

Comparative Analysis of Primer–Probe Sets for RT-qPCR of COVID-19 Causative Virus (SARS-CoV-2)

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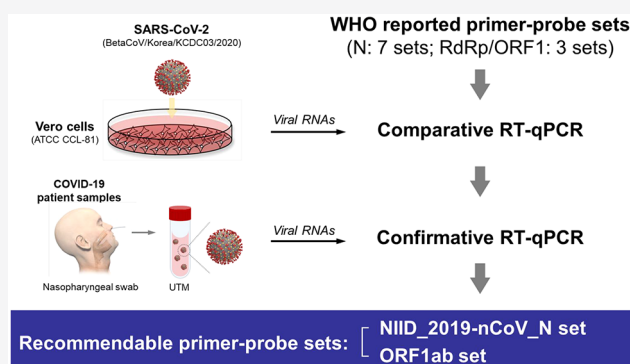
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ABSTRACT: Coronavirus disease 2019 (COVID-19) is a newly emerging human infectious disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2, also previously known as 2019-nCoV). Within 8 months of the outbreak, more than 10,000,000 cases of COVID-19 have been confirmed worldwide. Since human-to-human transmission occurs easily and the rate of human infection is rapidly increasing, sensitive and early diagnosis is essential to prevent a global outbreak. Recently, the World Health Organization (WHO) announced various primer–probe sets for SARS-CoV-2 developed at different institutions: China Center for Disease Control and Prevention (China CDC, China), Charité (Germany), The University of Hong Kong (HKU, Hong Kong), National Institute of Infectious Diseases in Japan (Japan NIID, Japan), National Institute of Health in Thailand (Thailand NIH, Thailand), and US CDC (USA). In this study, we compared the ability to detect SARS-CoV-2 RNA among seven primer–probe sets for the N gene and three primer–probe sets for the Orf1 gene. The results revealed that “NIID_2019-nCoV_N” from the Japan NIID and “ORF1ab” from China CDC represent a recommendable performance of RT-qPCR analysis for SARS-CoV-2 molecular diagnostics without nonspecific amplification and cross-reactivity for hCoV-229E, hCoV-OC43, and MERS-CoV RNA. Therefore, the appropriate combination of NIID_2019-nCoV_N (Japan NIID) and ORF1ab (China CDC) sets should be selected for sensitive and reliable SARS-CoV-2 molecular diagnostics.

KEYWORDS: SARS-CoV-2, real-time qPCR, molecular diagnosis, 2019-nCoV, COVID-19



The current outbreak of coronavirus disease 2019 (COVID-19), first reported to the World Health Organization (WHO) on December 31, 2019, involves 10,185,374 confirmed cases over 216 countries as of June 30, 2020.¹ The majority of COVID-19 patients develop pneumonia and exhibit symptoms including fever and cough.^{2,3} The genome sequence of the causative agent, a novel coronavirus, was shared through the Global Initiative on Sharing All Influenza Data (GISAID) platform beginning January 12, 2020. The sequences of the novel coronavirus (CoV) are highly similar to those of severe acute respiratory syndrome-related coronaviruses (SARSr-CoV), and like SARS-CoV, the virus uses ACE2 as the entry receptor.^{4–6} The Coronavirus Study Group of the International Committee on Taxonomy of Viruses designated the virus as Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2).⁷

Molecular diagnosis of COVID-19 is currently carried out by one-step quantitative RT-PCR (RT-qPCR) targeting SARS-CoV-2 using primers and probes developed by China CDC, Charité, HKU, Japan NIID, Thailand NIH, and US CDC; these

primer–probe sets were posted by the WHO.^{8–10} Clinical diagnosis methods including CT scanning have also been used to identify COVID-19 cases in Hubei province, China, starting on February 13, 2020.¹¹ Although the RT-qPCR assay served as a gold-standard method for detecting respiratory infectious viruses such as SARS-CoV and MERS-CoV,^{12–15} current RT-qPCR assays targeting SARS-CoV-2 have some limitations. First, due to the high similarity between SARS-CoV-2 and SARS-CoV, primer–probe sets cross-react. Second, the sensitivity of these assays may not be sufficient to confirm suspicious patients at early time points after admission. Indeed, several cases have been reported in which CT scan results were

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Table 1. Comparative Analysis of Ct Values Obtained from the Representative Experiment by Employing Each Primer–Probe Set

