Original Article

**Performance evaluation of real-time RT-PCR assays for detection of severe acute respiratory syndrome coronavirus-2 developed by the National Institute of Infectious Diseases, Japan.**

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Summary

Soon after the December 2019 outbreak of coronavirus disease 2019 in Wuhan, China, a protocol for real-time RT-PCR assay detection of severe acute respiratory syndrome coronavirus (SARS-CoV-2) was established by the National Institute of Infectious Diseases (NIID) in Japan. The protocol used Charité’s nucleocapsid (Sarbeco-N) and NIID’s nucleocapsid (NIID-N2) assays. During the following months, SARS-CoV-2 spread causing a global pandemic, and a variety of SARS-CoV-2 sequences were registered to public databases, such as the Global Initiative on Sharing All Influenza Data (GISAID). In this study, we evaluated the newly developed S2 assay (NIID-S2) to replace the Sarbeco-N assay and the performance of NIID-N2 and NIID-S2 assays, referring mismatches in the primer/probe targeted region. We found the analytical sensitivity and specificity of the NIID-S2 set were comparable to the NIID-N2 assay, and the detection rate for clinical specimens was identical to that of the NIID-N2 assay. Furthermore, among available sequences (approximately 192,000), the NIID-N2 and NIID-S2 sets had 2.6% and 1.2% mismatched sequences, respectively, although most of these mismatches did not affect the amplification efficiency, with the exception of the 3’ end of the NIID-N2 forward primer. These findings indicate that the previously developed NIID-N2 assay remains suitable for the detection SARS-CoV-2 with support of the newly developed
NIID-S2 set.
Introduction

The outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) originated in Wuhan, China, in December 2019 and has rapidly spread worldwide (1, 2). As of December 17, 2020, 72,851,747 confirmed cases and 1,643,339 deaths have been reported globally, with 187,103 confirmed cases and 2,739 deaths reported in Japan (3, 4). A real-time RT-PCR assay, termed the National Institute of Infectious Disease, Japan (NIID) assay (5), for the detection of SARS-CoV-2 was developed soon after the initial outbreak and was distributed to municipal and prefectural institutes, health centers, and quarantine stations for national surveillance. The protocol consisted of Corman’s Sarbeco-N assay (6) and in-house N2 assay (NIID-N2) (5) because primer and probe sets were selected by optimizing the operation on a LightCycler 480, which was the main instrument used for the national confirmation assay. During the year since the emergence of the outbreak, an enormous number of SARS-CoV-2 genomic sequences have been registered to public databases, such as the Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org/), and a variety of genomic sequences have been seen. Therefore, the NIID assay should be evaluated to ascertain its capacity to deal with mutations in targeted region sequences. Additionally, although Corman’s assay has been used by the
World Health Organization (WHO), the Sarbeco-N assay is no longer used (7). Certainly, the analytical sensitivity of Sarbeco-N was somewhat lower than our NIID-N2 assay (8), hence another assay equivalent to the NIID-N2 assay would be useful to replace the Sarbeco-N assay. In this study, the performance of the NIID-N2 assay was evaluated using representative variations of mutations in primer/probe region sequences alongside evaluation of a newly developed NIID-S2 assay targeting the spike gene.

