Chulalongkorn Medical Journal

Volume 66 | Issue 2 Article 1

4-1-2022

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Recommended Citation

Samiminemati, Afshin; Zeinoddini, Mehdi; Xodadadi, Nasrin; and Sheikhi, Fatemeh (2022) "Design and evaluation of an artificial positive control construct for detection of the novel coronavirus 2019 using triplex PCR," *Chulalongkorn Medical Journal*: Vol. 66: Iss. 2, Article 1.
DOI: 10.14456/clmj.2022.16
Available at: https://digital.car.chula.ac.th/clmjournal/vol66/iss2/1

Original article

Design and evaluation of an artificial positive control construct for detection of the novel coronavirus 2019 using triplex PCR

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Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is a novel-emerged strain with the person-to-person transmission that had never been found in humans. The main method for the identification of SARS-CoV2 is the genome-based reverse transcription polymerase chain reaction (RT-PCR) using expensive instrument (real time thermal cycler); therefore, viral RNA is required to perform PCR reaction. On the other hand, false-positive or negative results of RT-PCR detection of this virus are a challenge for all clinical laboratories. One smart and scientific strategy for solving this problem is designing an artificial positive control construct.

Objectives: To design and evaluate this positive control construct for the correct and standard detection of SARS-CoV2. To design an artificial construct, three specific viral genomic regions were chosen as the target, which contained open reading frame (ORF1ab), envelope (E), and nucleocapsid (N) of SARS-CoV2. Hence, 813bp-conserved regions were cloned into the *EcoRV* sequence of pBlueScriptII SK(+) plasmid. In the following, six specific primers were designed for the specific detection of these conserved regions. Finally, the performance of this synthetic construct, named pBlue-ORF-E-N, was simulated in triplex PCR assay using SnapGene software.

Results: The data showed that this synthetic construct was very applicable for detecting SARS-CoV2. Therefore, it is possible to apply a triplexPCR to identify the virus using this construct as a positive control and to compare with the native RT-PCR by extracted viral RNA.

Conclusion: TriplexPCR can be developed using this novel construct in the detection of the emerged virus in order to confirm the common RT-PCR detection used in all clinical laboratories without real time PCR machine.

Keywords: Novel coronavirus, artificial construct, triplex-PCR, detection.

On December 30 of 2019, a new coronavirus was emerged in Wuhan (Hubei Province, China) that had properties similar to severe acute respiratory syndrome coronaviruses (SARS-CoV) and caused the emergence of several pneumonia cases. Therefore, the 2019 novel coronavirus (2019-nCoV) and the developed disease were named SARS-CoV-2 and Coronavirus disease 2019 (COVID-19), respectively by the World Health Organization (WHO). This infection spread rapidly worldwide and today has become an outbreak in all countries.[1, 2] This outbreak caused significant mortality and morbidity in the world and over one million patients have infected with it.

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Received: July 22, 2021
Revised: November 15, 2021
Accepted: December 30, 2021

The SARS-CoV-2 strain was classified into beta coronaviruses and is genetically related to the Middle East Respiratory Syndrome coronavirus (MERS-CoV) and SARS-CoV.[3] On the other hand, the genome sequences of the SARS-CoV-2, SARS-CoV, and MERS-CoV were compared and showed that SARS-CoV-2 has a closer sequence identity to SARS-CoV than the MERS CoV. Also, the amino acid sequence of SARS-CoV-2 varies from the other coronaviruses exclusively in surface glycoprotein spike (S-protein) and also the regions of ORF1ab polyprotein.[4 - 6] The other related coronaviruses are spherical nanoparticles with about 150 to 160 nm in size associated with five sections, positive single-stranded RNA (+ssRNA), nucleocapsid protein (N), membrane glycoprotein (M), envelope protein (E), and glycoprotein spike (S) (Figure 1). This novel respiratory coronavirus unlike SARS and MERS has an additional glycoprotein with acetyl esterase and hemagglutination (HE) properties.[7, 8] There are many