reaction temperature (°C)	target	institute	name	1.5 × 10 ⁴ copies			1.5 × 10 ³ copies			1.5 × 10 ² copies			1.5 × 10 ¹ copies			NTC	
				mean Ct	STD	mean Ct	mean Ct	STD	mean Ct	STD	mean Ct	STD	mean Ct	STD	mean Ct	STD	mean Ct
60	N	China CDC HKU Japan NIID Thailand NIH US CDC	N HKU-N NIID_2019-nCoV_N WH-NIC N 2019-nCoV_N1 2019-nCoV_N2 2019-nCoV_N3 ORFlab RdRp_SARSr HKU-ORFlb-nsp14	24.01	0.062	26.96	0.049	0.049	30.46	0.534	34.86	0.119	N/A	N/A	N/A	N/A	N/A
				26.00	0.055	29.45	0.083	0.083	33.17	0.178	35.43	0.902	N/A	N/A	N/A	N/A	N/A
				23.09	0.053	26.56	0.150	0.150	29.5	0.199	33.13	0.954	N/A	N/A	N/A	N/A	N/A
				28.64	0.058	31.89	0.237	0.237	35.26	0.200	38.13	0.885	N/A	N/A	N/A	N/A	N/A
				24.25	0.185	27.50	0.095	0.095	30.57	0.445	34.71	0.770	N/A	N/A	N/A	N/A	N/A
				22.88	0.097	26.12	0.133	0.133	29.26	0.900	33.14	1.822	N/A	N/A	N/A	N/A	N/A
				22.64	0.082	26.01	0.112	0.112	29.42	0.260	33.09	0.857	N/A	N/A	N/A	N/A	N/A
				27.33	0.085	30.33	0.061	0.061	33.61	0.117	36.85	0.255	N/A	N/A	N/A	N/A	N/A
				31.89	0.115	35.14	0.050	0.050	38.57	0.707	43.00 ^b	1.959 ^b	N/A	N/A	N/A	N/A	N/A
				29.04	0.217	32.03	0.206	0.206	35.33	0.446	38.97	0.594	N/A	N/A	N/A	N/A	N/A
				25.07	0.129	28.59	0.165	0.165	33.72	0.785	35.49	0.140	38.06 ^a	-	-	-	-
				26.73	0.040	29.85	0.192	0.192	34.80	0.287	37.69	0.896	N/A	N/A	N/A	N/A	N/A
58	N	China CDC HKU Japan NIID Thailand NIH US CDC	N HKU-N NIID_2019-nCoV_N WH-NIC N 2019-nCoV_N1 2019-nCoV_N2 2019-nCoV_N3 ORFlab RdRp_SARSr HKU-ORFlb-nsp14	23.40	0.021	27.08	0.080	0.080	31.79	0.278	34.66	0.181	N/A	N/A	N/A	N/A	N/A
				26.96	0.078	30.37	0.093	0.093	34.91	0.379	35.74	0.465	36.98 ^b	0.667 ^b	0.667 ^b	0.667 ^b	0.667 ^b
				24.80	0.035	28.93	0.145	0.145	32.23	0.150	36.17	1.483	N/A	N/A	N/A	N/A	N/A
				24.37	0.040	28.08	0.491	0.491	31.37	0.433	35.05	0.577	N/A	N/A	N/A	N/A	N/A
				24.57	0.047	27.51	0.158	0.158	31.83	0.320	33.99	0.479	N/A	N/A	N/A	N/A	N/A
				24.12	0.064	27.81	0.104	0.104	32.13	0.631	35.21	0.951	N/A	N/A	N/A	N/A	N/A
				27.34	0.266	31.19	0.248	0.248	35.94 ^b	0.580 ^b	N/A	N/A	N/A	N/A	N/A	N/A	N/A
				24.56	0.053	28.12	0.153	0.153	32.84	0.046	35.51	0.725	N/A	N/A	N/A	N/A	N/A
				25.26	0.086	29.56	0.201	0.201	33.11	0.023	36.05	0.700	N/A	N/A	N/A	N/A	N/A
				26.03	0.153	30.89	0.307	0.307	33.71	0.230	36.32	0.497	N/A	N/A	N/A	N/A	N/A
				24.13	0.070	28.70	0.140	0.140	31.44	0.180	35.17	1.104	N/A	N/A	N/A	N/A	N/A
				26.09	0.021	30.83	0.363	0.363	33.32	0.317	35.79	0.430	N/A	N/A	N/A	N/A	N/A
55	N	China CDC HKU Japan NIID Thailand NIH US CDC	N HKU-N NIID_2019-nCoV_N WH-NIC N 2019-nCoV_N1 2019-nCoV_N2 2019-nCoV_N3 ORFlab RdRp_SARSr HKU-ORFlb-nsp14	24.84	0.232	29.85	0.364	0.364	32.17	0.075	35.51	0.905	N/A	N/A	N/A	N/A	N/A
				25.34	0.171	30.55	0.103	0.103	32.95	0.143	35.32	0.332	N/A	N/A	N/A	N/A	N/A
				24.53	0.046	28.99	0.176	0.176	31.58	0.349	34.89	0.870	37.11 ^a	-	-	-	-
				24.34	0.023	28.14	0.134	0.134	31.86	0.407	34.10	0.653	N/A	N/A	N/A	N/A	N/A
				28.24	0.141	33.12	0.612	0.612	36.09	0.097	38.00 ^b	1.089 ^b	N/A	N/A	N/A	N/A	N/A
				24.51	0.139	28.34	0.187	0.187	31.43	0.778	35.38	1.650	N/A	N/A	N/A	N/A	N/A

^aThe assay showed a positive signal from a single reaction of the triplicate. ^bThe assay showed positive signals from the two reactions of the triplicate.

