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High-quality RT-PCR with chemically modified RNA controls

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ABSTRACT

In detecting infectious diseases, such as coronavirus 2019 (COVID-19), real-time reverse-transcription polymerase chain reaction (RT-PCR) is one of the most important technologies for RNA detection and disease diagnosis. To achieve high quality assurance, appropriate positive and negative controls are critical for disease detection using RT-PCR kits. In this study, we have found that commercial kits often adopt DNAs instead of RNAs as the positive controls, which can't report the kit problems in reverse transcription, thereby increasing risk of the false negative results when testing patient samples. To face the challenge, we have proposed and developed the chemically modified RNAs, such as phosphoroselenaote and phosphorothioate RNAs (Se-RNA and S-RNA), as the controls. We have found that while demonstrating the high thermostability, biostability, chemostability and exclusivity (or specificity), both Se-RNA and S-RNA can be fine templates for reverse transcription, indicating their potentials as both positive and negative controls for RT-PCR kits.

1. Introduction

Novel coronavirus pneumonia is caused by severe acute respiratory syndrome coronavirus 2 (COVID-19 or SARS-CoV-2), which has infected over 18 million people and caused over seven hundred-thousand death [1,2]. The early diagnosis of COVID-19 is critical for prevention and control of this worldwide pandemic [3–5]. Real-time reverse-transcription polymerase chain reaction (RT-PCR) is generally regarded as the most powerful strategy for COVID-19 diagnosis [6,7]. However, recent studies have found that the current RT-PCR kits offer unsatisfactory results, due to high numbers of false negative results (approximately 40%) [7–10]. False negative result is extremely dangerous and may cause many problems in COVID-19 prevention and epidemic control. Since this pathogen is highly infectious and deadly, persons with false negative results can easily infect other people around them, causing serious wide-spreading of the virus and badly impacting on the population. Instead of the detection kits themselves, false negative results are generally attributed to improper sample collection, transportation, storage and/or handling. However, a problematic RT-PCR kit, such as a kit with inappropriate controls and failed reverse transcription, can indeed cause false negativity as well.

RT-PCR, which is one of the most widely-used RNA detection methods in foundmental research and disease diagnosis, is of two steps: reverse transcription (Step 1) and quantitative real-time polymerase chain reaction (Step 2) [11]. Though it can detect RNA virtually as rapid and sensitive as DNA, RNA is vulnerable to RNase degradation, causing its inherent instability [12]. To make matters worse, RNases are nearly ubiquitous in surrounding environment and hard to eliminate, and they can rapidly degrade RNAs, including RNA samples and RNA controls. Though RNAs should be the positive controls in theory, in commercial RT-PCR kits, DNAs are normally used as the positive controls in practice, to avoid the RNA bio-degradation and face the chanlenge of preparation a large amount of inactivated or recombinant virus in a short period of time. The problem is that the positive control DNAs can't inspect and report a RT-PCR kit with failed reverse transcription step, thereby causing high risk of false negative results in disease diagnosis on the basis of RNA detection, such as the false negativity in the novel coronavirus pneumonia diagnosis [13–16]. Thus, appropriate controls are essential for viral RNA detection with RT-PCR kits.

In principle, the kits with any problems should be identified and reported by the positive and/or negative controls. In this study, however, we discovered that a problematic kit with failed reverse

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transcription escaped the internal inspections, causing false negative results in COVID-19 RNA detection. Further, we demonstrated that the current commercial RT-PCR kits generally adopt DNAs as positive controls, thereby offering false assurance on the problematic kit with failed reverse transcription and increasing risk of false negative. Our alarming finding indicates that the usage of DNA positive controls is not an ideal strategy for RT-PCR kits, and it may be a better choice to use RNA positive controls.

Although canonical RNA is inherently unstable, the modified RNAs can be stable in nuclease resistance [17] and useful as potential therapeutics in disease treatment, such as hyperlipidemia, HCV, and others [18]. It is known that NTPαSe can be recognized by T7 RNA polymerase with high specificity and the transcribed phosphoroselenaote RNAs are nuclease-resistant [19–21]. Therefore, we have hypothesized that the modified RNAs by replacing the phosphate nonbridging oxygen with selenium or sulfur may be specifically and efficiently recorgnized by reverse transcriptase and work as RNA positive or negative controls of the RT-PCR kits. Following our hypothesis, we explored the potentials of phosphoroselenaote and phosphorothioate RNAs (Se-RNA and S-RNA) as both positive and negative controls. We have found that while demonstrating the high thermostability, biostability, chemostability and exclusivity (or specificity), both Se-RNA and S-RNA can be fine templates for reverse transcription, indicating their potentials as both positive and negative controls for RT-PCR kits.

