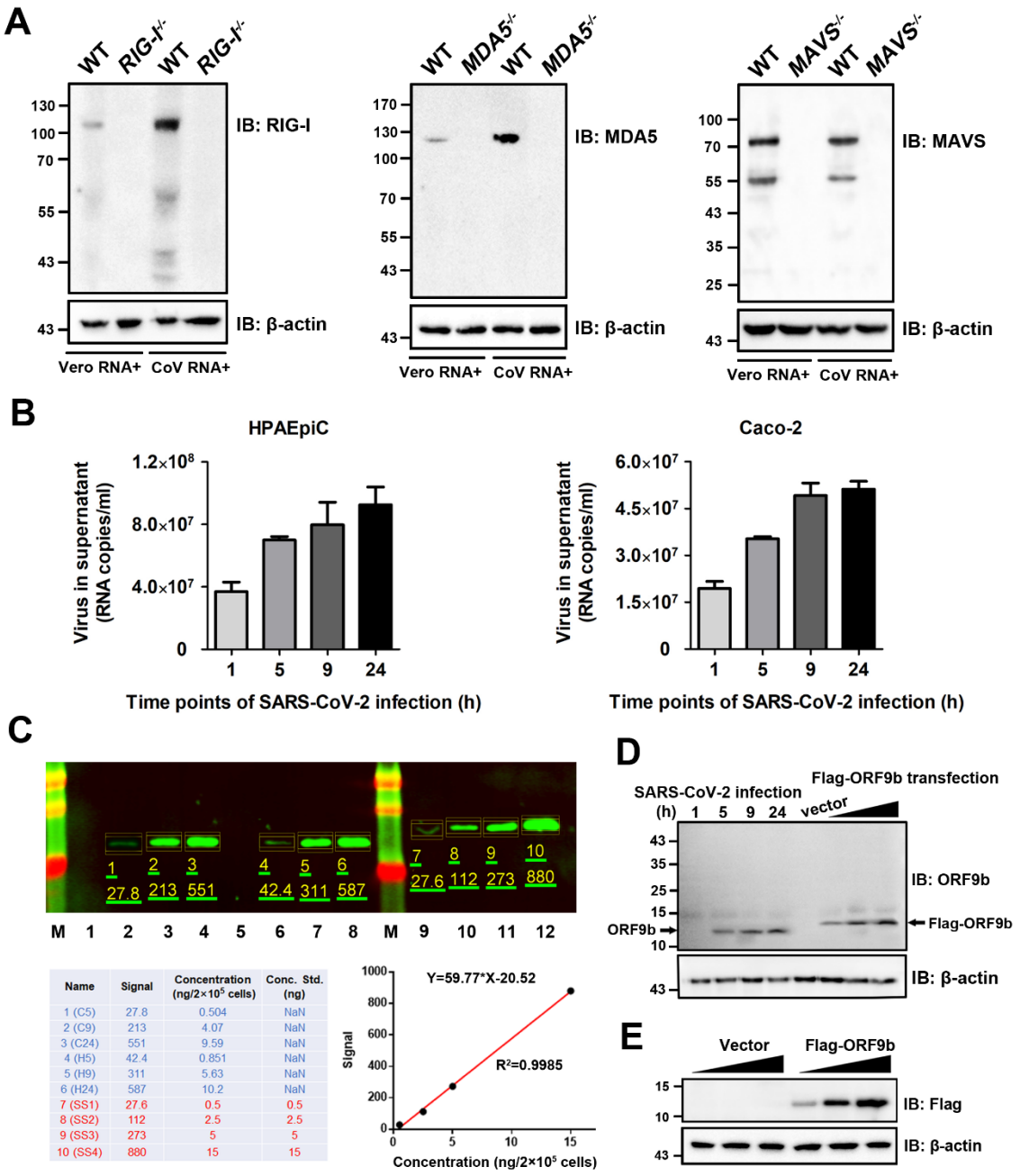

Figure S1. Endogenous and ectopic expression levels of different proteins in various human cell lines, related to Figure 1

(A) Wildtype (WT), *DDX58*$^{-/-}$ (*RIG-I*$^{-/-}$), *IFIH1*$^{-/-}$ (*MDA5*$^{-/-}$) and *MAVS*$^{-/-}$ HEK293T cells were transfected for 12 h with 100 ng RNA extracted from mock-infected Vero E6 cells, or 100 ng viral RNA isolated from SARS-CoV-2-infected Vero E6 cells. Whole cell lysates were prepared for immunoblotting using indicated antibodies. β-actin

was immunoblotted as loading control.

