Original Article

A Comprehensive Comparison of Rapid RNA Extraction Methods for Detection of SARS-CoV-2 as the Infectious Agent of the Upper Respiratory Tract using Direct RT-LAMP Assay

Maryam Moazami Goodarzi[1], Razieh Taghizadeh Pirposhteh[2], Hadi Ravan[3], Farnaz Vahidian[4], Omolbani Kheirkhah[5], Reza Fotouhi Ardakani[6], Fatemeh Fotouhi[7]

[1]Department of Research and Development, Production and Research Complex, Pasteur Institute of Iran, 3159915111 Karaj, Iran, [2]Virology Department, Pasteur Institute of Iran, Tehran 1316943551, Iran, [3]Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, 76169-14111 Kerman, Iran, [4]Department of Biology, Science and Arts University, Yazd, Iran, [5]Malaria and Vector Research Group (MVRG), Biotechnology Research Center (BRC), Pasteur Institute of Iran, Tehran, Iran, [6]Cellular and Molecular Research Center, Qom University of Medical Sciences, Qom, 3736175513, Iran, [7]Department of Influenza and other Respiratory Viruses, Pasteur Institute of Iran, Tehran, Iran

Abstract

Background: The current COVID-19 pandemic has highlighted the need for faster and more cost-effective diagnostic methods. The RNA extraction step in current diagnostic methods, such as real-time qPCR, increases the cost and time required for testing. Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) is a promising technique for developing diagnostic tests with desired sensitivity and specificity without the need for RNA extraction.

Materials and Methods: An RT-LAMP assay was developed to detect SARS-CoV-2 with a sensitivity of 0.5 copies of positive control plasmid per microliter in 40 min. Several rapid RNA extraction protocols were evaluated using different reagents, including bovine serum albumin, Triton X-100, Tween 20, proteinase K, guanidine hydrochloride, guanidinium isothiocyanate (GITC), and thermal treatment. Finally, the sensitivity and specificity of the developed direct RT-LAMP were determined using 150 upper respiratory tract samples.

Results: Method 10 was selected as the most efficient protocol for the RNA extraction step. The sensitivity and specificity of the developed direct RT-LAMP assay with clinical samples were estimated at 98.4% and 88.8%, respectively.

Conclusion: These results suggest that the combination of GITC and Triton X-100 detergent is a highly efficient method for RNA extraction and direct RT-LAMP detection of SARS-CoV-2 in clinical samples, providing a valuable tool for the rapid and cost-effective diagnosis of COVID-19.

Keywords: Rapid diagnostic tests, RT-LAMP assay, SARS-CoV-2

Address for correspondence: Dr. Razieh Taghizadeh Pirposhteh, Virology Department, Pasteur Institute of Iran, Tehran 1316943551, Iran.
E-mail: r_taghizadeh@pasteur.ac.ir
Submitted: 19-Feb-2023; **Revised:** 17-Jul-2023; **Accepted:** 19-Jul-2023; **Published:** 29-Nov-2023

Introduction

Since December 2019, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has rapidly spread across the world, resulting in more than 6,952,522 deaths from coronavirus disease (COVID-19) to date.[1]

SARS-CoV-2 as the leading cause of respiratory disease is transmitted through oral and nasal droplets.[2] Accurate and fast screening of infected individuals is critical for handling population mortality and hospital overload during outbreaks.[3,4] However, similar and diverse ranges of clinical symptoms make it difficult to discriminate COVID-19 from other respiratory infections, and it is critical to identify asymptomatic individuals carrying the virus. Moreover,

Access this article online

Quick Response Code:

Website: www.advbiores.net

DOI: 10.4103/abr.abr_63_23

How to cite this article: Moazami Goodarzi M, Taghizadeh Pirposhteh R, Ravan H, Vahidian F, Kheirkhah O, Fotouhi Ardakani R, *et al*. A comprehensive comparison of rapid RNA extraction methods for detection of SARS-CoV-2 as the infectious agent of the upper respiratory tract using direct RT-LAMP assay. Adv Biomed Res 2023;12:261.

running emergency measurements for prevention and therapy requires fast and accurate decision-making using high volumes of diagnostic testing.[5] Nucleic acid amplification tests are the first-line strategies for rapid and accurate detection. Among them, reverse transcriptase quantitative real-time PCR (RT-qPCR) is determined as the main standard test for SARS-CoV-2 diagnosis with acceptable sensitivity and specificity. However, this technique exhibited some limitations during the pandemic, such as the inability to screen a large number of individuals in a short time, requiring trained staff, needing costly equipment and facilities, and being time-consuming.[5] Moreover, the RT-qPCR needs an RNA extraction step, which is costly and time-consuming and may cause shortages in the supply of RNA purification kits. Also, some false-negative reports have been reported in qRT-PCR tests when the RNA concentration was not determined.[2]

Therefore, developing a faster, direct, and more versatile detection method with comparable sensitivity and specificity to RT-qPCR is critical for more efficient epidemiological management of the COVID-19 outbreak. The reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) can be employed as an alternative diagnostic technique. The Bst DNA polymerase, commonly used in LAMP amplification, possesses vigorous DNA strand displacement activity, which removes the need for different temperature cycling as seen in costly conventional PCR platforms. Moreover, owing to high polymerase robustness, this enzyme is highly resistant to inhibitors in clinical samples, and consequently, the RNA extraction step could be simplified, and also the possibility of combining reverse transcription and amplification in one step highlights the potential implications of this test.[6,7] According to these benefits, LAMP assays have been progressively respected for developing point-of-care tests.[8,9] Although the tolerance of Bst DNA polymerase to inhibitors is remarkable, previous research has shown that the use of chemical and physical treatments can significantly improve the efficiency of this test.[3,8,10]

The current study was primarily aimed to evaluate and compare the efficiency of RT-LAMP assay by using several rapid RNA extraction methods for expanding the best approach to detecting SARS-CoV-2 in clinical specimens. For this purpose, suitable primers were designed to detect the different genes of SARS-CoV-2. Ultimately, an RT-LAMP assay was optimized, and the limit of detection (LOD) of the optimized assay was determined using positive control plasmid. Several clinical samples with different CT values were treated based on the designed rapid RNA extraction protocols and compared in terms of RNA extraction and RT-LAMP reaction efficiency. Finally, the most efficient method was selected and the sensitivity and specificity of the developed direct RT-LAMP assay were evaluated using a higher number of clinical samples.

