Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 304 (2022) 114513

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Simultaneous detection of SARS-CoV-2 and identification of spike D614G mutation using point-of-care real-time polymerase chain reaction

So Yul Lee [a], Ji Su Lee [a], Jeong Jin Ahn [b], Seung Jun Kim [b], Heungsup Sung [c], Jin Won Huh [d], Seung Yong Hwang [a,*]

[a] Department of Molecular and Life Science, Hanyang University, 55, Hanyangdeahak-ro, Sangnok-gu, Ansan, Gyeonggi-do 15588, Republic of Korea
[b] GeneTrigger, 20, Magokjungang 5-ro 1-gil, Gangseo-gu, Seoul, Republic of Korea
[c] Department of Laboratory Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea
[d] Department of Pulmonary and Critical Care Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

ARTICLE INFO

Keywords:
SARS-CoV-2
Spike D614G mutation
Point-of-care
Real-time PCR
COVID-19
Molecular diagnosis

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is associated with high mortality and infectivity rates in humans since its emergence. Analysis using high-accuracy real-time polymerase chain reaction (PCR) is recommended for the detection of general respiratory viruses including SARS-CoV-2, but it takes a long time (e.g. ~ 6 h); moreover, on-site diagnosis is difficult owing to the need for skilled technicians and advanced laboratory facilities. Currently, the importance of point-of-care testing (POCT) is being emphasized for the rapid detection of SARS-CoV-2. Here, we developed a multiplex real-time reverse transcription PCR (rRT-PCR) analysis that not only detects SARS-CoV-2 but also D614G strains with higher contagiousness than wild types among SARS-CoV-2 mutants using probe-based rRT-PCR. Moreover, this method was applied to portable PCR equipment capable of POCT to confirm high detection sensitivity and specificity. Multiple assays were possible with fluorescence labeling of individual probes. Furthermore, using a microfluidic chip-based point-of-care testing rRT-PCR platform, detection time was reduced by more than half compared with the commonly used detection system. This demonstrates that our assay has 100% of high sensitivity and specificity and could thus aid in the rapid and simple screening of SARS-CoV-2 carrying the mutation. We present a rapid detection method for mutations in SARS-CoV-2.

In December 2019, a case of pneumonia with unknown etiology occurred in Wuhan, China, the causative agent of which was eventually identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is genetically distinct from SARS-CoV (Wu et al., 2020). The virus is associated with high mortality and infectivity rates in humans since its emergence. It has become a major health concern worldwide because of potential secondary and tertiary infections, even after the declaration of the coronavirus disease 2019 (COVID-19) pandemic in March 2020 (Vo et al., 2020). The infectivity of SARS-CoV-2 is higher than that of other major respiratory viruses, and the basic reproduction number is 3.3–5.47 (Zhao et al., 2020). This indicates that humans have no direct immunological exposure to SARS-CoV-2 and are thus highly vulnerable to infection with this virus. The virus has been continuously mutating, requiring the development of new vaccines at regular intervals, and scientists are concerned that SARS-CoV-2 will behave similar to the influenza virus (Dearlove et al., 2020; Zhao et al., 2020).

The World Health Organization (WHO) recommends the use of real-time polymerase chain reaction (PCR)—an assay that requires experienced staff and advanced laboratory facilities—for the molecular diagnosis of SARS-CoV-2 infection (Corman et al., 2020; Vo et al., 2020). The diagnosis of the virus using RT-PCR is rapid and sensitive, which are essential for appropriate treatment and alleviation of in-hospital transmission. Multiplex RT-PCR, which can detect multiple respiratory viruses simultaneously with a single PCR reaction, can enable timely treatment decisions and also cost-effective infection prevention (Kim et al., 2009; Liolios et al., 2001). Although this method is highly accurate, it is time consuming and requires nucleic acid extraction, both of which hamper rapid on-site diagnosis (Basile et al., 2018; Kohmer et al., 2021). The point-of-care (POC) test based on molecular amplification enables rapid early diagnosis and involves clinical pathology tests at a site close to the location where the patient is being treated. It shows

