Differential gene expression analysis of common target genes for the detection of SARS-CoV-2 using real time-PCR

Reza Valadan1,2, Soheila Golchin3, Reza Alizadeh-Navaei3, Mohammadreza Haghshenas4, Mehryar Zargari5, Tahoora Mousavi1,6* and Mohammad Ghamati7

Abstract
COVID‑19 currently is the main cause of the severe acute respiratory disease and fatal outcomes in human beings worldwide. Several genes are used as targets for the detection of SARS‑CoV‑2, including the RDRP, N, and E genes. The present study aimed to determine the RDRP, N, and E genes expressions of SARS‑CoV‑2 in clinical samples. For this purpose, 100 SARS‑CoV‑2 positive samples were collected from diagnostic laboratories of Mazandaran province, Iran. After RNA extraction, the real‑time reverse transcription PCR (real‑time RT‑PCR) assay was performed for differential gene expressions’ analysis of N, E, and RDRP. The threshold cycle (Ct) values for N, RDRP, and E targets of 100 clinical samples for identifying SARS‑CoV‑2 were then evaluated using quantitative real‑time PCR (qRT‑PCR). This result suggests N gene as a potential target for the detection of the SARS‑CoV‑2, since it was observed to be highly expressed in the nasopharyngeal or oropharynges of COVID‑19 patients (P < 0.0001). Herein, we showed that SARS‑CoV‑2 genes were differentially expressed in the host cells. Therefore, to reduce obtaining false negative results and to increase the sensitivity of the available diagnostic tests, the target genes should be carefully selected based on the most expressed genes in the cells.

Keywords: SARS‑CoV‑2, RT ‑PCR assay, RDRP, N, And E genes expressions

Introduction
COVID-19, which is currently known as the global pandemic of Coronavirus, is responsible for the severe acute respiratory disease and fatal outcomes in human beings worldwide (Korber et al. 2020). Coronaviruses as a group of enveloped viruses with positive-sense single-stranded RNA belong to the family Coronaviridae, which are able to spread between humans and animals (Holshue et al. 2020).

Unlike HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1 to cause moderate upper respiratory infection in human (Hu et al. 2014), three previous epidemics of β-coronaviruses such as Middle East respiratory syndrome-related Coronavirus (MERS-CoV), severe acute respiratory syndrome Coronavirus (SARS-CoV), and severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), have been potentially associated with acute respiratory distress syndrome (ARDS) with about 35.5%, 9.6%, and 6.76% mortality rates, respectively (Lu et al. 2020).

SARS-CoV-2 contains open reading frames (ORFs) that encode four structural proteins, including S-spike, M-membrane, E-envelope, and N-nucleocapsid. Of note, several genes are used as the targets gene for detection of SARS-CoV-2 such as the (RDRP and S), (N and S), and E genes. In this regard, studies have previously shown that N protein is produced in large quantities in infected cells, which is related to the processes of replication, translation, and transcription. Moreover, it causes
cell cycle deregulation, consequently inhibiting interferon production and inducing apoptosis (Astuti 2020). The RNA-dependent RNA polymerase, called ORF1ab, is responsible for viral transcription and replication. Therefore, RT-PCR based 2 target genes (ORF1ab and N) are the crucial targets for SARS-CoV-2 detection (Shen et al. 2021).

In order to have the best RT-PCR performance, the components of these targets should be optimized (Tombuloglu et al. 2021). Accordingly, reverse transcription polymerase chain reaction (RT-PCR) using fluorescent dyes is considered as a gold standard method for detecting bacterial and viral nucleic acid (DNA/RNA). RT-q PCR can also be used as a rapid and accurate assay for screening SARS-CoV-2 in throat samples, nasopharyngeal swabs, and feces (Chaimayo et al. 2020). A cohort study has shown that RT-PCR with sensitivity and specificity values of 70% and 95% could detect viruses in patients, even in those showing no symptoms (Arevalo-Rodriguez et al. 2020; Rutuja Sunil and Vasudeo Pandharinath 2021). However, a successful detection of this virus depends on some factors such as test time, early or late detection time, viral load, and sample collection procedure (Vickers 2017).