Materials and Methods

Designing LAMP primers and positive control plasmid

The nucleic acid sequences of the N gene (accession no.: NC_045512.2 (28274.29533)), E gene (accession no.: NC_045512.2 (26245.26472)), and RdRp gene (accession no.: NC_045512.2 (266.21555)) were obtained from Gene database of the National Center for Biotechnology Information (NCBI). The primers were designed by Primer Explorer 5 software and evaluated by Primer3, Integrated DNA Technologies (IDT) OligoAnalyzer™ Tool (Integrated DNA Technologies, Coralville, Iowa, USA), and Fast PCR (Primer Digital Ltd, Helsinki, Finland) software to assess the probability of primer interactions. Furthermore, primer sets were analyzed to assess the possibility of cross-reactivity with other pathogens similar or related to SARS using the basic, local alignment search tool for nucleotides (NCBI-primer blast). Nine sets of primers were chosen and ordered for each gene (including N, E, and RdRp) in HPLC purified grade and 0.04 μM concentration (TAG Copenhagen, Denmark). Due to the size and sequence of the RdRp gene, this set of primers had no forward and backward loop primers. A positive control plasmid was designed by choosing approximately 100 nucleotides around forward and reverse primers of the N, E, and RdRp genes, and the sequences were ordered in the pUC57 plasmid (Biomatic, Canada).

Collection of samples

Oropharyngeal and nasopharyngeal samples were collected from 150 cases using Dacron/Polyester swabs and placed in 3 mL of viral transport medium. Samples were stored at 4°C and used within the first 24–48 h after collection. All samples were tested for SARS-CoV-2 detection by RT-qPCR with the Sansure SARS-CoV-2 diagnosis Kit (Sansure Biotech, China) as the standard gold method. The samples were collected from each enrolled patient at the Resalat Nano Medical Laboratory.

Ethical statement

This study was reviewed and approved by the medical University Qom with ethical code IR.MUQ.REC.1399.194.

RT-LAMP reaction optimization

RT-LAMP assay was performed in a total volume of 25 μL consisting of 12.5 μL 2× WarmStart® LAMP Kit (DNA and RNA), 0.5 μL LAMP fluorescent dye, 2.5 μL 10 × primer mix, 9 μL PCR-grade water, and 1 μL sample (E1700L, NEB, Ipswich, USA). PCR grade water was used as a substitute for the sample in the non-template control. All reactions were carried out in a Corbett Rotor-Gene RG-6000 Real-Time PCR Analyzer (Qiagen, Germantown, MD, USA). The efficiency of the RT-LAMP reaction and the presence of undesired nonspecific primer interactions were evaluated using 2% agarose gel electrophoresis. To screen and select the optimum temperature for the RT-LAMP reaction, PCR reactions were incubated at three different temperatures (60°C, 62°C, and 64°C) for 60 min with continuous fluorescence detection. Reactions were considered positive if sample amplification (fluorescence signal above a given threshold)

was detected. The best primer sets for each gene (including N, E, and RdRp) were selected for further evaluation at the optimum temperature. To assess the best primer mix proportion for the RT-LAMP reaction, the primer sets mix was prepared by mixing equal volumes of F3 and B3 (2 μM), LF and LB (4 μM), and three different ratios of FIP and BIP primers (including 8 μM, 12 μM, and 16 μM). At least three reactions were performed to evaluate the primers and RT-LAMP reactions, including a positive control, negative RNA (to check nonspecific interactions), and a reaction without any sample to assess nonspecific interactions of primers.

Limit of detection of RT-LAMP primer sets

The sensitivity of selected primer sets (i.e., E and N primer sets) in optimum RT-LAMP conditions was assessed using several serial dilutions of positive control plasmid with a final concentration of 10,000, 1000, 100, 10, 1, and 0.5 copies/μL to determine the LOD of the optimized RT-LAMP assay. Each dilution was analyzed in duplicate.

Screening among various rapid RNA extraction methods

In this study, we compared the efficiency of 10 different RNA extraction methods for the direct detection of SARS-CoV-2 using RT-LAMP. The methods were designed to isolate high-quality RNA from clinical samples, and each method was evaluated based on its yield and RNA purity. To determine the appropriate concentration of chemicals for RNA extraction protocols, we conducted early screening experiments and information on the subject available in the literature. To perform these experiments, four SARS-CoV-2 samples were treated based on the 10 designed protocols, and the RNA concentrations were assessed using microvolume quantification by Nanodrop (Epoch, Germany), in triplicate.

The first method involved thermal treatment, where the samples were heated at 85°C for 10 min. The second method involved the use of proteinase K (1 mg/mL) at 60°C for 10 min, followed by a heating step at 85°C for an additional 10 min. The third method combined proteinase K (1 mg/mL) and Triton X-100 (2%), followed by the same heating steps as the second method. The fourth method used Triton X-100 (2%) at 85°C for 10 min, while the fifth method combined Triton X-100 (2%) and bovine serum albumin (BSA) at 2 mg/mL, also at 85°C for 10 min. The sixth method used BSA (2 mg/mL) alone at 85°C for 10 min.

The seventh method used Tween 20 at 2% at 85°C for 10 min. The eighth method used guanidinium isothiocyanate (GITC) (4 M) at 85°C for 10 min. The ninth method used guanidine hydrochloride (GuHCl) (4 M) at 85°C for 10 min. The final method combined GITC (4 M) and Triton X-100 (2%) at 85°C for 10 min. Subsequently, the treated samples were diluted or directly used (with noninhibitory concentration) into optimized RT-LAMP reactions to define the efficiency of different methods for direct detection of SARS-CoV-2 from clinical samples in the RT-LAMP assay.

Clinical sensitivity and specificity of developed direct RT-LAMP

The specificity of the selected primer in the RT-LAMP assay was tested using genomic RNA of influenza A and influenza B. Each of the genomic RNA was tested in duplicates.

The clinical sensitivity and specificity of the developed direct RT-LAMP method were evaluated using 150 nasopharyngeal swabs samples according to method number 10 of rapid RNA extraction. The sensitivity was calculated based on the formula: (number of true positives)/(number of true positives + number of false negatives) and the specificity was calculated as (number of true negatives)/(number of true negatives + number of false positives).

Results

Primers sets analysis and RT-LAMP assay optimization

In-silico analysis revealed that all the primer sets were very specific and did not show cross-reactivity with other pathogens that cause upper respiratory tract infections including rhinovirus, influenza A and B, parainfluenza, respiratory syncytial virus, adenovirus, metapneumovirus, and enterovirus, as well as bacteria, including *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Streptococcus pyogenes*, S. *pneumoniae*, *Bordetella pertussis*, and *Haemophilus influenzae*. Three different primer sets were tested for each target gene in the diagnostic assay. Based on the best detection of the target with the minimum amount of sample, reaction speed, and the least nonspecific primer interactions with themselves or other primer sets of a different targeted gene, the best primer set for each targeted gene was selected and their sequences are shown in Table 1.