* Corresponding author.
E-mail address: syhwang@hanyang.ac.kr (S.Y. Hwang).

https://doi.org/10.1016/j.jviromet.2022.114513
Received 26 November 2021; Received in revised form 2 March 2022; Accepted 3 March 2022
Available online 5 March 2022

excellent economic efficiency (Nelson et al., 2020). The importance of POC test has increased due to the current COVID-19 pandemic, and the analytical sensitivity and specificity of the test will continue to improve with the integration of new technologies to detect nucleic acids into the POC tests.

In this milieu, we developed a rRT-PCR detection method that simultaneously detects common SARS-CoV-2 and the SARS-CoV-2 D614 strain (a mutant with the replacement of aspartic acid with glycine at position 614 of the spike glycoprotein (S protein)), which has become a major circulating strain worldwide in a short period (Korber et al., 2020; Wang et al., 2021). We applied this method to RT-PCR equipment capable of point-of-care testing (POCT) and achieved high detection sensitivity and specificity for SARS-Cov-2 detection; this method can be used for rapid virus diagnosis.

For POCT, we designed a platform based on GENECHECKER Model UF-300 (GENESYSTEM Co., Ltd, Korea), a portable PCR device with dual detection channels (FAM/ROX), and a multiplex biochip (Rapi:chip™ PCR Chip for GENECHECKER®) applicable to this device. For the simultaneous detection of SARS-CoV-2 and its variant (D614G mutant), FAM and ROX fluorophores were attached to each probe during probe sequence design to enable target discrimination. In this platform, PCR mixture with sample is injected into a multiplex biochip consisting of 10 wells, and 10 samples can be tested in one reaction (Fig. 1).

SARS-CoV-2 RNA samples (n = 24) were provided by and used at Asan Medical Center (AMC), Seoul, Republic of Korea. Sputum or nasopharyngeal flocked swabs were collected from patients suspected with coronavirus disease (COVID-19) (FLOQSwab; Copán, Brescia, Italy). The swabs were stored in universal transport medium (Copán) at 4 °C, and an automated nucleic acid extraction system (eMAG; bioMérieux, Mar-cy-l'Etoile, France) was used for RNA extraction. RNA was eluted in the AMC laboratory from 200 μL of medium containing the collected RNA swabs using 50 μL of buffer (NUCLISENS easyMAG; bioMérieux, Marcyl'Etoile, France). Real-time PCR was performed with Allplex Respiratory Panel assays (Seegene, Seoul, Korea) to check the expression of RNA-dependent RNA-polymerase (*RdRp*), nucleocapsid (*N*), and envelope protein (*E*) genes, and COVID-19 cases were confirmed based on positive real-time PCR results (Table S1). To evaluate cross-reactivity, a cross-reactivity experiment was conducted using RNA samples from nine different types of respiratory viruses: human bocavirus (HBoV), human rhinovirus (HRV), respiratory syncytial B (RSVB), adenovirus (ADV), human metapneumovirus (HMPV), human parainfluenza virus type 1 (PIV1), human parainfluenza virus type 2 (PIV2), human parainfluenza virus type 3 (PIV3), and human parainfluenza virus type 4 (PIV4) (n = 1 each). All samples were confirmed to be positive for each virus through multiplex rRT-PCR with Allplex Respiratory Panel 1,2,3 (Seegene). Before the experiment, viral RNA samples were stored at − 80 °C.