(B) Quantitative analysis of viral RNA copies in SARS-CoV-2 infected cell supernatants. Supernatants containing SARS-CoV-2 were obtained from infected cells as described in Figure 1C. qPCR targeting S gene was conducted to quantify viral genome copies (per ml of cell culture) at the indicated time points of infection. Data are represented as means ±SDs calculated from three biological replicates in the same experiment.

(C) Quantitative analysis of ORF9b protein levels in cells during SARS-CoV-2 infection. As described in Figure 1C, an appropriate portion of lysates from SARS-CoV-2 infected cells were subjected to fluorescence quantification immunoblotting using anti-ORF9b and IRDye labeled secondary antibodies. The fluorescence image was shown, with the ORF9b immunoblots selected for quantification. Lane 1-4, SARS-CoV-2 infected Caco-2 cell lysates collected at 1, 5, 9, 24 hours post-infection (hpi). Lane 5-8, SARS-CoV-2 infected HPAEpiC cell lysates collected at 1, 5, 9, 24 hpi. Lane 9-12, serial dilutions (0.5 ng, 2.5 ng, 5 ng, 15 ng) of Flag-tagged ORF9b protein, which were used as the standard sample for accurate quantitative analysis. ORF9b protein levels in individual sample viral infected cell lysates were calculated by the standard curve method, and were converted into concentration (ng/1×10^6 cells). Data were obtained from a representative replicate from three biological replicates in the same experiment.

(D) Near-physiological expression of Flag-ORF9b in HPAEpiC cells. Lysates were obtained from HPAEpiC cells electrotransfected with empty vector or Flag-ORF9b expressing plasmid as described in Figures 1E-1G, and subjected to immunoblotting analysis using anti-ORF9b antibodies. Lysates from SARS-CoV-2 infected HPAEpiC (collected at 1, 5, 9, 24 hpi) were immunoblotted to show the physiological levels of ORF9b during viral infection. β-actin was immunoblotted as loading control. Immunoblots of ORF9b and Flag-ORF9b were indicated by arrows.

(E) Ectopic expression levels of ORF9b in 293T cells. Transfection of empty vector and Flag-ORF9b expressing vector was conducted as described in Figure 1H. Immunoblotting analysis of whole cell lysates was conducted.