The ORF1ab/RdRp, E, N, and S genes most commonly used targets for detection of SARS-CoV-2 so, there are some commercial RT-PCR kits for the diagnosis of COVID-19 such as Primer Design (England, RdRp), See-gene (Korea, RdRp, N, E), CerTest Biotec (Spain, ORF1ab, N), Altona Diagnostics (Germany, S, E), BGI (China, ORF1ab), KH Medical (Korea, RdRp, S), and R-Biopharm AG (Germany, E) with different qualities, which are available to be used for the diagnosis of SARS-CoV-2 (Puck et al. 2020). According to this point that diagnosis of SARS-CoV-2 infection with two or three targets lead to an increase in sensitivity and specificity and avoid a false negative result, so the present study attempted to analyze the RDRP, N, and E genes expressions of SARS-COV-2 using qRT-PCR through specific primer pairs in the obtained clinical samples.

Materials and methods
Simplex primer and probe design
The specific qRT-PCR primers and probe for the diagnosis of the target regions of the SARS-CoV-2 were designed using the following programs: PrimerPooler, PrimerPlex, and Primer3 (Tombuloglu et al. 2021). Moreover, 5’ Fluorescein amidites (FAM)-labeled probe was designed for the SARS-CoV-2 RdRp/ N/RP, as well as Hypoxanthine Phosphoribosyltransferase (HPRT) and Yakima yellow-labeled probe for the viral E gene, which were then synthesized (Fig. 1). The sequence of each primer or probe is shown in Table 1.

RNA extraction from the clinical samples
The study was approved by the Mazandaran University of Medical Sciences, Iran, with the number IR.MAZUMS.REC.1399.8671. For the purpose of this study, Nasopharyngeal and oropharyngeal swabs were collected from symptomatic patients, immediately diluted with viral transfer medium (VTM), and finally transferred to the COVID-19 laboratory at Mazandaran University of Medical Sciences for the detection of SARS-CoV-2. RNA extraction was performed in 100 positive samples using the RNJia virus kit (Jivan, Iran) in terms of the
manufacturer’s instructions. Subsequently, the differential gene expressions of N, E, and RdRp were performed using qRT-PCR.

**Real-time RT-PCR assay**

In this study, 20-µL reaction containing 4 µL of RNA, 10 µL of one step RT-PCR kit(add bio, korea), 2 µL of enzyme mixture, 0.5 µL of forward and reverse primers, 0.5 µL of each probe, and RNase/DNase-free ddH2O up to 20 µL, was setup. Final primers and probes concentrations in the reaction were adjusted using the following steps:

1. 1.025 pM for RdRP-F, and 0.25 pM for RdRP-R.
2. 2. 0.25 pM for E-F, and 0.25 pM for E-R.
3. 3. 0.25 pM for N-F, and 0.25 pM for N-R.
4. 4. 0.25 pM for HPRT-F, and 0.25 pM for HPRT-R.

The reaction was dispensed in 96-well microplates (MicroAmp™ Fast Optical 96-well reaction Plate 0.1 mL, Applied Biosystems) and then sealed with optical film (MicroAmp™ Optical Adhesive Film, Applied Biosystems). Of note, a negative control reaction (RNase/DNasefree ddH2O) was used to check the presence of any contamination. In addition, HPRT and RP genes were used as internal controls (Valadan et al. 2015b).

Thereafter, Quantitation experiments were conducted using RT-PCR instrument (StepOne™ Real-Time PCR System).

As well, qPCR was performed as follows:

1. Reverse transcription was performed for 20 min at 50 °C,
2. Inactivation of the reverse transcriptase was done for 10 min at 95 °C.
3. PCR amplification was performed with 40 cycles for 15 s at 95 °C and for 30 s at 58 °C using StepOne™ Real-Time PCR.