SARS-CoV-2 sequences were obtained through GISAID, and specific primer pairs and probe sets were designed by multiple sequence alignment with ClustalW multiple alignment in MAFFT (Multiple Alignment using Fast Fourier Transform) v7.0 software and BioEdit Sequence Alignment Editor. To improve the detection accuracy, OligoAnalyzer™ 3.1 (Integrated DNA Technologies, Inc.) was used to design primer pairs and probe sets against each target gene. These were confirmed using Basic Local Alignment Search Tool (BLAST) and synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA). The *RdRp* and *N* genes are the most conserved genes in SARS-CoV-2 (Bar-On et al., 2020). In this study, we designed primer pairs and probe sets to amplify the most conserved regions within the *RdRp* and *N* genes to facilitate more accurate detection of SARS-CoV-2. Probes were labeled with a FAM fluorophore at the 5′-terminus and a BHQ-1 quencher at the 3′-terminus. We also designed a primer pair and probe set to amplify the spike (*S*) gene containing G23403 to detect the D614G mutation. The probe was labeled with Cal Red 610 (ROX) fluorophore at the 5′-terminus and a BHQ-2 quencher at the 3′-terminus. The primers and probes designed in this study are listed in Table 1.

To confirm that the designed primer pair and probe set were specific to the associated target, a standard curve was generated to determine the detection limit. In this experiment, the main sequences of each target gene in the L and G clades were classified as templates by GISAID. As a standard for detection limit test of RT-PCR assay, amplified target gene sequence synthesis was performed in IDT. Reference sequences (L clade: EPI_ISL_402124, G clade: EPI_ISL_529551) were obtained from GISAID (Zhou et al., 2020). The synthetic genes were diluted to concentrations of 1.0×10^5–1.0×10^1 copies/μL to confirm the detection limit. For multiplex PCR, primer pairs and probe sets corresponding to each target gene were mixed. The test was conducted using GENECHECKER Model UF-300 (GENESYSTEM Co., Ltd., Daejeon, Republic of Korea), to confirm its field applicability. To verify the efficacy of a microfluidic chip-based POCT rRT-PCR platform, Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), which is mainly used for general RT-PCR, was used (Fig. S1). This system employed 20-μL reaction mixtures containing 10 μL of 2 × Premix Ex Taq ™ (Takara), 1 μM of each primer, 0.5 μM of the probe, 1 μL of serially diluted DNA template, and distilled water. GENECHECKER Model UF-300 was used with 10 μL reaction mixtures containing 5 μL of Rapi:Spec™ Probe Master Mix (GENESYSTEM Co., Ltd, Daejeon, Korea), 0.5 μM of each primer, 0.25 μM of the probe, 1 μL of serially diluted DNA template, and distilled water. The following real-time PCR conditions were used: 45 cycles of pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s. Data were analyzed using CFX Manager Software (Bio-Rad) and Gene Recorder software (GENESYSTEM Co., Ltd, Korea). The chip included in the portable equipment contained 10 wells, and the PCR was repeated twice for five samples. In the experiment, the specificity and sensitivity of *RdRp* and *N* target primer pairs and probe sets were confirmed in the L clade, chosen to represent viruses containing the target gene without the D614G mutation. As a result, only FAM fluorescence was detected and *RdRp* and *N* were confirmed by the quantitative amplification of the synthetic gene at 1.0×10^5–1.0×10^1 copies (Fig. 2A). This was evident from Ct measurement using a fluorescence amplification curve (Fig. 2A) and the regression equation (FAM: Y = 3.644x + 40.416) with a

