

## Melting Curve-based Assay as an Alternative Technique for the Accurate Detection of SARS-CoV-2

### Abstract

**Background:** Early and cost-effective diagnosis and monitoring of the infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are critically important to anticipate and control the disease. We aimed to set up a SYBR Green-based one-step real-time polymerase chain reaction (PCR) as a lower-cost alternative method to detect the virus. **Materials and Methods:** An in-house SYBR Green-based PCR assay targeting the envelope (E) and RNA-dependent RNA polymerase (RdRp) genes, was set up to diagnose the infection, and was compared with the reference probe-based PCR method. **Results:** When the commercial probe-based assay was considered as the reference method, SYBR Green-based PCR had a slightly lower sensitivity (81.98% and 86.25% for E and RdRp targets, respectively) and a good specificity (100% and 94.44% for E and RdRp targets, respectively). For both gene targets, three different melting temperature ( $T_m$ ) patterns were found in the PCRs of the nasopharyngeal/oropharyngeal swab samples, but no size polymorphism was seen in agarose gel electrophoresis. **Conclusion:** Further studies to improvement of the assay are needed to make it an inexpensive and reliable tool for the diagnosis of COVID-19.

**Keywords:** E-gene, RdRp-gene, SARS-CoV-2, SYBR Green, Diagnosis Biological Assay

### Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly contagious virus causing a range of mild-to-severe infections.<sup>[1]</sup> Since the emergence of this disease in late 2019, health care systems have experienced a variety of challenges including diagnosis, treatment, and prevention.<sup>[2]</sup> Although the majority of the infected people are asymptomatic<sup>[3]</sup> or represent mild symptoms with no significant patterns of computed tomography (CT) of the chest,<sup>[4]</sup> this disease has caused a high rate of spread and subsequent increased mortality rate. The virus may affect many organs. It binds to host receptors (angiotensin-converting enzyme 2) and penetrate by endocytosis or membrane fusion, viral contents are released inside the host cells, its RNA enters the nucleus, and viral messenger RNA is used to make viral proteins. Recruitment of inflammatory interleukin 6 secreting monocytes caused severe lung pathology in patients with COVID-19. Release of the virus in the lung cells leads to nonspecific symptoms such as fever, myalgia, headache, and respiratory symptoms.<sup>[5]</sup>

Reliable and cost-effective diagnosis is urgent to improve the management of the patients and to investigate the inter/intra society outbreaks. Regarding the emergency of proper diagnosis, different molecular detection methods have been conducted mainly based on polymerase chain reaction (PCR), real-time-PCR (rt-PCR),<sup>[6]</sup> multiplex PCR,<sup>[7]</sup> loop-mediated isothermal amplification,<sup>[8]</sup> aptamers,<sup>[9]</sup> and CRISPR-Cas.<sup>[10]</sup> Different approaches of rt-PCR are regarded as the methods of choice for the detection of coronavirus disease 2019 (COVID-19) from nasopharyngeal and oropharyngeal samples.<sup>[11,12]</sup> TaqMan probe-based reverse transcription real-time PCR assay, based on the detection of a specific complementary sequence of the pathogen in a specifically amplified region, has been accepted as the golden standard for the detection of the viral pathogen in clinical samples. However, in dye-based rt-PCR detection using SYBR Green or Eva Green, the presence of a specific amplified sequence is considered. Dissociation characteristics of double-stranded DNA during heating can be assessed by melting curve analysis, in which with raising the temperature, the

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DNA strands begin to dissociate that results in rising in the absorbance intensity. The dissociation between two strands of amplified DNA can be measured using SYBR Green, EvaGreen, or fluorophore-labeled DNA probes ([https://en.wikipedia.org/wiki/Melting\\_curve\\_analysis](https://en.wikipedia.org/wiki/Melting_curve_analysis)). Although the probe-based method indicates higher specificity than the SYBR Green-based technique, the latter is less expensive<sup>[13]</sup> and suitable for the detection of variable genotypes in pathogens.<sup>[14]</sup>

