

Detection of Specific RNA Targets by Multimerization

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Abstract—Detection of specific RNA targets via amplification-mediated techniques is widely used in fundamental studies and medicine due to essential role of RNA in transfer of genetic information and development of diseases. Here, we report on an approach for detection of RNA targets based on the particular type of isothermal amplification, namely, reaction of nucleic acid multimerization. The proposed technique requires only a single DNA polymerase possessing reverse transcriptase, DNA-dependent DNA polymerase, and strand-displacement activities. Reaction conditions that lead to efficient detection of the target RNAs through multimerization mechanism were determined. The approach was verified by using genetic material of the SARS-CoV-2 coronavirus as a model viral RNA. Reaction of multimerization allowed to differentiate the SARS-CoV-2 RNA-positive samples from the SARS-CoV-2 negative samples with high reliability. The proposed technique allows detection of RNA even in the samples, which were subjected to multiple freezing-thawing cycles.

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INTRODUCTION

RNA analysis is commonly conducted in the studies of gene expression and non-coding RNA [1], as well as for detection of viral pathogens [2]. In 2020, the RNA-containing virus SARS-CoV-2 caused the COVID-19 pandemic, which highlighted the need for monitoring epidemiological situation for the particularly dangerous pathogens [3]. At the beginning of the COVID-19 pandemic, the problem of “missed” and asymptomatic patients emphasized drawbacks of amplification techniques and revealed the demand to develop fast, sensitive, and inexpensive methods for detection of infectious agents [3, 4]. Although, polymerase chain reaction (PCR) remains the “gold standard” and the most used method in molecular diagnostics of infectious diseases, the need for reliable detection of SARS-CoV-2 led to the development of new approaches based on microfluidics, biosensor technologies, genome editing tools (CRISPR/Cas), etc. [5-7].

Abbreviations: MM, multimerization; NA, nucleic acids; PCR, polymerase chain reaction; RT, reverse transcription; T_t, threshold time.

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Considerable progress has been achieved in isothermal techniques as well [8-10], including approaches with no reverse transcription step (e.g., hybridization chain reaction, DNazyme digestion, RNA-supported ligation followed by rolling circle amplification, etc.) [11-13]. In general, isothermal methods are an excellent alternative to PCR that require no expensive thermal cycling equipment and are applicable for the analysis of various biomolecular targets [13, 14]. One of the promising types of isothermal amplification is multimerization reaction (MM) [15]. It was demonstrated that MM can be used to determine RNA targets with high accuracy [16]. The mechanism [17, 18] and the factors that influence course of this reaction [19-22] were studied in detail. It was reported [17] that MM starts after formation of the pseudo-cyclic DNA structures due to the partial denaturation of amplicons termini (so-called “DNA breathing”) followed by bending of the free 3'-ends and their annealing at the opposite part of the DNA duplex. We have shown that this phenomenon occurs due to ionic interactions between the phosphate backbone of the synthesized DNA strands and the surface amino groups of polymerase (lysine and arginine) [18]. MM efficiently proceeds only under certain reaction conditions [19]

and results in formation of the products, which represent tandem nucleotide repeats that appear as a ladder on electrophoretic gels.

Like for a PCR assay, detection of specific RNA via isothermal techniques requires the use of the RNA-dependent DNA polymerases (reverse transcriptases) for cDNA (DNA copy) synthesis. Besides, due to the low stability of RNA molecules, analysis of the RNA-containing samples require compliance with certain laboratory requirements. In the case of SARS-CoV-2, non-compliance often led to unreliable results [23-25]. Detection of RNA through isothermal amplification using Bst DNA polymerase could simplify the assay and provide increase of the assay reliability. Reverse transcriptase activity of the Bst DNA polymerase [26] makes it suitable for direct detection of RNA.

This study aimed to demonstrate applicability of multimerization reaction, representing a new type of isothermal amplification, for the direct detection of specific RNA, using SARS-CoV-2 viral RNA as a model target.