Materials and Methods

Viruses

Wuhan-related Japanese isolates of SARS-CoV-2 (AI/I-004/2020, GISAID EPI_ISL_407084; TY/WK-521/2020, EPI_ISL_408667; TY/WK-501/2020, EPI_ISL_408666 (9)), and Euro and America-related Japanese SARS-CoV-2 isolates (QH-328-073, QH-329-037, QH-406-006, QH-406-007; TY5-329; TY5-332, TY5-337, TY5-339, TY5-340) were used. These were isolated in NIID and the viral isolation was performed using VeroE6/TMPRSS2 cells (10). AI/I-004/2020, TY/WK-521/2020, and TY/WK-501/2020 isolates were isolated from specimens collected from migrants from Wuhan in January–February 2020. QH-328-073, QH-329-037, QH-406-006, and QH-406-007 isolates were isolated from specimens collected from international travelers
(France, France, USA and USA, respectively) in March–April 2020. TY5-329 to 340 isolates were isolated from specimens collected during the Tokyo-derived COVID-19 cluster in June 2020, which were Japanese endemic isolates derived from European SARS-CoV-2 (11). SARS coronavirus (Frankfurt strain) was supplied by Dr. J. Ziebuhr, University of Würzburg, Germany. MERS-CoV EMC strain was kindly provided by Dr. Ron A. M. Fouchier, Erasmus Medical Center, Rotterdam, the Netherlands. Human orthopneumoviruses (respiratory syncytial virus [RSV], Long, A2, B WV/14617/85 [B1 wild type], and CH/18537) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Human coronavirus (HCoV)-229E isolates ATCC VR-740, Sendai-H/1121/04, and Niigata/01/08 (12) were used. HCoV-NL63 (Amsterdam I) was supplied by Dr. Lia van der Hoek, University of Amsterdam, the Netherlands. HCoV-OC43 ATCC VR-1558 was used. Human respiroviruses (parainfluenza viruses [PIV] 1 [strain C35] and 3 [strain C243]) were obtained from ATCC. Adenoviruses (ADVs) (serotype 3, strain G.B.; serotype 4, strain RI-67; and serotype 7, strain Gomen) were also obtained from ATCC. Influenza viruses (flu; A/California/7/2009 [H1N1pdm], A/Victoria/210/2009 [H3N2], and B/Brisbane/60/2008), stored in NIID, were used. Viruses were propagated and titrated using HEp-2, HeLa, RD, Vero, VeroE6, LLC-Mk2, MDCK, Vero/TMPRSS2 cells (13) or VeroE6/TMPRSS2 (10). Clinical isolates of HCoV-
OC43 (Tokyo/SGH-36/2014, LC315646; Tokyo/SGH-61/2014, LC315647; Tokyo/SGH-06/2015, LC315648; Tokyo/SGH-65/2016, LC315649), HKU1 (Tokyo/SGH-15/2014, LC315650; Tokyo/SGH-18/2016, LC315651), and NL63 (Tokyo/SGH-15/2017, LC488390; Tokyo/SGH-24/2018, LC488388) were isolated and propagated using human bronchial tracheal epithelial cells (Lifeline Cell Technology, Frederick, MD), cultured, and allowed to differentiate at an air-liquid interface, as previously described (14). Copy numbers were calculated via virus-specific real-time RT-PCR (15). Clinical isolates of RSV (A/NIID/2347/14, LC474556; A/NIID/2367/14, LC474557; A/NIID/2470/14, LC474558; B/NIID/2472/14, LC474559; B/NIID/2474/14, LC474560), isolated in HEp-2 cells, were also used (16). A clinical isolate of human metapneumovirus (hMPV; IA10-2003) was obtained from ZeptoMetrix (https://www.zeptometrix.com/). Three clinical isolates of previously reported hMPV (Sendai/0256/2015, Sendai/414/2013, and Sendai/1052/2011) were used (10), as were clinical isolates of PIVs (PIV1/NIID/79081/1/2019, PIV1/NIID/79082/2/2019, PIV2/NIID/56606/2/2019, PIV2/NIID/56607/1/2019, PIV3/NIID/79133/1/2019), isolated in NIID using Vero/TMPRSS2 cells.

Specimens and nucleic acid extraction
Pharyngeal swabs, nasal swabs, and sputum obtained during national surveillance tests were used. Nasopharyngeal and nasal swabs obtained from Discovery Life Sciences (Los Osos, CA) were also used. These were used with the approval of the Research and Ethical Committee for the Use of Human Subjects of the National Institute of Infectious Diseases, Japan (approval #1001 and 1091). TRIzol LS, TRIzol reagent (Thermo Fisher Scientific, Waltham, MA), and/or QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany) were used for nucleic acid extractions from specimens and virus stocks in accordance with the manufacturer’s instructions. Extracted RNA was diluted with nuclease-free water containing 10 μg/ml yeast RNA (R6750, Sigma-Aldrich, St. Louis, MO) as carrier.

