〈Regular Article〉

Real-time RT-PCR detection method for SARS-CoV-2

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ABSTRACT　Emerging in December, 2019, SARS-CoV-2 is spreading worldwide, endangering the citizens of the globe. The authors sought to develop a feasible real-time RT-PCR method to detect RNA of the virus. The fluorescent probe the authors designed proved its potency and utility, by detecting *in vitro* transcribed RNA sequence at an estimated concentration approximating a single copy per reaction, used along with 3 sets of primers that flank the probe sequence. Though not tested against actual viral RNA, or clinical specimens, this method can serve as another choice of rapid detection of the plague-causing virus.

doi：10.11482/KMJ-E202046097　*(Accepted on August 25, 2020)*

Key words：COVID-19，SARS-CoV-2，Real-time RT-PCR，Rapid diagnosis

INTRODUCTION

Since its emergence in December, 2019[1)], infections including fatal pneumonia coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is endangering the citizens of the globe[2)]. Japan, a neighboring country of the People's Republic of China, where the plague made its first appearance, is currently under threat of the plague as well. A number of detection methods for SARS-CoV-2, mostly based on nucleic acid amplification, are reported in the literature[3－5)]. Still, their results are sometimes misinterpreted.

The authors sought to develop another method to detect SARS-CoV-2, using real-time RT-PCR, to serve as a means of further confirmation of ambiguous results.

STUDY DESIGN

Primers/Probe for Real-Time RT-PCR

All sequences of oligonucleotides employed in this study are presented in the Table 1.

Four reverse primers (RevB-E) to be paired with the forward primer (Fwd1) sequence adopted from previous literature[3)] as well as a candidate probe sequence (Probe) were designed. Two more primer sets of shorter amplicons, employing the same probe were also designed (Fwd2 and RevF, Fwd3 and RevG). The specificity of all primer pairs were confirmed by Primer BLAST search (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi) against NCBI Transcript Reference Sequences. All pairs except Fwd1/RevA were evaluated as specific to the intended template sequence; concerning pair Fwd1/RevA, potential target sequence from

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Table 1. Oligonucleotides employed in this study

Name	Sequence (5'→3')	Corresponding positions*
Fwd1†	TTGGCAAAATTCAAGACTCACTTT	24354-24377
RevA†	TGTGGTTCATAAAAATTCCTTTGTG	24900-24876
RevB	ACCAGTGTGTGCCATTTGAA	24869-24850
RevC	AACCAGTGTGTGCCATTTGA	24870-24851
RevD	ACAAACCAGTGTGTGCCATT	24873-24854
RevE	GTTACAAACCAGTGTGTGCCA	24876-24856
Fwd2	GCTTCTGCTAATCTTGCTGCT	24620-24640
RevF	AGTTCTTTTCTTGTGCAGGGAC	24785-24764
Fwd3	AGTTGATTTTTGTGGAAAGGGCT	24679-24701
RevG	ACAAAGACACCTTCACGAGGA	24849-24829
Probe	VIC-CAGTCAGCACCTCATGGT-MGB	24722-24739
ivtFwd	GATAATACGACTCACTATA*G*GGTACAATCACTTCTGGTTGGA	24199-24221
ivtRev	ATCTGGTGATGTATGATTCTTAAAATATTTATCTAACTCCT	25051-25011

underline indicates T7 RNA polymerase promoter sequence; *Italic* indicates first nucleotide of *in vitro* transcribed RNA sequence

* according to Reference sequence MN908947.3

† adopted from Reference[3]

an arthropod was suggested. Primers were ordered from Eurofin Genomics K.K. (Tokyo, Japan), and the common fluorescent probe was ordered from Life Technologies Japan Ltd. (Tokyo, Japan).

POSITIVE CONTROLS

Having no access to positive clinical specimens nor cultured virus, the authors used artificial gene synthesis to obtain an alternative. A plasmid harboring a small portion of the virus genome (nts 24199-25054 according to MN908947.3) was ordered from Eurofin Genomics. A trace amount of this plasmid served as a template for PCR amplification using a forward primer with a T7 RNA polymerase promoter sequence (ivtFwd) and a reverse primer (ivtRev); PrimeScript HS premix (TaKaRa Bio, Inc., Shiga, Japan) was used for this amplification. The PCR product was purified by gel extraction using NucleoSpin Gel and PCR Clean-up (Machery-Nagel GmbH, Düren, Germany), reconstituted in manufacturer-provided Elution buffer (5mM Tris/HCl, pH 8.5). The DNA concentration of this eluate was estimated by NanoDrop One (Thermo Fisher Scientific, Inc., Madison, WI, USA). A portion of this eluate served as the template for *in vitro* transcription. Using MEGA script T7 kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instruction, positive control RNA was synthesized in a 50 μL reaction. The reaction was further processed using MEGA clear kit (Thermo Fisher Scientific, Inc.) according to the manufacturer-supplied instructions, to remove contaminants from the transcribed RNA. A small aliquot of the cleared transcribed RNA was electrophoresed on Agilent 4200 TapeStation (Agilent Technologies, Inc., Santa Clara, CA, USA), which showed a single band approximating the estimated size (856nts). The concentration of the cleared transcribed RNA was determined by NanoDrop One (Thermo Fisher Scientific, Inc.), which was converted into molecules (RNA copies) per volume (1,705.4 ng/μL $\approx 3.54 \times 10^{12}$ copies/μL); the RNA solution was first diluted to 1×10^{12} copies/μL using THE RNA storage solution (Thermo Fisher Scientific, Inc.), from which 10-fold serial dilutions down to 1×10^{0} copies/μL were prepared using 0.5 μg/μL Poly(A) solution as diluent.

