

**Table S1.** List of SARS-CoV-2 isolates used in the multiple sequence alignment focused on primer/probe binding sites

| Sequence number       | Isolate name                              | Collection date | Country      |
|-----------------------|-------------------------------------------|-----------------|--------------|
| <b><u>GenBank</u></b> |                                           |                 |              |
| MN908947.3            | Wuhan-Hu-1                                | Dec-2019        | China        |
| MT019529.1            | BetaCoV/Wuhan/IPBCAMS-WH-01/2019          | 23-Dec-2019     | China        |
| MN996527.1            | WIV02                                     | 30-Dec-2019     | China        |
| MN988668.1            | 2019-nCoV WHU01                           | 02-Jan-2020     | China        |
| MN938384.1            | 2019-nCoV_HKU-SZ-002a_2020                | 10-Jan-2020     | China        |
| MN975262.1            | 2019-nCoV_HKU-SZ-005b_2020                | 11-Jan-2020     | China        |
| MT039873.1            | HZ-1                                      | 20-Jan-2020     | China        |
| MT121215.1            | SARS-CoV-2/human/CHN/SH01/2020            | 02-Feb-2020     | China        |
| LC528232.1            | SARS-CoV-2/Hu/DP/Kng/19-020               | 10-Feb-2020     | Japan        |
| MT304475.1            | SARS-CoV-2/human/KOR/BA-ACH_2718/2020     | 29-Feb-2020     | South Korea  |
| MT066175.1            | SARS-CoV-2/NTU01/TWN/human/2020           | 31-Jan-2020     | Taiwan       |
| MT192772.1            | SARS-CoV-2/human/VNM/nCoV-19-01S/2020     | 22-Jan-2020     | Vietnam      |
| MT072688.1            | SARS-CoV-2/human/NPL/61-TW/2020           | 13-Jan-2020     | Nepal        |
| MT276598.1            | SARS-CoV-2/human/ISR/ISR_IT0320/2020      | Mar-2020        | Israel       |
| MT262993.1            | SARS-Cov-2/human/PAK/Mangal/2020          | 12-Mar-2020     | Pakistan     |
| MT012098.1            | SARS-CoV-2/human/IND/29/2020              | 27-Jan-2020     | India        |
| MT007544.1            | Australia/VIC01/2020                      | 25-Jan-2020     | Australia    |
| MT451786.1            | SARS-CoV-2/human/AUS/VIC1178/2020         | 10-Apr-2020     | Australia    |
| MN985325.1            | 2019-nCoV/USA-WA1/2020                    | 19-Jan-2020     | USA          |
| MT039888.1            | 2019-nCoV/USA-MA1/2020                    | 29-Jan-2020     | USA          |
| MT106054.1            | 2019-nCoV/USA-TX1/2020                    | 11-Feb-2020     | USA          |
| MT159717.1            | 2019-nCoV/USA-CruiseA-1/2020              | 17-Feb-2020     | USA          |
| MT152824.1            | SARS-CoV-2/human/USA/WA2/2020             | 24-Feb-2020     | USA          |
| MT246461.1            | SARS-CoV-2/human/USA/WA-UW204/2020        | 13-Mar-2020     | USA          |
| MT126808.1            | SARS-CoV-2/human/BRA/SP02/2020            | 28-Feb-2020     | Brazil       |
| MT256924.1            | SARS-CoV-2/human/COL/79256_Antioquia/2020 | 11-Mar-2020     | Colombia     |
| MT263074.1            | SARS-CoV-2/human/PER/Peru-10/2020         | 10-Mar-2020     | Peru         |
| MT020781.2            | nCoV-FIN-29-Jan-2020                      | 29-Jan-2020     | Finland      |
| MT093571.1            | SARS-CoV-2/human/SWE/01/2020              | 07-Feb-2020     | Sweden       |
| MT066156.1            | SARS-CoV-2/human/ITA/INMI1/2020           | 30-Jan-2020     | Italy        |
| MT198652.2            | SARS-CoV-2/human/ESP/Valencia003/2020     | 05-Mar-2020     | Spain        |
| MT470179.1            | SARS-CoV-2/human/France/40002VJ/2020      | Mar-2020        | France       |
| MT358643.1            | SARS-CoV-2/human/DEU/FFM7/2020            | Feb-2020        | Germany      |
| MT324062.1            | SARS-CoV-2/human/ZAF/R03006/2020          | 07-Mar-2020     | South Africa |