Real-time RT-PCR for detection of SARS-CoV-2

The NIID-N2 assay was used as previously described (5). Primers and probes for detection of the spike (S) gene were designed using Primer 3 software (ver. 4.0, http://bioinfo.ut.ee/primer3-0.4.0/) based on the sequence of a Japanese viral isolate (Japan/TY/WK-521/2020, EPI_ISL_408667), as follows: SARS-CoV-2-NIID-S-F1 (8), 5’-CAGTCAGCACCTCATGGTGTA-3’; SARS-CoV-2-NIID-S-R3, 5’-GGCAGGAGCAGTTGTGAAGT-3’; SARS-CoV-2-NIID-S-P2. This set was a modification of the previously reported S set (8) and was designated as the S2 set. The
primer and probe concentrations were as follows: NIID-S-F1, 400 nM; NIID-S-R3, 700 nM; NIID-S-P2, 200 nM. The real-time RT-PCR was performed using QuantiTect Probe RT-PCR kits (Qiagen) and LightCycler systems (480, 96 and nano; Roche, Basel, Switzerland) or a QuantStudio5 system (QS5, Thermo Fisher Scientific), and the cycling condition and reaction components were as previously described (5). Cycle values (Cp for LightCycler and Ct for QS5) <40 for both N2 and S2 sets were considered as positive. Nuclease-free water containing yeast RNA was used as negative control. Positive control SARS-CoV-2 RNAs were transcribed from a T7 promotor-incorporated PCR template by Megascript T7 Transcription Kit (Thermo Fisher Scientific) (5). For the validation of mismatches, S and N region sequences of SARS-CoV-2 (WK-521 isolate) were subcloned to the cloning vector pMW119 (Nippon Gene, Tokyo, Japan) and the mutations were inserted by site-directed mutagenesis using specific primers and PrimeSTAR MAX DNA polymerase (Takara-Bio, Shiga, Japan). The gene recombination experiments were performed with the approval of the Research Promotion Bureau of the Ministry of Education, Culture, Sports, Science and Technology (no. 269 in 2020). These were used as above as the PCR template for RNA transcription. For the N gene, the 1260 bp template was amplified using following primers: forward, 5’-TAATACGACTCACTATAGGGATGTCTGATAATGGACCCCAA-3’; reverse, 5’-
TTAGGCCTGAGTTGAGTCAGCA-3’. For the S gene, the 1336 bp template was amplified using following primers, 5’-TAATACGACTCACTATAGGGCCATCAAAACCAAGCAAGAGG-3’; reverse, 5’-AGCAGGATCCACAAGAACAACAG-3’. Transcribed RNA was quantitated by measuring absorbance, and copy numbers were calculated, followed by serial dilution with nuclease free water containing yeast RNA. The copy number of viral RNA was calculated by standard curve made by measuring control RNA, and the analytical sensitivity was calculated by the Reed-Müench method.

Collation of genomic sequences of SARS-CoV-2

SARS-CoV-2 genomic sequences were collated from EpiCoV in the GISAID database (https://www.gisaid.org/). The data were collected checking “complete”, “high coverage” and “low coverage excl” checkboxes and input “human” in the host field, separating by submission date to allow data analysis (approximately 2000–9000 sequences). Each sequence was aligned based on the Wuhan-Hu-1 isolate (GenBank: MN908947) using the multiple alignment program for amino acid or nucleotide sequences (MAFFT version 7, https://mafft.cbrc.jp/alignment/server/add_fragments.html?frommanual) (17). Mismatches in targeted region sequences were analyzed using SEQUENCHER software.
(Gene Codes, Ann Arbor, MI) with these alignments. The targeted region sequences of Euro-related isolates (TY5-332–340) were read by Sanger sequencing (Fasmac Co., Ltd., Kanagawa, Japan). Sequences containing “N” in the targeted region were excluded from the analysis.

Results

Analytical sensitivity and specificity of NIID-S2 assay

First, the newly developed NIID-S2 assay was evaluated. Analytical sensitivity was evaluated using serially diluted RNA of Japanese SARS-CoV-2 isolates (Table 1). The targeted sequences of NIID-N2 and NIID-S2 sets were conserved and the analytical sensitivity showed several copies/reaction for all, suggesting the analytical sensitivity of the NIID-S2 set was comparable to the NIID-N2 set (Table 1). Second, the specificity of the NIID-S2 set was evaluated using other respiratory viruses used in the previous studies (8, 16). Identical to the NIID-N2 set, NIID-S2 showed no cross reaction with other respiratory viruses, including HCoVs (data not shown). Third, the practicality of the NIID-S2 set was evaluated using clinical specimens in comparison with the results of the NIID-N2 assay (Table 2). The NIID-S2 set showed identical results to the NIID-N2 set; that is, 27 negatives for SARS-CoV-2 negative specimens, 14 positives for SARS-CoV-2
positive specimens, and 16 negatives for other respiratory virus-detected specimens. These findings indicate that the performance of the NIID-S2 set is comparable to the NIID-N2 assay.