**Statistical analysis**

The obtained results were examined by determining the amplification curve of the target gene and the housekeeping gene. Continuous variables are indicated as means (standard deviation, SD). All the statistical analyses were performed using GraphPad Prism 8 software and p-values less than 0.001 were considered as statistically significant.

**Results**

In the present study, 100 respiratory samples were collected from nasopharyngeal (NP) and throat swabs in health-care centers of Mazandaran, Iran, from December 2020 to September 2021. Thereafter, Real-time RT-PCR, using E, RDRP, and N targets, was performed for genome detection of SARS-CoV-2. Firstly, all the primers and probes were analyzed by simplex qRT-PCR. Prior to preparing the reactions, the qRT-PCR instrument was properly calibrated in order to achieve the best fluorescent signal. The simplex reactions were then performed in triplicate for three viral E, N, and RDRP genes as well as internal control genes (HPRT and RP). The criteria for the diagnosis of positive, negative, and suspicious

| Target | Sequence(5′-3′) | Label | Reference |
|--------|----------------|-------|-----------|
| 2019-nCoV_N1-F | GAC CCC AAA ATC AGC GAA AT | None | (Jung et al. 2020) |
| 2019-nCoV_N1-R | CT GGT TAC TGC CGT TGG ACC ACC-BHQ1 | FAM, BHQ1 | (Bruce et al. 2020) |
| E_Sarbeco_F1 | ACAGGGTACCTAATAGGTTG | None | (Corman et al. 2020a) |
| E_Sarbeco_R1 | ATATTGCAGTACGACGACACA | None | (Corman et al. 2020a) |
| E_Sarbeco_P | Yakkima yellow-ACTTAGCCCATCTCTTCTCTGCCTG-GCTTGACACCA | Yakkima yellow, BHQ1 | (Corman et al. 2020a) |
| 2019-nCoV_RDRP-F1 | GTGARATGGCTAGTGGGTGCGCG | None | (Corman et al. 2020a) |
| 2019-nCoV_RDRP-R | CARATGTTAACACCTATATGCAATA | None | (Corman et al. 2020a) |
| 2019-nCoV_RDRP-P | FAM-CAGGTGGAACCTCATTACGGGATGC-BHQ1 | FAM, BHQ1 | (Corman et al. 2020a) |
| HPRT-F | GGACTTATTGACGGACCACGTG | None | (Valadan et al. 2015a) |
| HPRT-R | GCTTCTCAGTCTCTGATAAAACTCAC | None | (Valadan et al. 2015a) |
| HPRT-P | FAM-CCTCCCATCTCTCCTCATCACATCTC-BHQ1 | FAM, BHQ1 | (Valadan et al. 2015a) |
| RP-F | AGA TTT GGA CCT GGG AGC G | None | (Gregianini et al. 2019) |
| RP-R | GAG CGG CTG TCT CCA CAA GT | None | (Gregianini et al. 2019) |
| RP-P | FAM – TCT TGA CCT GAA GGC TCT GCG CG – BHQ-1 | FAM, BHQ1 | (Gregianini et al. 2019) |
COVID-19 samples were as follows: (0 < Ct < 37.00), (NO Ct or Ct ≥ 40.00), and (37.00 ≤ Ct < 40.00), respectively.

The average cycle threshold (Ct) and ∆Ct value with standard deviations (SD) are shown in Tables 2 and 3, and the comparative Ct performances of each assay are shown in Figs. 2 and 3. In this research, HPRT and RP genes were used as internal controls. Indeed HPRT and RP had significantly increased expression level compared to other targets (including N, E, and RDRP) (P < 0.0001). Our findings showed that no detectable difference exists between HPRT and RP internal controls. According to the comparison of ∆Ct values among N, E, and RDRP targets, the N gene expression level was found to be higher than that of E and RDRP genes. (P < 0.0001). As shown in Fig. 4, there is no significant difference between E and N targets (0.611). The result of our study suggest N gene as the most sensitive target compared to E and RDRP for SARS-CoV-2 detection using RT-PCR.