**Table S2.** Test results for determining the limit of detection of the in-house developed single-tube nested (STN) real-time RT-PCR assays with genomic RNA extracted from a SARS-CoV-2 culture isolate

| Virus titer<br>(TCID <sub>50</sub> /mL) | Cp (Intra-run) |        |        | Cp (Inter-run) |        |        |
|-----------------------------------------|----------------|--------|--------|----------------|--------|--------|
|                                         | Test 1         | Test 2 | Test 3 | Test 1         | Test 2 | Test 3 |
| STN COVID-19-RdRp/Hel assay             |                |        |        |                |        |        |
| 1.8 x 10 <sup>1</sup>                   | 20.79          | 20.40  | 20.86  | 19.46          | 19.25  | 19.19  |
| 1.8 x 10 <sup>0</sup>                   | 24.34          | 23.52  | 25.27  | 22.56          | 21.45  | 21.98  |
| 1.8 x 10 <sup>-1</sup>                  | 26.17          | 25.76  | 23.96  | 25.34          | 23.99  | 25.18  |
| 1.8 x 10 <sup>-2</sup>                  | 28.37          | -      | -      | -              | -      | 27.49  |
| 1.8 x 10 <sup>-3</sup>                  | 37.87          | -      | -      | -              | 27.62  | -      |
| STN COVID-19-N assay                    |                |        |        |                |        |        |
| 1.8 x 10 <sup>1</sup>                   | 21.91          | 19.86  | 22.53  | 19.59          | 19.80  | 19.08  |
| 1.8 x 10 <sup>0</sup>                   | 27.69          | 22.17  | 25.25  | 22.67          | 24.01  | 24.11  |
| 1.8 x 10 <sup>-1</sup>                  | 28.75          | 31.26  | 29.19  | 27.99          | 27.56  | 28.80  |
| 1.8 x 10 <sup>-2</sup>                  | -              | -      | -      | 30.29          | -      | 29.91  |
| 1.8 x 10 <sup>-3</sup>                  | -              | -      | -      | -              | -      | -      |

**Table S3.** Performance of the STN COVID-19-RdRp/Hel and STN COVID-19-N assays on follow-up specimens from COVID-19 patients that tested negative by non-nested COVID-19-RdRp/Hel assay

| <b>Respiratory specimens (NPA/NPS/TS/saliva)</b>                        | <b>No. of positive specimens (%)<br/>(n=91)</b> |
|-------------------------------------------------------------------------|-------------------------------------------------|
| Positive by both STN assays                                             | 9 (9.9)                                         |
| Positive by STN COVID-19-RdRp/Hel assay only                            | 3 (3.3)                                         |
| Positive by STN COVID-19-N assay only                                   | 9 (9.9)                                         |
| NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; TS, throat swab |                                                 |
| <b>Non-respiratory specimens<br/>(rectal swabs/stool/plasma)</b>        | <b>No. of positive specimens (%)<br/>(n=17)</b> |
| Positive by both STN assays                                             | 3 (17.6)                                        |
| Positive by STN COVID-19-RdRp/Hel assay only                            | 1 (5.9)                                         |
| Positive by STN COVID-19-N assay only                                   | 3 (17.6)                                        |

**Table S4.** Evaluation of the paired NPS and saliva specimens from the confirmed COVID-19 cases with discrepant results by the non-nested COVID-19-RdRp/Hel assay using the STN real-time RT-PCR assays

| Patient | Specimen | Cp value                                  |                                 |                          |
|---------|----------|-------------------------------------------|---------------------------------|--------------------------|
|         |          | Non-nested<br>COVID-19-<br>RdRp/Hel assay | STN COVID-19-<br>RdRp/Hel assay | STN COVID-<br>19-N assay |
| A       | NPS      | 35.20                                     | 20.64                           | 20.67                    |
|         | Saliva   | -                                         | -                               | -                        |
| B       | NPS      | -                                         | -                               | -                        |
|         | Saliva   | 28.80                                     | 17.33                           | 17.91                    |
| C       | NPS      | -                                         | -                               | -                        |
|         | Saliva   | 23.10                                     | 13.83                           | 14.36                    |
| D       | NPS      | 32.31                                     | 19.90                           | 21.04                    |
|         | Saliva   | -                                         | -                               | -                        |
| E       | NPS      | 36.96                                     | 28.84                           | 31.22                    |
|         | Saliva   | -                                         | -                               | -                        |
| F       | NPS      | -                                         | -                               | -                        |
|         | Saliva   | 28.10                                     | 18.38                           | 16.81                    |
| G       | NPS      | 36.26                                     | 23.21                           | 31.08                    |
|         | Saliva   | -                                         | -                               | -                        |
| H       | NPS      | 35.37                                     | 22.10                           | 28.71                    |
|         | Saliva   | -                                         | 22.18                           | 20.23                    |