MATERIALS AND METHODS

Reagents. The following reagents were used: Bst 2.0 DNA polymerase and Isothermal buffer (New England Biolabs, UK), dNTP (Biolabmix, Russia), dsGreen intercalating dye (Lumiprobe, Russia), dithiothreitol (DTT), ammonium persulfate, acrylamide, N,N'-methylenebisacrylamide, Tris, disodium N,N,N',N'-ethylenediaminetetraacetate, and N,N,N',N'-tetramethylethylenediamine (Sigma, USA). All solutions were prepared with highly purified water (>18 MΩ, Millipore, USA).

Nucleic acids (NA). Genetic material of SARS-CoV-2 coronavirus was obtained from nasopharyngeal swabs of the COVID-19 patients ($n = 60$), using M-Sorb-OOM-96 extraction kit (Syntol, Russia). COVID-19 diagnosis was confirmed by RT-PCR assay using a RT-PCR-SARS-CoV-2 detection kit (Syntol). Results of PCR of nasopharyngeal swab extracts from the patients with SARS-CoV-2-uncertain diagnosis ($n = 50$) or with negative diagnosis ($n = 50$), and from the healthy individuals ($n = 25$) were used as well. Four types of Rmix samples were prepared by mixing 20 corresponding RNA extracts (30 ul each): Rmix(+) (SARS-CoV-2-positives), Rmix(?) (SARS-CoV-2-uncertain), Rmix(-) (SARS-CoV-2-negatives), and Rmix(H) (from healthy individuals) samples, respectively. Rmix(+) was divided into five aliquots further subjected to 2, 5, 10, or 20 freeze-thaw cycles at -20°C , resulting in the samples designated as Rmix0 (no freezing), Rmix2, Rmix5, Rmix10, and Rmix20 samples, respectively.

Oligonucleotides. Oligonucleotide primers and artificial RNA target Qt (table) were designed using OligoAnalyzer (Integrated DNA Technologies, USA) and purchased from Syntol. Nucleotide sequences of S, N, and ORF1a genes of the SARS-CoV-2 coronavirus were chosen as amplification targets.

Multimerization reaction. All samples for amplification were prepared in a UVC/T-M-AR PCR box (Biosan, Lithuania). Working space, dispensers, and plasticware were preliminarily irradiated with UV light for 20 min, amplification was carried out in an iQ5 thermal cycler (Bio-Rad Laboratories, USA). Reaction mixtures (20 ul) contained 5 pmol of each primer, 0.25 mM dNTP, $1\times$ Isothermal buffer, $0.2\times$ dsGreen intercalating dye,

Oligonucleotides

Gene	Name	Sequence, 5'→3'	Size, nt
<i>S</i>	F-S1	GTTATCAGACTCAGACTAATTCTCCTC	27
	R-S1	TTGACTAGCTACACTACGTGCCC	23
	F-S2	GTCACAGACTCAGACTAATTCTCCTC*	26
	R-S2	TGACTGACTAGCTACACTACGTGCCC	26
	Qt	GTTaucagacucagacuaauucuccgcccggggcacguaguguagcuaguCAA**	53
<i>N</i>	F-N	TGACGCTTCAGCGTTCTTCGGAATGTC*	27
	R-N	GTCAAGGTGTGACTTCCATGCCAATG	23
<i>ORF1a</i>	F-O	TGACAAAAGTATTCTACACTCCAGGGAC	28
	R-O	GTCAAAATGACTCTTACCAGTACCAGGTG	27

* 5'-terminal nucleotides that do not have homology with the nucleotide sequence of SARS-CoV-2 are in bold.

** Capital letters – deoxyribonucleotides, lowercase letters – ribonucleotides.

10 mM DTT, 3 a.u. of Bst 2.0 DNA polymerase, and 1 μ l of nasopharyngeal swab extract, or Rmix sample, or non-template control. Amplification program included the following steps: (i) 70°C, 30 s; (ii) 65°C – 60 s; (iii) 60°C – 3 h. In some cases, amplification products were analyzed by electrophoresis in 10% polyacrylamide gels followed by staining with ethidium bromide and visualization in a GelDoc XR System (Bio-Rad Laboratories).