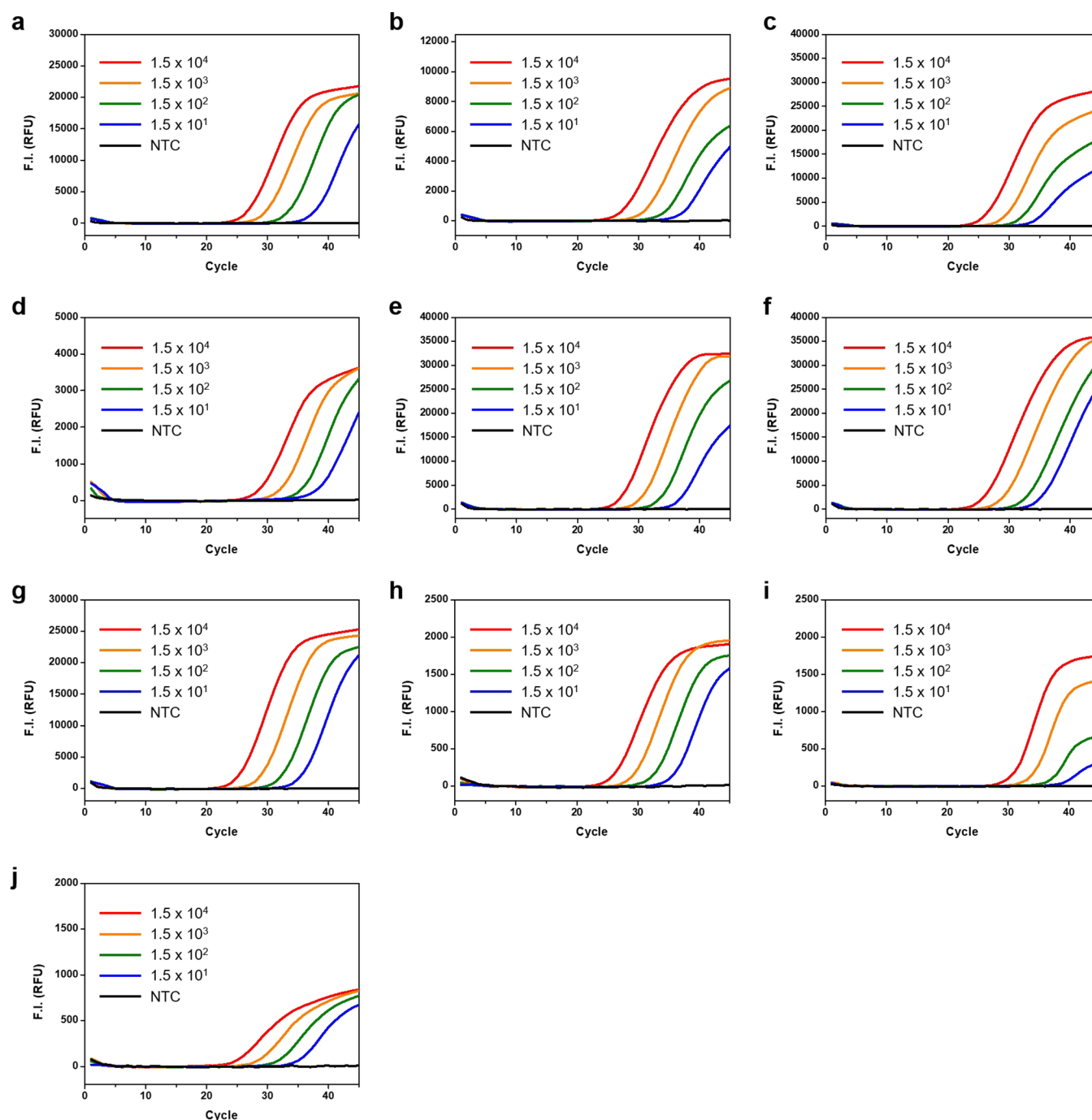


Figure 1. Representative amplification curves of fluorescence intensity against PCR cycle with each primer–probe set (amplification is performed at 60 °C). (a) N (China CDC), (b) HKU-N (HKU), (c) NIID_2019-nCoV_N (Japan NIID), (d) WH-NIC N (Thailand NIH), (e) 2019-nCoV_N1 (US CDC), (f) 2019-nCoV_N2 (US CDC), (g) 2019-nCoV_N3 (US CDC), (h) ORF1ab (China CDC), (i) RdRp_SARSr (Charité), and (j) HKU-ORF1b-nsp14 (HKU).

positive but RT-PCR results were negative at initial presentation.¹⁶ The performance of molecular diagnostics might depend on primers, probes, and reagents. Previous studies have been reported to compare some primer–probe sets listed in the WHO for the molecular diagnosis of SARS-CoV-2.^{17,18}

In this study, we performed RT-qPCR analysis with previously reported specific primer–probe sets targeting the RdRp/Orf1 and N region of SARS-CoV-2. We report here our results comparing the performance of SARS-CoV-2 molecular detection using three different primer–probe sets for the RdRp/

Orf1 region and seven different primer–probe sets for the N region.

RESULTS AND DISCUSSION

Validation of the RT-qPCR Assay. The negative control did not yield a Ct value, indicating that the reaction was performed aseptically. The standard curve from the E gene primer–probe set confirmed that the reaction was performed correctly. The R^2 value of the standard curve was 0.999, and the calculated amplification efficiency was 101.6%, indicating that the RT-qPCR reaction was performed under optimal con-

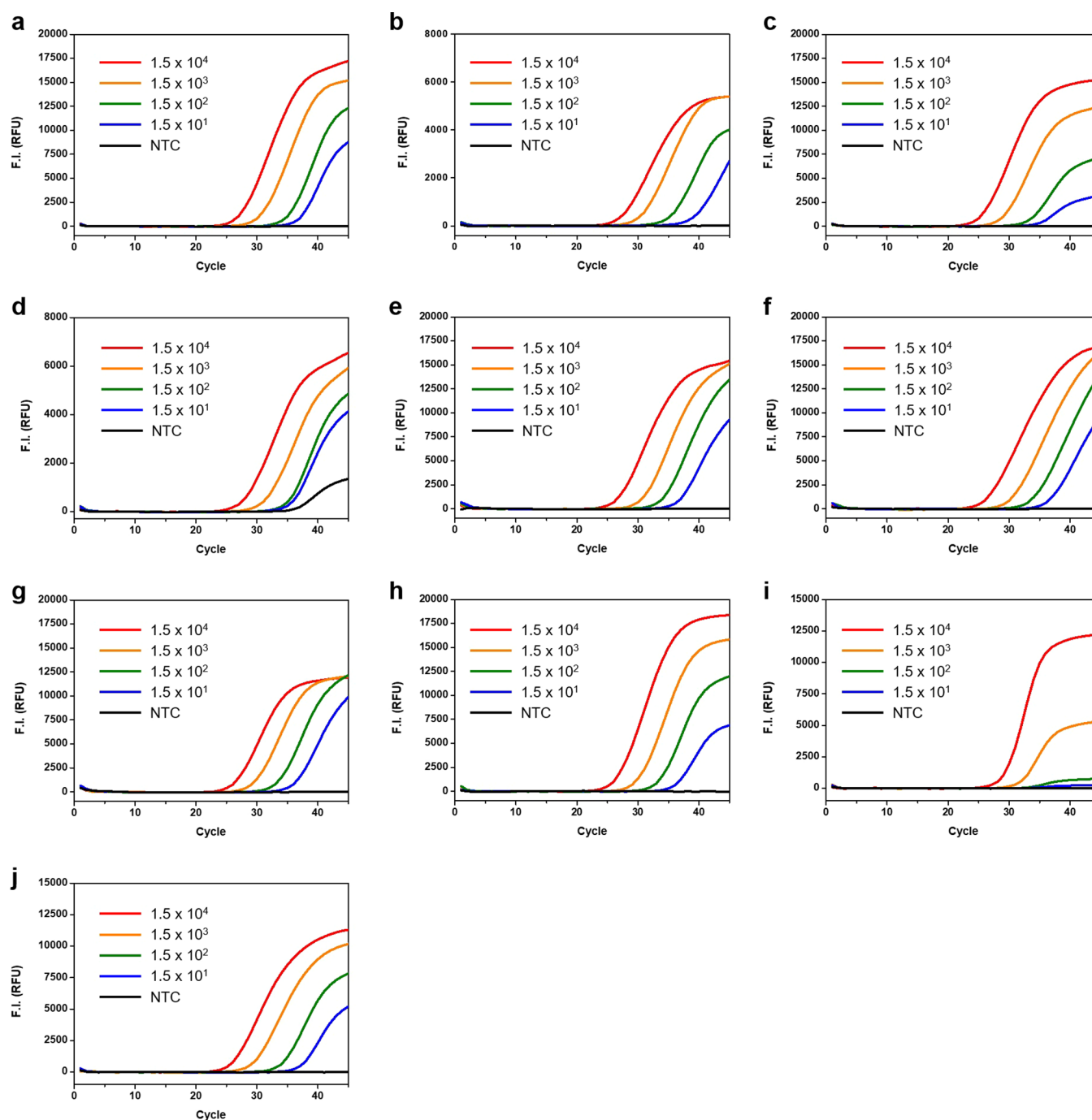


Figure 2. Representative amplification curves of fluorescence intensity against PCR cycle with each primer–probe set (amplification is performed at 58 °C). (a) N (China CDC), (b) HKU-N (HKU), (c) NIID_2019-nCoV_N (Japan NIID), (d) WH-NIC N (Thailand NIH), (e) 2019-nCoV_N1 (US CDC), (f) 2019-nCoV_N2 (US CDC), (g) 2019-nCoV_N3 (US CDC), (h) ORF1ab (China CDC), (i) RdRp_SARSr (Charité), and (j) HKU-ORF1b-nsp14 (HKU).

ditions. The viral concentrations of supernatant and cell lysate were determined using the E gene-based assay (Table 1).