-, negative; NPS, nasopharyngeal swab

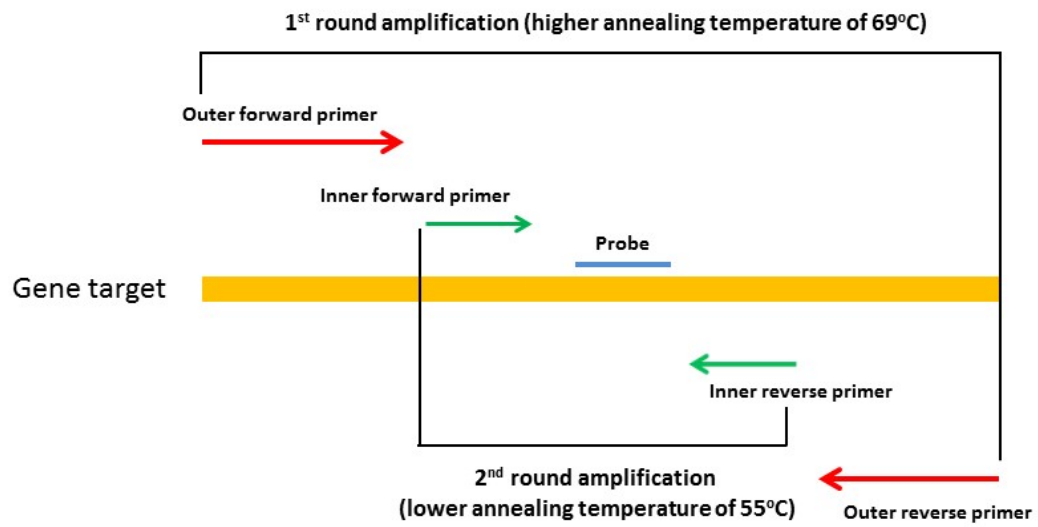
# STN COVID-19-RdRp/Hel assay

|                                           | Outer forward                          | Inner forward        | Probe                       | Inner reverse *           | Outer reverse *                       |
|-------------------------------------------|----------------------------------------|----------------------|-----------------------------|---------------------------|---------------------------------------|
| COVID-19-RdRp/Hel single-tube nested      | AGGTATTGGGAACTGAGTTTATGAGGCTATGTACACAC | CGCATACAGTCTTFCAGGCT | TTAAGATGTGGTCTCTGCAACGTAGAC | GAACATGTGCATGTCAACATCACAC | GTCTTGTCTGTAAATCGGATGTTTGCATGCTCCAGGT |
| Wuhan-Hu-1                                | .....                                  | .....                | .....                       | .....                     | .....                                 |
| BetaCoV/Wuhan/IPSCAMS-WH-01/2019          | .....                                  | .....                | .....                       | .....                     | .....                                 |
| WIV02                                     | .....                                  | .....                | .....                       | .....                     | .....                                 |
| 2019-nCoV WHU01                           | .....                                  | .....                | .....                       | .....                     | .....                                 |
| 2019-nCoV HKU-S2-002a/2020                | .....                                  | .....                | .....                       | .....                     | .....                                 |
| 2019-nCoV HKU-S2-005b/2020                | .....                                  | .....                | .....                       | .....                     | .....                                 |
| H2-1                                      | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/CHN/SH01/2020            | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/Hu/DP/Kang/19-020              | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/KOR/BA-ACN 2718/2020     | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/NTU01/TWN/human/2020           | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/TXM/nCoV-19-018/2020     | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/NZL/61-TW/2020           | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/ISR/ISR IT0320/2020      | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/PAR/Mangal/2020          | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/IND/29/2020              | .....                                  | .....                | .....                       | .....                     | .....                                 |
| Australia/VIC01/2020                      | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/AUS/VIC1178/2020         | .....                                  | .....                | .....                       | .....                     | .....                                 |
| 2019-nCoV/USA-WA1/2020                    | .....                                  | .....                | .....                       | .....                     | .....                                 |
| 2019-nCoV/USA-WA1/2020                    | .....                                  | .....                | .....                       | .....                     | .....                                 |
| 2019-nCoV/USA-TX1/2020                    | .....                                  | .....                | .....                       | .....                     | .....                                 |
| 2019-nCoV/USA-CruiseA-1/2020              | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/USA/WA2/2020             | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/USA/WA-TM204/2020        | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/BRA/SP02/2020            | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/COL/79256 Antioquia/2020 | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/PER/Peru-10/2020         | .....                                  | .....                | .....                       | .....                     | .....                                 |
| nCoV-FIN-29-Jan-2020                      | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/SWE/01/2020              | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/ITA/INM11/2020           | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/ESP/Valencia003/2020     | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/France/40002VJ/2020      | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/DEU/FFM7/2020            | .....                                  | .....                | .....                       | .....                     | .....                                 |
| SARS-CoV-2/human/ZAF/RO3006/2020          | .....                                  | .....                | .....                       | .....                     | .....                                 |