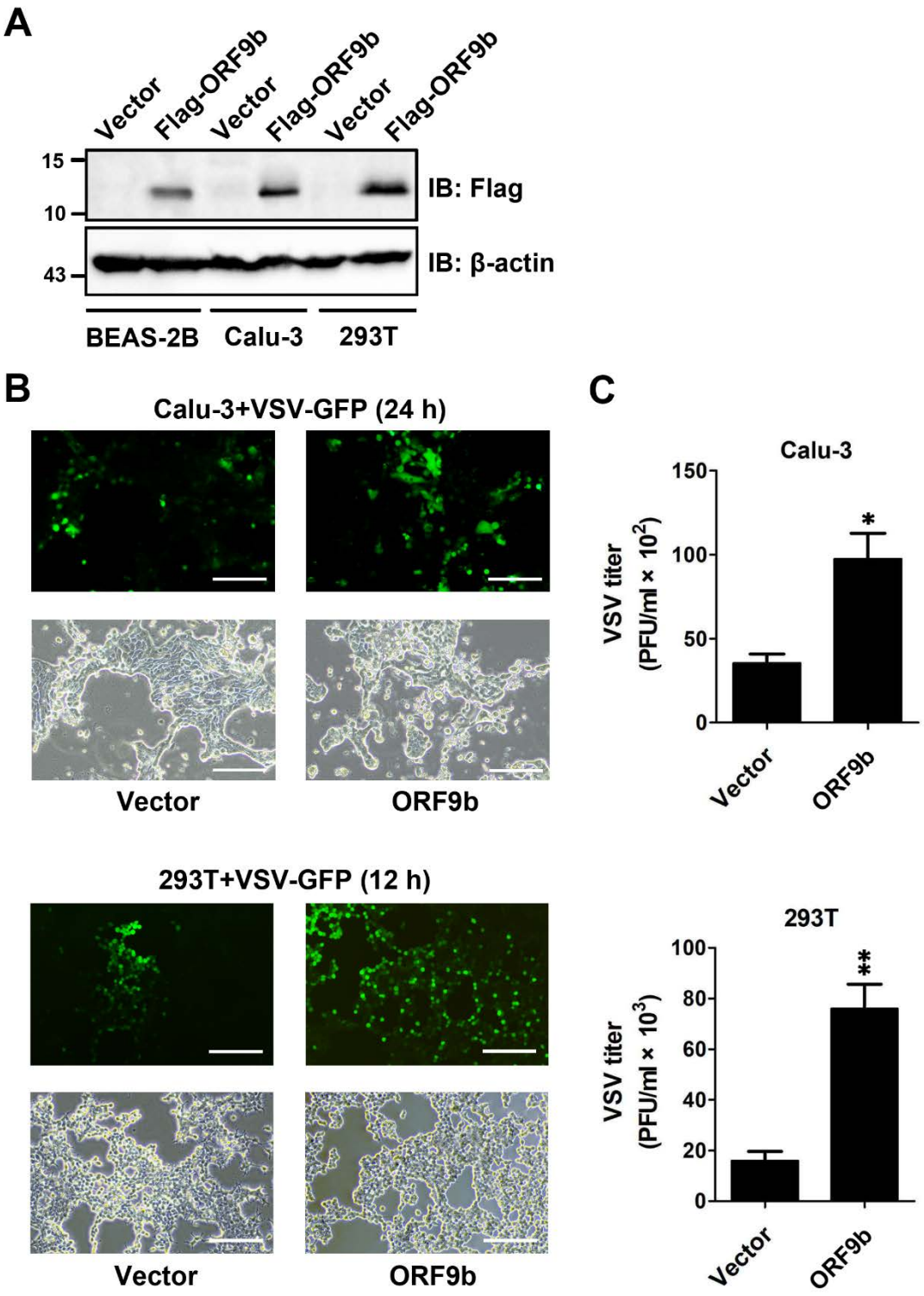

Figure S2. SARS-CoV-2 ORF9b rescues viral growth in diverse human cell lines, related to Figure 2

(A) Ectopic expression levels of ORF9b in various human cell lines, related to

Figure 2A. At 36 h post-transfection of Flag-ORF9b expressing vector, whole cell lysates from BEAS-2B, Calu-3 and 293T cells were subjected to immunoblotting. **(B)** Calu-3 and 293T cells were transfected with empty vector and Flag-ORF9b expressing plasmid for 24 h, and then infected with VSV-GFP for the indicated time. Fluorescent images were taken to examine VSV proliferation. Scale bar, 50 μm. **(C)** As described in Figure 2D, plaque assay was conducted to quantitate VSV titers. Data are represented as means ±SDs calculated from three independent experiments (*$p < 0.05$, **$p < 0.01$; t test).