**Evaluation of nucleotide mismatches in NIID-N2 and NIID-S2 assay.**

During the nearly 12 months since the emergence of SARS-CoV-2, a large number of full-length genomic sequences have been deposited in public databases. GISAID is one such database and the number of registered sequences is much larger than that of the National Center for Biotechnology Information (NCBI) (around 256,000 vs. around 30,000 as of 10th December, 2020). Therefore, sequence data were collected from the EpiCoV of GISAID. Data were narrowed down by quality and 192,325 sequences were available (Fig. 1a), and mutations in primer/probe target sequences were analyzed. Many mismatches were detected but most of them were scattered, and common mutations (more than 100 sequences) are listed in Fig. 1b. As for the NIID-N2 set, substitutions of G to T at nucleotide (nt) 16 (N2F_G16T) and C to T at nt20 (N2F_C20T) from the 5′ end were seen in the forward primer. In the reverse primer, substitutions of G to A at nt9 (N2Rver3_G9A) and G to T at nt11 (N2Rver3_G11T), while in the probe, substitution of G to T at nt6 (N2P_G6T) from the 5′ end were seen. In the NIID-S2 set, substitution of T
to G at nt9 (S2_R3_T9C) from the 5′ end was seen. In the probe, substitutions of G to T at nt2 (S2_P_G2T), G to T at nt 20 (S2_P_G20T), and T to C at nt22 (S2_P_T22C) from the 5′ end were seen. In the NIID-N2 set, 4933 mismatches (2.57%) were seen and N2P_G6T accounted for more than half (3105, 1.62%) (Table 3). In the NIID-S2 set, 2321 mismatches (1.21%) were seen in total, but the above four mismatches accounted for more than 60% (1421, 0.75%) (Table 3). The number of available sequences increased from around September 2020, and mismatches also increased along with the increase in sequence registration (Fig. 2). Although the number of mismatches increased, their occurrence rate remained stable around 1% in the NIID-S2 set but increased in the NIID-N2 set since September 2020, caused by increased incidence of N2P_G6T. The effects of these mutations on the amplification reaction were evaluated using control RNA made by in vitro transcription from mutated template (Table 3). The sensitivities of the assay were not affected by the existence of mutations except N2F_C20T. N2F_C20T mutation was just at the 3′ end and it affected the analytical sensitivity (59.3 copies/reaction). However, when the forward primer was replaced with one that had the C20T mutation at the 3′ end, the analytical sensitivity recovered to 1.4 copies/reaction, suggesting that this substitution decreased the amplification efficiency approximately 40-fold. However, the mutation did not abolish the amplification completely. Among the various mutations, N2P_G6T
showed no difference in calculated Cp value and the analytical sensitivity remained less than five copies/reaction (Table 3). However, the mutation affected the fluorescence value and the shape of the amplification curve (Fig. 3). The N2P_G6T substitution made the curve more shallow in the LightCycler device compared with wild-type sequence (Fig. 3a). On the QS5 machine, the shape of the amplification curve and the analytical sensitivity were unaffected but the Ct values increased compared with no mutation (Fig. 2b). The degenerate probe that contained K in 6th nucleotide was evaluated but it induced non-specific signals in negative targets, suggesting the mixed primer should not be used in practical use (data not shown).

**Discussion**

This study first describes the newly developed NIID-S2 assay for detection of SARS-CoV-2. The analytical sensitivity and specificity of NIID-S2 were comparable to the NIID-N2 assay and the performance in practical use with clinical specimens was identical to that of NIID-N2. These findings suggest that the NIID-S2 assay is sufficient to be included in the protocol for national surveillance of SARS-CoV-2 in Japan.

The SARS-CoV-2 pandemic shows no sign of ending, with huge numbers of cases being recorded daily (3, 4). Accordingly, many full-length genomic sequences have been
decoded around the world and deposited in official databases. During this coronaviral pandemic, GISAID has played a central role as the main database for genetical analysis of SARS-CoV-2. Therefore, in this study, sequence data were collected from the EpiCoV of GISAID and 192,325 sequences were available by narrowed down by quality (as of 10th December, 2020). Among these sequences, more than half (117,200) were registered from Europe and most data were from the UK (85,085). Together with sequences from North America (39,501), these two regions accounted for 81.4% of sequences. Most sequences registered as “Oceania” were from Australia. The number of sequences registered from Asia and the Middle East was 18,191 and data from Japan accounted for half of these (9385). Thus, although 74% of COVID-19 cases were reported from Americas and Europe, the bias for sequence origin in the GISAID database should be noted (3).