2. Materials and methods

2.1. Sample preparation

A plasmid DNA containing target sequences of COVID-19 (pCOVID-19) was commercially synthetized by Sangon Biotech (Sangon, Shanghai, China). Then, DNA fragment containing target COVID-19 sequence was prepared with T7 promotor via PCR. Finally, the target COVID-19 RNA was *in vitro* transcribed by HiScribe T7 High Yield RNA Synthesis Kit (NEB Co. Beijing, China), and purified by MEGAclear Transcription Clean-Up Kit (Thermo Fisher, MA, USA). The COVID-19 RNAs were used as samples for RT-PCR. The clinical COVID-19 RNA samples were also used as samples for RT-PCR.

2.2. S-RNA and Se-RNA transcription

To transcribe Se-RNA and S-RNA, DNA template (210 bp in N gene of COVID-19) was cloned, with T7 promoter, from pCOVID-19 by PCR (primer F: 5'-taatacgactcactatagCTCTTCTCGTTCCTCATC-3'; primer B: 5'-GCAGCAGATTTCTTAGTG-3'). Se-RNA and S-RNA were then transcribed with HiScribe T7 High Yield RNA Synthesis Kit by using all four NTPαSes and NTPαSs (SeNtInAll, Chengdu, China), respectively. After transcription, each product (1 μL) was analyzed by denaturing polyacrylamide gel electrophoresis (12% PAGE) and stained with GelRed (Sangon Biotech, Shanghai, China) and visualized by the ChemiDoc™ XRS Imaging System (Bio-Rad Co., California, USA).

2.3. cDNA preparation by using S-RNA and Se-RNA

To investigate whether Se- and S-RNAs can be efficiently recognized as templates by reverse transcriptase, reverse transcription experiments were carried out by using the modified RNA and MLV reverse transcriptase (HaiGene, Haerbin, China). The reactions were performed with the transcriptrd Se-RNA or S-RNA (final concentration: 1 μM), 5'-FAM labeled primer (1 μM), dNTP (100 μM), 10 U MLV reverse transcriptase, 2 U RNase Inhibitor and 1 × RT Buffer, incubated at 55 °C for 60 min. Then, the reactions were quenched with urea-saturated-loading buffer and the products were analyzed by denaturing PAGE (12%).

2.4. RT-PCR

For COVID-19 RNA detection, positive control and negative control were analyzed with a commercial RT-PCR kit according to the manufacture's instruction, in a LightCycler96 Real-Time PCR System (Roche, Shanghai, China). Briefly, prepare appropriate amounts of reaction mixtures (including buffer, enzyme, primer, and probe) according to the number of samples, positive controls and negative controls. Then, pipet template (5 μL) and reaction mixture (20 μL) to each reaction tube, followed by mixing and spinning down the reaction mixtures. Finally, the RT-PCR amplification was performed according to the manufacture's instruction.

2.5. Analysis of the positive controls in the six commercial kits

To investigate whether the positive control of commercial RT-PCR kit is DNAs or RNAs, six commercial RT-PCR kits were collected. Then, their positive controls were digested with RNase A or DNase I (HaiGene, Haerbin, China), followed by RT-PCR analysis with the corresponding RT-PCR kits. The digestion of positive control was carried out in a reaction mixture (20 μL), containing positive controls (17 μL), RNase A or DNase I (1 μL) and reaction buffer (2 μL), and incubated at 37 °C for 30 min, followed by inactivation at 80 °C for 5 min.

3. Results and discussion

3.1. Identification of positive controls as DNAs in commercial RT-PCR kits

We randomly chose six commercial RT-PCR kits (A-F; Fig. 1) for COVID-19 virus detection in market, for this study. In order to investigate their positive controls, we digested them with RNase A or DNase I, followed by analysis with their RT-PCR kits, respectively. We found that all of them can be digested by DNase I, while resisting to RNase A digestion (Fig. 1A–F). This experiment result has indicated that these positive controls were DNAs.