After selecting of the best primer sets for the detection of SARS-CoV-2 N, E, and RdRp genes, it was concluded that almost all these primer sets have the best RT-LAMP efficiency at temperatures around 62°C. So, this temperature was used for further reaction optimization measurements. The performed RT-LAMP reactions ran in agarose gel electrophoresis and the results revealed different band patterns for each primer's ratios. The best primer ratio with the highest reaction efficiency and lowest primer interactions for N, E, and RdRp-specific primers were selected. The best proportion of primer was different in each set. The chosen primer ratio for the N gene was 1/8/2 ratio (for F (B) 3/F (B) IP/F (B) Loop, respectively). While the ratio 1/4/2 amplified without nonspecific interactions, the chosen ratio had a sharper LAMP pattern. For the E gene, we had nonspecific interaction with negative RNA or primer–primer interactions in a ratio higher than 1/6/2, and it was the best-amplified reaction also for the RdRp gene; we only had the RT-LAMP reaction in a ratio of 1/8/2 [Figure 1].

In real-time PCR, none of the RdRp primer sets amplified the targeted region efficiently and no sharp ladder-like patterns were observed in gel electrophoresis. It may be due to the

Table 1: Three selected primer sets from the first screening experiments

Primer name	Sequence 5' to 3'
Primer set of N gene	
F3	GCCAAAAGGCTTCTACGC
B3	AGTTTGGCCTTGTTGTTGTT
FIP	CCTACTGCTGCCTGGAGTTGAAGTCAAGCCTCTTCTCGTTC
BIP	GCTAGAATGGCTGGCAATGGCCATTTTGCTCTCAAGCTGGT
FLoop	TTTCTTGAACTGTTGCGACTACGTG
Bloop	GCTTTGCTGCTGCTTGACAGAT
Primer set of E gene	
F3	ACTTATGTGTACTCATTCGTTTCGGA
B3	ATCAGGAACTCTAGAAGAATTCAGAT
FIP	CGCAGTAAGGATGGCTAGTGTAACAGAGACAGGTACGTTAATAGTTAATAGC
BIP	TCGATTGTGTGCGTACTGCTGCGAGAGTAAACGTAAAAAGAAGGTT
FLoop	CACGAAAGCAAGAAAAAGAAGTACGCTA
Bloop	TTGTTAACGTGAGTCTTGTAAAACCTTCTT
Primer set of RdRp	
F3	ATGACCAATAGACAGTTTCATCAA
B3	TGCAGTTAACGCAGTTGTAGTG
FIP	TGCCAACCACCATAGAATTTGCAATTATTGAAATCAATAGCCGCC
BIP	CCTCACCTTATGGGTTGGGATT-CCATAATTCTAAGCATGTTAGGCAT

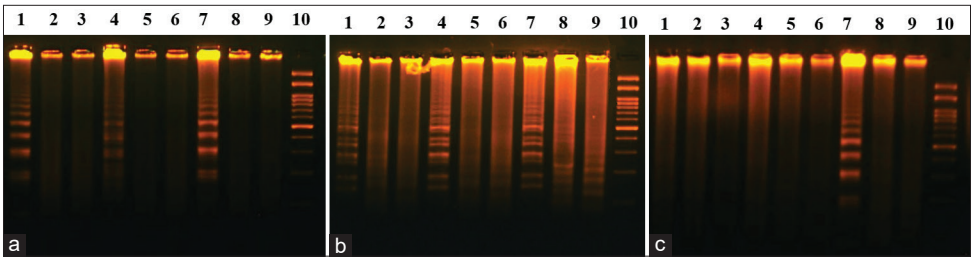


Figure 1: The different primer sets including N, E, and RdRp genes with three different ratios including 1/4/2 (columns 1–3), 1/6/2 (columns 4–6), and 1/8/2 (columns 7–9) are demonstrated in part a, b, and c, respectively. Columns 1,4, and 7 represent RT-LAMP reactions with the positive control, columns 2, 5, and 8 represent reactions with negative RNA, and columns 3, 6, and 9 are RT-LAMP reactions without RNA samples. Column 10 refers to the 1 Kb DNA ladder

absence of loop primers in these sets. Therefore, these primer sets were omitted from further studies.

Limit of detection

Sensitivity of candidate primer sets (E and N) in different concentrations of positive control plasmid showed that the N gene primer set is able to detect 0.5 copies/μL. The amplification plots for all concentrations are shown in Figure 2. The result showed that the E primer set is able to detect at least 10 copies/μL and related amplification plots are demonstrated in Figure 2b. As in the RT-LAMP reaction, the presence of several primer sets increases the possibility of interaction between them, and also evaluations revealed the lower sensitivity for E primer sets with no synergistic effect; this primer is also removed from the test and RT-LAMP reaction was finalized with 1 set of N gene primer.

Screening among various rapid RNA extraction methods

According to other direct-RT-LAMP studies, the most usual method is thermal treatments.[11] Also, several simple chemical treatments were applied to increase the efficiency of rapid RNA extraction from the nasopharyngeal swab. All these different methods were utilized for rapid RNA extraction and the average of released RNA is demonstrated in Table 2. These methods were analyzed with GraphPad Prism software (San Diego, CA, USA). One-way ANOVA and multiple comparisons were used to compare the mean of every method with the mean of every other extraction method. The result showed that all additional chemicals increased the amount of released RNA compared with thermal treatment alone. Methods 2, 6, and 9 were a little more efficient than thermal treatment. Among all methods, numbers 3, 4, 5, 8, and 10 released the highest RNA amount. Significant differences were not observed between methods 3, 4, 5, and 8 but these five methods were significantly more efficient than thermal treatment with a P value <0.0001. Among these five methods, the number 10 was the most efficient method in comparison with others. In direct RT-LAMP experiments, the materials used in the extraction process remain until the end of the visual detection step and could affect the speed of reaction or interfere with visualizing mechanism. This fact leads to the suitability of every efficient extraction method for specific direct-RT-LAMP assay. The presence of the Triton X-100, individually as a