### Table 2

The Ct value emerged in RT-PCR assay for the SARS-CoV-2

| Characteristics       | Result                  |
|-----------------------|-------------------------|
| Ct-value of E         | 26 ± 6.53               |
| Mean ± SD (min, max)  | (14.24, 39.24)          |
| Ct-value of N         | 20.19 ± 5.93            |
| Mean ± SD (min, max)  | (9.72, 33.82)           |
| Ct-value of RDRP      | 26.92 ± 7.04            |
| Mean ± SD (min, max)  | (13.85, 40)             |
| Ct-value of HPRT      | 31.34 ± 2.75            |
| Mean ± SD (min, max)  | (26.94, 40)             |
| Ct-value of RP        | 24.43 ± 1.63            |
| Mean ± SD (min, max)  | (20.56, 27.94)          |

### Table 3

The ∆Ct value emerged in RT-PCR assay for the SARS-CoV-2

| Characteristics       | ∆Ct value (HPRT control) | ∆Ct value (RP control) |
|-----------------------|--------------------------|------------------------|
| E gene                | −5.34 ± 6.76             | 1.57 ± 6.74            |
| Mean ± SD (min, max)  | (−15.15, 0)              | (−8.71, 19.07)         |
| N gene                | −11.15 ± 6.28            | −4.23 ± 6.13           |
| Mean ± SD (min, max)  | (−22.45, 0.43)           | (−13.23, 13.65)        |
| RDRP gene             | −4.42 ± 7.27             | 2.49 ± 7.32            |
| Mean ± SD (min, max)  | (−16.91, 11.42)          | (−8.94, 17.59)         |

**Discussion**

In this study, Ct values for the N, RDRP, and E targets were evaluated using qRT-PCR in order to detect SARS-CoV-2 in 100 clinical samples. It was observed that N gene has less Ct values (23.73 ± 6.99) than those of E and RDRP. Moreover, our results show a significant difference among the E, N, and RDRP groups.

The diagnosis of SARS-CoV-2 using molecular tests is known as the gold standard method for the diagnosis of COVID-19 infection. Of note, the RT-PCR is a sensitive assay for the detection of SARS-CoV-2 RNA in clinical specimens (Chaimayo et al. 2020). The study showed that after the onset of the disease’s symptoms, the SARS-CoV-2 viral load can be immediately observed in the upper respiratory tract and the antigen can also be detected in the first phase. However, some factors such as clinical manifestations, duration of disease to laboratory test, type of clinical sample, and sample collection procedure (technique process) can be effective on interpreting the results (Zou et al. 2020).
In general, many developed laboratory methods use various tools, reagents, and targets in order to identify SRRS-COV-2 (LeBlanc et al. 2020). RDRP, E, and N are three targets proposed by WHO for the SARS-COV-2 identification (Corman et al. 2020a). As well, the E gene is the first line screening, the RDRP gene is used as confirmatory test, and the N gene is used for a confirmatory testing, all of which are used in identifying the coronavirus. A previous study has shown that the RdRP_SARSr-P2 target could be specific for the coronavirus, and other probes are suitable for the detection of other types of coronavirus, and if false positive results are obtained regarding the diagnosis of Covid-19, it may possibly indicate that patients with mild symptoms are infected with other types of corona virus (Kakhki et al. 2020b). Besides, evidence suggests that other targets such as ORF8 and specific primers / probes, may act as additional confirmatory tests in the diagnosis of SARS-COV-2 (kamali Kakhki et al. 2020a).

Houda et al. in their study have evaluated three genes of RDRP, N, and E in 187 COVID-19 samples and found gene expression as 22% and 40% in N and N, E genes, respectively. They have also shown that 6% of patients with both E and N genes and 14% of those with N gene still remained positive after a 12-day treatment period (Benrahma et al. 2020). In addition, a study of 114 respiratory specimens has revealed that the N Ct value was more specific for laboratory diagnosis of SARS-CoV-2 (Abbasi et al. 2022).