REAL-TIME RT-PCR REACTIONS

Real-Time RT-PCR reaction tests were carried out on CFX96 Detection System (Bio-Rad Laboratories,

Hercules, CA, USA). The tests were prepared using One Step PrimeScript III RT-qPCR Mix (TaKaRa Bio, Inc.), in a final reaction volume of 20 μL; the final concentrations of forward primer, reverse primer, and fluorescent probe were 0.2 μM each; 4 μL of template solution was applied to each reaction. Thermal conditions were: 52℃ 5min; 95℃ 10s; 60 cycles of 95℃ 5s and 60℃ 30s. These concentrations and temperatures are the default values given in the manufacturer-provided protocol. To assess the chances of unintended rise in fluorescence, amplification cycles were increased to 60 cycles.

For selecting candidate primers, serial dilutions of positive control RNAs of $1\times10^{3,2,1,0}$ copies/ μL were used; for detection limit confirmation, serial 2-fold dilutions of 8,4,2,1×10^{0} copies per reaction were prepared and subjected to detection. In this case the last two concentrations were carried out in triplicates and negative control reactions in duplicates.

RESULTS

Among the 7 primer pairs tested, all but the pair Fwd3/RevG proved their potency in detecting RNA of 4×10^{0} copies per 20 μL reaction (Fig. 1A, 1B). Furthermore, no rise in fluorescence was detected in negative control reactions by 60 amplification cycles. Three primer pairs Fwd1/RevA (product 547bp), Fwd1/RevC (product 517bp), Fwd2/RevF (product 166bp) were further tested for detection limits, all of which succeeded in discovering at least one reaction among triplicates of 1×10^{0} copy per reaction within 41 amplification cycles (Fig. 1C~1H).

DISCUSSIONS

We have succeeded in developing another real-time RT-PCR method to detect SARS-CoV-2 RNA sequence. Though a number of similar methods are reported in the literature[3-5], our method proved its potency in detecting one copy per reaction among triplicates. Since the actual number of template molecules under concentrations approximating single molecules per reaction is known to follow a Poisson distribution, 13.5% of reactions of an estimated two copies per reaction, 36.8% of one copy per reaction may have no templates. Thus, negative reactions among the triplicates are not to be considered as differences in the primer sets' potentials but rather as a natural behavior of single molecule reactions. Furthermore, no unexpected reaction was observed even after 60 amplification cycles, suggesting a low possibility of false positive reactions.

The reagent being a premix of buffer, dNTP, DNA polymerase and reverse transcriptase, remaining unfrozen at -20℃, the reaction could be set up rapidly and feasibly, with less complication. This feature might reduce laboratory workers' burdens, as well as chances of human error throughout the detection procedure.

The fluorescent probe and reaction condition is shared among the primer pairs. Thus, in the case of suspected mutations of the viral sequence at annealing position of a certain primer pair, other primer pairs can readily serve a complementary role to each other. Shared fluorescent probe reduces the monetary cost and time for preparing another backup assay; shared reaction condition abolishes the risk of confusion among multiple thermal cycling programs.

One of the successful primer pairs (Fwd1/RevA) was adopted from the literature[3], which is currently used as the outer primer set of a nested RT-PCR detection procedure employing gel electrophoresis listed in the domestic SARS-CoV-2 detection manual[4]. Employing the aforementioned fluorescent probe might help abolish the second PCR and electrophoresis procedures in laboratories formerly employing the nested PCR procedure, without wasting the outer primers.