Following the complete genome sequence analysis of SARS-CoV-2,<sup>[15]</sup> several diagnostic strategies have been developed targeting specific sequences in the structural spike (S), nucleocapsid (N), membrane (M), envelope (E), or nonstructural protein-coding genes (nsp) required for virus replication (i.e., nsp12 or RNA-dependent RNA polymerase [RdRp], nsp13 or helicase [hel], and open reading frames [ORF] 1ab).<sup>[16]</sup> Considering the high specificity of the probe-based method on one hand, and the mutation capacity of the RNA virus, its anomalous transmission, and its spread (which positively affects an increased number of derivative subspecies) on the other hand, probe-based detection may encounter false-negative results because of the mutation in probe-target sequences.<sup>[17]</sup>

In the present study, we aimed to set up a SYBR Green-based one-step reverse transcription real-time PCR method as an alternative for probe-based real-time PCR to detect the COVID-19 virus. This lower-cost approach was compared with the routine TaqMan probe-based rt-PCR approach. The method can be used in conditions that supplying specific and/or expensive probes reagents or kits are difficult.

## Materials and Methods

### Clinical samples

This study included a total of 200 clinical specimens consisting of 100 samples from hospitalized patients with a COVID-19 and 100 samples from suspected patients who were in contact with affected individuals or indicated some clinical signs of COVID-19 and referred to healthcare centers affiliated to Isfahan University of Medical Sciences,

Isfahan, Iran. The nasopharyngeal/oropharyngeal swab samples were collected in hospitals and health centers and kept at collection tubes containing a virus transport medium. All samples and experiments were processed at COVID-19 diagnostic laboratory, Research Core Facility Lab, Isfahan University of Medical Sciences, Isfahan, Iran.

### Probe-based reverse transcription real-time PCR assay

The RNA was extracted and purified using a viral RNA isolation kit (ROJE, Iran) according to the manufacturer's instructions, and stored at  $-20^{\circ}\text{C}$  until to use. A 10  $\mu\text{L}$  aliquot of extracted RNAs were subjected to SARS-CoV-2-specific probe-based one-step rt-PCR kit (Sansure Biotech, China), targeting the N gene and ORF-1ab region, according to the manufacturer's protocol. The amplification was performed with a cycle of 30 min at  $50^{\circ}\text{C}$  for reverse transcription and 1 min at  $95^{\circ}\text{C}$  for primary denaturation, followed by 45 cycles of  $95^{\circ}\text{C}$  (15 s) and  $60^{\circ}\text{C}$  (30 s), by the LightCycler® 96 Instrument (Roche Life Science, Germany). According to the manufacturer's protocol, CT <40 in both genes was considered positive.

### SYBR Green-based real-time-polymerase chain reaction assay

The SYBR Green-based PCR was conducted on the same extracted RNAs used for probe-based PCR, using the reverse transcription enzyme included in the PCR reagent set of COVID-19 kits (Da An gene, China), and the SolisGreen qPCR Mix (Solis BioDyne, Estonia) in one tube. The specific primers for the target genes E,<sup>[18]</sup> RdRp,<sup>[19]</sup> and ORF-1ab<sup>[20]</sup> of the SARS-CoV-2 were used separately [Table 1]. A total reaction mixture of 15  $\mu\text{L}$  was prepared for each sample containing 5  $\mu\text{L}$  of the RNA template, 0.3  $\mu\text{L}$  (0.2  $\mu\text{M}$ ) of each primer, 3  $\mu\text{L}$  of 5X master mix, and 1  $\mu\text{L}$  of reverse transcriptase enzyme. The thermal program was 30 min at  $50^{\circ}\text{C}$  for reverse transcription and 15 min at  $95^{\circ}\text{C}$  to inactivate the enzyme as well as to activate the Taq DNA polymerase, followed by 45 cycles of  $95^{\circ}\text{C}$  for 15 s, and  $57^{\circ}\text{C}$  for 45 s. At the end of the program, melting curve analysis ranging from  $60-95^{\circ}\text{C}$  with acquiring fluorescence data every  $0.3^{\circ}\text{C}$