However RT-qPCR has a high levels of specificity and sensitivity, but sensitivity of COVID-19 RT-PCR diagnostic kits could be associated to the specimen conditions such as transportation or storage, sample preservation times, and the quality of the kits (Bezier et al. 2020). COVID-19 RT-PCR diagnostic kits with high analytical specificity and sensitivity could help reduce the impact of false-negative results and significantly improve the identification of COVID-19 patients (Shen et al. 2021).

Another study has shown that the one-step real-time RT-PCR can detect SARS-CoV-2 RNA in clinical specimens with a low detection sensitivity (Michel et al. 2021). Since January 2020, protocols, tests, and reagents have been developed and introduced for the detection of SARS-COV-2. These laboratory tests that use SARS-CoV-2 RNA for the detection of COVID-19, were
compared with commercial kits. A previous study using RT-PCR and two primers (N1 and N2) for SARS-COV-2 identification (Shirato et al. 2020) has shown that N2 primer has high specificity and sensitivity in this regard. These primers were also assessed using the following commercial kits: LN S & W-E, LN S & W-N, and LMW & RDRP (Hoehl et al. 2020). The results showed that the commercial LN S & W-N kit containing N primer was able to detect the virus better than the LN S & W-E (25 copies detected) and LMW & RDRP kits. It was observed that the LN S & WE targets are strongly conserved in the E gene region on SARS-COV and SARS-COV-2, while the N2 targets are a single region of N gene on SARS-COV-2 virus, so N2 is highly sensitive and specific for the detection of SARS-CoV-2 (Corman et al. 2020b).

This study showed that selection of different targets with high expression lead to increased sensitivity of diagnostic kits, therefore, to reduce false negative results and to increase the sensitivity, diagnostic tests should be designed based on the targets that have the most differential expression. Correspondingly, RT-PCR method using of N, E, and RDRP targets is known as a reliable and accurate method for SARS-CoV-2 identification that can be used in infection's prevention and control, and in diagnostic laboratories and medical centers.

Acknowledgements
we thank all health workers who fight against COVID-19.

Author contributions
RV participated in experimental design, SG carried out real time PCR, R A‑N performed the statistical analysis, MH participated in data collection, MZ and RV participated in experimental design, SG carried out real time PCR, R A‑N