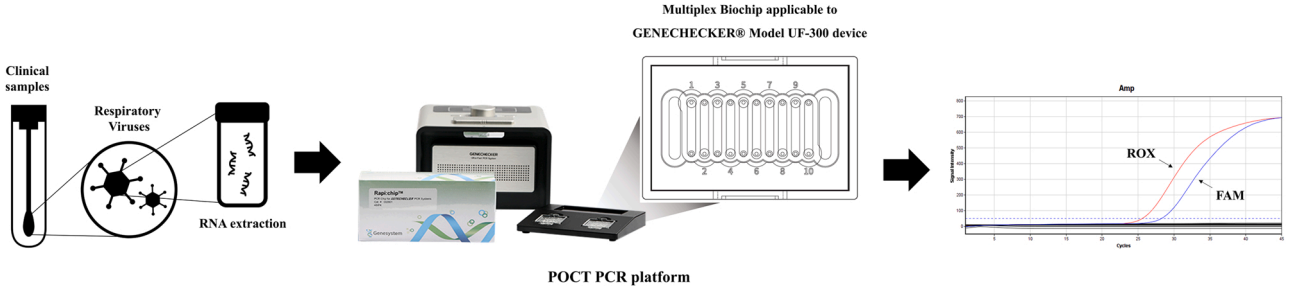

Fig. 1. Overall scheme of the developed platform. As sample, RNA extracted from virus is applied to portable PCR equipment and multiplex biochip. The results can be confirmed through two fluorophores (FAM/ROX).

Table 1
List of primer pairs and probe sets for SARS-CoV-2 detection.

Target	Type	Sequence (5′>3′)	Tm (°C)	Amplicon length (bp)
RdRp	Forward	TGGTCATGTGTGGCGGT	63.7	103
	Reverse	ACAGCTTGACAAATGTTAAAAACACTATT	63.7	
	Probe	FAM-AACCTCATCAGGAGATGCCACAAC-BHQ-1	66.4	
N	Forward	GCCAACAACAACAAGGCCA	63.8	93
	Reverse	TGTATGCTTTAGTGGCAGTACGT	64	
	Probe	FAM-CTGTCACTAAGAAATCTGCTGCTGAGG-BHQ-1	66.4	
S	Forward	AACACCAGGAACAAATACTTCTAACC	63.4	99
	Reverse	GGAGTAAGTTGATCTGCATGAATAGC	63.5	
	Probe	CAL RED 610-TTCTTTATCAGGGTGTTAACTGC-BHQ-2	61	

