The primary outbreak of coronavirus induced disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China in December 2019, and is now rapidly spreading worldwide (1,2). The genomic sequence of the SARS-CoV-2 was first released on January 10, 2020 (GenBank accession number: MN908947). As of February 22, 2020, 77,794 confirmed cases and 2,376 deaths from COVID-19 have been reported (3). Although >98% of the COVID-19 cases were detected in China, 28 countries have already experienced COVID-19 cases, with 1,402 confirmed cases outside of China (3). Japan reported the first COVID-19 case on January 15, 2020, and as of February 21, 2020, Japan had 69 domestically confirmed cases and 10 returnees from Wuhan, who came back through government-chartered flights (4).

Moreover, 14 asymptomatic cases have also been reported (4). Furthermore, as of February 18, 2020, 531 cases (14.3% of the total number of individuals on board a quarantined cruise ship on 5 February) were confirmed having the SARS-CoV-2 infection (5).

Therefore, availability of reliable diagnostic assay(s) is crucial if we want to understand the situation regarding the current disease outbreak and implement effective counter-measures against COVID-19. We have recently published a protocol of real-time RT-PCR using two primer sets, N set no.1 (N set) and N set no.2 (N2 set), both of which can specifically detect the SARS-CoV-2 nucleocapsid (N) gene (6). Both sets have unique positive controls containing marker sequences, which can differentiate cross-contamination signals from N gene-specific signals (6). The protocol was validated using the QuantiTect Probe RT–PCR Kit (Qiagen, Hilden Germany), because most of the local public institutes in Japan are equipped with this kit. The primers and probe for the N set were designed previously by Corman et al. (7). In the assay using the QuantiTect Probe RT–PCR Kit, the sensitivity of our original N2 set (6) was found to be higher than that of the N set. Other assays of Corman did not work so well with this reagent. Thus, on January 24, 2020, the laboratory diagnosis method at the National Institute of Infectious Diseases Surveillance Center, Department of Virology III, and Influenza Virus Research Center, National Institute of Infectious Diseases, Murayama Branch, Tokyo; and Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan
Real-Time PCR for SARS-CoV-2

Table 1. Analysis of SARS-CoV-2 RNAs purified from isolated viruses

| copies/well | LightMix N-gene | N2 | N | E-gene |
|------------|-----------------|----|---|--------|
| 2.5        | 0/2*            | 0/2| 1/2| 0/2    |
| 25         | 1/2             | 0/2| 2/2| 0/2    |
| 250        | 2/2             | 0/2| 2/2| 2/2    |
| 2500       | 2/2             | 2/2| 2/2| 2/2    |
| Negative control | 0/2  | 0/2| 0/2| 0/2 |
| Positive control | 2/2  | 2/2| 2/2| 2/2 |

*: Positive number of wells / Total number of wells.

Table 2. Analysis using clinical specimens from COVID-19-suspected individuals

| sample ID | LightMix N-gene | E-gene | N2 |
|-----------|-----------------|--------|----|
| 1         | –               | –      | –  |
| 2         | –               | –      | –  |
| 3         | –               | –      | –  |
| 4         | –               | –      | –  |
| 5         | –               | –      | –  |
| 6         | –               | –      | –  |
| 7         | –               | –      | –  |
| 8         | –               | –      | –  |
| 9         | –               | –      | –  |
| 10        | –               | –      | –  |
| 11        | –               | –      | –  |
| 12        | –               | –      | –  |
| 13        | –               | –      | –  |
| 14        | –               | –      | –  |
| 15        | –               | –      | –  |
| 16        | –               | +      | +  |
| 17        | +               | +      | +  |
| 18        | –               | +      | +  |
| 19        | +               | +      | +  |
| 20        | +               | +      | +  |
| 21        | +               | +      | +  |
| 22        | +               | +      | +  |
| 23        | +               | +      | +  |
| 24        | +               | +      | +  |
| 25        | +               | +      | +  |
| NC        | –               | –      | –  |
| PC        | +               | +      | +  |

+, positive result; –, negative result; NC, negative control; PC, positive control.

of Infectious Diseases (Japan) was set up using the N and N2 sets. On January 30–31, 2020, both the reagents and the protocol (6) were distributed to the local public health institutes and quarantine depots in Japan, and SARS-CoV-2 laboratory diagnostic testing was initiated nationwide.