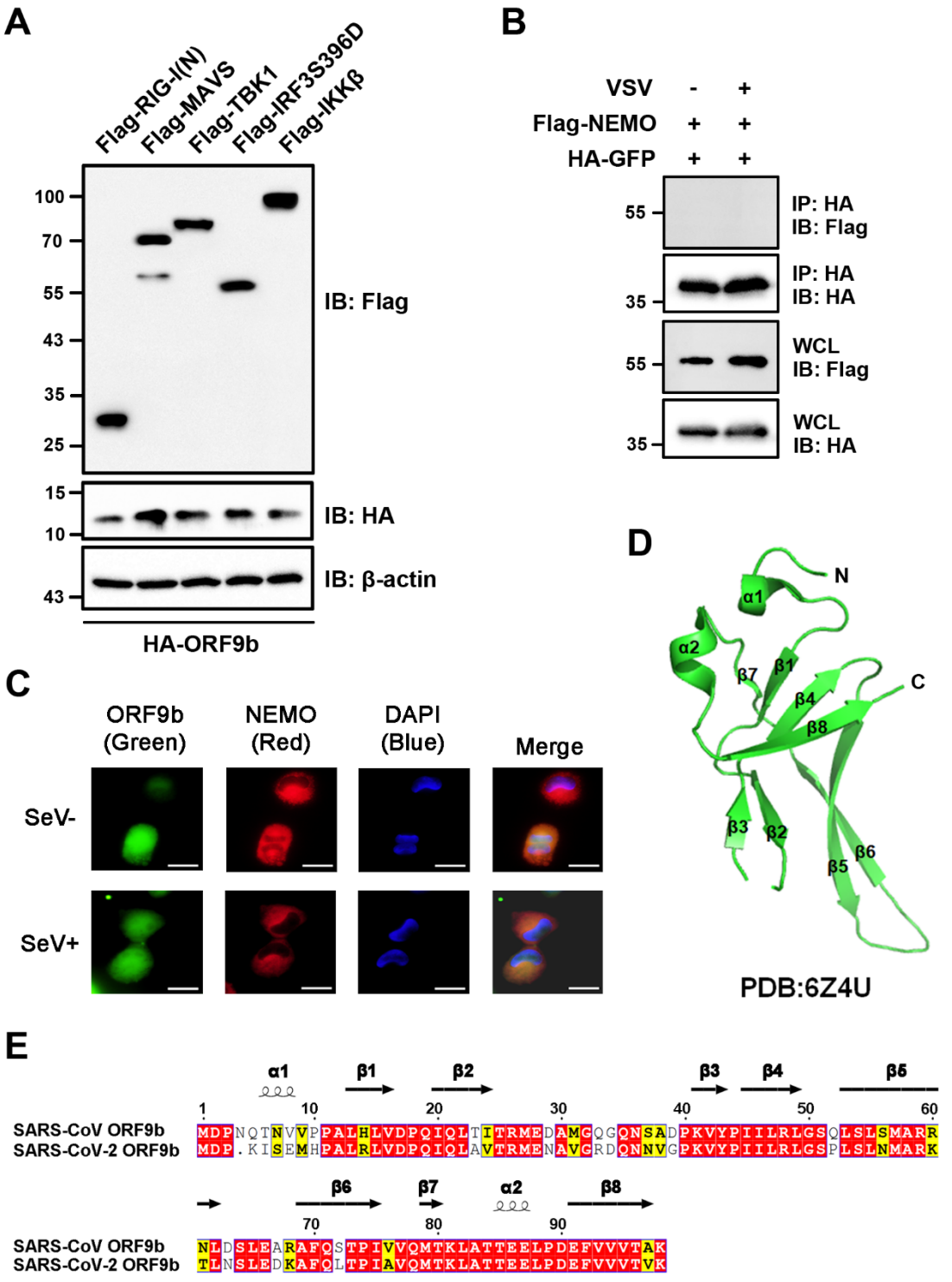

Figure S3. Ectopic expression levels of the RIG-I-MAVS signaling components, parallel experiments for cellular colocalization, and structural data of ORF9b protein, related to Figure 3

(A) Transfection of vectors expressing various signaling components and HA-ORF9b into 293T cells was conducted as described in Figure 3A. Whole cell lysates were prepared for immunoblotting analysis using indicated antibodies.

(B) As described in Figure 3D, plasmid encoding Flag-NEMO together with vector expressing HA-GFP were transfected into 293T cells for 24 h. Cell were then infected with or without VSV for 12 h. Immunoprecipitation was conducted using anti-HA beads.

(C) NCI-H1299 cells were co-transfected with vector expressing HA-GFP for 24 h, and were stimulated with or without SeV. After immunofluorescent staining of cells as described in Figure 3E, the fluorescent images were taken. Scale bar, 10 μm.

(D) The overall structure of SARS-CoV-2 ORF9b in cartoon diagram, related to Figure 3F. PDB file regarding the structure data of SARS-CoV-2 ORF9b protein was downloaded from the RSCB server (https://www.rcsb.org/). Structure was analyzed and drawn by PyMOL.

(E) Sequence and secondary structural analysis of ORF9b proteins from SARS-CoV and SARS-CoV-2, related to Figure 3F. FASTA files regarding the amino acids sequences of ORF9b were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). Alignment and structural analysis were conducted by ESPript (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Highly conservative amino acids were labeled with red character. Alpha helices and beta sheets are shown as “α” and “β”, respectively. α1 (a.a. 5-7), β1 (a.a. 12-15), β2 (a.a. 19-23), β3 (a.a. 40-42), β4 (a.a. 44-48), β5 (a.a. 52-61), β6 (a.a. 68-74), β7 (a.a. 78-79), α2 (a.a. 84-86), β8 (a.a. 90-96).

