Comparative analysis of primer-probe sets for RT-qPCR of COVID-19 causative virus (SARS-CoV-2)

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Supporting materials and methods

**Polyacrylamide gel electrophoresis of PCR products**

For electrophoresis of qRT-PCR products, polyacrylamide gel electrophoresis was performed. Briefly, 7.5% polyacrylamide gel was prepared with 1x TAE buffer (40mM Tris-Acetate, 1mM EDTA, diluted from 50X TAE, Biosesang, South Korea). Total 5 μL of each qRT-PCR product mixed with 6X Loading Dye (Biofact, South Korea) was loaded. The gel was stained in 1x TAE buffer containing 1x RedSafe Nucleic Acid Staining Solution (Intron biotechnology, South Korea) for 30 minutes at ambient temperature. The gel image was captured using ChemiDoc Touch Imaging System (Bio-Rad).

**Melting curve analysis**

For the melting curve analysis of qRT-PCR products, the same reagent, instrument, and method are used for qRT-PCR reaction with the following modifications. The reaction solutions were prepared with 1x SYBR Green I dye (Invitrogen) and probes were not added. Melting curves were made from melting steps from each extension temperature to 95°C.

**SARS-CoV-2 genome alignment**

The high-quality and complete SARS-CoV-genome sequenced up to 23 April 2020 retrieved from GISAID. The unique genome sequences were selected from the retrieved sequences using CDHIT [ref: Limin Fu, Beifang Niu, Zhengwei Zhu, Sitao Wu and Weizhong Li, CD-HIT: accelerated for clustering the next generation sequencing data. Bioinformatics, (2012), 28 (23): 3150-3152. doi: 10.1093/bioinformatics/bts565]. The genome sequences with more than one ambiguous sequences were removed using prinseq [ref: Schmieder R and Edwards R: Quality control and preprocessing of metagenomic datasets. Bioinformatics 2011, 27:863-864]. The processed genome sequences were aligned with MAFFT v7.450 [ref: Katoh, Standley 2013 (Molecular Biology and Evolution 30:772-780)] and the mismatches in primer and probe sites were determined.
Figure S1. Standard curve analyzed by known amounts of in vitro transcribed RNA standard (E gene) for quantification of (a) the first batch, and (b) the second batch of SARS-CoV-2 RNA.
Table S1. Comparative analysis of Ct values obtained from a repeated experiment by employing the second batch of SARS-CoV-2 RNA.