References
Abbas H, Tabaraei A, Hosseini SM, Khosravi A, Razavi Nikoo H (2022) Real‑time PCR Ct value in SARS‑CoV‑2 detection: RdRp or N gene? Infection 50(2):537–540
Arevalo‑Rodriguez J, Buitrago‑Garcia D, Simancas‑Racines D, Zambrano‑Achig P, Del Campo R, Ciapponi A, Sued O, Martinez‑Garcia I, Rutjes W, Low A, Bossuyt NM, Perez‑Molina PA, Zamora J J (2020) False‑negative results of initial RT‑PCR assays for COVID‑19: a systematic review. PLOs ONE 15(12):e0242958
Astuni L, Irafi Y (2020) Severe Acute Respiratory Syndrome Coronavirus 2 (SARS‑CoV‑2): An overview of viral structure and host response. Diabetes Metab Syndr 14(4):407–412
Benahmna H, Diawara I, Smyej I, Rahou J, Misekaoui N, Benmessaud R, Arouo K, Jaras Kh, Moujid FZ, Adam Z, Nahir S, Aouuzal Z, Elgueezar H, Jeddane L, Ousti F, EL Bakkouri J, Negari Ch (2020) Epidemiological description and analysis of RdRp, E and N genes dynamic by RT‑PCR of SARS‑Cov‑2 in Moroccan population: Experience of the National Reference Laboratory (LNR)‑UM6SS. medRxiv
Bezeir C, Anthonie G, Charki A (2020) Reliability of real‑time RT‑PCR tests to detect SARS‑CoV‑2: A literature review. Int J Metrol Qual Eng 11:13
Bruce EA, Tishe S, Hoffman J, Laaguiy Ph J, Gerrard L, Diehl DA, Leonard SGB, Huston Ch DD, Kirkpatrick D, Grothers BW, Dragon J, Bottjen J (2020) J RT‑qPCR detection of SARS‑CoV‑2 RNA from patient nasopharyngeal swab using Qiagen RTeasy kits or directly via omission of an RNA extraction step. BioRxiv
Chaimoyo Ch, Kaewnpahan B, Tanieng L, Athipanyasip N, Sirirujphat R, Chayakulkleeeme M, Mangkasikwina N, Sutthent R, Puangpunngarn M, Tharmviboonsn T, Pongraweewan O, Chuthapiphat S, Sritivatanavoon Y, Kanttakamalakul W, Horthongkham N (2020a) Rapid SARS‑Cov‑2 antigen detection assay in comparison with real‑time RT‑PCR assay for laboratory diagnosis of COVID‑19 in Thailand. Virol J 17(1):171
Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DKW, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DGC, Haagmans BL, van der Vee B, van den Brink Sh, Wijman L, Goderski G, Ronnete JL, Ellis J, Zambon M, Peiris M, Gossens H, Reusken Ch, Koopmans MFC, Drosten Ch (2020b) Detection of 2019 novel coronavirus (2019‑nCoV) by real‑time RT‑PCR. Eurosurveillance 25(3):23
Corman V, Bleicker T, Brünink S, Drosten Ch (2020a) Diagnostic detection of 2019‑nCoV by real‑time RT‑PCR. World Health Organization 17:1–13
Gregianini TS, Santos Varella IR, Fisch P, Garay Martins L, Veiga ABG (2019) Dual and triple infections with influenza A and B viruses: a case‑control study in Southern Brazil. J Infect Dis 220(6):961–968
Hoehl S, Rabenau H, Berger A, Kortenbusch M, Cinatl J, Bokhassar D, Behrens P, Böddinghaus B, Gotsch U, Naujoks F, Neumann P, Schork J, Tiarks‑Jungk P, Wälczok A, Eickmann M, Vehreschild MJG, Kainn T, Wolf G, Gottschalk T, Ciesek R S (2020) Evidence of SARS‑Cov‑2 infection in returning travelers from Wuhan, China. N Eng J Med 382(13):1278–1280
Holshue ML, Lover NL, Spitters Ch, Tural A, Patel A, Tongs S, Pallansch M A, Biggs HAM (2020) First case of 2019 novel coronavirus in the United States. N Eng J Med 382.929–936
Jung YJ, Park GS, Moon JH, Ju K, Beak SH, Kim S, Changkyun Park E, Park D, Lee JH, Ch WB, Lee JJ, Maeng JS, Kim SJ, Il Kim S, Kim BT, Lee MJ, Kim HG (2020) Comparative analysis of primer–probe sets for RT‑qPCR of COVID‑19 causative virus (SARS‑CoV‑2). ACS Infect Dis 6:2513–2523
Kamali Kakhki R, Aryan E, Mekhzt S, Sankian M (2020a) Development of a cost‑effective line probe assay for rapid detection and differentiation of mycobacterium species: a pilot study. Rep Biochem Mol Biol 8(4):383–393
Kamali Kakhki R, Kamali Kakhki M, Neshani A (2020b) COVID-19 target: A specific target for novel coronavirus detection. Gene Rep 20:100740

Korber B, Fischer WM, Gnanakaran S, Yoon H, Theiler J, Abfalterer W, Hengartner N, Giorgi EE, Bhattacharya T, Foley B, Havlir DM, Partridge LG, Perez LG, Tang H, Moon-Walker A, Whelan SP, Labranche CC, Saphire EO, Montefiori DC (2020) Tracking changes in SARS-CoV-2 Spike: evidence that D614G increases infectivity of the COVID-19 virus. Cell 182(4):812–827