RESULTS AND DISCUSSION

Amplification of any RNA starts from the synthesis of its cDNA during a reverse transcription reaction, which is usually carried out with a separate enzyme (reverse transcriptase) at a relatively low temperature before main amplification. However, it would be convenient to amplify RNA without a reverse transcription step, i.e., by using a single enzyme that would have both reverse transcriptase and regular DNA polymerase activity. Recently, it has been shown that Hemo KlenTaq, which is posed as a DNA-dependent DNA polymerase, possesses reverse transcriptase activity and facilitates successful ampli-

cation of RNA targets [27]. As for isothermal amplification, a large fragment of Bst DNA polymerase (Bst exo⁻) could be of great interest due to the strong strand-displacement activity, moderate thermal stability, and high processivity. Reverse transcriptase activity (manifested at temperatures up to 72°C) has been reported for the Bst 3.0 DNA polymerase (<https://neb.com/products/>), but it is likely that all commercially available forms of Bst exo⁻ possess such activity [26].

Unequivocal course of the multimerization reaction allows its application for the detection of NA-targets [16]. If a pair of specific primers could be designed complementary to the sites on a single-stranded nucleic acid (NA) (such as RNA) sequence in the target, which are located close to each other, one could expect successful amplification through multimerization mechanism as shown in Fig. 1.

In the first step of the reaction, one of the primers (denoted as R in Fig. 1) is annealed to the RNA strand and extended by DNA polymerase with reverse transcriptase activity, resulting in formation of a double-stranded DNA/RNA heteroduplex. Next, due to the strands “breathing”, it becomes possible to anneal the second