N Assays. The Ct values of N (China CDC), HKU-N (HKU), NIID_2019-nCoV_N (Japan NIID), WH-NIC N (Thailand NIH), and 2019-nCoV_N1, -N2, and -N3 (US CDC) obtained from a low concentration of RNA (15 copies/reaction) were 34.86, 35.43, 33.13, 38.13, 34.71, 33.14, and 33.09, respectively (Table 1). The Ct values of 2019-nCoV_N2 and -N3 (US CDC) and NIID_2019-nCoV_N (Japan NIID) sets were similar to each other, and these sets could be regarded as the most sensitive. The assay based on 2019-nCoV_N1 (USA

CDC) and N (China CDC) was moderately sensitive: these sets had higher Ct values than the most sensitive sets, but the Ct values obtained from the low concentration (15 copies/reaction) were still within the cutoff value ($C_t < 37$). The WH-NIC N (Thailand NIH) set was less sensitive than other sets, with a Ct value at low concentration (15 copies/reaction) close to the cutoff value ($C_t < 38$). The R^2 values from N (China CDC), HKU-N (HKU), NIID_2019-nCoV_N (Japan NIID), WH-NIC N (Thailand NIH), and 2019-nCoV_N1, -N2, and -N3 (US CDC) were 0.989, 0.980, 0.987, 0.987, 0.986, 0.952, and 0.991, respectively. The calculated amplification efficiencies

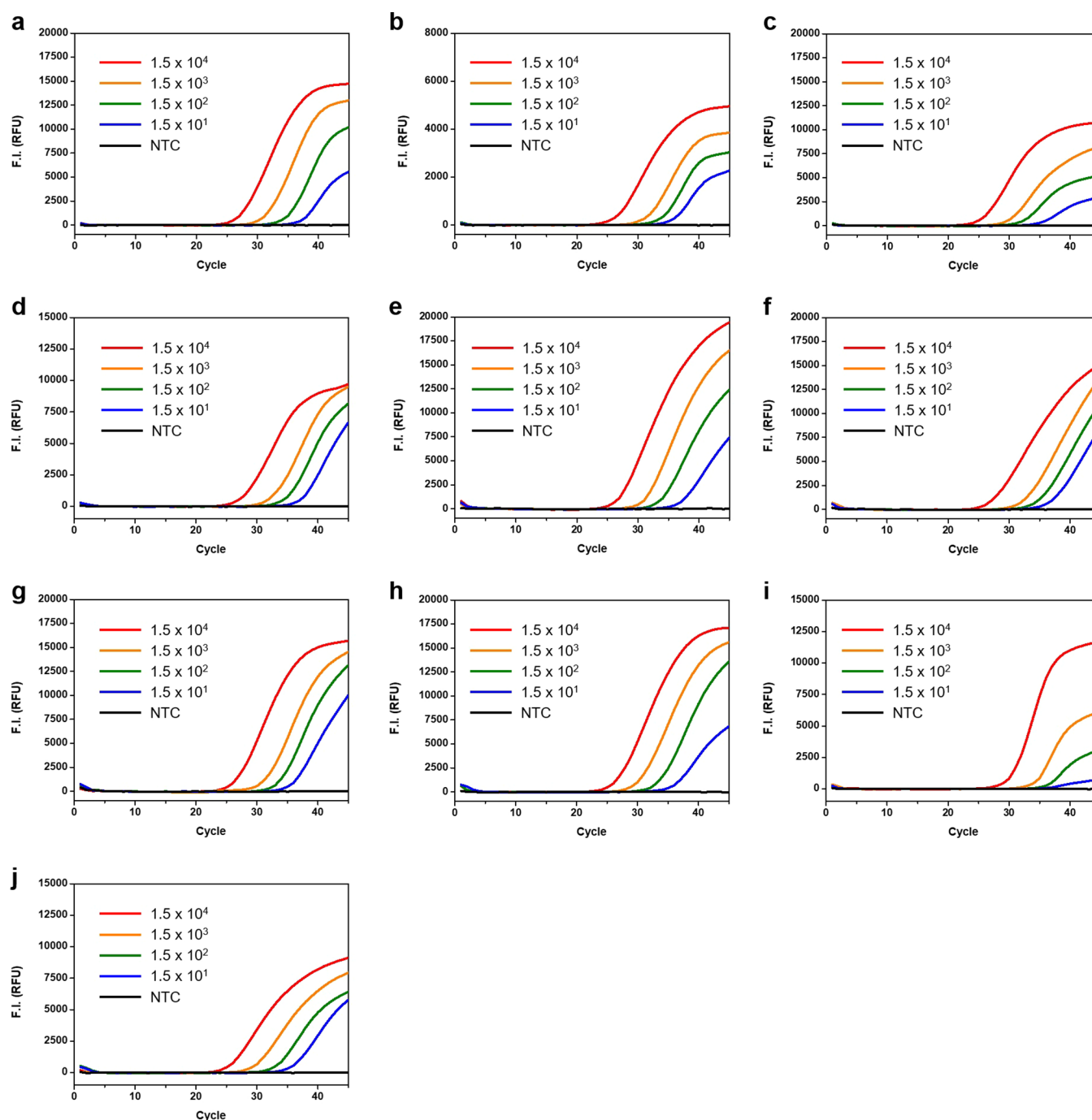


Figure 3. Representative amplification curves of fluorescence intensity against PCR cycle with each primer–probe set (amplification is performed at 55 °C). (a) N (China CDC), (b) HKU-N (HKU), (c) NIID_2019-nCoV_N (Japan NIID), (d) WH-NIC N (Thailand NIH), (e) 2019-nCoV_N1 (US CDC), (f) 2019-nCoV_N2 (US CDC), (g) 2019-nCoV_N3 (US CDC), (h) ORF1ab (China CDC), (i) RdRp_SARSr (Charité), and (j) HKU-ORF1b-nsp14 (HKU)

of N (China CDC), HKU-N (HKU), NIID_2019-nCoV_N (Japan NIID), WH-NIC N (Thailand NIH), and 2019-nCoV_N1, -N2, and -N3 (US CDC) were 89.4, 105.3, 100.7, 106.2, 95.2, 97.3, and 93.9, respectively.