3.2. False negative results of COVID-19 viral RNA detection with a problematic commercial kit

Following the digestion study of the positive controls in these kits, we identified a problematic kit (named as Batch-1 of kit A; Table 1 and Fig. 2). We performed the detection on COVID-19 viral RNA samples with Batch-1, while its positive control DNA was used as reference. We found that though the DNA control reported positive result as usual, Batch-1 failed to report the positive results on these positive viral RNA samples, indicating that the reverse transcription in Step 1 didn't work, but the DNA polymerization and PCR in Step 2 functioned normally. Further, integrity of these RNA samples was confirmed by kit B (Table 1 and Fig. 2). Our experimental results have demonstrated that the problematic kit carrying DNA positive control can cause false negativity, indicating that RNAs instead of DNAs should be more appropriate as positive controls.

3.3. The modified RNA can be efficiently recognized by reverse transcriptase, working as fine positive controls as canonical RNA

Although in theory, RNAs should be ideal positive controls for RT-PCR, DNAs are used as the positive controls in practice, since RNAs can be contaminated and decomposed by RNases. Thus, the chemically modified RNA controls with high stability and bioactivity are desired for RT-PCR detection. We successfully demonstrated that all four selenium- and sulfur-modified NTPs (NTPαSe and NTPαS) can be enzymatically polymerized by T7 RNA polymerase (Fig. 3A), into RNAs with enhanced stability [19–21]. We also found that Se-RNA and S-RNA can be efficiently recognized by reverse transcriptase, generating cDNAs (Fig. 3B).

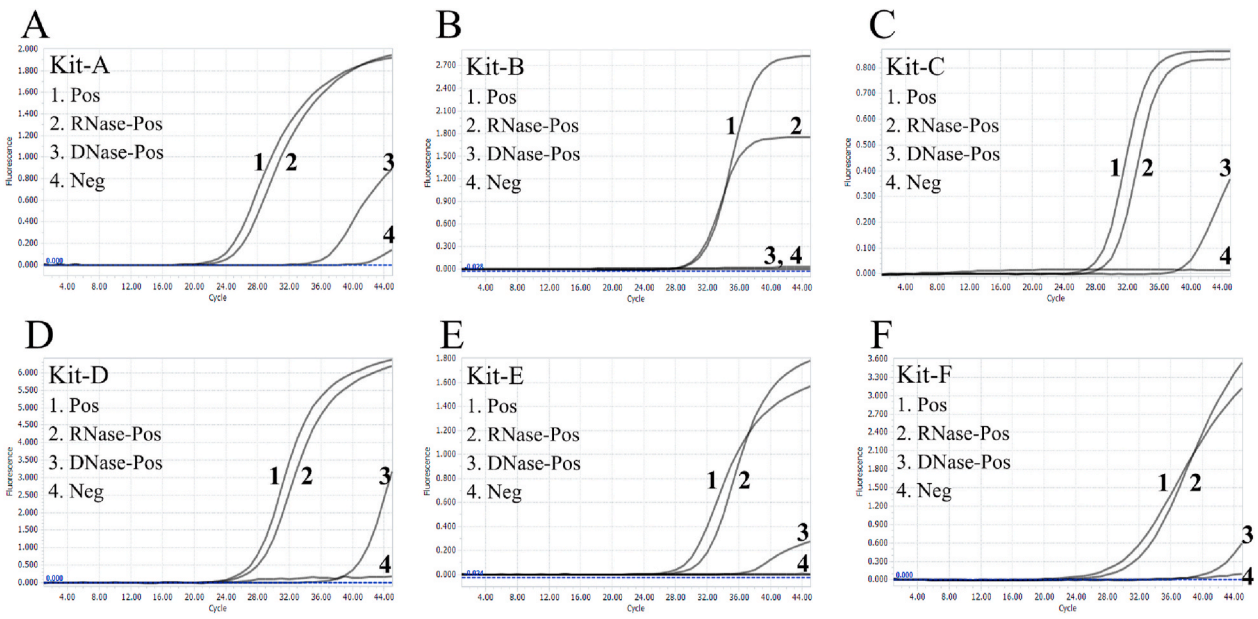

Fig. 1. DNAs were chosen as positive controls in the commercial kits for COVID-19 detection. Figure A to F refer to six commercial RT-PCR kits. In each figure, positive and negative controls were from corresponding kit, respectively; Curve 1: RT-PCR with undigested positive control; Curve 2: RT-PCR with positive control digested with RNase A; Curve 3: RT-PCR with positive control digested with DNase I; Curve 4: negative control.