# STN COVID-19-N assay

|                                           | Outer forward                | Inner forward      | Probe                  | Inner reverse *        | Outer reverse *                          |
|-------------------------------------------|------------------------------|--------------------|------------------------|------------------------|------------------------------------------|
| COVID-19-N single-tube nested             | AATTCACAACTTGGCCGCCAGCGCTTCA | CGGTTCTTCGAAATGTGG | AACGTGGTGGACCTACACAGST | CAAAATGGGATGCAAGATCCAA | TCAAAGATCAAGTCATTTTCGTGAATAGCAATATGACGCA |
| Wuhan-Hu-1                                | .....                        | .....              | .....                  | .....                  | .....                                    |
| BetaCoV/Wuhan/IPSCAMS-WH-01/2019          | .....                        | .....              | .....                  | .....                  | .....                                    |
| WIV02                                     | .....                        | .....              | .....                  | .....                  | .....                                    |
| 2019-nCoV WHU01                           | .....                        | .....              | .....                  | .....                  | .....                                    |
| 2019-nCoV HKU-S2-002a/2020                | .....                        | .....              | .....                  | .....                  | .....                                    |
| 2019-nCoV HKU-S2-005b/2020                | .....                        | .....              | .....                  | .....                  | .....                                    |
| H2-1                                      | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/CHN/SH01/2020            | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/Hu/DP/Kang/19-020              | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/KOR/BA-ACN 2718/2020     | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/NTU01/TWN/human/2020           | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/TXM/nCoV-19-018/2020     | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/NZL/61-TW/2020           | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/ISR/ISR IT0320/2020      | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/PAR/Mangal/2020          | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/IND/29/2020              | .....                        | .....              | .....                  | .....                  | .....                                    |
| Australia/VIC01/2020                      | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/AUS/VIC1178/2020         | .....                        | .....              | .....                  | .....                  | .....                                    |
| 2019-nCoV/USA-WA1/2020                    | .....                        | .....              | .....                  | .....                  | .....                                    |
| 2019-nCoV/USA-WA1/2020                    | .....                        | .....              | .....                  | .....                  | .....                                    |
| 2019-nCoV/USA-TX1/2020                    | .....                        | .....              | .....                  | .....                  | .....                                    |
| 2019-nCoV/USA-CruiseA-1/2020              | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/USA/WA2/2020             | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/USA/WA-TM204/2020        | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/BRA/SP02/2020            | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/COL/79256 Antioquia/2020 | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/PER/Peru-10/2020         | .....                        | .....              | .....                  | .....                  | .....                                    |
| nCoV-FIN-29-Jan-2020                      | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/SWE/01/2020              | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/ITA/INM11/2020           | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/ESP/Valencia003/2020     | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/France/40002VJ/2020      | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/DEU/FFM7/2020            | .....                        | .....              | .....                  | .....                  | .....                                    |
| SARS-CoV-2/human/ZAF/RO3006/2020          | .....                        | .....              | .....                  | .....                  | .....                                    |

**Figure S1.** Multiple sequence alignments using the primer/probe sequences of the STN assays and the gene sequences of global SARS-CoV-2 isolates. \*reverse-complement of reverse primer sequence



**Figure S2.** A schematic diagram of the in-house STN assay for the detection of SARS-CoV-2