According to the mean Ct of RT-qPCR analysis for the N assay in this study, 2019-nCoV_N1 and -N3 (US CDC) seemed to be sensitive amplifications (Table 1). However, nonspecific amplification with those sets was partly observed. The nonspecific amplification was confirmed by the melting curve analysis (Figure S5a). The electrophoresis analysis showed nonspecific amplification at lower positions (Lane 1, Figure

S5b) than the result of specific amplification with the 2019-nCoV_N1 (US CDC) set. The RT-qPCR analysis for the N assay using the NIID_2019-nCoV_N (Japan NIID) set showed stable and specific amplification at the recommended extension temperature, 60 °C (Table 1 and Figure 1c), although there was a mismatched sequence in the region of the reverse primer. We had previously compared the performance of RT-qPCR analysis for the N assay using the original and corrected set (sequence of reverse primer (5' → 3'): TGG CAC CTG TGT AGG TCA AC) of NIID_2019-nCoV_N set. Interestingly, there was no significant difference in amplification between the original and

Table 2. Comparative analysis of Ct values for each RNA extracted from clinical samples of COVID-19 patients

patients	RNA copies/reaction	N				RdRp/Orf1			
		NIID_2019-nCoV_N (Japan NIID)		2019-nCoV_N2 (US CDC)		ORF1ab (China CDC)		HKU-ORF1b-nsp14 (HKU)	
		Mean Ct	STD	Mean Ct	STD	Mean Ct	STD	Mean Ct	STD
patient 1 (P1)	5.3×10^5	22.22	0.012	23.42	0.183	25.17	0.191	25.29	0.225
	5.3×10^4	26.86	0.017	27.13	0.117	29.85	0.195	30.31	0.154
	5.3×10^3	31.88	0.242	30.99	0.102	35.50	0.644	36.00 ^b	0.771 ^b
	5.3×10^2	35.60	0.544	33.65	0.564	37.37 ^a		36.72 ^a	
	5.3×10^1	N/A	N/A	38.27	1.421	N/A	N/A	N/A	N/A
	5.3×10^0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
patient 2 (P2)	NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4.3×10^3	30.26	0.029	33.47	0.116	31.93	0.324	31.34	0.164
	4.3×10^2	33.14	0.310	37.63	0.136	37.61	2.780	35.04	1.000
	4.3×10^1	36.19 ^b	0.021 ^b	39.34 ^b	0.806 ^b	39.66 ^a		37.37 ^a	
	4.3×10^0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4.3×10^{-1}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
patient 3 (P3)	4.3×10^{-2}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	5.9×10^3	29.88	0.252	31.18	0.168	32.06	0.260	31.48	0.794
	5.9×10^2	33.37	0.137	35.65	0.607	36.89	0.893	36.68 ^b	0.325 ^b
	5.9×10^1	N/A	N/A	38.51	0.480	N/A	N/A	N/A	N/A
	5.9×10^0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
patient 4 (P4)	5.9×10^{-1}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	5.9×10^{-2}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	8.7×10^3	28.63	0.183	31.74	0.404	29.52	0.232	29.33	0.181
	8.7×10^2	32.17	0.365	36.43	1.703	33.46	0.359	31.94	0.515
	8.7×10^1	35.43 ^a		41.15 ^b	2.885 ^b	36.29 ^a		36.09 ^a	
patient 5 (P5)	8.7×10^0	N/A	N/A	40.76 ^a		N/A	N/A	N/A	N/A
	8.7×10^{-1}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	8.7×10^{-2}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4.0×10^2	32.67	0.818	41.88 ^a		33.80	0.614	32.95	0.601
	4.0×10^1	35.59	0.128	N/A	N/A	34.41 ^a		36.08 ^b	0.120 ^b
patient 6 (P6)	4.0×10^0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4.0×10^{-1}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4.0×10^{-2}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	4.0×10^{-3}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6.5×10^1	36.71 ^a		37.20 ^b	0.205 ^b	36.08	0.965	37.52 ^b	1.252 ^b
	6.5×10^0	N/A	N/A	N/A	N/A	N/A	N/A	36.04 ^a	
	6.5×10^{-1}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6.5×10^{-2}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6.5×10^{-3}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	6.5×10^{-4}	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	NTC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

^aThe assay showed a positive signal from a single reaction of the triplicate. ^bThe assay showed positive signals from the two reactions of the triplicate.

corrected set of NIID_2019-nCoV_N (data not shown). Not only NIID_2019-nCoV_N (Japan NIID) but also 2019-nCoV_N2 (US CDC) showed proper amplification at all reaction temperatures without nonspecific amplification (Table 1 and Figures 1, 2, and 3). The extension temperature (55 °C) of the 2019-nCoV_N2 set was previously recommended from the US CDC. The mean Ct values of RT-qPCR analysis using the 2019-nCoV_N2 set showed that 60 °C was slightly more effective than 55 and 58 °C in the present study. Therefore, NIID_2019-nCoV_N (Japan NIID) and 2019-nCoV_N2 (US CDC) sets should be optimal for SARS-CoV-2 by RT-qPCR assay of the N gene.

RdRp/Orf1 Assays. The mean Ct values of RdRp_SARSr (Charité), HKU-ORF1b-nsp14 (HKU), and ORF1ab (China CDC) obtained from a low concentration of RNA (15 copies/reaction) were 43.00, 38.97, and 36.85, respectively (Table 1). The assay with the RdRp_SARSr (Charité) set yielded a positive signal from the two reactions of the triplicate set at a concentration of 15 copies/reaction. The assay with HKU-ORF1b-nsp14 (HKU) and ORF1ab (China CDC) sets yielded positive signals at a concentration of 1.5 copies/reaction (data not shown). The R^2 values from RdRp_SARSr (Charité), HKU-ORF1b-nsp14 (HKU), and ORF1ab (China CDC) were 0.983, 0.997, and 0.997, respectively. The calculated amplification