| Reaction temperature | Target | Institute | Name | 1.5 x 10^3 copies | 1.5 x 10^4 copies | 1.5 x 10^5 copies | 1.5 x 10^6 copies | NTC |
|----------------------|--------|-----------|------|-------------------|-------------------|-------------------|-------------------|-----|
|                      |        |           | Mean Ct | STD | Mean Ct | STD | Mean Ct | STD | Mean Ct | STD | Mean Ct | STD |
| 60 °C                | N      | China CDC | N      | 26.50 | 0.072 | 29.80 | 0.070 | 32.40 | 0.118 | 36.09 | 0.149 | 37.53 | 1.529 |
|                      |        | HKU       | HKU-N  | 26.88 | 0.044 | 30.21 | 0.062 | 33.32 | 0.079 | 36.47 | 0.183 | 40.03* | -     |
|                      |        | Japan NIID | NIID_2019-nCOV_N | 24.05 | 0.006 | 27.09 | 0.058 | 30.20 | 0.108 | 33.82 | 0.282 | N/A | N/A |
|                      |        | Thailand NIH | WH-NIC_N | 29.08 | 0.060 | 32.40 | 0.076 | 34.75 | 0.104 | 38.93 | 1.271 | 44.55** | 0.141** |
|                      |        | US CDC | 2019-nCoV_N1 | 25.86 | 0.234 | 28.94 | 0.068 | 32.68 | 0.242 | 36.09 | 0.903 | N/A | N/A |
|                      |        |        | 2019-nCoV_N2 | 24.19 | 0.045 | 27.22 | 0.040 | 30.64 | 0.038 | 34.55 | 0.120 | N/A | N/A |
|                      |        |        | 2019-nCoV_N3 | 24.22 | 0.030 | 27.40 | 0.072 | 30.60 | 0.095 | 34.00 | 0.557 | N/A | N/A |
|                      |        | China CDC | ORF1ab | 24.43 | 0.025 | 28.01 | 0.042 | 30.61 | 0.102 | 34.15 | 0.559 | N/A | N/A |
|                      |        | Charité | RdRp_SARSr | 28.96 | 0.071 | 32.34 | 0.167 | 35.06 | 0.221 | 37.40 | 1.176 | 42.88** | 2.291*** |
|                      |        | HKU | HKU-ORF1b-nsp14 | 25.53 | 0.127 | 28.91 | 0.055 | 31.74 | 0.062 | 36.14 | 1.293 | N/A | N/A |
| 58 °C                | N      | China CDC | N      | 27.17 | 0.076 | 30.95 | 0.122 | 33.09 | 0.040 | 37.57 | 0.288 | 36.74* | -     |
|                      |        | HKU       | HKU-N  | 27.70 | 0.114 | 31.50 | 0.047 | 34.10 | 0.165 | 39.18 | 1.028 | N/A | N/A |
|                      |        | Japan NIID | NIID_2019-nCOV_N | 25.05 | 0.084 | 28.84 | 0.157 | 31.09 | 0.064 | 34.96 | 0.429 | N/A | N/A |
|                      |        | Thailand NIH | WH-NIC_N | 29.05 | 0.122 | 33.05 | 0.036 | 35.04 | 0.036 | 39.49 | 1.739 | 40.04** | 1.414** |
|                      |        | US CDC | 2019-nCoV_N1 | 26.96 | 0.02 | 30.84 | 0.095 | 32.95 | 0.422 | 36.13 | 1.525 | N/A | N/A |
|                      |        |        | 2019-nCoV_N2 | 25.26 | 0.066 | 29.20 | 0.191 | 31.30 | 0.046 | 35.79 | 1.325 | N/A | N/A |
|                      |        |        | 2019-nCoV_N3 | 25.32 | 0.067 | 29.18 | 0.050 | 31.20 | 0.135 | 35.37 | 0.952 | N/A | N/A |
|                      |        | China CDC | ORF1ab | 25.25 | 0.060 | 28.98 | 0.137 | 31.39 | 0.348 | 34.76 | 0.635 | N/A | N/A |
|                      |        | Charité | RdRp_SARSr | 29.54 | 0.092 | 33.59 | 0.390 | 35.27 | 0.521 | 37.47 | 0.370 | N/A | N/A |
|                      |        | HKU | HKU-ORF1b-nsp14 | 25.83 | 0.044 | 29.63 | 0.080 | 32.01 | 0.072 | 35.12 | 0.522 | N/A | N/A |
| 55 °C                | N      | China CDC | N      | 26.3 | 0.182 | 29.67 | 0.307 | 32.92 | 0.231 | 35.63 | 1.081 | 37.68** | 0.622** |
|                      |        | HKU       | HKU-N  | 25.89 | 0.056 | 29.36 | 0.161 | 32.95 | 0.234 | 37.55 | 0.630 | 38.36* | -     |
|                      |        | Japan NIID | NIID_2019-nCOV_N | 23.76 | 0.107 | 27.05 | 0.038 | 30.27 | 0.029 | 34.73 | 1.287 | N/A | N/A |
|             |                  |      |      |      |      |      |      |      |
|-------------|------------------|------|------|------|------|------|------|------|
| Thailand NIH | WH-NIC N         | 27.80| 0.188| 31.21| 0.090| 34.37| 0.221| 36.71| 0.547| 38.11| 0.648|
| US CDC      | 2019-nCoV_N1     | 27.15| 0.069| 30.88| 0.312| 33.13| 0.350| 37.41| 0.366| 37.54*|
|             | 2019-nCoV_N2     | 26.38| 0.012| 30.97| 0.093| 32.5  | 0.137| 36.37| 0.100| N/A   | N/A   |
|             | 2019-nCoV_N3     | 24.78| 0.615| 27.53| 0.042| 30.55| 0.095| 35.83| 1.421| N/A   | N/A   |
| China CDC   | ORF1ab           | 24.83| 0.075| 28.39| 0.082| 32.13| 0.193| 35.27| 0.237| N/A   | N/A   |
| Charité     | RdRp_SARSr       | 29.77| 0.150| 32.69| 0.431| 37.19| 0.571| 37.39| 1.105| N/A   | N/A   |
| HKU         | HKU-ORF1b-nsp14  | 25.14| 0.035| 28.16| 0.061| 31.58| 0.501| 34.87| 0.460| N/A   | N/A   |