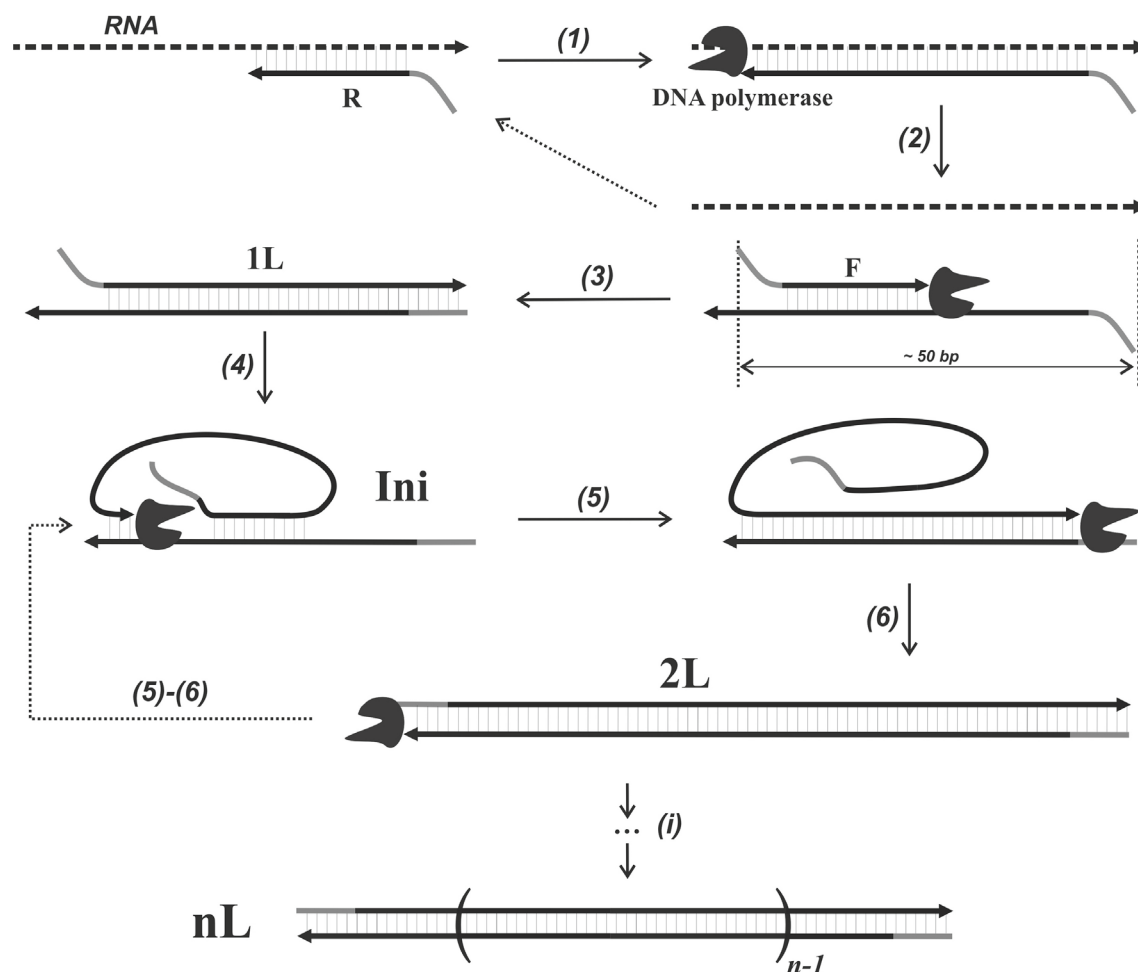


Fig. 1. Scheme of RNA detection using multimerization reaction [F and R, primers; Ini, initiation complex; L, the length of monomeric dsDNA (double-stranded DNA) unit; (1)–(i), stages (steps) of the reaction].

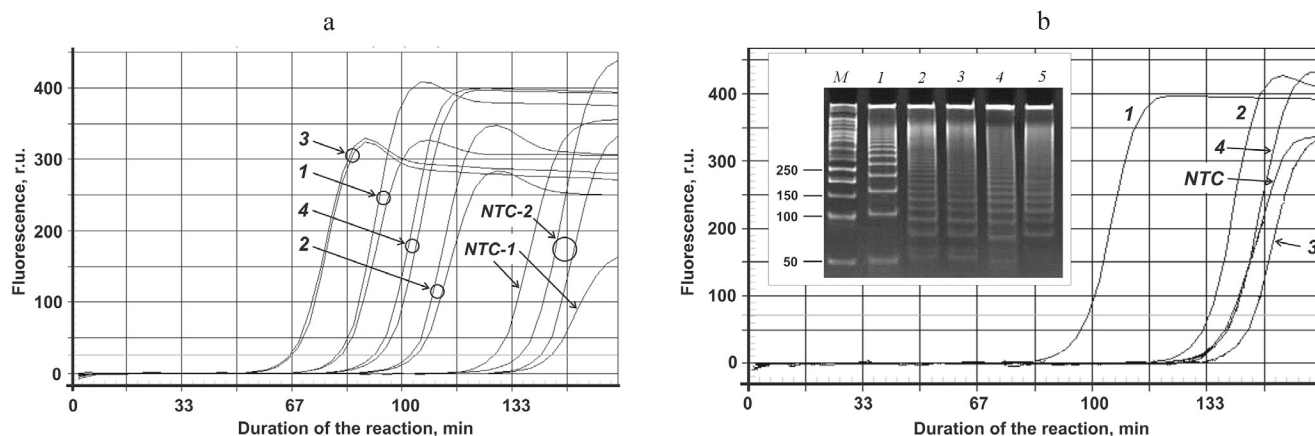


Fig. 2. Time course of multimerization reaction depending on the primer structures and type of RNA. a) Effect of primer structures and type of RNA target on the rate of multimerization: 1) F-S1/R-S1 primer pair and artificial (Qt) RNA, 2) F-S1/R-S1 and Rmix(+), 3) F-S2/R-S2 primer pair and Qt, 4) F-S2/R-S2 and Rmix(+); NTC-1, non-template control for F-S1/R-S1; NTC-2, non-template control for F-S2/R-S2. b) Amplification curves and electrophoretic analysis (inset) for: 1) Rmix(+), 2) Rmix(?), 3) Rmix(-), and 4) Rmix(H) samples; NTC, non-template control (data for F-S2/R-S2 primer pair and only one of the two repeats are given).