**Table 1: The sequence of each primer and the relevant amplicon sizes, the sensitivity and specificity of each primer pair used in this study**

Target gene	Primers Sequence	Product size (bp)	Tm patterns (number of samples having same Tm)			Sensitivity in comparison with probe-rt PCR (%)	Specificity in comparison with probe-rt PCR (%)	
E	Forward	ACAGGTACGTTAATAGTTAATAGCGT	113	77.5-78.8	78.81-79.8	79.81-80.8	81.98	100
	Reverse	ATATTGCAGCAGTACGCACACA		(22)	(69)	(51)		
RdRp	Forward	TGTAAACCAGGTGGAAC	156	78.5-79.5	79.51-80.5	80.51-81.5	86.25	94.44
	Reverse	CTGTGTTGTAGATTGCG		(14)	(83)	(56)		
ORF1ab	Forward	CCCTGTGGGTTTTACTACTTAA	119	82			45	100
	Reverse	ACGATTGTGCATCAGCTGA						

Tm: Melting temperature, PCR: Polymerase chain reaction, rt: Real-time

was performed. All reactions were performed using the LightCycler® 96 Instrument (Roche, Germany).

### Agarose gel electrophoresis

To obtain an objective judgment on any possible size polymorphism or additional amplicons, a total of 14 random samples representing each relevant pattern and having different melting temperatures ( $T_m$ ) in SYBR Green RT-rt-PCR, were subjected to 2% agarose gel electrophoresis. The gels were stained with 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide and visualized under ultraviolet light.

### Statistical analysis

The sensitivity and specificity of SYBR Green-based RT-rt-PCR in the detection of COVID-19 by two primer sets were determined using Fisher's test using Graphpad Prism 6. A  $P < 0.05$  was considered statistically significant.

### Results

Flow diagram depicting the methods and results is shown in Figure 1. The samples were from seven different age groups of the patients consisted of under 19 years of age ( $n = 14$ ), 20–29 years ( $n = 31$ ), 30–39 years ( $n = 51$ ), 40–49 years ( $n = 32$ ), 50–59 years ( $n = 24$ ), 60–69 years ( $n = 23$ ), and over 70 years of age ( $n = 25$ ). Overall, 24 cases of 25 hospitalized patients were aged  $>70$  years, and 37 of 51 patients referred from healthcare centers were 30–39 years. The majority (58.5%,  $n = 117$ ) of the study population was men.

Based on the probe-based RT-rt-PCR method, 182 positive and 18 negative samples were selected to be re-tested with the SYBR Green-based PCR assay. Table 1 indicates the

sequence of each primer and the relevant amplicon sizes, the sensitivity and specificity of the tests performed by each primer pair, and the number of each pattern obtained from melting curve analysis. Among 18 samples that were negative in the probe-based PCR test, only one sample become positive using the RdRp primers in the SYBR Green assay. Considering the probe-based assay as a reference method, the sensitivity of the SYBR Green assay was 81.98% and 86.25% when the targets E and RdRp were used, respectively. The assessment of SYBR Green test by ORF-1ab was discontinued following preliminary low sensitivity results. The mean CT values for ORF-1ab and N genes in the commercial probe-based assay were 26.37 and 23.24, respectively, whereas in the SYBR Green-based method, the corresponding values were 26.12 and 24.79 for E and RdRp genes, respectively. After performing the tests for ORF-1ab, the results were negative for the samples with CTs higher than 28 in the probe-based reference assay.