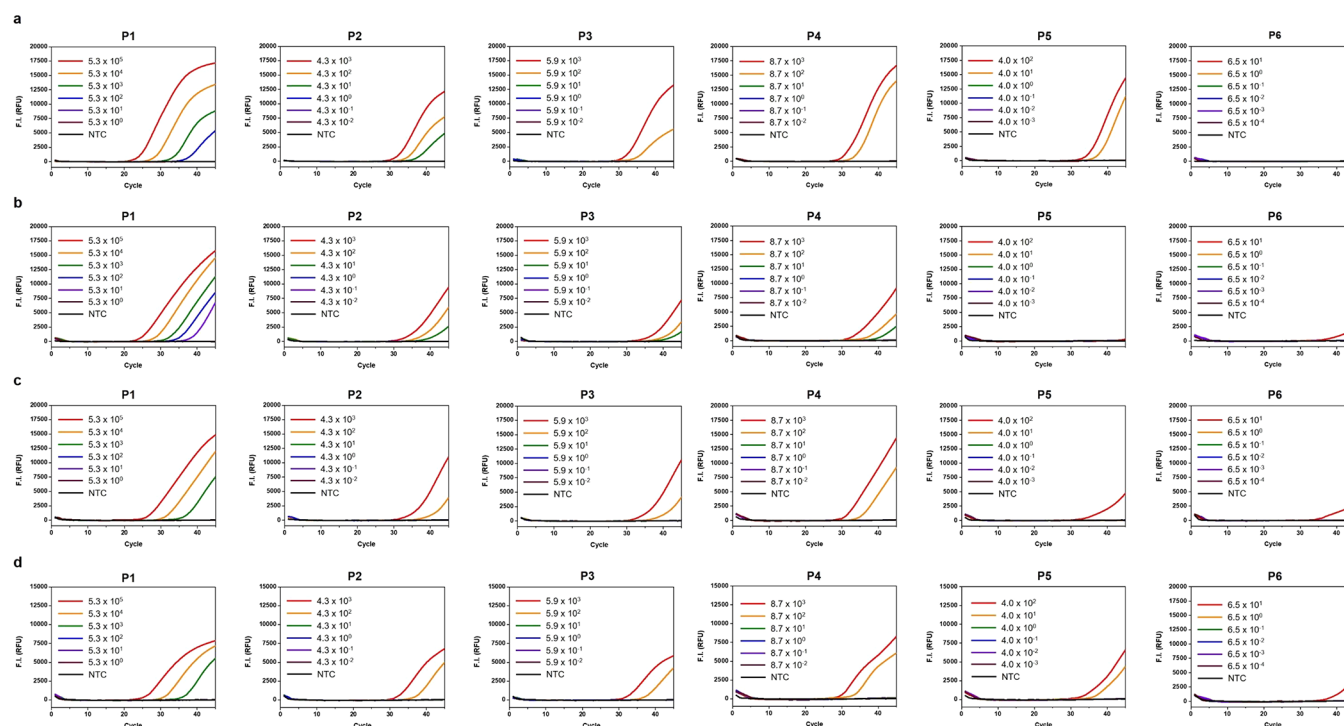


Figure 4. Amplification curves of fluorescence intensity against the PCR cycle with the RNAs extracted from clinical samples of COVID-19 patients. (a) NIID_2019-nCoV_N (Japan NIID), (b) 2019-nCoV_N2 (US CDC), (c) ORF1ab (China CDC), and (d) HKU-ORF1b-nsp14 (HKU).

efficiencies of RdRp_SARSr (Charité), HKU-ORF1b-nsp14 (HKU), and ORF1ab (China CDC) were 101.6%, 96.1%, and 109.8%, respectively.

According to the mean Ct values of RT-qPCR analysis for the RdRp/Orf1 assay in this study, ORF1ab (China CDC) seemed to have sensitive amplification (Table 1). In the case of the ORF1ab (China CDC) set, the extension temperature was not recommended by the China CDC. From the mean Ct values of RT-qPCR analysis, the ORF1ab set showed slightly higher specific and sensitive amplification at 55 °C than at 58 and 60 °C. Not only ORF1ab (China CDC) but also HKU-ORF1b-nsp14 (HKU) showed proper amplification at all reaction temperatures without nonspecific amplification (Table 1 and Figures 1b, 2b, and 3b). The extension temperature (60 °C) of the HKU-ORF1b-nsp14 set was previously recommended from HKU. The mean Ct values of RT-qPCR analysis using the HKU-ORF1b-nsp14 set showed that 55 °C was slightly more effective than 58 and 60 °C in the present study. On the other hand, the RdRp_SARSr (Charité) set shows less effective amplification than the other two primer sets at all reaction temperatures including their recommended extension temperature (58 °C). Unexpected amplifications from NTC samples were observed with the RdRp_SARSr (Charité) set. The electrophoresis and melting curve analysis showed nonspecific amplification at lower positions (Lane 5, Figure S5b) and temperatures (Figure S5a) than the result of specific amplification with the RdRp_SARSr (Charité) set. Only the RdRp_SARSr (Charité) set yielded positive signals from the two reactions of the triplicate at a concentration of 15 copies/reaction, whereas the other two primer sets showed stable positive signals. Therefore, the ORF1ab (China CDC) set should be optimal for the RdRp/Orf1 assay.

Laboratory Confirmation Using Clinical Samples. To confirm reliable primer–probe sets for the N and RdRp/Orf1 assays, the upper respiratory tract specimens of COVID-19

patients ($n = 6$) and healthy subjects ($n = 9$) were used for RT-qPCR analysis (Table 2, Figure 4, Table S2, and Figure S6). The mean Ct value of the NIID_2019-nCoV_N set was slightly smaller than that of the 2019-nCoV_N2 set. The limit of the detection level (4.0×10^1 copies/reaction in Patient 5) of the NIID_2019-nCoV_N set in the triplicate assay was better than that of the 2019-nCoV_N2 set (Table 2). The 2019-nCoV_N2 set caused nonspecific amplification in the upper respiratory tract specimens of the healthy subject (N5) to occur (Figure S6b). Therefore, the NIID_2019-nCoV_N (Japan NIID) sets should be optimal for laboratory confirmation of SARS-CoV-2 by the RT-qPCR assay of the N gene without cross-reactivity for hCoV-229E, hCoV-OC43, and MERS-CoV RNA (Figure S7a). The sequence variation at the binding sites of the NIID_2019-nCoV_N (Japan NIID) primer–probe set was investigated with 3,323 SARS-CoV-2 genomes from GISAID. The frequency of mismatch nucleotide was from 0.03% to 0.42% at 15 positions, excluding the mismatched sequence in the reverse primer (C $\rightarrow$ G substitution) already mentioned above (Table S3).