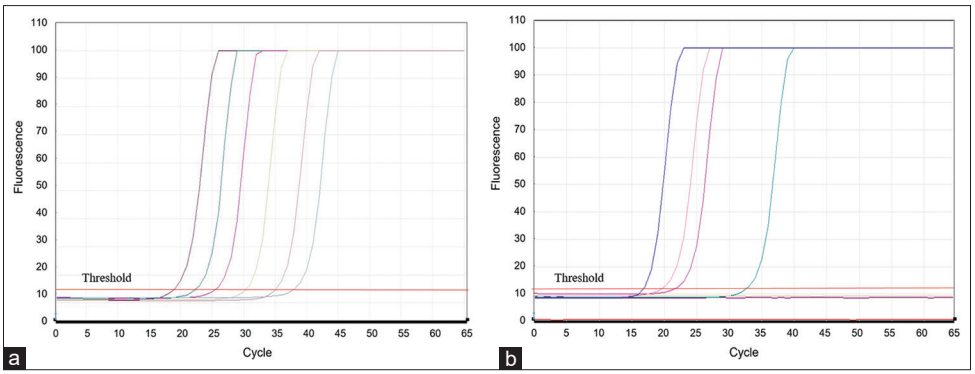


Figure 2: Sensitivity of primer sets for (a) N gene and (b) E gene. Amplification plots for positive control plasmid with final copy number concentrations of 10,000 copies/μL, 1000 copies/μL, 100 copies/μL, 10 copies/μL, 1 copies/μL, and 0.5 copies/μL are demonstrated

Table 2: The 10 methods of quick RNA extraction

Number of method	Chemical treatment	Heat treatment	Mean of RNA concentration (ng/uL)
1	-	10 min 85°C	≈20
2	proteinase K (1 mg/mL)	10 min 85°C	≈87
3	proteinase K (1 mg/mL) + Triton X100 (2%)	10 min 85°C	≈738
4	Triton X100 (2%)	10 min 85°C	≈702
5	Triton X100 (2%) + BSA (2 mg/mL)	10 min 85°C	≈910
6	BSA (2 mg/mL)	10 min 85°C	≈71
7	Tween 20 (2%)	10 min 85°C	≈118
8	GITC (4 M)	10 min 85°C	≈513
9	GuHCl (4 M)	10 min 85°C	≈ 63
10	GITC (4 M) + Triton X100 (2%)	10 min 85°C	≈1184

detergent in methods 3, 4, 5, and 10, remarkably increased the effectiveness of quick extraction methods in comparison with other detergents or materials [Figure 3].

Evaluation of the effects of different extraction methods on RT-LAMP reaction

To evaluate the effects of extracted RNA on RT-LAMP reaction efficiency, two nasopharyngeal samples with cycles of threshold 24 and 28 (CT values determined by Sansour) were collected. RNA was extracted using the 10 methods described above and one additional extraction was performed using RNA extraction kit (Payeshgene, Tehran, Iran) as a control. Subsequently, the treated samples were directly used in the reaction based on two limiting factors: the maximum allowed volume of sample in the final reaction volume and the material's noninhibitory concentration. Finally, the maximum volumes of each treatment for using in the reaction were calculated based on noninhibitory concentration obtained from former experiments (data not shown), and the results are illustrated in Table 3.

RT-LAMP reactions were performed with RNA samples derived from rapid extraction methods under optimal conditions (Section 2.2) in duplicate for both samples with different CTs. The results are shown in Figure 4 and Figure S1 in supporting information.

Among the methods used for rapid RNA extraction, the efficiency of method no. 1 (thermal treatment) was lower than all other methods and specifically lower than the control reaction (with P value <0.0001). In sample with CT 24, one of the two duplicate reactions within a time interval of 60 min was not amplified. Extraction method no. 2, which was associated with proteinase K enzyme, significantly reduced the reaction efficiency compared with the control reaction (P value = 0.0017). However, most previous studies have focused on these two methods for direct RT-LAMP. Methods 7, 8, and 9 were also less effective than the control reaction and did not significantly improve direct RT-LAMP performance. Among all three methods, Method 8 released an acceptable amount of RNA compared to the other methods, but its RT-LAMP reaction was weaker than the control reaction.

Methods numbers 3, 5, 6, and 10 were not significantly different from the control method (RNA extracted by commercial kit). On the other hand, the efficiency of these four methods was not different from each other. However, in more replicates, especially in higher CT values, method number 10 showed the best performance. Finally, according to the data related to RNA extraction reported in the above section, method number 10 was selected to perform sensitivity and specificity tests [Figure 5].

Some of the materials and methods used in this research may be evaluated separately in other previous studies. Due to differences in the viral load of the samples and variations in the procedures, concentration, and quality of the utilized

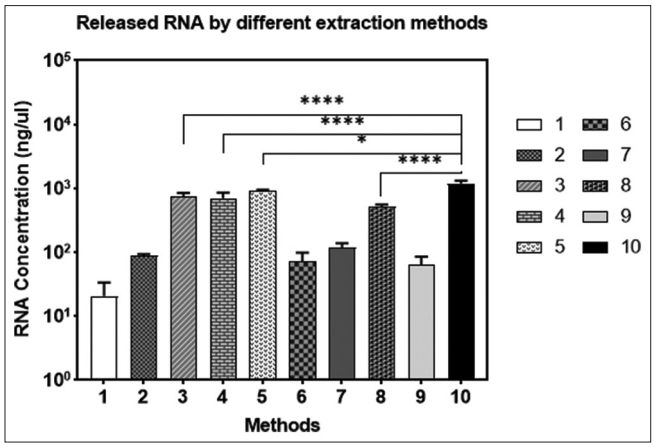


Figure 3: The average of released RNA by different rapid RNA extraction methods. Number of 1–10, respectively, are thermal treatment alone, proteinase K (1 mg/mL), proteinase K (1 mg/mL) + Triton X-100 (2%), Triton X-100 (2%), Triton X-100 (2%) + BSA (2 mg/mL), BSA (2 mg/mL), Tween 20 (2%), GITC (4 M), GuHCl (4 M), and GITC (4 M) +Triton X-100 (2%). ****P value < 0.0001, *P value = 0.01

materials and kits, determining the effectiveness of methods and comparing them with each other between various studies becomes complicated.

Nevertheless, to reach comprehensive insight, based on the most common methods and materials, 10 different rapid RNA extraction protocols were optimized and performed simultaneously on each specific sample.

Clinical sensitivity and specificity of developed Direct-RT-LAMP method

To calculate the clinical sensitivity and specificity of the finalized direct RT-LAMP method (method number 10), RT-qPCR was used as the gold standard. A number of 150 nasopharyngeal samples were tested including 114 RT-qPCR-positive samples (including 64 samples with CT ≤30, 51 samples with 30 < CT ≤ 35) and 36 RT-qPCR-negative samples.