The melting temperature analysis of the SYBR Green PCR assessing E and RdRp genes revealed three defined  $T_m$  patterns in both genes. Using the E gene, rt-PCR of the positive samples showed three distinct  $T_m$  patterns, i.e., 77.5–78.8, 78.81–79.8, and 79.81–80.8, while using the RdRp gene, the  $T_m$  patterns were 78.5–79.5, 79.51–80.5, and 80.51–81.5 [Figures 2a and b]. To confirm the stability of the  $T_m$  patterns, the tests were repeated for 24 samples having different patterns of both E and RdRp genes including 22 positive and two negative samples, and the same results were observed. In spite of existing different  $T_m$  patterns in melting curve analysis, no fragments with a size different with 113 bp and 156 bp (for E and RdRp genes, respectively) were observed in agarose gel electrophoresis [Figures 2c and d].

### Discussion

Novel  $\beta$ -coronavirus (SARS-CoV-2 infection) has emerged as the most challenging worldwide catastrophe. It is highly contagious with a wide range of severity causing death mostly in susceptible individuals, which rapidly develop among communities worldwide.<sup>[21-23]</sup> Rapid and reliable diagnostic tools are critical for detecting the infected people and for isolation and treatment of the patients.<sup>[24]</sup> In parallel with the clinical signs and symptoms, as well as paraclinical signs and symptoms, a variety of commercially available probe-based rt-PCRs have been designed and are widely used to provide reliable detection of SARS-CoV-2. The increasing number of samples and subsequent unaffordable costs needed for molecular tests, necessitate introducing alternate, and cost-benefit approaches to diagnostic strategies. To meet this purpose, attempts have been led to assess the efficiency of SYBR Green detection for SARS-CoV-2.<sup>[25-27]</sup> Due to the lower cost and processing speed, this methodology can be utilized on a larger scale, providing peace of mind for those being tested and their peers, also guidance for social isolation protocols.<sup>[28]</sup>

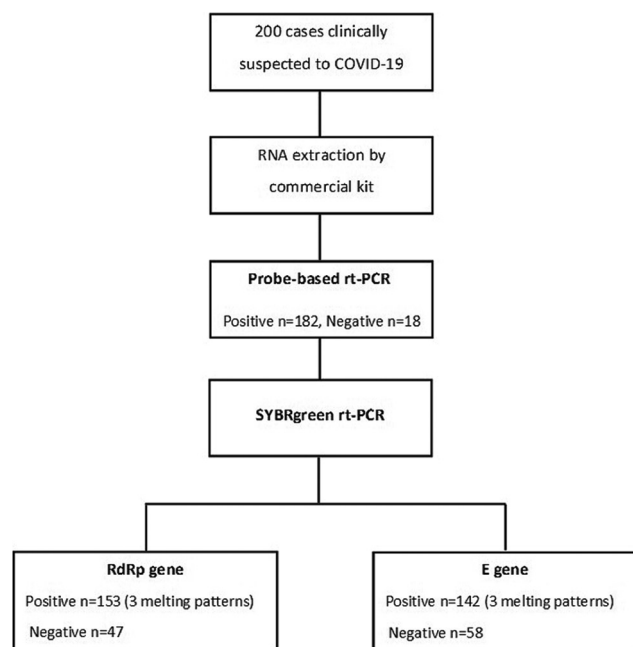
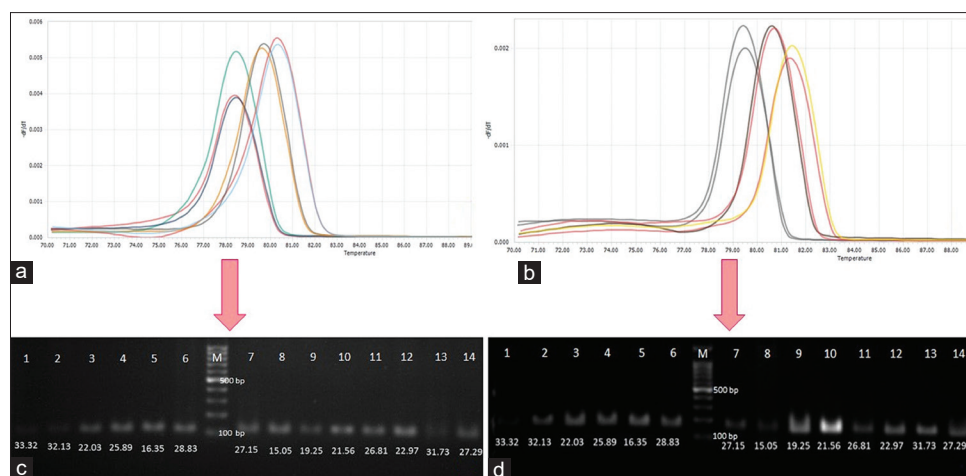