In the case of the RdRp/Orf1 assay using clinical samples, there was no significant difference in mean Ct values between ORF1ab and HKU-ORF1b-nsp14 sets, but the ORF1ab set showed more stable amplification in the triplicate assay than the HKU-ORF1b-nsp14 set (Table 2 and Figure 4). Therefore, the ORF1ab (China CDC) set should be optimal for laboratory confirmation of SARS-CoV-2 for the RdRp/Orf1 assay without cross-reactivity for hCoV-229E, hCoV-OC43, and MERS-CoV RNA (Figure S7b). According to the previous study by Vogels et al., 2 mismatches ($>0.1\%$) have been reported at the binding sites of the ORF1ab (China CDC) primer–probe set.¹⁷ The calculated frequency from the comparison of 3,323 SARS-CoV-2 genomes was 0.03% at 6 positions and 1.53% at the 26th position (T $\rightarrow$ G substitution) of the probe binding site (Table S3). Although there has been no critical problem for sensitivity

Table 3. Information of Primers and Probes Analyzed in the Study

target	institute	name	type	sequence (5' → 3')	position	recommended extension temperature (°C)	reference
N	China CDC	N-F	F	GGG GAA CTT CTC CTG CTA GAA T	28881–28902	N/A	20
		N-R	R	CAG ACA TTT TGC TCT CAA GCT G	28958–28979		
		N-P	P	FAM–TTG CTG CTG CTT GAC AGA TT–BHQ1 ^a	28934–28953		
	HKU	HKU-NF	F	TAA TCA GAC AAG GAA CTG ATT A	29145–29166	60	9
		HKU-NR	R	CGA AGG TGT GAC TTC CAT G	29235–29254		
		HKU-NP	P	FAM–GCA AAT TGT GCA ATT TGC GG–BHQ1 ^a	29177–29196		
	Japan NIID	NIID_2019-nCoV_N_F2	F	AAA TTT TGG GGA CCA GGA AC	29125–29144	60	21
		NIID_2019-nCoV_N_R2	R	TGG CAG CTG TGT AGG TCA AC ^b	29263–29282		
		NIID_2019-nCoV_N_P2	P	FAM–ATG TCG CGC ATT GGC ATG GA–BHQ1 ^a	29222–29241		
	Thailand NIH	WH-NIC N-F	F	CGT TTG GTG GAC CCT CAG AT	28320–28339	55	22
		WH-NIC N-R	R	CCC CAC TGC GTT CTC CAT T	28358–28376		
		WH-NIC N-P	P	FAM–CAA CTG GCA GTA ACC A–BHQ1 ^a	28341–28356		
	US CDC	2019-nCoV_N1-F	F	GAC CCC AAA ATC AGC GAA AT	28287–28306	55	23
		2019-nCoV_N1-R	R	TCT GGT TAC TGC CAG TTG AAT CTG	28335–28358		
		2019-nCoV_N1-P	P	FAM–ACC CCG CAT TAC GTT TGG TGG ACC–BHQ1 ^a	28309–28332		
		2019-nCoV_N2-F	F	TTA CAA ACA TTG GCC GCA AA	29164–29183	55	
		2019-nCoV_N2-R	R	GCG CGA CAT TCC GAA GAA	29213–29230		
		2019-nCoV_N2-P	P	FAM–ACA ATT TGC CCC CAG CGC TTC AG–BHQ1 ^a	29188–29210		
		2019-nCoV_N3-F	F	GGG AGC CTT GAA TAC ACC AAA A	28681–28702	55	
		2019-nCoV_N3-R	R	TGT AGC ACG ATT GCA GCA TTG	28732–28752		
		2019-nCoV_N3-P	P	FAM–AYC ACA TTG GCA CCC GCA ATC CTG–BHQ1 ^a	28704–28727		
RdRp/Orf1	China CDC	ORF1ab-F	F	CCC TGT GGG TTT TAC ACT TAA	13342–13362	N/A	20
		ORF1ab-R	R	ACG ATT GTG CAT CAG CTG A	13442–13460		
		ORF1ab-P	P	FAM–CCG TCT GCG GTA TGT GGA AAG GTT ATG G–BHQ1 ^a	13377–13404		
	Charité	RdRp_SARSr-F	F	GTG ARA TGG TCA TGT GTG GCG G	15431–15452	58	10
		RdRp_SARSr-R	R	CAR ATG TTA AAS ACA CTA TTA GCA TA	15505–15530		
		RdRp_SARSr-P2	P	FAM–CAG GTG GAA CCT CAT CAG GAG ATG C–BHQ1 ^a	15470–15494		
	HKU	HKU-ORF1b-nsp14F	F	TGG GGY TTT ACR GGT AAC CT	18778–18797	60	9
		HKU-ORF1b-nsp14R	R	AAC RCG CTT AAC AAA GCA CTC	18889–18909		
		HKU-ORF1b-nsp14P	P	FAM–TAG TTG TGA TGC WAT CAT GAC TAG–BHQ1 ^a	18849–18872		

^aFAM: 6-carboxyfluorescein; BHQ-1: Black Hole Quencher-1. ^bThe underlined letter within NIID_2019-nCoV_NR2 indicates a mismatched site to the PCR template.

of those primer–probe sets in this present study, these mismatches may affect sensitivity of RT-qPCR analysis.

CONCLUSIONS

Various primer–probe sets have been reported for the detection of SARS-CoV-2 by the RT-qPCR assay. However, the sensitivity of these assays may not be sufficient to confirm suspicious patients in the early stage of SARS-CoV-2 infection. Previous studies have been reported to compare some primer–probe sets listed in the WHO for the molecular diagnosis of SARS-CoV-2.^{17,18} In this study, we performed RT-qPCR analysis with previously reported specific primer–probe sets targeting the RdRp/Orf1 and N region of SARS-CoV-2. The NIID_2019-nCoV_N (Japan NIID) set and the ORF1ab (China CDC) set among various primer–probe sets (Table 3) posted by the WHO represent a recommendable performance of RT-qPCR analysis for SARS-CoV-2 molecular diagnostics without non-

specific amplification and cross-reactivity for hCoV-229E, hCoV-OC43, and MERS-CoV RNA. Although some degeneracies were found at the binding sites of these primer–probe sets, there has been no critical problem for sensitivity of our recommended primer–probe sets. More precise primer–probe sets for target genes will be needed through a comparison of abundant genomes of SARS-CoV-2.

METHODS

Primer Information on qPCR. For comparative analysis of laboratory confirmation for SARS-CoV-2, ten primer–probe sets were selected on the basis of sequence information from six different national institutions: the Centers for Disease Control and Prevention (CDC) (USA); Charité – Universitätsmedizin Berlin Institute of Virology (Germany), the University of Hong Kong (Hong Kong); the National Institute of Infectious Disease, Department of Virology III (Japan); China CDC