* The assay showed positive signal from the single reaction of triplicate.
** The assay showed positive signals from the two reactions of triplicate.
Figure S2. Amplification curves of fluorescence intensity against PCR cycle with the second batch of SARS-CoV-2 RNA (Amplification is performed at 60°C). (a) N (China CDC), (b) HKU-N (HKU), (c) NIID_2019-nCOV_N (Japan NIID), (d) WH-NIC N (Thailand NIH), (e) 2019-nCoV_N1 (US CDC), (f) 2019-nCoV_N2 (US CDC), (g) 2019-nCoV_N3 (US CDC), (h) ORF1ab (China CDC), (i) RdRp_SARSr (Charité), and (j) HKU-ORF1b-nsp14 (HKU)
Figure S3. Amplification curves of fluorescence intensity against PCR cycle with the second batch of SARS-CoV-2 RNA (Amplification is performed at 58°C). (a) N (China CDC), (b) HKU-N (HKU), (c) NIID_2019-nCOV_N (Japan NIID), (d) WH-NIC N (Thailand NIH), (e) 2019-nCoV_N1 (US CDC), (f) 2019-nCoV_N2 (US CDC), (g) 2019-nCoV_N3 (US CDC), (h) ORF1ab (China CDC), (i) RdRp_SARSr (Charité), and (j) HKU-ORF1b-nsp14 (HKU)
Figure S4. Amplification curves of fluorescence intensity against PCR cycle with the second batch of SARS-CoV-2 RNA (Amplification is performed at 55°C). (a) N (China CDC), (b) HKU-N (HKU), (c) NIID_2019-nCOV_N (Japan NIID), (d) WH-NIC N (Thailand NIH), (e) 2019-nCoV_N1 (US CDC), (f) 2019-nCoV_N2 (US CDC), (g) 2019-nCoV_N3 (US CDC), (h) ORF1ab (China CDC), (i) RdRp_SARSr (Charité), and (j) HKU-ORF1b-nsp14 (HKU)
Figure S5. (a) Melting curve analysis and (b) polyacrylamide gel image of PCR products with primer-probe sets that show positive signals in the NTC samples. M: DNA ladder; 1: NTC sample with 2019-nCoV_N1 (US CDC); 2: PCR product with 2019-nCoV_N1 (US CDC); 3: NTC sample with N (China CDC); 4: PCR product with N (China CDC); 5: NTC sample with RdRp_SARSr (Charité); 6: PCR product with RdRp_SARSr (Charité)
Table S2. Clinical characteristics of laboratory-confirmed patients with COVID-19.