Table 1
False negative detection of COVID-19 RNA with commercial kit.

Kit/Sample	COVID-19 RNA sample 1	COVID-19 RNA sample 2	Positive control (from kit)	Negative control
Kit A (Batch-1; Ct)	>45	>45	34.2	>45
Kit A (Batch-2; Ct)	23.5	26.1	33.6	>45
Kit B (Ct)	22.9	25.6	31.5	>45

These experiments have been repeated for five times, and the results were consistent.

Further, we demonstrated that both Se-RNA and S-RNA can be amplified and detected by RT-PCR (Fig. 3C). Furthermore, we found that the detection limit of Se-RNA and S-RNA was similar to that of canonical RNA (O-RNA) (Table 2), indicating the Se/S-RNA potential as the positive controls.

3.4. The modified RNAs are thermo-stable, nuclease-resistant and chemo-stable

Since the stability (both biostability and thermostability) is the most important property for the positive RNA controls, we investigated the Se-RNA and S-RNA under various conditions, compared with the O-RNA. After 1-h incubations under various temperatures (55–95 °C), while the canonical RNA decomposed, the modified RNAs didn't show significant changes in RT-PCR detection and the Ct values, demonstrating their excellent thermostability (Fig. 4A–C). We found that after the incubation, while O-RNA was significantly decomposed at higher temperature, Se-RNA was barely affected and S-RNA was also stable, following their thermostability order: Se-RNA>S-RNA»O-RNA.

Further, when treated with RNase T1, serum and saliva, Se-RNA and S-RNA were barely affected by the nuclease degradation, while canonical RNA was digested (Fig. 4D–F), following their biostability order: Se-RNA>S-RNA»O-RNA. Furthermore, the Se-RNA, S-RNA and O-RNA were kept for several weeks at 4 °C (Fig. 4G–I), demonstrating their chemo-stability order: Se-RNA>S-RNA»O-RNA. Our experiments clearly revealed that the modified Se-RNA and S-RNA are stable and suitable as positive controls for the RT-PCR kits.

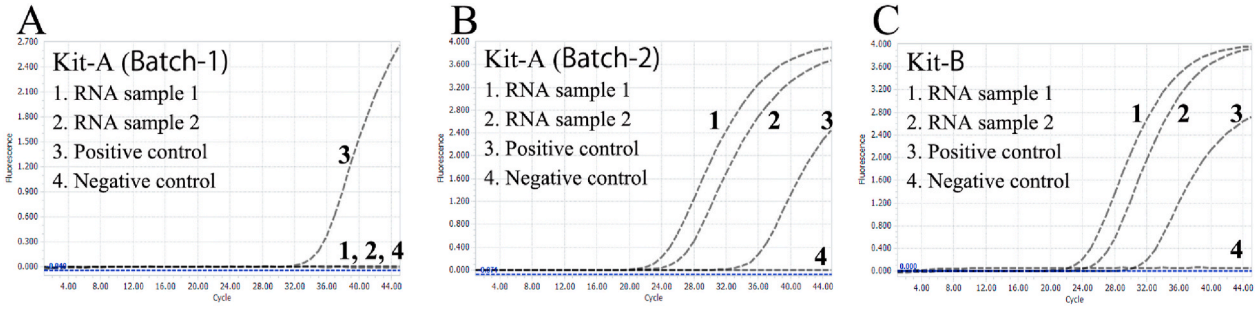

Fig. 2. False negative results caused by the problematic commercial kit (Table 1). In each figure, Curve 1: COVID-19 positive RNA sample 1; Curve 2: COVID-19 positive RNA sample 2; Curve 3: positive control of corresponding kit; Curve 4: negative control of corresponding kit.