Among 114 total RT-qPCR-positive samples, 88 samples were detected as positive by our direct RT-LAMP assay. For the samples with CT ≤35, the total sensitivity and specificity was

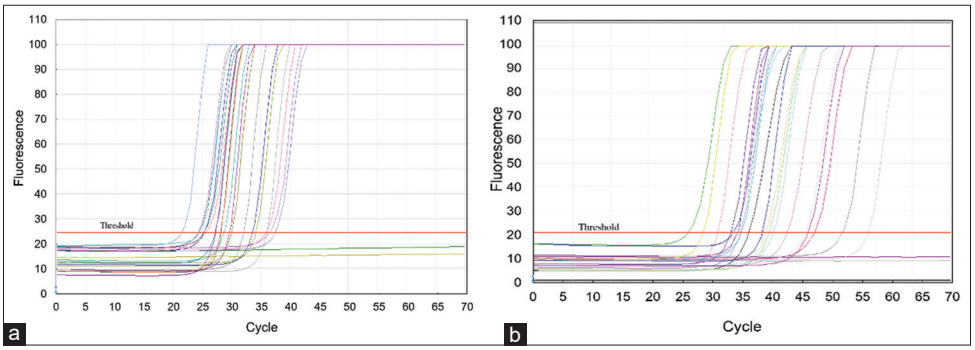


Figure 4: The duplicated amplification plots of direct RT-LAMP reactions with SARS-CoV-2 samples of (a) sample with CT 24 and (b) sample with CT 28 treated by 10 different rapid RNA extraction protocols. A number of 1–10, respectively, belong to the same sample treated by thermal treatment alone and with proteinase K (1 mg/mL), proteinase K (1 mg/mL) + Triton X-100 (2%), Triton X-100 (2%), Triton X-100 (2%) + BSA (2 mg/mL), BSA (2 mg/mL), Tween 20 (2%), GITC (4 M), GuHCl (4 M), and GITC (4 M) +Triton X-100 (2%)

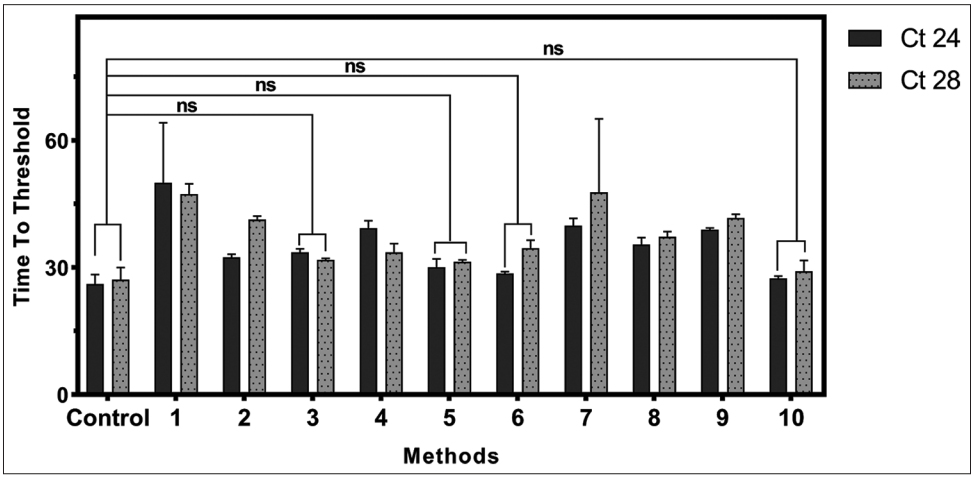


Figure 5: The mean time to the threshold for direct- RT-LAMP reactions with SARS-COV-2 samples treated by 10 different rapid RNA extraction protocols. A number of 1–10, respectively, belong to the same sample treated by thermal treatment alone and with proteinase K (1 mg/mL), proteinase K (1 mg/mL) + Triton X-100 (2%), Triton X-100 (2%), Triton X-100 (2%) + BSA (2 mg/mL), BSA (2 mg/mL), Tween 20 (2%), GITC (4 M), GuHCl (4 M) and GITC (4 M) +Triton X-100 (2%). ns: non-significant differences

Table 3: Amount of treated samples with 10 rapid RNA extraction protocols in 25 μL RT-LAMP reaction

Method	Control	1	2	3	4	5	6	7	8	9	10
Volume of sample in reaction (μL)	0.625	8.3	8.3	0.208	0.208	0.208	0.208	0.208	0.208	0.208	0.208

Table 4: Sensitivity and specificity of developed direct RT-LAMP assay for clinical samples

LAMP result		No. of samples with RT-qPCR result			Sensitivity (95% CI)	Specificity (95% CI)
		Positive	Negative	Total		
Positive	CT≤30	63	1	64	98.4 (95.3–101.4)	88.8 (78.6–99.15)
	30<CT≤35	26	25	51	50.98 (37.26–64.7)	88.8 (78.6–99.15)
	CT≤35	88	26	114	77.19 (69.4–84.8)	88.8 (78.6–99.15)
Negative		4	32	36		
Total		92	58	150		

77.19 (77.19%, 95% CI: 69.4–84.8%) and 88.8 (88.8%, 95% CI: 78.62–99.15%), respectively. Moreover, results for different ranges of CT values were shown in Table 4. For CT values less than 30, the sensitivity was 98.4 (95.3%, 95% CI: 95.3–101.4%), and for samples, with CT values only between 30 and 35, this assessment was 50.98, 95% CI: 37.26–64.7% [Table 4]. Furthermore, the technique detected 32 samples from 35 RT-qPCR-negative samples as true negative and 4 negative samples had nonspecific amplification (false positive). These results confirm that the specificity of the finalized direct RT-LAMP assay is 88.8% (88.8%, 95% CI: 78.62–99.15%) [Table 4].

Discussion

The COVID-19 pandemic has had a significant impact on the world,[12] highlighting the urgent need for fast and accurate diagnostic tests for mass screening of infected individuals. While RT-PCR is the gold standard for diagnostic tests, it suffers from several limitations, including the need for RNA extraction before amplification, which prolongs detection time and may result in false negatives.[13,14]