reported methods for fast and selective detection of this virus that most of them are reverse transcription polymerase chain reaction (RT-PCR)-based techniques.[9–11] In these assays the virus is necessary to be utilized in the experiment.[12] This virus is belonging to biosafety level 3 (BSL 3) so it requires special laboratory conditions for experiment.[13] Also, false-positive or negative results of the RT-PCR detection of this virus are a challenge for all of the clinical laboratories. While, this problem could be solved by utilizing artificial positive controls including the specific and conserved region of various genes from this pathogen. [14, 15] These conserved viral genomic regions could be synthesized artificially and cloned into the plasmid vectors and used as a positive control in other genome-based methods such as real-time PCR, multiplex PCR, nested PCR, and isothermal methods.[16] In this work, we designed an artificial construct and suitable primers for specific and direct diagnosis of SARS-CoV-2 for the control assessment of real-time RT-PCR. Also, according to the specific and selective region of SARS-CoV-2 the conserved region of this virus in ORF1ab, E, and N genes were selected and used for the triplex PCR assay that showed a higher specificity than monoplex PCR assay in both conventional and quantitative PCR methods. Therefore, we designed a new construct for the detection of three specific genes of SARS-Cov-2 in a single tube and at the same time.

Materials and methods

Gene selection and primer design

Firstly the open reading frame (ORF1ab), envelope producer gene (E), and nucleocapsid gene (N) were chosen as the target gene according to the current literature. In follow, specific primers were designed according to the purpose of the study. For this gene sequences were obtained from the National Center for Biotechnology Information (NCBI) database and the sequence alignment was performed using Mega blast 6 offline software. Then, the conserved regions of each gene were used for primer designing. A schematic overview of virus genome mapping is shown in Figure 1A. Three sets of specific primers were designed by Gene Runner oligo tools and Oligoanalyzer online software to apply triplex PCR reactions. The primer names and their sequence properties are shown in Table 1.

Artificial construct design

The related construct was designed by SnapGene offline software. For this, a specific region of each gene fragment was designed. This sequence has the following characteristics, gene fragments were as follows, ORF1ab, E, and N gene segments (Figure 1B). In the next step, restriction sites were added to the sequence in order to separate each gene amplicon from the other or digest the whole of the sequence from the plasmid vector. These restriction sites involve the *BamHI* restriction site at the 5/ end of the designed sequence and two *SmaI* restriction sites located at the end of the ORF1ab gene segment and the end of the E gene. While the HindIII restriction site placed at the 3/ end of the sequence (Figure 1C). Finally, the designed sequence was cloned into a plasmid vector to amplify and make the gene library. This section of the study was simulated by SnapGene software using a restriction cloning tool.

Table 1. Primer properties.

Primer name	Sequences (5/—3/)	Length (mer)	Amplicon size (bp)
ORF1ab-F	TGCCTCAACTTGAACAGC	18	265
ORF1ab-R	AGCTCGTCGCCTAAGTCAA	19	
E - F	TTTCGGAAGAGACAGGTAC	19	200
E - R	AGGAACTCTAGAAGAATTCAGA	22	
N - F	ACTGCGTCTTGGTTCACC	18	324
N - R	TGCAGCATTGTTAGCAGGAT	20	