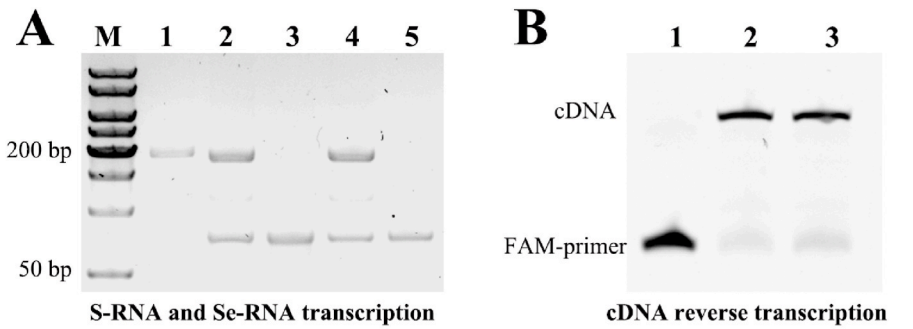

Fig. 3. Bioactivity of Se-RNA and S-RNA. (A) Se-RNA and S-RNA transcription. Lane 1: DNA template; Lane 2: transcripted Se-RNA before DNase I digestion; Lane 3: transcripted Se-RNA after DNase I digestion; Lane 4: transcripted S-RNA before DNase I digestion; Lane 5: transcripted S-RNA after DNase I digestion. (B) Both Se-RNA and S-RNA can be efficiently recognized by MLV reverse transcriptase. Lane 1, FAM labeled primer; Lane 2: S-RNA was used as the template for cDNA reverse transcription; Lane 3: Se-RNA as the templale. (C) Se-RNA and S-RNA can be efficiently amplified and detected by RT-PCR.

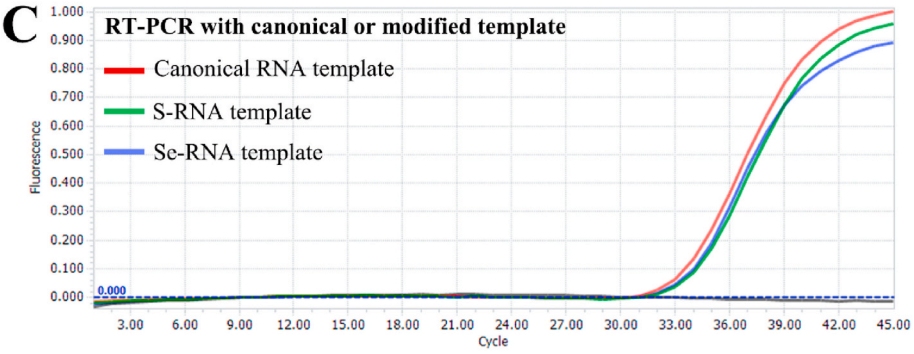

Table 2
Detection sensitivity of the canonical and modified RNAs.

Template	1000 copies	500	100	50
O-RNA (Ct)	31.2	33.5	35.8	36.0
S-RNA (Ct)	32.9	33.7	35.0	37.1
Se-RNA (Ct)	32.5	33.9	34.5	36.4

3.5. The modified RNAs are preferable negative controls for RT-PCR kits

RT-PCR detection, without strict control, design and care, can easily cause aerosol and/or surface cross-contaminations and signal mis-detection. The negative controls are designed for monitoring the contaminations and mis-detection. Though high-purity water is a convenient and common choice for the negative control, non-specific RNAs are better choices in theory, especially monitoring mis-detection. When non-specific RNAs are chosen, in addition to the stability requirement (similar as the positive controls), the exclusivity (or exclusive specificity) is critical for the negative control. In order to investigate the exclusivity of negative RNA controls, we designed several sets of non-specific primer pairs to investigate the Se-RNA, S-RNA and O-RNA (Table 3). We have found that the modified RNAs (Se-RNA and S-RNA) are generally more exclusive than the canonical RNA (O-RNA), indicated by their higher Ct values than the O-RNA ones. This higher exclusivity is consistent with the higher specificity of dNTPαSe [19]. Obviously, our experimental results have illustrated that as the negative controls, the modified RNAs are statistically more advantageous than the canonical ones. These findings have indicated that the Se-RNA and S-RNA are preferable negative controls for RT-PCR kits, and they can help decreasing the false negative results in RT-PCR detection.