primer (denoted as F) to the synthesized cDNA strand, which provides an amplicon containing dsDNA (double-stranded DNA) fragment with the size 1L (steps 2 and 3). Primers F and R contain 5'-tails which are not homologous with the RNA target but are complementary to each other. These dangling 5'-ends increase DNA "breathing", facilitate initiation complex (Ini) formation (step 4), and ensure further accumulation of multimeric products (steps 5-*i*). Generation of duplexes with the length of $>2L$ shift the reaction to an exponential mode due to increase in the number of annealing sites for both primers. It should be noted that RNA molecules are best suited for MM because they are single stranded and promote easy annealing of the first (R) primer.

Multimerization starts from the Ini formation, that is likely a random and very rare event occurring with low probability under certain conditions. It seems that for the MM to begin, presence of any double-stranded NA and primers for its amplification is sufficient. We have previously shown that accumulation of the detectable amount of MM products begins within 25-70 min from the start of the reaction, depending on concentration of the template and reaction conditions (i.e., "specific" MM) [19]. In the absence of a template, or primer dimers (i.e., for high-quality primers) the reaction takes more time (>100 min) and, hence, can be called "nonspecific" MM. It was also demonstrated that the highest efficiency of MM is observed for the DNA polymerase Bst 2.0 and DNA templates of about 50 nt long at the reduced concentration of SYBR Green I intercalating dye [19]. It should be also noted that based on our experience addition of 10 mM DTT noticeably increases efficiency of MM (data not shown).

Taking into account the mechanism of MM [17, 18], another way to increase MM efficiency is design of the primers. It has been shown, that the use of primers with

complementary 5'-termini improves MM [16]. In this study, two primer pairs were designed for the model amplification experiments: the conventional primer pair F-S1/R-S1, and the primers with four-nucleotide complementary 5'-ends (F-S2/R-S2). Effect of the primers structures on MM was assessed using the artificial RNA template Qt, which contains three deoxyribonucleotides at the 5'- and 3'-ends, providing higher resistance of this RNA to ribonuclease digestion. Use of the F-S1/R-S1 primers resulted in lower MM efficiency [threshold time (T_t) ~ 70 min] compared to the F-S2/R-S2 primers ($T_t \sim 50$ min) (Fig. 2a, curves 1 and 3, respectively).

It turned out that MM proceeds at higher rate with the artificial RNA (Fig. 2a, curves 1 and 2, or 3 and 4). This is probably due to the finite length of the Qt oligoribonucleotide, which ensures rapid formation of Ini and promotes an earlier start of MM. It should be noted that, compared to PCR, multimerization curves demonstrate lower convergence of sample replicates (Fig. 2a, curves 1, 2, and 4). For MM, dispersion of T_t values slightly increases with the decrease in the number of target copies [16]. This relative deviation from nonlinearity is explained by the feature of MM reaction. As was mentioned above, start of the MM reaction is a random event, and as the number of target copies decreases, more time is required for the reaction to start resulting in a larger difference in the start of the exponential stage of the reaction between the replicates.

Thus, based on the obtained and previously reported data, the following conditions to increase efficiency of the "specific" MM, can be recommended: (i) use of two primers with relatively short 5'-tails ($\sim 4-5$ nt), which are complementary to each other, (ii) primers must facilitate formation of the primary monomeric dsDNA unit with the length of $\sim 50-55$ bp, (iii) use of Bst 2.0 DNA polymerase and Isothermal buffer, addition of up to 10 mM