To further validate the laboratory diagnostic abilities of the N and N2 sets, their sensitivities were compared with those of the following commercially available detection kits that were used for previous research: a) LightMix Modular SARS and Wuhan CoV E-gene (LM S&W-E); b) LightMix Modular SARS and Wuhan CoV N-gene (LM S&W-N); and c) LightMix Modular Wuhan CoV RdRP-gene (LM W–RdRP) (TIB MOLBIOL, Berlin Germany) (8). The LM S&W-N and -E kits were shown to widely detect SARS-like betacoronaviruses, including bat coronaviruses, whereas the LM W-RdRP kit could specifically detect the SARS-CoV-2 (9).

First, the sensitivities of N and N2 sets, and LM S&W-E, LM S&W-N, and LM W-RdRP kits were compared using the SARS-CoV-2 viral RNAs. The viral RNAs were prepared from the isolate (GISAID no. EPI_ISL_4070840) obtained from the clinical specimens of COVID-19 patients (10). They were extracted from viral culture supernatants using the TRIzol LS reagent (Thermo Fisher Scientific, Waltham, MA, USA), and the viral RNA copy numbers were calculated by real-time RT-PCR assays. The LM S&W-E, LM S&W-N, and LM W-RdRP assays were conducted according to the manufacturer’s instructions, whereas the real-time RT-PCR assays using the N and N2 sets were performed using the QuantiTect Probe RT–PCR Kit (6). Both assays were conducted on the LightCycler 480 (Roche, Basel, Switzerland). In these assays, the LM S&W-E kit could detect as low as 2.5 copies of viral RNA in one of the two wells used in the plate (Table 1). The LM S&W-E and N2 sets detected 25 copies of viral RNA in the corresponding two wells. However, the LM S&W-N and N sets required a higher number of viral RNA copies for detection than those in the LM S&W-E and N2 sets (Table 1). Also, the LM W-RdRP set was less sensitive than the other primer/probe sets, suggesting that this kit was not suitable for practical use. These results also suggest the practical use of the LM S&W-E and LM S&W-N kits in current scenario because of their high sensitivities.

Further analysis was conducted with viral RNA extracted from clinical specimens using real-time RT-PCR assays and the LM S&W-E, LM S&W-N, and N2 sets, and with the samples collected from 25 COVID-19-suspected individuals. These clinical specimens were collected as public health diagnostic activities and used under the approval of Ethical Committee of our institute (refer to the Ethical statement). The results using the LM S&W-E and N2 sets were consistent with each other, whereas two samples, which were positive using the LM S&W-E and N2 sets, yielded negative results with the LM S&W-N set (Table 2). For the true negative samples, all the assays showed the same result (all samples were negative). This suggests that the LM S&W-E and N2
sets exhibit similar detection sensitivities towards the SARS-CoV-2 in clinical specimens. The LM S&W-E set targets the highly conserved region of the E gene in the SARS-CoV and SARS-CoV-2 (7), while the N2 set targets a unique region in the SARS-CoV-2 N gene. Moreover, no cross-reactivity of the N2 set with the SARS-CoV was identified (data not shown). Therefore, the N2 set has been implied to exhibit high specificity and sensitivity for SARS-CoV-2 detection. To date, many clinical specimens have been analyzed using the N and N2 sets in Japan, and undoubtedly the positivity rate of N2 set has been found to be higher than that of N set. Cases that were tested positive with only the N2 set have often been associated with samples containing very low viral RNA copy number, suggesting that the N2 set is much more sensitive than the N set.

Conclusively, this study has shown that the protocol using the N and N2 sets is comparable to the commercially available kits, LM S&W-E and LM S&W-N, and is reliable for SARS-CoV-2 detection, and thus can be implemented for laboratory diagnosis of COVID-19. (This study was approved by the Ethics Committee of National Institute of Infectious Diseases at approved number 1091).

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Conflict of interest None to declare.

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