Figure 1: Flow diagram depicting methods and results of the present study





**Figure 2: Variable melting curve patterns generated in melting curves of SYBR green real time-PCR for the detection of SARS-CoV-2 and the electrophoresis of the PCR products: (a) E gene, (b) RdRp gene, (c) amplified fragment of different melting temperature patterns of E gene, and (d) amplified fragment of different melting temperature patterns in the RdRp gene. M: Molecular size marker.**

Moreover, the high rate of contamination in the ingredients of commercially available kits imposes more costs on the diagnosis process, which reward insisting on in-house less expensive workflows. As SARS-CoV-2 is presented mostly in the upper and lower respiratory systems, nasopharyngeal and oropharyngeal swabs are the best sample types for clinical diagnosis of the virus.<sup>[29,30]</sup>

In this study, in-house SYBR Green rt-PCR was compared with commercial probe-based rt-PCR to diagnose SARS-CoV-2 in 200 randomly chosen samples. Compared to commercial probe-based PCR, our in-house method indicated a 100% and 94.44% specificity for E and RdRp genes, respectively; however, the RdRp gene revealed a higher sensitivity compared to E gene (86.25 vs. 81.98%). This indicates acceptable reliability of the SYBR Green rt-PCR approach, in comparison with the probe-based techniques, and proclaims the value of conducting more studies to approve the usage in clinical diagnosis. Nevertheless, its relatively lower sensitivity might be partly due to the fact that after testing the samples with the routine probe-based method, the extracted RNAs were conserved at the freezer and the SYBR Green tests were performed after several months. Melting curve analysis of the rt-PCR has been introduced as a primary approach in molecular diagnosing of different species, serotypes, or genotypes in many organisms. Through the utilization of this approach and a unique set of primers, a variety of melting curve shapes or melting temperatures are capable of discriminating between different amplicons.<sup>[14,31]</sup>

The high mutation capacity of the coronaviruses<sup>[32]</sup> is one of the challenging features of the current pandemic. This would lead to variations in virus characteristics, virulence, pathogenesis, and subsequent challenges in predicting therapeutic and vaccine preparation strategies as previously reported on coronaviruses.<sup>[33,34]</sup> The

extraordinary virulence capacity of SARS-CoV-2 and the ensuing immense number of infected individuals, extremely increase this mutation chance and enriches the genetic pool of the virus. Although further investigations are needed to clarify the probable association between different variants and the severity of the disease, different melting temperature patterns investigated in this study can be indicative of variable GC content,<sup>[26]</sup> or the mutations carried by the virus in the population. However, further studies and sequencing analyses are required before making the judgment.

## Conclusion

As the conclusion, an in-house SYBR Green one-step rt-PCR assay using two E and RdRp genes, was compared with a commercial probe-based method to evaluate the probable replacement of the workflow in detecting SARS-CoV-2 in the clinical diagnosing process. The resultant findings indicated an acceptable correspondence with the commercial probe-based rt-PCR, and it looks that it could provide melting temperature differences and consequent evidence regarding probable viral variants in the population and the possible association with the infection contingency and severity.

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## Ethical conduct of research

Ethical approval of the study was obtained from the ethics committee of Isfahan University of Medical Sciences, Isfahan, Iran (IR. MUI. RESEARCH. REC.1398.778).

## Conflicts of interest

There are no conflicts of interest.

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