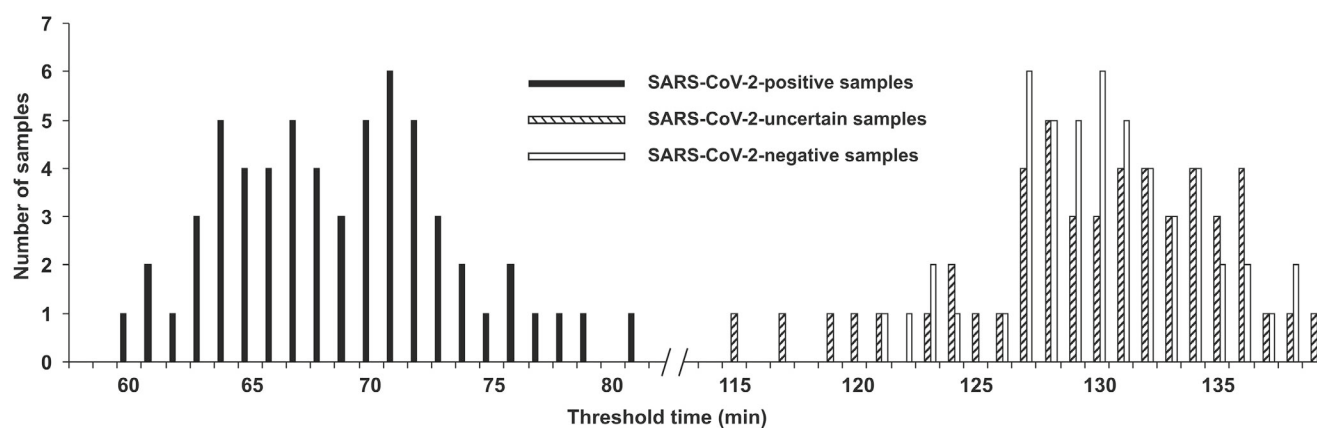


Fig. 3. Distribution of Tt values for the SARS-CoV-2-positive samples ($n = 60$), SARS-CoV-2-uncertain samples ($n = 50$), and SARS-CoV-2-negative samples ($n = 50$) (data for F-S2/R-S2 primer pair are given).