Analysis of primer/probe sequence mismatches using deposited SARS-CoV-2 sequences showed that a wide variety of mismatches exist; however, the most common mutations did not affect detection efficiency by both NIID-N2 and NIID-S2 assays, except the mutation in the 3’ end of the NIID-N2 assay forward primer (N2F_C20T). Although the N2F_C20T mutation decreased the analytical sensitivity, the NIID-N2 set still detected this mutated sequence. The occurrence rate of the N2F_C20T mutation was
0.17% and was likely this low. Although, the N2F_C20T mutation was initially seen in sequences from Spain and Portugal, it spread around the world, and no certain origin was ascertained, and 36 sequences were deposited in the GISAID database from Japan. Usage of NIID-S2 can support the detection of SARS-CoV-2 containing this mutation by NIID-N2 assay.

The N2P_G6T mutation did not affect the analytical sensitivity of the assay, though it affected the shape of amplification curve on the device that used the 2nd derivative max method, and affected Ct values on the device that used the crossing-point method for calculation of results. As described above, 3105 N2P_G6T sequences were registered on GISAID as of 10th December, 2020, with almost all of them (3024, 97.4%) being from the UK, indicating a British origin for sequences carrying this mutation; therefore, if a case has direct or indirect epidemiological links to the UK, attention should be paid to the amplification process. However, using NIID-S2 and NIID-N2 together resolve this concern.

**Competing interests**

No author has any competing interest.
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Figure legends.

Figure 1. Analysis of mismatches in sequences of the NIID assays primers/probes. Full-
length sequences of SARS-CoV-2 were collated from EpiCoV of the GISAID database. The data were collected checking “complete”, “high coverage” and “low coverage excl” checkboxes and input “human” in host field. The alignments were generated by using MAFFT with Wuhan-Hu-1 isolate (GenBank: MN908947) as the contrast. Mismatches in the targeted region sequences were detected using SEQUENCHER software (Gene Codes, Ann Arbor, MI).

a) Registered and available sequence numbers on 10th December 2020.

b) The position of mismatches in primers/probes of the NIID assays. Mutations registered in more than 100 sequences are listed.

Figure 2. Time-course of the number of registered sequences. Left graphs indicate NIID-N2 assay; right graphs indicate NIID-S2 assay. Sequence data were collected as described in figure legend 1. Upper panel shows available sequence numbers; middle panel shows number of mismatches; lower panel shows occurrence rate of mutations against total number of available sequences.

Figure 3. Images of amplification curves during amplification of the N2P_G6T mutation.

a) A LightCycler 96 was used for the amplification of serially diluted control RNA
template (2500, 250, 25, 2.5 copies/reaction). Upper panel shows original (non-mutated) template; middle panel shows the N2P_G6T template; lower panel shows merged image.

b) A QS5 machine was used for comparison amplifications. Left panel shows original template; right panel shows N2P_G6T template.
Table 1. Analytical sensitivities of NIID assays for Japanese SARS-CoV-2 isolates.

| Origin of isolate | Name of isolate | Analytical sensitivity (copies/reaction)* |
|-------------------|-----------------|------------------------------------------|
|                   |                 | NIID-N2 set | NIID-S2 set |
| Wuhan             | AI/I-004/2020   | 7.9         | 4.4         |
|                   | TY/WK-521/2020  | 1.4         | 1.4         |
|                   | TY/WK-501/2020  | 1.4         | 4.4         |
|                   | Wuhan-total*    | 2.5         | 3.0         |
| Europe            | QH-328-037      | 1.7         | 7.9         |
|                   | QH-329-073      | 3.7         | 1.7         |
|                   | QH-406-006      | 3.7         | 3.7         |
|                   | QH-406-007      | 3.7         | 1.7         |
|                   | Euro-total      | 3.0         | 3.0         |
| Tokyo             | TY5-329         | 4.4         | 7.9         |
|                   | TY5-332         | 2.5         | 4.4         |
|                   | TY5-337         | 4.4         | 5.4         |
|                   | TY5-339         | 1.4         | 7.9         |
|                   | TY5-340         | 1.4         | 4.4         |
|                   | Tokyo-total     | 2.5         | 5.8         |

*Analytical sensitivities were shown as actual copies/reaction.