One promising diagnostic trend during the COVID-19 outbreak was developing an RT-LAMP-based detection assay.[15] The tolerance of LAMP Bst polymerase to inhibitors is one of the strengths of this technique for direct detection of SARS-CoV-2 from specimens.[16] Many researchers tried to develop direct RT-LAMP reactions and achieved different levels of sensitivity and specificity. However, in direct RT-LAMP reactions, all components of the sample, including salt, pH, protein, and DNA, are transferred directly from extraction to amplification, which may affect reaction sensitivity, specificity, test robustness, and visual detection mechanism. For accurate diagnostic studies, RNA molecules should be released intact and free of all cellular proteins and also be preserved in a safe storage condition during the extraction and processing steps. Various physical and chemical treatments can influence reaction speed and efficacy through different mechanisms, such as the amount of released RNA, increasing polymerase enzyme activity by crowding effects, and inactivating RNase or other interfering proteins in reaction environments. However, due to differences in viral load, variation in performing rapid RNA extraction procedures, concentration, and quality of materials and kits used, determining the effectiveness of methods and comparing them between various studies becomes complicated. In this study, we assessed the efficiency of RT-LAMP assays using 10 rapid RNA extraction methods on the same SARS-CoV-2 clinical specimens at two levels: amount of released RNA and qPCR reaction efficiency. The study by Lalli *et al*.[11] developed a rapid colorimetric assay for SARS-CoV-2 detection from saliva samples using RT-LAMP without the need for RNA purification. They reported an accuracy of more than 90% using only thermal treatment for RNA extraction. However, our results showed that using only thermal treatment resulted in the lowest RNA concentration. We found that combining thermal treatment with proteinase K increased the efficiency of this method. This difference in results may be due to differences in the sample types or the specific protocols used in the two studies. Nonetheless, our findings highlight the importance of optimizing RNA extraction methods for SARS-CoV-2 detection using RT-LAMP to improve the sensitivity and accuracy of diagnostic tests. This finding is consistent with other studies that have shown that heat treatment alone is not efficient enough and produces higher shifts in CTs.[17,18]

Some studies have demonstrated that combining proteinase K with thermal treatment improves reaction performance by inactivating nucleases and disrupting inhibitor proteins in specimens. For example, Genoud *et al*.[17] demonstrated that using thermal treatment alone for SARS-CoV-2 detection by RT-qPCR is not efficient and demonstrated that using a treatment with proteinase K followed by heat inactivation (PK + HID method) improved their RT-qPCR.[18–21] However, in our study, proteinase K did not have a significant effect compared to thermal treatment for RNA release. This may be due to the fact that protease enzymatic reactions need time to inhibit RNases.[22–24] Proteinase K in method 2 did not significantly increase RNA release but significantly increased reaction speed compared to thermal treatment (P value = 0.0323).

The presence of proteinase K along with Triton X-100 (method 3) significantly increased reaction speed compared

to method 1 (thermal treatment), which was more pronounced (*P* value = 0.0002) compared to these materials alone (methods 2 and 3). Proteinase K has shown discrepant results for increasing sensitivity compared to heat treatments alone in previous studies.[11,18]

The nonionic surfactant Triton X-100 was used for its RNase inactivation properties and non-inhibiting effects in amplification assays. This is because the surfactant solubilizes proteins rather than completely denaturing them, thereby retaining the activity of RT-LAMP enzymes.[23] Triton X-100 is known to be very efficient in disintegrating lipid-enveloped viruses like SARS-CoV-2, which is in strong agreement with our finding related to the effectiveness of Triton X-100 in releasing RNA.[25–27] However, Triton X-100 is a comparatively mild detergent that is unable to completely inactivate and denature proteins bound to cytoplasmic RNA. Failure to inactivate interfering proteins can slow reaction speed even in the presence of high levels of RNA.[28]

Method 5 containing Triton X-100 and BSA was an effective method, showing the same result as the control experiments, and was significantly better than method 1 (*P* value < 0.0001). This may be due to the enhancing effects of BSA as a crowding agent, which reduces the inhibitory effect on the reaction, stabilizes the enzymes, and prevents nucleic acids from binding to the reaction containers.[29] Perhaps the enhancer effect of BSA along with the specific effects of proteinase K removes the inhibitory effect of interferer proteins and helps improve the efficacy of the LAMP reactions that do not occur with BSA or Triton X-100 alone (methods 4 and 6). However, the combination of BSA and proteinase K with Triton X-100 detergent may help better RNA release, which in turn gives us the possibility of increasing dilution factor.

Guanidine salts as chaotropic agents have commonly been used for optimizing extraction conditions due to the low cost of the reagent, great potential in purifying nucleic acids, and high ability for denaturation and deactivating RNases.[23,30] According to the results of this study, GITC had more ability to release RNA in the reaction while GuHCl was a weaker property. This finding is in great agreement with previous studies that mentioned that GITC is more effective than GuHCl, which has long been used for RNA preparation studies due to the rapid denaturing of all cellular proteins (protein removal RNA) and RNases.[22,31] Finally, method 10 which is containing GITC and Triton X-100 was repeatedly the most effective method.

Conclusion

Developing a direct RT-LAMP assay requires an efficient rapid RNA extraction method with sufficient RNA in the noninhibitory concentration of chemicals. Conducting a comprehensive study using specific clinical samples to determine the effectiveness of methods and comparing them is inevitable. In conclusion, our study highlights the importance of selecting the appropriate RNA extraction method for direct RT-LAMP assays to ensure accurate diagnostic results. We suggest that the incorporation of concurrent chemical and thermal treatment in SARS-CoV-2 clinical samples for effective cell lysis and removing the inhibitory effect of sample impurity could be synergistic. Finally, as expected, the combination of GITC as the best candidate for removing interfering proteins and RNases with Triton X-100 detergent due to its ability to disintegrate virus structure presented the highest efficiency for RNA extraction and direct amplification of SARS-CoV-2 from clinical samples.

Acknowledgments

This study was conducted with the financial support of the Payesh Gene Rasti company with an ethical code of IR.MUQ.REC.1399.194. These financial and scientific supports are gratefully acknowledged.

Financial support and sponsorship

Funding acquisition; Razieh Taghizadeh Pirposhteh, Maryam Moazami Goodarzi.