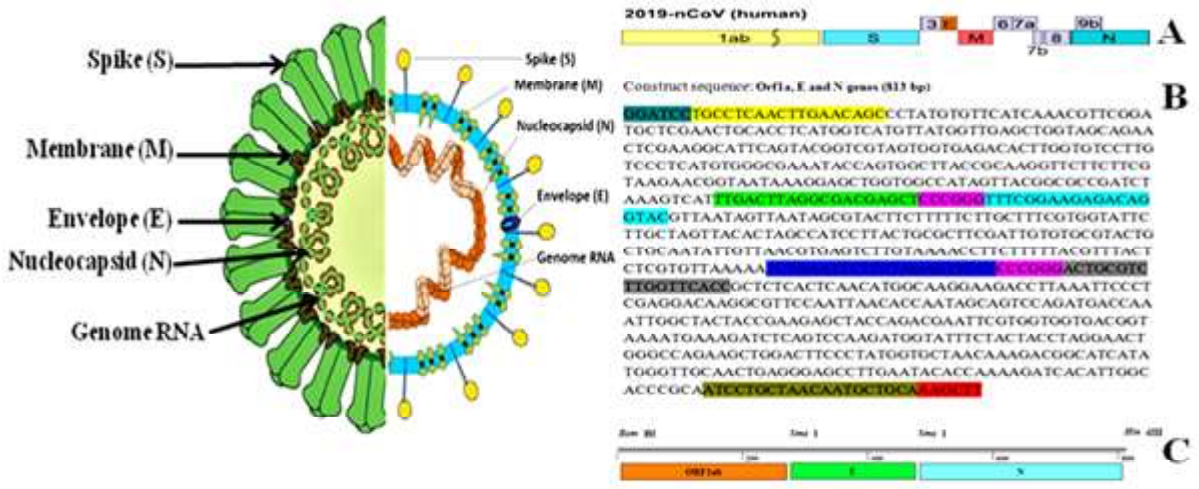

Figure 1. Left: SARS-CoV-2 schematic diagram. Right: (**A**) Schematic view of the genome mapping of SARA-CoV-2. (**B**) Designed sequence containing conserved regions of three genes of SARA-CoV-2. (**C**) Schematic view of the selected genes of this work provided by DNASTAR software.

PCR reaction simulation

In the first step the conserved regions of the gene segments were carefully analyzed using SnapGene software. In the next step the designed primers were given to the software to simulate the PCR amplification in order to ensure the size of amplicons. Also the NCBI primer blast was used to ensure the primers and amplicon size in the native virus sequences. In follow and after primer synthesize and preparation (Table 1), triplex PCR assay was carried out according to simulated process. For this, in order to confirm any of the pairs of ORF1ab, E and N primers, the monoplex PCR reaction was performed in a volume of 5 µL as follows, 1 µM (0.25 µl) from each primer and 3.5 µl master mix of Taq DNA polymerase (amplicon PCR kit, Denmark) and 100 ng (1 µl) DNA template. The PCR was carried out according to program below, Initial denaturation at 95°C for 4 min, 30 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Also the triplex PCR mixture was adjusted to 5 µL and was carried out with 0.25 µl from each primer at one step tube. Finally, the amplified products were analyzed on 2.0% agarose gel electrophoresis.

Results

Simulation of the artificial construct

The artificial construct, which can be used as a positive control of PCR amplification is shown in Figure 2A. This synthetic construct contains the conserved genomic region of SARS-CoV-2, which can be amplified using specific primers. Although this construct was designed for triplex PCR reactions for the virus detection in a single tube, it may also be used for the monoplex PCR reaction.

Simulation of PCR reaction by the designed primers

PCR reaction was simulated using SnapGene software. Each pair of primers detects a specific region in the construct and native virus. These primers and their amplifiable regions are shown in Figure 1B. Also, agarose gel electrophoresis was simulated and the PCR products of each gene segment are shown in a separate well according to Figure 2B.

Gene amplification

Using specific primer we detected ORF1ab, E and N fragment gene from *SARS-CoV-2* individually according to monoplex PCR (Figure 3B). The presence of 265, 200 and 324 bp amplicon in agarose gel confirm that ORF1ab, E and N fragment gene available in the sample, respectively. After ensuring that each pairs of primer were suitable for amplification we used from three pairs primers in one step PCR (multiplex) reaction, according to the procedure offered in the materials and methods. The PCR products were evaluated on the agarose gel and the presence of specific fragment size in agarose gel confirms that each three fragment was amplified (Figure 3A).