**Total analytical sensitivities were calculated using all data every isolate origin.
Table 2. Evaluation of NIID assays using clinical specimens.

| No. | Detection result | Specimen type | (Cp value) |
|-----|------------------|----------------|------------|
|     |                  |                | NIID-N2 set | NIID-S2 set |
| 1   | Not detected     | Pharyngeal swab| -          | -           |
| 2   | Not detected     | Pharyngeal swab| -          | -           |
| 3   | Not detected     | Pharyngeal swab| -          | -           |
| 4   | Not detected     | Pharyngeal swab| -          | -           |
| 5   | Not detected     | Pharyngeal swab| -          | -           |
| 6   | Not detected     | Pharyngeal swab| -          | -           |
| 7   | Not detected     | Pharyngeal swab| -          | -           |
| 8   | Not detected     | Pharyngeal swab| -          | -           |
| 9   | Not detected     | Pharyngeal swab| -          | -           |
| 10  | Not detected     | Pharyngeal swab| -          | -           |
| 11  | Not detected     | Pharyngeal swab| -          | -           |
| 12  | Not detected     | Pharyngeal swab| -          | -           |
| 13  | Not detected     | Pharyngeal swab| -          | -           |
| 14  | Not detected     | Pharyngeal swab| -          | -           |
| 15  | Not detected     | Pharyngeal swab| -          | -           |
| 16  | Not detected     | Pharyngeal swab| -          | -           |
| 17  | Not detected     | Pharyngeal swab| -          | -           |
| 18  | Not detected     | Pharyngeal swab| -          | -           |
| 19  | Not detected     | Pharyngeal swab| -          | -           |
| 20  | Not detected     | Pharyngeal swab| -          | -           |
| 21  | Not detected     | Pharyngeal swab| -          | -           |
| 22  | Not detected     | Pharyngeal swab| -          | -           |
| 23  | Not detected     | Pharyngeal swab| -          | -           |
| 24  | Not detected     | Pharyngeal swab| -          | -           |
| 25  | Not detected     | Nasal swab     | -          | -           |
| 26  | Not detected     | Sputum         | -          | -           |
| 27  | Not detected     | Sputum         | -          | -           |
| 28  | SARS-CoV-2       | Pharyngeal swab| 23.3       | 24.15       |
| 29  | SARS-CoV-2       | Pharyngeal swab| 25.1       | 25.87       |
| 30  | SARS-CoV-2       | Pharyngeal swab| 27.4       | 28.15       |
| 31  | SARS-CoV-2       | Pharyngeal swab| 29.7       | 30.57       |
| 32  | SARS-CoV-2       | Pharyngeal swab| 29.7       | 30.46       |
|   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|
| 33 | SARS-CoV-2 | Pharyngeal swab | 28.9 | 30.43 |   |   |
| 34 | SARS-CoV-2 | Pharyngeal swab | 30.5 | 32.16 |   |   |
| 35 | SARS-CoV-2 | Pharyngeal swab | 32.7 | 33.54 |   |   |
| 36 | SARS-CoV-2 | Pharyngeal swab | 35.9 | 36.14 |   |   |
| 37 | SARS-CoV-2 | Pharyngeal swab | 36.2 | 38.53 |   |   |
| 38 | SARS-CoV-2 | Nasopharyngeal swab | 34.9 | 35.0 |   |   |
| 39 | SARS-CoV-2 | Pharyngeal swab | 33.7 | 33.9 |   |   |
| 40 | SARS-CoV-2 | Pharyngeal swab | 28.7 | 28.1 |   |   |
| 41 | SARS-CoV-2 | Sputum | 22.5 | 28.1 |   |   |
| 42 | ADV C | Nasopharyngeal swab | - | - |   |   |
| 43 | ADV C | Nasopharyngeal swab | - | - |   |   |
| 44 | hMPV | Nasopharyngeal swab | - | - |   |   |
| 45 | hMPV | Nasopharyngeal swab | - | - |   |   |
| 46 | Influenza A | Nasopharyngeal swab | - | - |   |   |
| 47 | Influenza A | Nasopharyngeal swab | - | - |   |   |
| 48 | PIV1 | Nasopharyngeal swab | - | - |   |   |
| 49 | PIV1 | Nasopharyngeal swab | - | - |   |   |
| 50 | PIV2 | Nasopharyngeal swab | - | - |   |   |
| 51 | PIV2 | Nasopharyngeal swab | - | - |   |   |
| 52 | PIV3 | Nasopharyngeal swab | - | - |   |   |
| 53 | PIV3 | Nasopharyngeal swab | - | - |   |   |
| 54 | RSV A | Nasopharyngeal swab | - | - |   |   |
| 55 | RSV B | Nasopharyngeal swab | - | - |   |   |
| 56 | RSV A | Nasal swab | - | - |   |   |
| 57 | RSV B | Nasal swab | - | - |   |   |