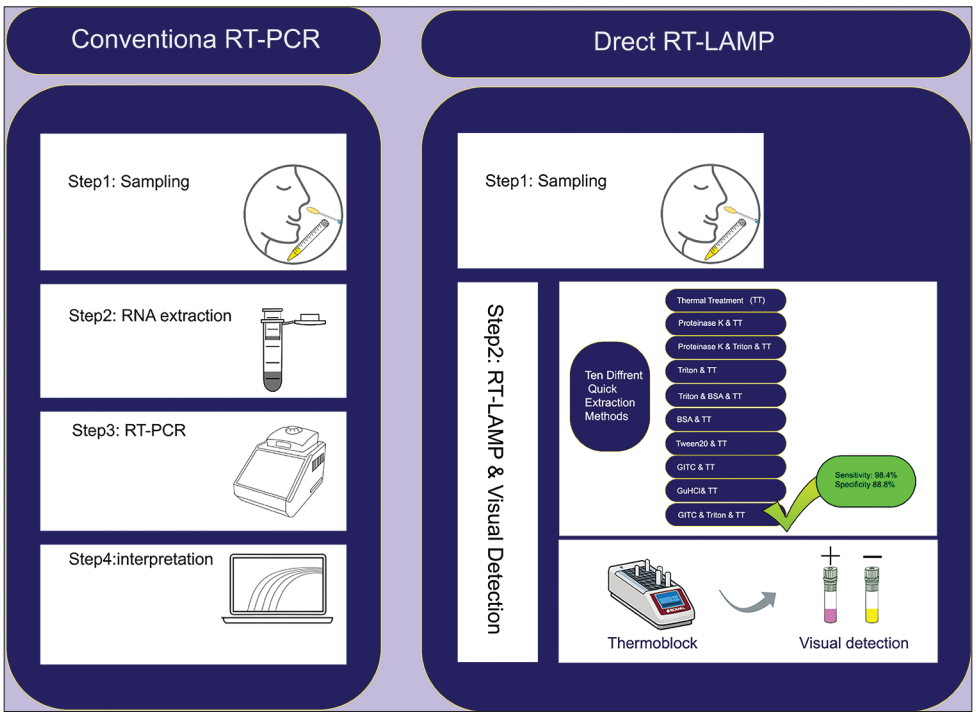
Conflicts of interest

There are no conflicts of interest.

References

1. WHO. 2022. Available from: https://covid19.who.int. 26 July 2023.
2. Rödel J, Egerer R, Suleyman A, Sommer-Schmid B, Baier M, Henke A, *et al*. Use of the variplex™ SARS-CoV-2 RT-LAMP as a rapid molecular assay to complement RT-PCR for COVID-19 diagnosis. J Clin Virol 2020; 132:104616.
3. Garcia-Venzor A, Rueda-Zarazua B, Marquez-Garcia E, Maldonado V, Moncada-Morales A, Olivera H, *et al*. SARS-CoV-2 direct detection without RNA isolation with Loop-Mediated Isothermal Amplification (LAMP) and CRISPR-Cas12. Front Med 2021; 8:627679.
4. Babu US, Harrison LM, Mammel MK, Bigley EC 3rd, Hiett KL, Balan KV. A loop-mediated isothermal amplification (LAMP) assay for the consensus detection of human pathogenic Campylobacter species. J Microbiol Methods 2020; 176:106009.
5. Institute of Medicine, Board on Global Health, Forum on Microbial Threats. Infectious Disease Movement in a Borderless World: Workshop Summary. National Academies Press; 2010. 322 p.
6. Wei S, Kohl E, Djandji A, Morgan S, Whittier S, Mansukhani M, *et al*. Direct diagnostic testing of SARS-CoV-2 without the need for prior RNA extraction. Sci Rep 2021;11:2402.
7. Ganguli A, Mostafa A, Berger J, Aydin MY, Sun F, Stewart de Ramirez SA, *et al*. Rapid isothermal amplification and portable detection system for SARS-CoV-2. Proc Natl Acad Sci U S A 2020;117:22727-22735.
8. Alekseenko A, Barrett D, Pareja-Sanchez Y, Howard RJ, Strandback E, Ampah-Korsah H, *et al*. Direct detection of SARS-CoV-2 using non-commercial RT-LAMP reagents on heat-inactivated samples. Sci Rep 2021; 11:1820.
9. Ortiz-Cartagena C, Fernández-García L, Blasco L, Pacios O, Bleriot I, López M, *et al*. Reverse transcription-loop-mediated isothermal amplification-CRISPR-Cas13a technology as a promising diagnostic tool for SARS-CoV-2. Microbiol Spectr 2022;10:e0239822.
10. Dao Thi VL, Herbst K, Boerner K, Meurer M, Kremer LPM, Kirrmaier D, *et al*. A colorimetric RT-LAMP assay and LAMP-sequencing for detecting SARS-CoV-2 RNA in clinical samples. Sci Transl Med 2020;12: eabc7075.
11. Lalli MA, Langmade JS, Chen X, Fronick CC, Sawyer CS, Burcea LC, *et al*. Rapid and extraction-free detection of SARS-CoV-2 from saliva by colorimetric reverse-transcription loop-mediated isothermal amplification. Clin Chem 2021; 67:415–24.
12. Salgado-Benvindo C, Thaler M, Tas A, Ogando NS, Bredenbeek PJ,

Ninaber DK, *et al*. Suramin inhibits SARS-CoV-2 infection in cell culture by interfering with early steps of the replication cycle. Antimicrob Agents Chemother 2020;64: e00900-20.
13. CDC. CDC's Diagnostic Test for COVID-19 Only and Supplies. Centers for Disease Control and Prevention. 2022. Available from: https://www.cdc.gov/coronavirus/2019-ncov/lab/virus-requests.html. [Last accessed on 2022 Oct 18].
14. Arevalo-Rodriguez I, Buitrago-Garcia D, Simancas-Racines D, Zambrano-Achig P, Campo R Del, Ciapponi A, *et al*. False-negative results of initial RT-PCR assays for COVID-19: A systematic review. PLoS One 2020;15: e0242958.
15. Mukhopadhyay CD, Sharma P, Sinha K, Rajarshi K. Recent trends in analytical and digital techniques for the detection of the SARS-Cov-2. Biophys Chem 2021; 270:106538.
16. Thompson D, Lei Y. Mini review: Recent progress in RT-LAMP enabled COVID-19 detection. Vol. 2, Sensors and Actuators Reports. Elsevier; 2020.
17. Genoud V, Stortz M, Waisman A, Berardino BG, Verneri P, Dansey V, *et al*. Extraction-free protocol combining proteinase K and heat inactivation for detection of SARS-CoV-2 by RT-qPCR. PLoS One 2021;16: e0247792.
18. Shukla A, Gangwar M, Sharma G, Prakash P, Nath G. Vitality of Proteinase K in rRTPCR Detection of SARS-CoV2 Bypassing RNA Extraction. Front Cell Infect Microbiol 2021;11:717068.
19. Yu F, Qiu T, Zeng Y, Wang Y, Zheng S, Chen X, *et al*. Comparative evaluation of three preprocessing methods for extraction and detection of influenza A virus nucleic acids from sputum. Front Med. 2018;5:56.
20. Sung H, Yong D, Ki CS, Kim JS, Seong MW, Lee H, *et al*. Comparative evaluation of three homogenization methods for isolating middle east respiratory syndrome coronavirus nucleic acids from sputum samples for real-time reverse transcription PCR. Ann Lab Med 2016; 36:457-62.
21. Cook N. Viruses in Food and Water: Risks, Surveillance and Control. Elsevier; 2013. 560 p.
22. Hartshorn C, Anshelevich A, Wangh LJ. Rapid, single-tube method for quantitative preparation and analysis of RNA and DNA in samples as small as one cell. BMC Biotechnol 2005; 5:2.
23. Bender AT, Sullivan BP, Lillis L, Posner JD. Enzymatic and chemical-based methods to inactivate endogenous blood ribonucleases for nucleic acid diagnostics. J Mol Diagn 2020;22:1030–40.
24. Lever MA, Torti A, Eickenbusch P, Michaud AB, Šantl-Temkiv T, Jørgensen BB. A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. Front Microbiol 2015; 6:476.
25. Scallan MF, Dempsey C, MacSharry J, O'Callaghan I, O'Connor PM, Horgan CP, *et al*. Validation of a lysis buffer containing 4 M Guanidinium thiocyanate (GITC)/Triton X-100 for extraction of SARS-CoV-2 RNA for COVID-19 testing: Comparison of formulated lysis buffers containing 4 to 6 M GITC, Roche external lysis buffer and qiagen RTL lysis buffer. bioRxiv 2020.
26. Roberts PL. Virus inactivation by solvent/detergent treatment using Triton X-100 in a high purity factor VIII. Biologicals 2008; 36:330–5.
27. Weidner L, Laner-Plamberger S, Horner D, Pistorius C, Jurkin J, Karbiener M, *et al*. Sample buffer containing guanidine-hydrochloride combines biological safety and RNA preservation for SARS-CoV-2 molecular diagnostics. Diagnostics (Basel) 2022; 12:1186.
28. Bertonati C, Honig B, Alexov E. Poisson-Boltzmann calculations of nonspecific salt effects on protein-protein binding free energies. Biophys J 2007; 92:1891–9.
29. Ge X, Xu J. Macromolecular crowding effects on transcription and translation are regulated by free magnesium ion. Biotechnol Appl Biochem 2020; 67:117–22.
30. Zainabadi K, Dhayabaran V, Moideen K, Krishnaswamy P. An efficient and cost-effective method for purification of small sized DNAs and RNAs from human urine. PLoS One 2019;14: e0210813.
31. Urbaniak J, Janowski D, Jacewski B. Isolation of nucleic acids using silicon dioxide powder as a tool for environmental monitoring. Environ Monit Assess 2019; 191:732.