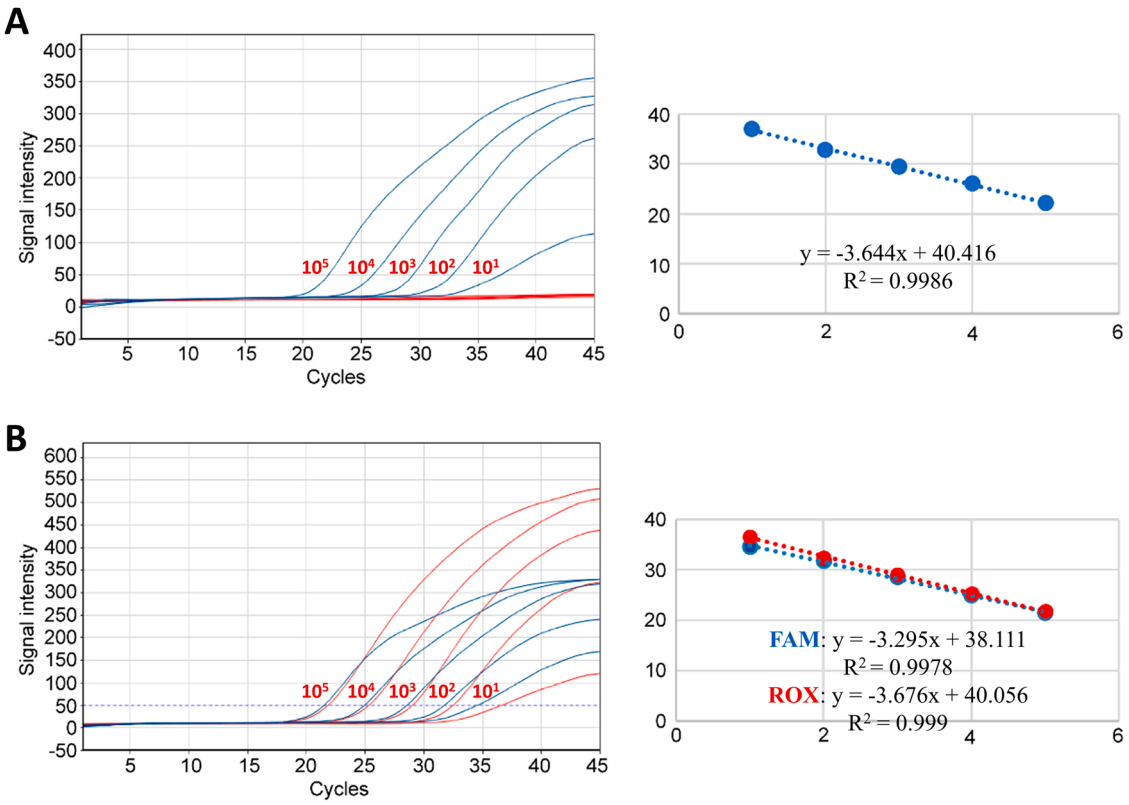

Fig. 2. Standard curve setting – (A) standard curve using specific primer pairs and probes for *RdRp* and *N*; (B) standard curve using specific primer pairs and probes for *RdRp*, *N*, and *S*.

regression coefficient (R^2) of 0.9986 using the Ct value and the template copy number. The specificity and sensitivity of *S* target primer pairs and probe set were confirmed in the G clade, representing viruses containing the target gene with the D614G mutation. As a result, both FAM and Cal Red 610 (ROX) fluorophores were detected and confirmed by quantitative amplification of the synthetic gene at 1.0×10^5–1.0×10^1 copies (Fig. 2B). This was evident from the Ct measurement using a fluorescence amplification curve (Fig. 2B) and the regression equations (FAM: $Y = -3.295x + 38.111$) with R^2 of 0.9978 and (ROX: $Y = -3.676x + 40.056$) with R^2 of 0.999 using the Ct value and the template copy number.

To confirm field applicability of the primer pairs and probe sets produced in this study, RNA samples (n = 16) of SARS-CoV-2 provided by AMC were used to screen for mutations. For multiplex PCR, primer pair and probe sets corresponding to each target gene were mixed. First, the Bio-Rad CFX96 Touch Real-Time PCR Detection System was used (Bio-Rad; Fig. S2) with a 20-μL reaction mixture containing 10 μL of 2 × SensiFAST Probe No-ROX One-Step Mix (cat. no. BIO-76001; Bioline, London, UK), 1 μM of each primer, 0.5 μM of the probe, 4 μL of RNA sample, and 1 μL of distilled water. GENECHECKER Model UF-300 was used with 10 μL of reaction mixtures containing 5 μL of Rapi:Spec™ Probe Master Mix with RTase (GENESYSTEM Co., Ltd, Korea), 0.5 μM of each primer, 0.25 μM of the probe, 4 μL of RNA sample. The following real-time RT-PCR conditions were employed: 45 cycles of reverse transcription at 45 °C for 10 min, pre-denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s. FAM and Cal Red 610 fluorophores were detected in all RNA samples (Fig. 3), indicating that samples contained the D614G mutation. We also conducted experiments on *RdRp* and *N* gene through additional samples (n = 8) with high Ct values (Table S2). These results were 100% consistent with the patient data received from AMC; thus, the field application was considered successful. The cross-reactivity assay using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and GENECHECKER Model UF-300 (GENESYSTEM Co., Ltd, Korea) with nine different respiratory viruses (HBoV, HRV, RSVB, ADV, HMPV, PIV1, PIV2, PIV3, and PIV4) suggested no cross-reactivity (Figs. S3 and S4), confirming that the primer pairs and probe sets were specific to SARS-CoV-2.