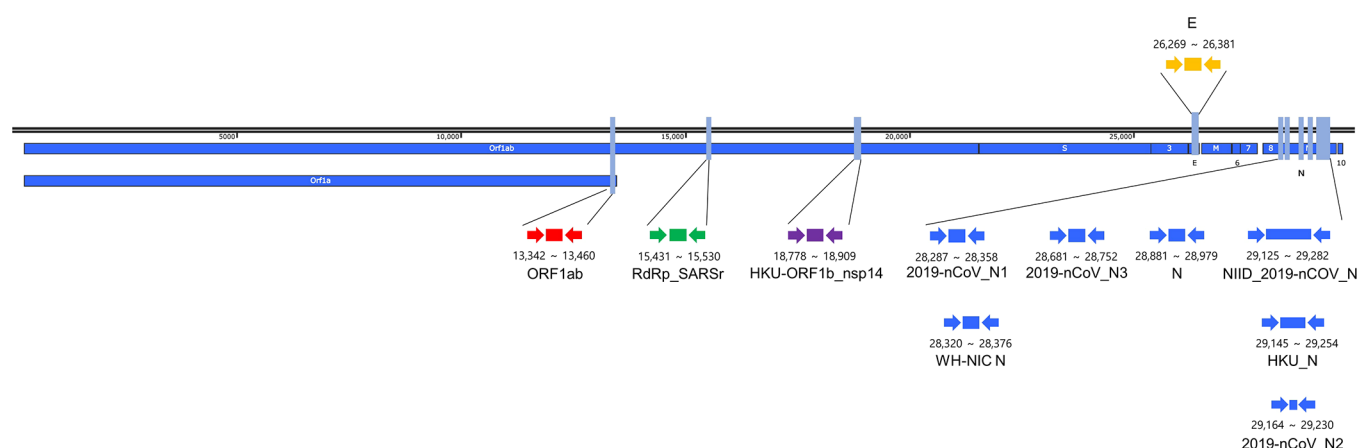


Figure 5. Relative positions of RT-qPCR primer–probe sets in the SARS-CoV-2 genome. Target gene and primer sequences were obtained from the WHO Web site (<http://www.who.int>). The numbers below the amplicons indicate genome positions in SARS-CoV-2 (GenBank MN908947.3). The sets were published by China CDC (Orf1ab and N), Charité – Universitätsmedizin Berlin Institute of Virology in Germany (RdRp_SARSr and E), the University of Hong Kong (HKU-ORF1b_nsp14 and HKU-N), USA CDC (2019-nCoV_N1, -N2, and -N3), the National Institute of Health in Thailand (WH-NIC N), and the National Institute of Infectious Disease in Japan (NIID_2019-nCoV_N). Orf1: open reading frame 1; RdRp: RNA-dependent RNA polymerase; Nsp14: nonstructural protein 14 gene; S: spike protein gene; E: envelop protein gene; N: nucleocapsid protein gene.

(China); the National Institute of Health (Thailand). All DNA oligonucleotides were synthesized by Neoprobe (Daejeon, South Korea). Sequences of the primer–probe sets and their locations in the viral RNA (GenBank MN908947.3) are listed in Figure 5 and Table 3. Seven of the ten sets were derived from the N gene and the other three, from the Orf1 gene (RdRp, ORF 1b-Nsp14, and ORF 1-Nsp10). All DNA oligonucleotides were resuspended in nuclease-free water before use.

Viral RNA Preparation. Infection experiments were performed in a biosafety level-3 (BSL-3) laboratory. African green monkey kidney Vero cells (ATCC CCL-81) were infected with a clinical isolate of SARS-CoV-2 (BetaCoV/Korea/KCDC03/2020 provided by Korea CDC). After 72 h, culture medium containing mature infectious virions (virus medium) was collected, and viral RNA was isolated from the culture medium using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany). Human coronavirus (hCoV)-229E, hCoV-OC43, and Middle East respiratory syndrome (MERS)-CoV RNA were prepared, as previously described.¹⁹

Preparation of *In Vitro* Transcribed RNA Standard. The coding sequence of the SARS-CoV-2 Envelope (E) protein, cloned into pET21a, was PCR-amplified with the T7 promoter primer (5′-AATACGACTCACTATAG-3′, MacroGen Inc., Seoul, South Korea) and T7 terminator primer (5′-GCTAGTTATTGCTCAGCGG-3′, MacroGen Inc.) using the AccuPower PCR PreMix (-dye) kit (Bioneer Inc., Daejeon, South Korea). The PCR product was then used as the *in vitro* transcription template for the MEGAscript T7 Transcription Kit (Invitrogen Inc., CA, USA). The copy number of *in vitro* transcribed RNA was calculated from RNA concentration measured with a Quantus Fluorometer (Promega, Madison, WI, USA). Standardized amounts of *in vitro* transcribed RNA were amplified by RT-qPCR using the E primers to produce a standard curve.

RT-qPCR Analysis in N and RdRp/Orf1. Extracted nucleic acid samples were tested for comparative analysis of SARS-CoV-2 by RT-qPCR. The N and Orf1 regions of SARS-CoV-2 were used as the target sequences for SARS-CoV-2-specific genes. Briefly, 10 μ L of purified viral RNA was amplified in a 20 μ L reaction solution containing 1 $\times$ TaqPath 1-Step Multiplex Master Mix (Thermo Fisher Scientific INC., MA, USA) and 300

nM of primers and probes for target detection. RT-qPCR was performed in a CFX 96 touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The RT-qPCR conditions applied in this study were as follows: UNG incubation, RT incubation, and enzyme activation were performed in series at 25 $^{\circ}$ C for 2 min, 55 $^{\circ}$ C for 10 min, and 94 $^{\circ}$ C for 3 min, respectively. Thermal cycling was then performed at 94 $^{\circ}$ C for 15 s (denaturation) and 60 $^{\circ}$ C for 30 s (annealing and amplification) for 45 cycles. All confirmatory RT-qPCR assays were performed more than three times. All mean Ct values were calculated from the three reactions of each triplicated set.

Clinical Sample Preparation. The upper respiratory tract specimens (nasal- and oropharyngeal swabs) of COVID-19 patients ($n = 6$) and healthy subjects ($n = 9$) used in this study (Table S2) were collected from subjects as part of the registered protocols approved by the Institutional Review Board (IRB) of Jeonbuk National University Hospital. All patients provided written informed consent (IRB registration number: CUH 2020-02-050-008). The clinical samples were stored in transport medium (eNAT; COPAN, Murrieta, CA). All samples were inactivated by heating at 100 $^{\circ}$ C for 10 min and stored at -80° C for further analysis. Viral RNA was isolated from the clinical samples using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany). The copy number of extracted RNA from the clinical samples was calculated by the method described above.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00464>.

Polyacrylamide gel electrophoresis, melting curve analysis, SARS-CoV-2 genome alignment protocols, standard curves for quantification of SARS-CoV-2 RNA, additional comparative analysis data of Ct values, amplification curves of each primer–probe set, melting curve and polyacrylamide gel analysis data, clinical characteristics of COVID-19 patients, and PCR test data with the RNAs extracted from clinical samples of healthy subjects and other coronaviruses (PDF)

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Notes

The authors declare no competing financial interest.

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