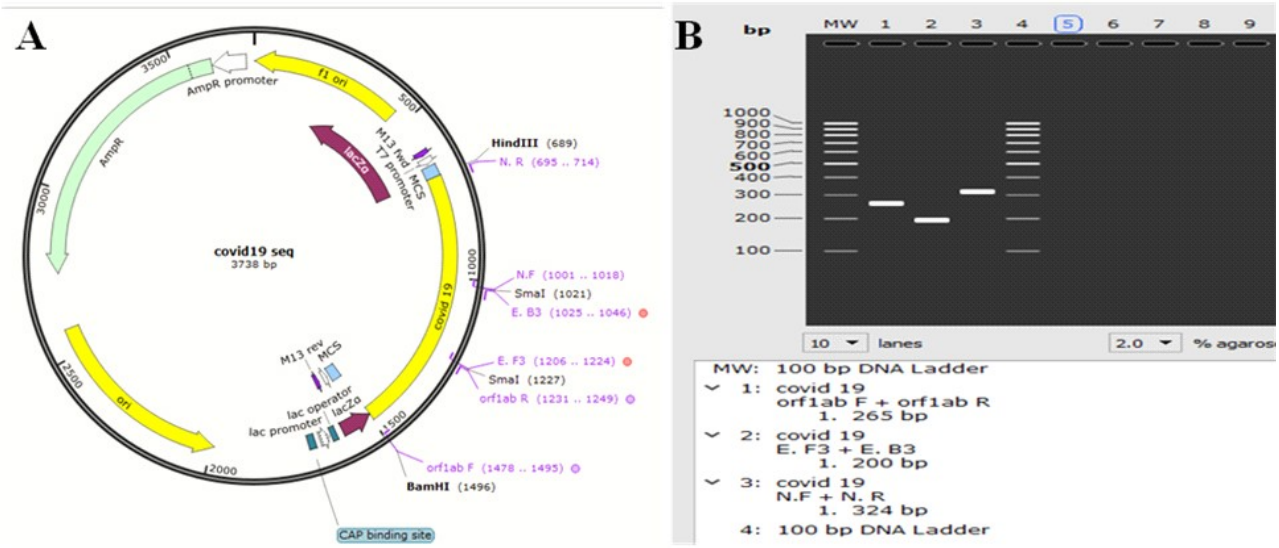

Figure 2. (**A**) Simulation of the designed artificial construct of SARS-CoV-2 using SnapGene software. (**B**) Simulation of PCR product analysis using the 2% gel agarose electrophoresis via SnapGene software. Lane M: 100bp DNA size marker. Lane 1: 265 bp amplicon of ORF1ab. Lane 2: 200 bp for E gene amplicon. Lane 3. 324 bp for N gene amplicon. Lane 4: 100 bp DNA size marker.