In this study, we demonstrated simultaneous detection of SARS-CoV-2 and identification of the highly infectious D614G mutation using

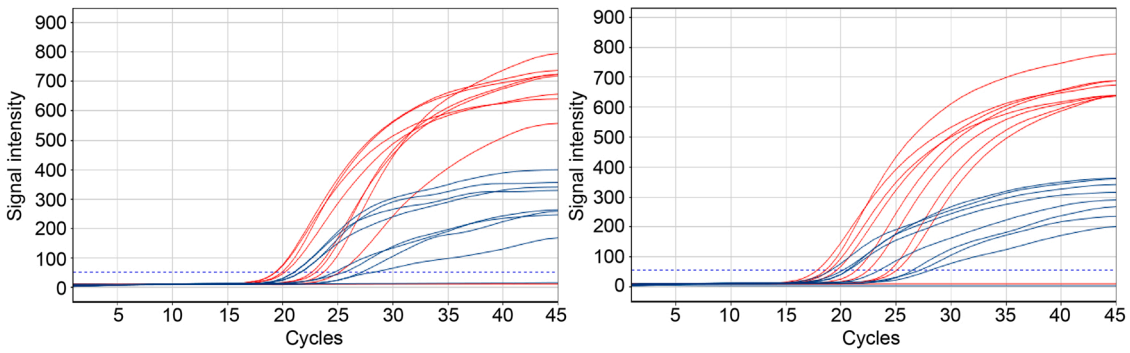

Fig. 3. Diagnostic results based on the real-time PCR analysis of RNA samples from SARS-CoV-2-positive samples (n = 16) to evaluate the field applicability of the primer pairs and probe sets set.

specific primer pairs and probe sets in a newly designed portable chip-based real-time PCR device. The experiment confirmed that the primer pairs and probe sets detected up to 10^1 copies/reaction of the synthetic gene in a portable RT-PCR device. For on-site diagnosis, a POCT real-time PCR platform was used to facilitate on-site detection and diagnosis within 40 min. A recent Cochrane review of 64 studies found that rapid tests correctly identified, on an average, 58% of individuals who were infected with the virus but asymptomatic, suggesting that more than one in three cases were missed. Because SARS-CoV-2 is constantly mutating, more potent strains are expected to emerge over time, and it was estimated that one SARS-CoV-2 mutation is established in the population every 11 days. With the new method described herein, it would be possible to identify the exact time when mutations such as D614G occur, allowing the detection methods to be rapidly adjusted to the specific mutation, which is crucial for epidemiological surveillance.

Institutional Review Board Statement

This study was approved by the Institutional Review Board of Asan Medical Center (IRB No.: 2020-0312).

CRediT authorship contribution statement

So Yul Lee: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, **Ji Su Lee:** Funding acquisition, **Jeong Jin Ahn:** Methodology, Supervision, Formal analysis, Project administration, Funding acquisition, **Seung Jun Kim:** Supervision, **Heungsup Sung:** Investigation, **Jin Won Huh:** Investigation, **Seung Yong Hwang:** Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgment

Funding: This work was supported by the Korea Health Industry Development Institute (KHIDI) through the Infectious Disease Prevention and Treatment Technology Development Program, funded by the Ministry of Health and Welfare (MOHW) [grant number HI20C0061].

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2022.114513.