The RT-PCR study was carried out with eight randomly-designed primer pairs on Se-, S- and O-RNAs as negative controls, respectively. The Ct value ($\geq$36) means the negative detection.

4. Conclusion

In this study, we reported that RT-PCR kit with problems in reverse transcription caused false negative results in COVID-19 diagnosis. Due to the common usage of DNA-based positive controls, this problematic RT-PCR kit displayed normal positive results in its internal inspections. However, when real positive COVID-19 RNA samples were used, this kit reported negative results, which were false negative. To investigate the positive controls of the commercial kits, we randomly sampled six RT-PCR kits. We found that the positive controls were DNAs, suggesting that DNAs were often used as the positive controls of commercial RT-PCR kits.

Given RNA instability, expensiveness and the difficulty of preparation a large amount of inactivated and recombinant virus in a short time, DNAs have been reasonable choices as positive controls in commercial kits, which are used for emerging infectious disease detection, such as COVID-19 pandemic. However, this strategy with DNA-based positive controls can't report the kit problems in reverse transcription. As the result, kits with this type of problems can still offer normal results for inspection with DNA positive controls, falsely assuring the working conditions of the kits. This type of failed kits will offer inaccurate results on the positive clinical samples, thereby causing false negativity. It's worth noting that the current COVID-19 diagnosis has offered the high number of false negative reports [7–10], which are often attributed to other problems, instead of the detection kits. From our research, we have found that the false negativity can be caused by problematic RT-PCR kits. It's worth noting that this source of false negativity may be underestimated and overlooked.

Although in theory, RNAs (especially inactivated or recombinant viruses) are more appropriate as the positive controls, DNA positive controls are frequently chosen for commercial RT-PCR kits, due to their stability, economical efficiency and easy preparation. To enhance the stability of RNAs, many chemical modification strategies have been developed, such as 2'-modified functionality, 5'-phosphonate modification and locked nucleic acids [18]. However, it is unknown whether these stability-enhanced RNAs are appropriate as the positive controls. Our previous study has demonstrated that phosphoroselenaote NTPs can be efficiently recognized by T7 RNA polymerase [20]. Thus, we have hypothesized that phosphoroselenaote and phosphorothioate RNAs (Se-RNA and S-RNA) can be recognized by reverse transcriptase and may