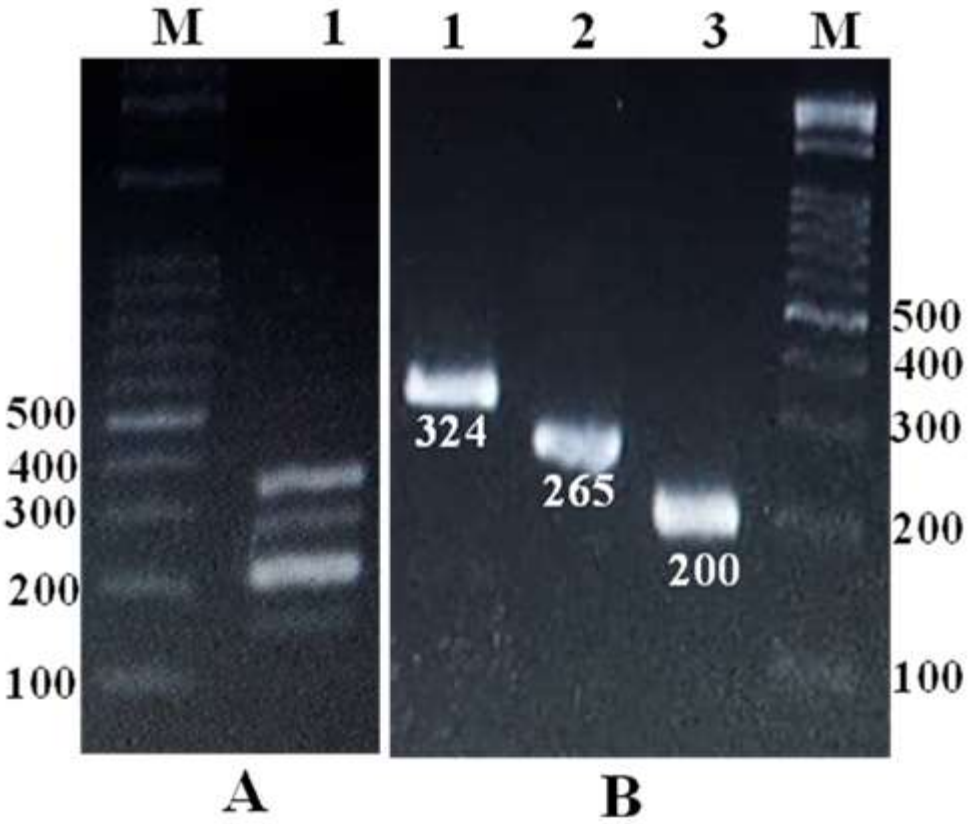

Figure 3. (**A**) The triplex PCR product analysis (1), 100 bp DNA leader (M). (**B**) The PCR product analysis for N (1), ORF1ab (2) and E (3) fragment gene using agarose gel electrophoresis.

Discussion

Today, COVID-19 has become a major obstacle to human health in the world. This epidemic spread from the most eastern point of the world to most countries.[17] The prevalence of this disease is increasing extremely, which has led to the closure of various centers around the world and the expansion of quarantine in many countries. Currently, various real-time RT-PCR kits developed for the diagnosis of this virus in nasopharyngeal swabs, alveolar lavage fluid, sputum, and blood samples to be used in laboratories and clinical centers.[16-18] In this field, radiological scans like thin slice chest Computed Tomography, play an important role in the analysis of the infectious disease at primary stages. The radiological methods have some problems and false responses because they are not specific to diagnosing this virus.[19, 20] The specificity of the PCR method can be increased by applying some changes in the basic reaction. For example, in multiplex PCR assay, various genes have been chosen as the target and several primers have been designed for them. The present study reported that the multiplex PCR reaction is more specific than conventional PCR assays.[21] Also, the multiplex PCR assay may be utilized for quantitative PCR and increase the sensitivity and specificity of the reaction.[22] Applying this method allows for amplifying multiple genes in a single tube for the reduce reaction time. Multiple diagnoses of various microbes at the same time are the other advantage of this method.[23] All molecular methods detect a specific region in the genome of microorganisms. Artificial constructs include a plasmid vector and a DNA sequence that contains conserved genomic regions and restriction sites for digestion. This tool allows us to provide a safe detection for highly infectious elements in common laboratories as a positive control of amplification. This method is useful to check the designed primers and also PCR amplification program to develop a new PCR method for the detection of microorganisms. Another advantage of this method is that it is completely safe and can be preferred to diagnose dangerous pathogens in laboratories with basic biosafety requirements. In this field, in 2019 Pourmahdi N, *et al*. identified *Yersinia pestis* and *Francisella tularensis* by the multiplex-PCR assay using a synthetic construct.[24] In another study, Caasi DR, *et al*. designed a PCR positive control for Barley yellow dwarf virus, Soilborne wheat mosaic virus, Triticum mosaic virus, and Wheat streak mosaic virus.[25] Moreover, in a previous study, Sohni Y, *et al*. constructed control of amplification for *Bacillus anthracis* by cloning synthetic segments into a plasmid vector.[26] Also, Samimi NA, *et al*. constructed a positive control for *Coxiella burnetti* by cloning the PCR products of specific genes into TA-cloning vectors.[27] They applied the nested-multiplex PCR assay on their plasmid constructs.[28] In this work, we suggested a new designed artificial construct for fast and reliable detection of SARS-CoV-2 in diagnostic laboratories. This construct can be used as a positive control for the triplex PCR assay. As mentioned, this approach is safe for testing the designed primers to develop a triplex PCR method for accurate detection of this virus. However, the virus is highly infectious and biosafety level 3 laboratory condition is necessary for RNA extraction. This construct lets us optimize the reaction in general molecular laboratories. The triplex assay evaluated in this investigation, could be used in order to positive control assay at all of the clinical laboratories.

Conclusion

The results of this work showed that the designed synthetic construct is applicable for simple detection of SARS-CoV-2. Also, it is possible to apply a triplex PCR for identifying this virus using this new construct. In conclusion, false-positive results of the common RT-PCR detection of this virus can be assessed using this novel construct and it can be developed for this emerged virus in all clinical laboratories without real time PCR instrument.

Acknowledgements

The authors would like to thank the research council of Malek-Ashtar University of Technology (MUT) for the financial support of this investigation.

Conflict of interests

The authors declare no conflict of interest.

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