*not detected*
Table 3. Effect of mutations in primer/probe regions on analytical sensitivities of NIID assays.

|                | Analytical sensitivities | Number of mismatches*** | Existence rate (%)**** |
|----------------|--------------------------|--------------------------|------------------------|
|                | N2 (Ver.3)               | N2F_C20T**               |                        |
| Control template | N2 original (Ver.3)      | 2.6*                     | N/A                    | 4933                   | 2.57                   |
|                | N2F_G16T                 | 2.5                      | N/A                    | 129                    | 0.07                   |
|                | N2F_C20T                 | 59.3                     | 1.4                    | 325                    | 0.17                   |
|                | N2Rver3_G9A              | 4.4                      | N/A                    | 348                    | 0.18                   |
|                | N2Rver3_G11T             | 4.4                      | N/A                    | 123                    | 0.06                   |
|                | N2P_G6T                  | 0.8                      | N/A                    | 3105                   | 1.62                   |
|                | S2 original              | 1.1                      |                        | 2321                   |                        |
| Control template | S2_R3_T9C               | 1.4                      |                        | 376                    | 0.20                   |
|                | S2_P2_G2T                | 0.8                      |                        | 245                    | 0.13                   |
|                | S2_P2_G20T               | 0.8                      |                        | 325                    | 0.17                   |
|                | S2_P2_T22C               | 2.5                      |                        | 476                    | 0.25                   |

*The analytical sensitivities were shown as actual copies/reaction

**N2F_20T, forward primer.

***The data on 10th December 2020.

****Existence rate (%) of mutated sequence numbers against for available sequence numbers.
**Fig. 1**

### a)

| Registered sequence numbers | 256429 |
|----------------------------|--------|
| Available sequences numbers | 192325 |

| Geography              | Numbers | Existence Rate (%) |
|------------------------|---------|--------------------|
| Euro                   | 117200  | 60.9               |
| United Kingdom         | 85085   |                    |
| North America          | 39501   | 20.5               |
| USA                    | 36562   |                    |
| South America          | 1585    | 0.8                |
| Asia + Middle East     | 18191   | 9.5                |
| Japan                  | 9385    |                    |
| Oceania                | 13185   | 6.9                |
| Australia              | 12471   |                    |
| Africa                 | 2593    | 1.3                |

### b)

**NIID-N2 set**

**Sequence (5' to 3')**

- **NIID_2019-nCOV_N_F2**
  - N2F_G16T: AAATTGGGGGACCAGGAAC
  - N2F_C20T: AAATTGGGGGACCAGGAAT

- **NIID_2019-nCOV_N_R2ver3**
  - N2Rver3_G9A: TGGCACCTGTGCTGCTGCAAC
  - N2Rver3_G11T: TGGCACCTGTAGGTCAAC

- **NIID_2019-nCOV_N_P2**
  - N2P_G6T: ATGTCCGCATTGGCATCGGA

**NIID-S2 set**

**Sequence (5' to 3')**

- **SARS-CoV2_NIID_S_F1**
  - CAGTCAGCAGCCTCATGAGTGTA

- **SARS-CoV2_NIID_S_R3**
  - S_R3_T9C: AACCAGTGTGTGCGCATTTGA

- **SARS-CoV2_NIID_S_P2**
  - S_P2_G2T: TGCTCCTGCCATTGGTCTGATGAG
  - S_P2_G20T: TGCTCCTGCCATTGGTCTGATGAG
  - S_P2_T22C: TGCTCCTGCCATTGGTCTGATGACGG
Fig. 2

- Available sequence numbers (NIID-N2 all)
  - Number of mismatches (NIID-N2 all)
    - Existence rate of mismatches (NIID-N2 all)
  - Available sequence numbers (NIID_S2_all)
    - Number of mismatches (NIID_S2_all)
      - Existence rate of mismatches (NIID_S2_all)
Fig. 3

(a) Control RNA (Original)

(b) Control RNA (N2P_G6T)