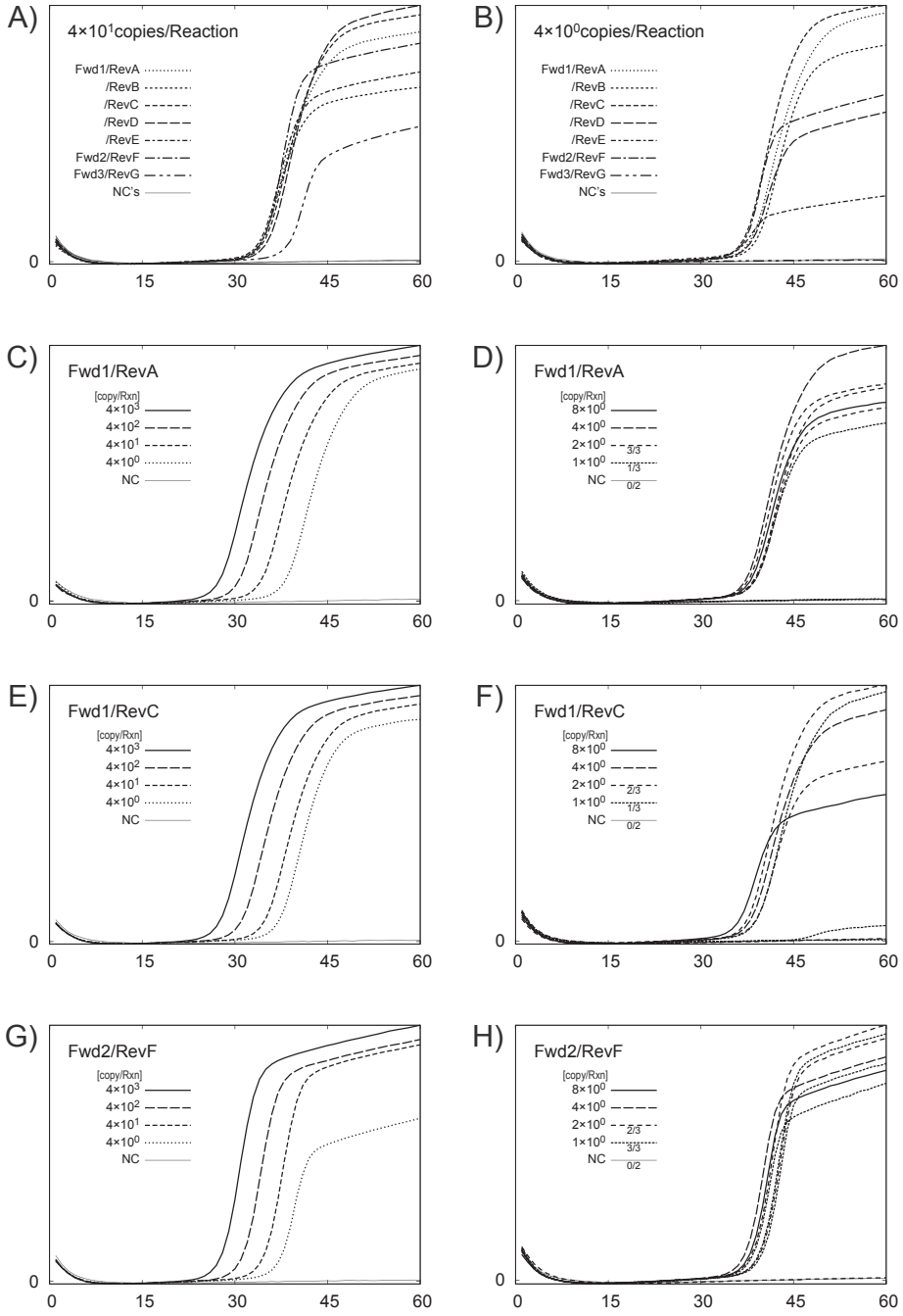

Fig. 1. Amplification plot of each primer set.
X-axis, amplification cycles; Y-axis, relative fluorescent units; NC, negative control.
Panels A), B) 7sets against 4×10^1, 4×10^0 copies per reaction respectively; C), D), primer Fwd1/RevA; E), F) primer Fwd1/RevC; G), H) primer Fwd2/RevF. Panels C), E), G) against controls 4×10^3 to 4×10^0 copies per reaction; D), F), H) against controls 8×10^0 to 1×10^0 copies per reaction. The numbers *n/m* in the legends of panels D), F), H) denotes the number of positive reactions among multiplicates.
Panels A), B), C), E), G) are plots from a single run; D), F), H) from another single run.

The authors are aware of the following limitations. The assay was tested against artificial RNA controls, since experiments using actual viral RNA controls could not be carried out, due to inaccessibility. Although a few clinical specimens from both our institute and the Kawasaki Medical School General Medical Center were provisionally analyzed by the assay, no specimens proved positive, which was completely concordant with the authorized analysis results from the Prefectural Institute for Environmental Science and Public Health. Furthermore, institutional ethical policies hindered the authors from immediately carrying out specific studies employing nucleic acid extracts derived from clinical specimens known to be positive for other causative agents of respiratory infections. Evaluations utilizing such clinical specimens are warranted.

The reaction conditions might not be optimal, though the default reaction conditions from the manufacturer-provided protocol proved practical.

The concentration of our artificial control RNA, determined by optical density, might be misunderstood to be prone to overestimation among colleagues used to fluorescence-based quantitation. Overestimation tends to occur when RNA concentrations are faint, and/or with contaminants, which is unlikely in our case which quantitated concentrated RNA of 1.7 μg/ μL, cleared from contaminants using silica-based columns. Furthermore, overestimated control RNA concentration will underestimate the detection limit, the prudent side for detecting trace amounts of target molecules.

The emerging disease caused by SARS-CoV-2 is still spreading over the globe, across boundaries. Demands for clinically available tests are rising. Our method, bearing capability of detecting as trace as a single copy target per reaction, and robustness/ flexibility against target sequence mutations, might serve as another means of meeting such demands.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. Reiji Kimura for technical assistance.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare concerning the content of the manuscript.

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