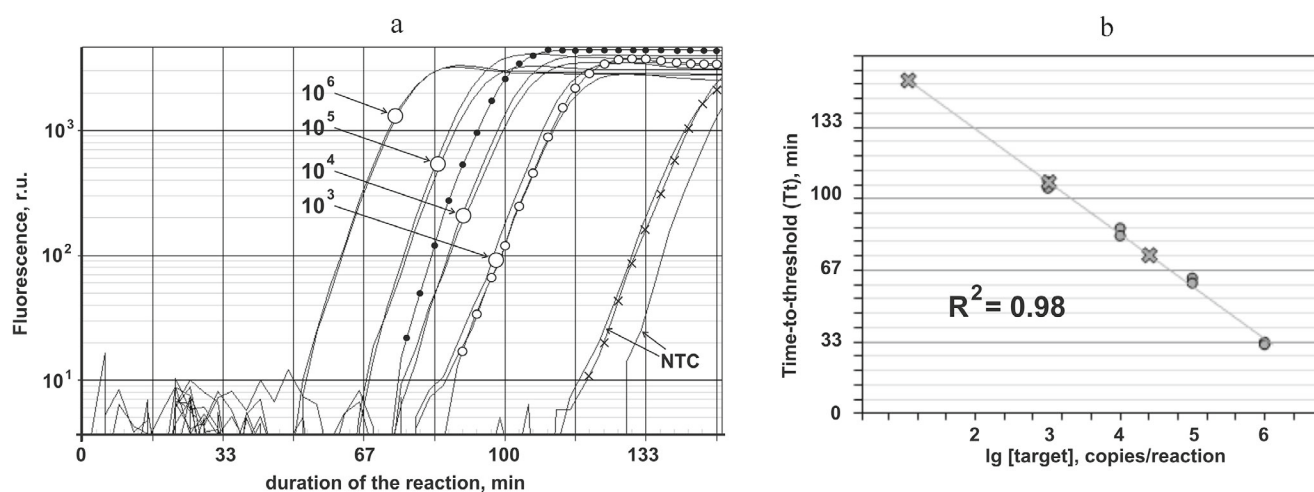


Fig. 4. Analytical performance of the MM-based detection of viral RNA (data for F-S2/R-S2 primer pair are given). a) Example of quantitative analysis of SARS-CoV-2 coronavirus RNA (primer pair F1/R1, logarithmic scale): amplification curves of Rmix(+) (dark circles), Rmix(?) (white circles), Rmix(-) (crosses) samples and calibration samples (solid lines) containing 10^3 - 10^6 copies of Qt target. b) Calibration plot (calibration samples contained 10^3 , 10^4 , 10^5 , and 10^6 target copies/ul).

DTT and of the reduced amount of intercalating dye, reaction temperature $\sim 60^\circ\text{C}$.

Applicability of MM for the detection of specific RNA was studied using genetic material of the SARS-CoV-2 coronavirus. The SARS-CoV-2-positive, SARS-CoV-2-negative sample, and samples with inconclusive PCR results (SARS-CoV-2-uncertain) as well as samples from the healthy individuals were used. Three primer pairs were designed for amplification of three different genes of this pathogen to assess the influence of primary structure of the targets on MM efficiency. All these specific primer pairs contained complementary 5'-motifs. Experiments showed no effect of the target structure on the course of MM, therefore, only the results for the F-S2/R-S2 pair of primers are given below. Using optimized preparations, amplification was found to occur in all samples, both for the SARS-CoV-2-positive samples [Rmix(+)] and the controls [Rmix(?), Rmix(-), and

Rmix(H)]. However, for the Rmix(+) samples, Tt values were in the range of 60-90 min from the start of the reaction, while for the controls they exceeded 110-120 min (Fig. 2b). For the Rmix(+) samples, electrophoresis revealed the presence of characteristic bands on the gels corresponding to multimeric DNA products with the sizes multiple to the expected one (~ 55 bp). As for the rest of the samples, multimers were formed as well, but the size of the products did not match the size of the primary amplicon indicating that nonspecific MM occurred in this case (Fig. 2b).

Analysis of the amplification data obtained for individual specimens (total $n = 160$) showed feasibility to discriminate specific and non-specific MM. Tt values for the SARS-CoV-2-positive samples were within the range of 60-80 min; at the same time, Tt values > 115 min were determined for other test groups indicating absence of the target (Fig. 3).

It turned out that the obtained T_t values correlate well with the cycle threshold values (C_t) obtained for these specimens in PCR-amplification (data not shown). The range of T_t values for the SARS-CoV-2-positive samples and the samples without target RNA did not overlap, which allowed to discriminate these types of samples and to detect pathogenic RNA with high reliability. Thus, the value of $T_t \approx 110$ min could be considered as a threshold of analytical significance for the used reaction conditions.

To determine whether the SARS-CoV-2 RNA could be quantified using multimerization reaction, Q_t was used as a calibration template. Reference samples, containing 10^3 - 10^6 copies of Q_t , were obtained by serial dilutions of the stock solution. It should be noted that the copy number of RNA target in the test samples was unknown. However, it was previously reported that the nasopharyngeal swab extracts from the COVID-19-positive individuals contain on average about 10^4 - 10^5 copies of the target per 1 μ l of solution, depending on the type of biomaterial, viral load, RNA isolation technique, and the method of samples handling [28-31]. MM experiments allowed to quantify coronavirus RNA in the samples from the SARS-CoV-2-positive patients. For the most samples, RNA copy number was within the 10^4 - 10^5 range (Fig. 4a).

In general, the obtained results correlated well with the data reported earlier for the quantitative RT-PCR assays performed without specific fluorogenic probes [3, 4, 32-34], and indicated detection limit at pM level. However, for the samples with lower viral load, detection of the specific targets via multimerization could be considered as a semi-quantitative assay due to the lower convergence of the T_t values of the replicates (Fig. 4b).