Graphical abstract

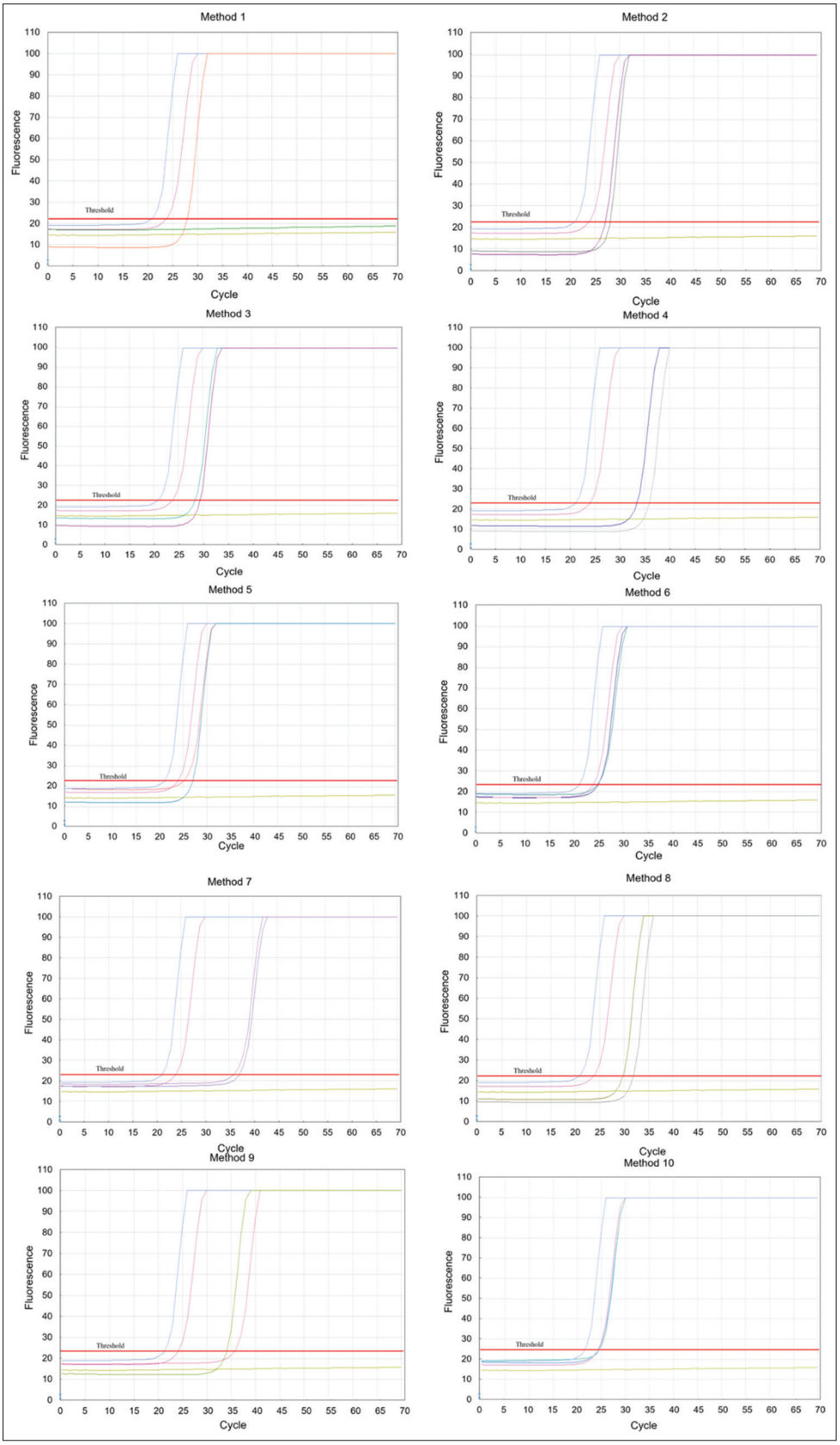


Figure S1: Real-time amplification plots for each method of 10 different methods with dual repeat. The test is performed on a sample with CT 24. Method number of 1-10 respectively belong to the same sample treated by thermal treatment alone and with proteinase K (1mg/ml), proteinase K (1mg/ml)+ Triton X-100 (2%), Triton X-100 (2%), Triton X-100 (2%)+ BSA (2mg/ml), BSA (2mg/ml), Tween 20 (2%), GITC (4M), GuHCl (4M) and GITC (4M)+Triton X-100 (2%)