|                  | Patient 1 (P1) | Patient 2 (P2) | Patient 3 (P3) | Patient 4 (P4) | Patient 5 (P5) | Patient 6 (P6) |
|------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Age (years)/Sex  | 72/M           | 57/M           | 72/F           | 38/M           | 21/F           | 32/F           |
| Fever            | -              | +              | +              | -              | +              | +              |
| Chills           | -              | +              | -              | -              | -              | +              |
| Cough            | +              | +              | +              | -              | -              | -              |
| Dyspnea          | -              | +              | +              | -              | -              | -              |
| Diarrhea         | -              | -              | -              | -              | -              | -              |
| **Laboratory findings** |              |                |                |                |                |                |
| WBC              | 3,570          | 3,710          | 6,720          | 5,810          | 5,800          | 4,280          |
| Hemoglobin       | 10.3           | 12.9           | 16.1           | 14             | 14.1           | 11.3           |
| Platelet         | 157,000        | 42,000         | 190,000        | 254,000        | 304,000        | 261,000        |
| AST              | 23             | 57             | 32             | 39             | 23             | 20             |
| ALT              | 22             | 55             | 13             | 68             | 26             | 7              |
| Creatinine       | 0.83           | 0.77           | 0.55           | 0.92           | 0.69           | 0.5            |
| **Used drug**    |                |                |                |                |                |                |
| Lopinavir/ritonavir | +             | +              | +              | +              | +              | +              |
| Hydroxychloroquine | +             | +              | +              | +              | +              | +              |
| **Outcome**      | Cured          | Cured          | Cured          | Cured          | Cured          | Cured          |
Figure S6. Amplification curves of fluorescence intensity against PCR cycle with the RNAs extracted from clinical samples of healthy subjects. (a) NIID_2019-nCOV_N (Japan NIID), (b) 2019-nCoV_N2 (US CDC), (c) ORF1ab (China CDC), and (d) HKU-ORF1b-nsp14 (HKU)
Figure S7. Cross-reactivity test of the RT-qPCR assays for other coronaviruses. (a) NIID_2019-nCOV_N (Japan NIID), (b) ORF1ab (China CDC).
Table S3. Mismatch nucleotides at the binding sites of the recommended primer-probe sets.

| Target     | Institute     | Name              | Type | Primer/probe position (5' → 3') | Primer/probe nucleotide (*RC) | Mismatch nucleotide | Number of mismatch | Frequency (%) |
|------------|---------------|-------------------|------|---------------------------------|-------------------------------|---------------------|--------------------|---------------|
|            |               | NIID_2019-nCOV_N_F2 | F    | 4                               | T                             | C                   | 2/3,323            | 0.06          |
|            |               |                   |      | 13                              | C                             | T                   | 2/3,323            | 0.06          |
|            |               |                   |      | 16                              | G                             | A,T                 | 3/3,323            | 0.09          |
|            |               |                   |      | 20                              | C                             | T                   | 14/3,323           | 0.42          |
|            | Japan         | NIID_2019-nCOV_N_R2 | R    | 4                               | G*                            | T                   | 1/3,323            | 0.03          |
|            |               |                   |      | 7                               | C*                            | T                   | 2/3,323            | 0.06          |
|            |               |                   |      | 10                              | C*                            | T                   | 2/3,323            | 0.06          |
|            |               |                   |      | 12                              | C*                            | T                   | 1/3,323            | 0.03          |
|            |               |                   |      | 15                              | C*                            | G                   | 3,323/3,323        | 100           |
|            |               |                   |      | 20                              | A*                            | T                   | 1/3,323            | 0.03          |
|            |               | NIID_2019-nCOV_N_P2 | P    | 6                               | G                             | A,T                 | 2/3,323            | 0.06          |
|            |               |                   |      | 8                               | G                             | A                   | 1/3,323            | 0.03          |
|            |               |                   |      | 9                               | C                             | T                   | 1/3,323            | 0.03          |
|            |               |                   |      | 13                              | G                             | C                   | 1/3,323            | 0.03          |
|            |               |                   |      | 17                              | T                             | C                   | 1/3,323            | 0.03          |
|            |               |                   |      | 18                              | G                             | A,T                 | 3/3,323            | 0.09          |
|            |               | ORF1ab-F           | F    | 4                               | T                             | C                   | 1/3,323            | 0.03          |
|            |               |                   |      | 17                              | C                             | T                   | 1/3,323            | 0.03          |
|            |               | ORF1ab-R           | R    | 9                               | T*                            | C                   | 1/3,323            | 0.03          |
|            |               |                   |      | 15                              | A*                            | G                   | 1/3,323            | 0.03          |
|            | China         | ORF1ab-P           | P    | 2                               | C                             | T                   | 1/3,323            | 0.03          |
|            | CDC           |                   |      | 8                               | C                             | T                   | 1/3,323            | 0.03          |
|            |               |                   |      | 26                              | T                             | G                   | 51/3,323           | 1.53          |

*RC : Reverse complement
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