The proposed method has a number of advantages due to the use of primers complimentary to the sequences located in close proximity, which enable amplification of short NA sequences like degraded DNA [35] or miRNA [16] with high specificity, sensitivity, and reliability. For the RNA targets, degradation is a more serious problem due to its lower stability compared to DNA, and use of the primers targeting closely located sequences is justified for analysis of the relatively short RNA fragments suitable for amplification. Moreover, if DNA polymerase possesses weak reverse transcriptase activity, amplification of the shorter sequences is preferable. To evaluate applicability of MM for the analysis of degraded RNA, model samples of coronavirus RNA were prepared by subjecting the Rmix(+) sample to freeze-thaw cycles facilitating RNA fragmentation. The experiments with RNA samples showed that multiple freeze-thaw cycles, in general, caused a slight decrease in the number of amplifiable RNA targets, which resulted in the increase of T_t values (Fig. 5).

Surprisingly, a slight decrease in the T_t value was observed for the Rmix2 sample that undergone freezing for 2 times, which was probably due to increase in the

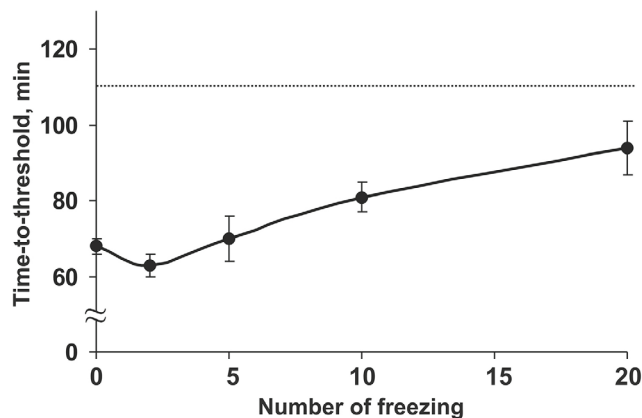


Fig. 5. Effect of multiple freezing-thawing of Rmix(+) solution on the rate of multimerization reaction. Dotted line at $T_t = 110$ min denotes threshold of analytical significance (data for F-S2/R-S2 primer pair are given).

MM efficiency caused by generation of the truncated molecules during freezing-thawing. Increase in the number of freeze-thaw cycles led to a gradual increase in T_t , but even for the Rmix20 sample, T_t did not exceed 110 min, which indicated the possibility to detect specific RNA with high reliability. Thus, proximity of the primers allows to detect degraded RNA, which could allow using not so strict requirements for transportation and storage of the RNA-containing materials.

CONCLUSION

The obtained results demonstrate that multimerization of nucleic acids can be used for detection of viral RNA. For this, efficiency and specificity of multimerization must be improved through special primer design involving insertion of 5'-terminal complementary motifs, and use of certain reaction components, namely, Bst 2.0 DNA polymerase, dithiothreitol, and reduced amount of intercalating dye. Multimerization-based amplification proceeds under isothermal conditions and excludes addition of common reverse transcriptase into the reaction mixture; only a single enzyme with reverse transcriptase and strand-displacement activities is needed. MM allows to differentiate NA-positive samples from NA-negative or non-template controls, providing detection of the targets with high reliability. The proposed approach is based on the use of primers annealed close to each other on the target, which ensures efficient amplification and detection of RNA target even in the samples, which underwent multiple freezing. For the samples with low copy number of the targets, detection via multimerization could be considered as a semi-quantitative assay due to the lower convergence of the T_t values of the replicates.

Contributions. Assol R. Sakhabutdinova – carried out experiments, wrote the manuscript; Alexey V. Chemeris –

discussed the results of experiments, edited the manuscript; Ravil R. Garafutdinov – conceived and supervised the study, edited the manuscript.

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Ethics declarations. The authors declare no conflicts of interest in financial or any other sphere. This article does not contain any studies involving human participants or animals performed by any of the authors. Depersonalized samples containing isolated SARS-CoV-2 RNA material, rather than the coronavirus, were used.

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