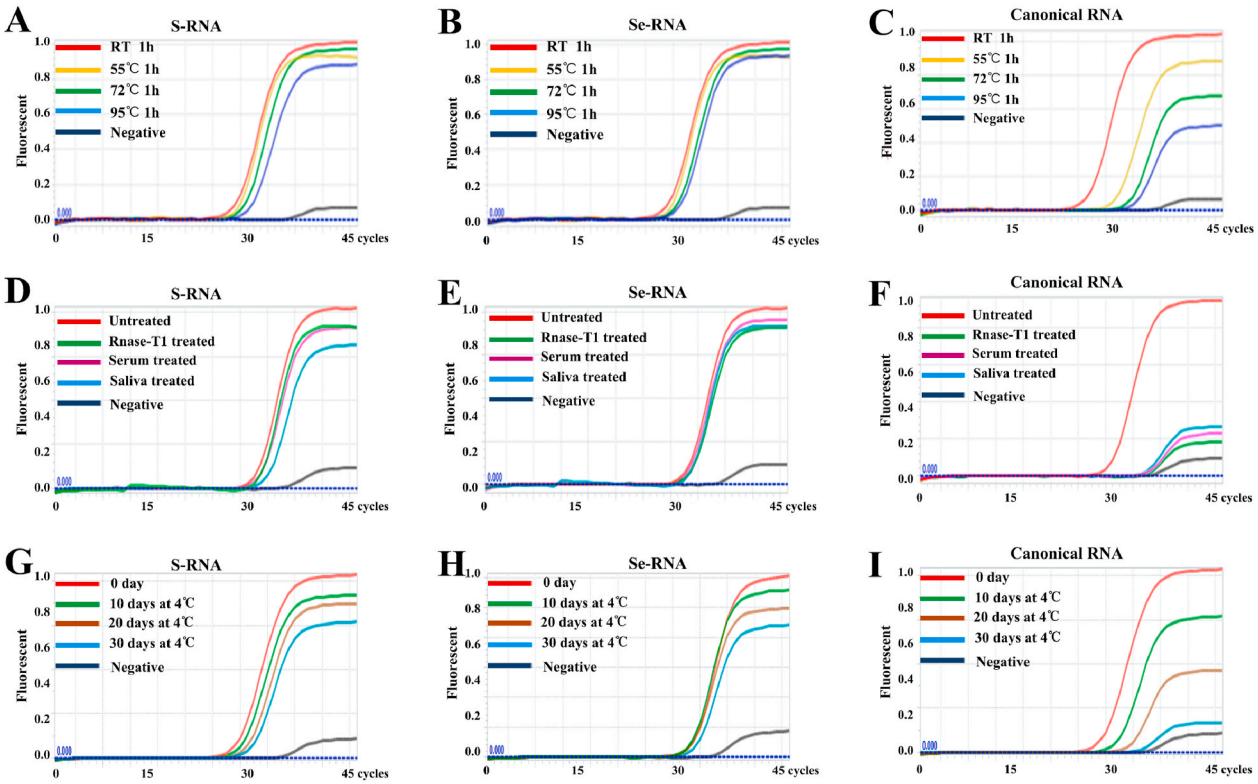

Fig. 4. Enhanced stability of Se-RNA and S-RNA. Figure A–C refer to the thermostability study of Se-RNA, S-RNA and O-RNA at room temperature, 55, 72 and 95 °C for 1 h, respectively. Figure D–F refer to the biostability study of Se-RNA, S-RNA and O-RNA with RNase-T1, serum and saliva, respectively. Figure G–I refer to the chemo-stability study of Se-RNA, S-RNA and O-RNA stored at 4 °C for 0–30 days, respectively.

Table 3
Se-RNA and S-RNA as negative controls for RT-PCR.

Negative Control	Primer Pair 1	2	3	4	5	6	7	8
O-RNA (Ct)	35	35	40	34	33	39	41	>45
S-RNA (Ct)	37	42	>45	41	35	>45	>45	>45
Se-RNA (Ct)	>45	44	>45	36	37	40	>45	>45

be appropriate as positive controls in RT-PCR kits. Consistent with our previous study, we have found that the Se-RNAs and S-RNAs can be not only of high stability, but also efficiently recognized by reverse transcriptase. These results have demonstrated that Se-RNA and S-RNA are excellent alternatives to replace DNAs as positive controls in commercial RT-PCR kits.

Further, since using water as the negative control is not ideal for RT-PCR detection, especially for monitoring mis-detection. Non-specific RNA would be a better choice as negative control. Considering RNA bio-instability, RNA modification is necessary. Our experimental results have showed that the modified RNAs (Se-RNA and S-RNA) are more stable and exclusive (or specific), thereby reducing false negative results and working as preferable negative controls of RT-PCR.

In conclusion, we have found that problematic commercial RT-PCR kit can cause false detection results and the commonly-used DNA positive controls can lead to false negativity, as they can't report the problems in reverse transcription. This increases risk of false negativity in COVID-19 diagnosis. Further, we have discovered that the chemically modified RNAs (Se-RNA and S-RNA) have excellent stability and bioactivity, thereby demonstrating their potentials as both RNA positive and negative controls in RT-PCR kits. In summary, we have developed a novel strategy for RT-PCR detection without false negativity caused by the controls.

Novelty statement

This paper focused on the deficiencies in current commercial RT-PCR kits. We found that when COVID-19 pandemic breaks out, due to the difficulty of preparation a large amount of RNA-based positive control in a short period of time, DNA was widely adopted as positive control in commercial RT-PCR kits. DNA-based control cannot report a kit with problems in reverse transcription, thereby increasing risk of false negative. To face the challenge, we developed chemically modified RNA, which showed high thermostability, biostability, chemostability and specificity, and can be fine templates for RT-PCR. Furthermore, due to its easy preparation, the modified RNAs are fine and feasible alternatives as the positive controls in emergency circumstances. In conclusion, chemically modified RNAs has great potential for the quality improvement of commercial RT-PCR kits.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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