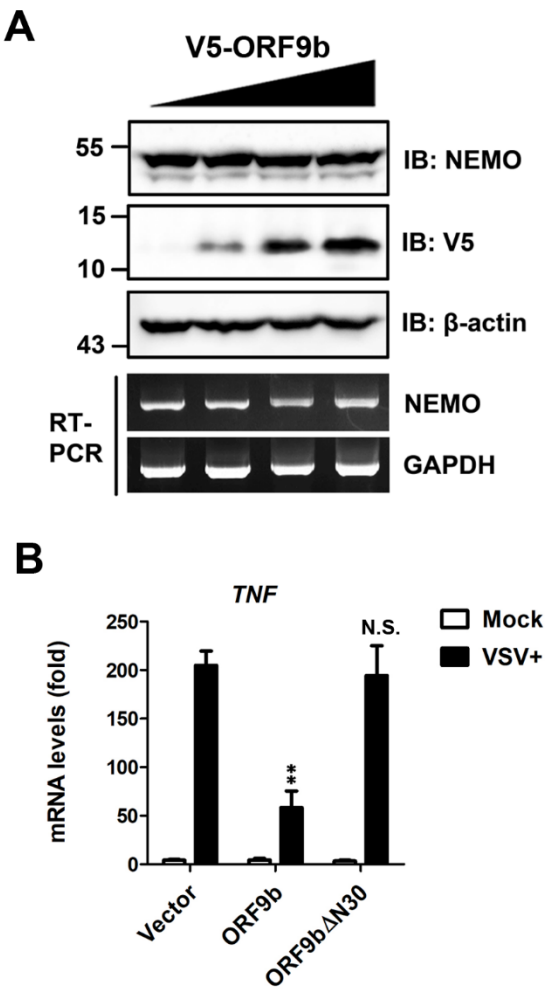

Figure S4. Effects of ORF9b on the mRNA and protein levels of endogenous NEMO, as well as the virally induced *TNF* expression, related to Figure 4

(A) 293T cells were transfected with V5-ORF9b expressing vector for 36 h. Cells were subjected to immunoblotting and reverse transcription-PCR (RT-PCR) for measuring the protein levels and mRNA levels of endogenous NEMO, respectively. GAPDH was analyzed as an internal control.

(B) Experiment was conducted as described in Figure 4F-4G. qPCR was conducted to determine the effects of ORF9b on virally induced expression of *TNF.* Data are represented as means ±SDs calculated from three independent experiments (**$p < 0.01$, N.S., non-significant; t test).

Table S1. Sequence of primers for gene cloning and qPCR, Related to STAR Methods.

Gene ID / Name	Sequence (5' to 3')	Purpose
pcDNA-Flag-For	CCAAGCTTATGGACTACAAGGACGACGATGACAAGGGT	Protein expression
pcDNA-Flag-Rev	GCTCTAGATTAGGTACCCTTGTCATCGTCGTC	
pcDNA-HA-For	GGGGTACCATGGGATACCCATACGACGTCCCAGACTACGC	
pcDNA-HA-Rev	GCTCTAGATTACTCGAGAGCGTAGTCTGGGACGTCG	
pcDNA-V5-For	GGGGTACCATGGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCG	
pcDNA-V5-Rev	GCTCTAGATTACTCGAGCGTAGAATCGAGACCGAGGAGAGGGTTA	
pcDNA-Flag-ORF9b-For	GGGGTACCATGGACCCCAAAATCAGCGAAATG	
pcDNA-Flag-ORF9b-Rev	GCTCTAGATTATTTTACCGTCACCACCACGAA	
pcDNA-HA-ORF9b-For	CCCTCGAGGACCCCAAAATCAGCGAAATGC	
pcDNA-HA-ORF9b-Rev	GCTCTAGATTATTTTACCGTCACCACCACGAA	
pcDNA-HA-ORF9b-GFP-For	CCCTCGAGGACCCCAAAATCAGCGAAATGC	
pcDNA-HA-ORF9b-GFP-Rev	GCTCTAGATTATTTGTATAGTTCATCCATGCCA	
pcDNA-HA-GFP-For	CCCTCGAGAGTAAAGGAGAAGAACTTTTCACTG	
pcDNA-HA-GFP-Rev	GCTCTAGATTATTTGTATAGTTCATCCATGCCA	
pcDNA-HA-ORF9bΔN30-For	CCCTCGAGCGCGATCAAAACAACGTCGGC	
pcDNA-HA-ORF9bΔN30-Rev	GCTCTAGATTATTTTACCGTCACCACCACGAA	
pcDNA-HA-ORF9bΔ41-62-For1	CCCTCGAGGACCCCAAAATCAGCGAAATGC	
pcDNA-HA-ORF9bΔ41-62-Rev1	GTCTTCCAGGGACTTGGGGCCGACGTTGTTTTG	
pcDNA-HA-ORF9bΔ41-62-For2	CGGCCCCAAGTCCCTGGAAGACAAGGCGTTCCAAT	
pcDNA-HA-ORF9bΔ41-62-Rev2	GCTCTAGATTATTTTACCGTCACCACCACGAA	
pcDNA-HA-ORF9bΔC30-For	CCCTCGAGGACCCCAAAATCAGCGAAATGC	
pcDNA-HA-ORF9bΔC30-Rev	GCTCTAGATTACTTGTCTTCCAGGGAATTTAAG	
pcDNA-V5-ORF9b-For	CCCTCGAGGACCCCAAAATCAGCGAAATGC	
pcDNA-V5-ORF9b-Rev	GCTCTAGATTATTTTACCGTCACCACCACGAA	
pcDNA-V5-ORF9bΔN30-For	CCCTCGAGCGCGATCAAAACAACGTCGGC	
pcDNA-V5-ORF9bΔN30-Rev	GCTCTAGATTATTTTACCGTCACCACCACGAA	
pcDNA-Flag-RIG-I(N)-For	GGGGTACCATGGACTACAAGGACGACGATGACAAGATGACCACCGAGCAGCGACGCA	
pcDNA-Flag-RIG-I(N). Rev	CCCTCGAGTCAAAGCTCTAATTGGTAATTTCTT	
pcDNA-Flag-RIG-I-For	GGGGTACCATGGACTACAAGGACGACGATGACAAG	