References

Bar-On, Y.M., Flamholz, A., Phillips, R., Milo, R., 2020. SARS-CoV-2 (COVID-19) by the numbers. Elife 9.
Basile, K., Kok, J., Dwyer, D.E., 2018. Point-of-care diagnostics for respiratory viral infections. Expert Rev. Mol. Diagn. 18, 75–83.
Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L., Mulders, D.G., Haagmans, B.L., van der Veer, B., van den Brink, S., Wijsman, L., Goderski, G., Romette, J.-L., Ellis, J., Zambon, M., Peiris, M., Goossens, H., Reusken, C., Koopmans, M.P., Drosten, C., 2020. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 25, 2000045.
Dearlove, B., Lewitus, E., Bai, H., Li, Y., Reeves, D.B., Joyce, M.G., Scott, P.T., Amare, M. F., Vasan, S., Michael, N.L., Modjarrad, K., Rolland, M., 2020. A SARS-CoV-2 vaccine candidate would likely match all currently circulating variants. Proc. Natl. Acad. Sci. USA 117, 23652–23662.
Kim, S.R., Ki, C.S., Lee, N.Y., 2009. Rapid detection and identification of 12 respiratory viruses using a dual priming oligonucleotide system-based multiplex PCR assay. J. Virol. Methods 156, 111–116.
Kohmer, N., Rabenau, H.F., Hoehl, S., Kortenbusch, M., Ciesek, S., Berger, A., 2021. Comparative analysis of point-of-care, high-throughput and laboratory-developed SARS-CoV-2 nucleic acid amplification tests (NATs). J. Virol. Methods 291, 114102.
Korber, B., Fischer, W.M., Gnanakaran, S., Yoon, H., Theiler, J., Abfalterer, W., Hengartner, N., Giorgi, E.E., Bhattacharya, T., Foley, B., Hastie, K.M., Parker, M.D., Partridge, D.G., Evans, C.M., Freeman, T.M., de Silva, T.I., Sheffield, C.-G.G., McDanal, C., Perez, L.G., Tang, H., Moon-Walker, A., Whelan, S.P., LaBranche, C.C., Saphire, E.O., Montefiori, D.C., 2020. Tracking changes in SARS-CoV-2 spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell 182, 812–827 e19.
Liolios, L., Jenney, A., Spelman, D., Kotsimbos, T., Catton, M., Wesselingh, S., 2001. Comparison of a multiplex reverse transcription-PCR-enzyme hybridization assay with conventional viral culture and immunofluorescence techniques for the detection of seven viral respiratory pathogens. J. Clin. Microbiol. 39, 2779–2783.
Nelson, P.P., Rath, B.A., Fragkou, P.C., Antalis, E., Tsiodras, S., Skevaki, C., 2020. Current and future point-of-care tests for emerging and new respiratory viruses and future perspectives. Front. Cell Infect. Microbiol. 10.
Vo, V.G., Bagyinszky, E., Shim, K., Park, Y.S., An, S.S.A., 2020. Additional diagnostic testing of the 2019 novel coronavirus (SARS-CoV-2). Mol. Cell Toxicol. 1–3.
Wang, C., Zheng, Y., Niu, Z., Jiang, X., Sun, Q., 2021. The virological impacts of SARS-CoV-2 D614G mutation. J. Mol. Cell Biol.
Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., Hu, Y., Tao, Z.-W., Tian, J.-H., Pei, Y.-Y., Yuan, M.-L., Zhang, Y.-L., Dai, F.-H., Liu, Y., Wang, Q.-M., Zheng, J.-J., Xu, L., Holmes, E.C., Zhang, Y.-Z., 2020. A new coronavirus associated with human respiratory disease in China. Nature 579, 265–269.
Zhao, S., Lin, Q., Ran, J., Musa, S.S., Yang, G., Wang, W., Lou, Y., Gao, D., Yang, L., He, D., Wang, M.H., 2020. Preliminary estimation of the basic reproduction number of novel coronavirus (2019-nCoV) in China, from 2019 to 2020: A data-driven analysis in the early phase of the outbreak. Int. J. Infect. Dis. 92, 214–217.
Zhou, P., Yang, X.L., Wang, X.G., Hu, B., Zhang, L., Zhang, W., Si, H.R., Zhu, Y., Li, B., Huang, C.L., Chen, H.D., Chen, J., Luo, Y., Guo, H., Jiang, R.D., Liu, M.Q., Chen, Y., Shen, X.R., Wang, X., Zheng, X.S., Zhao, K., Chen, Q.J., Deng, F., Liu, L.L., Yan, B., Zhan, F.X., Wang, Y.Y., Xiao, G.F., Shi, Z.L., 2020. A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579, 270–273.