LeBlanc JJ, Gubbay J-B, Lif Y, Needleg R, Radons Arnesonf S, Marcinsof D, Charest H, Desnoyerg G, Dustj K, Fattouhk R, Garceau R, Germanl G, Hatchette TF, Kozak RA, Kradjenn M, Kuschaft T, Lango ALS, Levett R, Mazzulli T, McDonald R, Mubareka S, Prystajeckyn N, Rutherfordd C, Smiejaq M, Yusg Y, Zahariadisg G, Zelyas N, Bastaenf N (2020) Real-time PCR-based SARS-CoV-2 detection in Canadian laboratories. J Clin Virol 128:104433

Lu L, Zhong W, Biai Z, Li Z, Zhang K, Liang B, Zhong Y, Hu M, Lin L, Liu J, Lin X, Huang J, Yang X, Zhang X, Huang Z (2020) A comparison of mortality-related risk factors of COVID-19, SARS, and MERS: A systematic review and meta-analysis. J Infect 81(4):e18–e25

Michel J, Neumann M, Krause E, Rinner T, Muzenieks T, Grossesgesse M, Hille G, Schwarz F, Puyksens A, Förster S, Biere B, Bourquain D, Domingo C, Brinkmann A, Schaele L, Schrick L, Nitsche A (2021) Resource-efficient internally controlled in-house real-time PCR detection of SARS-CoV-2. Virol J 18(1):110

Puck BVK, van der Veer B, van den Brink Sh, Wijsman L, de Jonge J, van den Brandt A, Molenkamp R, Reusken CBEM, Meijer A (2020) Comparison of seven commercial RT-PCR diagnostic kits for COVID-19. J Clin Virol 128:104412

Qin Hu, Lu R, Peng K, Duan X, Wang Y, Zhao Y, Wang W, Lou Y, Tan W (2014) Prevalence and genetic diversity analysis of human coronavirus OC43 among adult patients with acute respiratory infections in Beijing, 2012. PLoS ONE 9(7):e100781

Rutuja Sunil P, Vasudeo Pandharinath Z (2021) Development of RT-PCR Based Diagnosis of SARS-CoV-2. Biotechnology to Combat COVID-19 (IntechOpen:96823)

Shen L, Cui Sh, Zhang D, Lin Ch, Chen L, Wang Q (2021) Comparison of four commercial RT-PCR diagnostic kits for COVID-19 in China. J Clin Lab Anal 35(1):e23605

Shirato K, Nao N, Kato H, Takayama I, Saito Sh, Kato F, Katoh H, Sakata M, Nakatsu Y, Mori Y, Kageyama T, Matsuyama Sh, Takeda M (2020) Development of genetic diagnostic methods for novel coronavirus 2019 (nCoV-2019) in Japan. Jap J of Infect Dis 73(4):304–307

Tombuloglu H, Sabt H, Al-Suhaimi E, Al Jindan R, Alkharsah R, Kh (2021) Development of multiplex real-time RT-PCR assay for the detection of SARS-CoV-2. PLoS ONE 16(4):e0250942

Valadan R, Amjadi O, Tehrani M, Rafiee A, Hedayatzadeh-Omran A, Alizadeh-Navaei R (2015a) Pseudogene-free amplification of HPRT1 in quantitative reverse transcriptase polymerase chain reaction. Anal Biochem 485:46–48

Valadan R, Hedayatzadeh-Omran A, Naghavi Alhosseini-Abyazani M, Amjadi O, Rafiee A, Tehrani M, Alizadeh-Navaei R (2015b) Data supporting the design and evaluation of a universal primer pair for pseudogene-free amplification of HPRT1 in real-time PCR. Data brief. 4:384–89

Vickers NJ (2017) Animal communication: when i’m calling you, will you answer too? Curr bio 27(14):R713–R15

Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Zh, Yu J, Kang M, Song Y, Xia L, Guo Q, Song T, He J, Ven H-L, Peirs M, Wu J (2020) SARS-CoV-2 viral load in upper respiratory specimens of infected patients. N Eng J Med 382(12):1177–1179

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.