	ATGACCACCGAGCAGCGACGCA	
pcDNA-Flag-RIG-I-Rev	CCCTCGAGTCATTTGGACATTTCTGCTGGAT	
pcDNA-Flag-MAVS-For	GGGGTACCATGCCGTTTGCTGAAGACAAGACCT	
pcDNA-Flag-MAVS-Rev	GCTCTAGACTAGTGCAGACGCCGCCGGTACAGCA	
pcDNA-Flag-NEMO-For	GGGGTACCATGGCCCTTGTGATCCAGGTG	
pcDNA-Flag-NEMO-Rev	GCTCTAGACTACTCAATGCACTCCATGACAT	
pcDNA-Flag-TBK1-For	GGGGTACCATGCAGAGCACTTCTAATCATCTGTG	
pcDNA-Flag-TBK1-Rev	GCTCTAGACTAAAGACAGTCAACGTTGCGAAGGC	
pcDNA-Flag-IRF3-For	GGGGTACCATGGGAACCCCAAAGCCACGGATCCTG	
pcDNA-Flag-IRF3-Rev	GCTCTAGATCAGCTCTCCCCAGGGCCCTGG	
pcDNA-Flag-IRF3S396D-For1	GGGGTACCATGGGAACCCCAAAGCCACGGATCCTG	
pcDNA-Flag-IRF3S396D-Rev1	TGGCTGTTGTCAATGTGCAGGTCCACAGTATTCT	
pcDNA-Flag-IRF3S396D-For2	ACCTGCACATTGACAACAGCCACCCACTCTCCCTC	
pcDNA-Flag-IRF3S396D-Rev2	GCTCTAGATCAGCTCTCCCCAGGGCCCTGG	
pcDNA-Flag-IKKβ-For	GGGGTACCATGAGCTGGTCACCTTCCCTGACAAC	
pcDNA-Flag-IKKβ-Rev	GCTCTAGATTATGAGGCCTGCTCCAGGCAGCTG	
SARS-CoV-2 *S*-For	CAATGGTTTAACAGGCACAGG	qPCR
SARS-CoV-2 *S*-Rev	CTCAAGTGTCTGTGGATCACG	
IFNB1-For	CAGCAGTTCCAGAAGGAGGA	
IFNB1-Rev	AGCCAGGAGGTTCTCAACAA	
GAPDH-For	AGAAGGCTGGGGCTCATTTG	
GAPDH-Rev	AGGGGCCATCCACAGTCTTC	
IL-6-For	GAGAAAGGAGACATGTAACAAGAGTAAC	
IL-6-Rev	ACTCATCTGCACAGCTCTGGC	
TNF-For	CCTCTCTCTAATCAGCCCTCTG	
TNF-Rev	GAGGACCTGGGAGTAGATGAG	
ISG15-For	CGCAGATCACCCAGAAGATCG	
ISG15-Rev	TTCGTCGCATTTGTCCACCA	
MCP-1-For	CAGCCAGATGCAATCAATGCC	
MCP-1-Rev	TGGAATCCTGAACCCACTTCT	
IP-10-For	GTGGCATTCAAGGAGTACCTC	
IP-10-Rev	TGATGGCCTTCGATTCTGGATT	