The high-temperature inactivation process prior to viral nucleic acid extraction significantly reduces the quantity of detectable viral nucleic acid templates.

Authors: Zhang Qinxin, Zhao Qingshun, Zhao Qingshun

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Abstract

SARS-CoV-2 is the pathogen of viral infectious pneumonia (COVID-19). However, the false negative rate of SARS-CoV-2 nucleic acid detection in clinical practice is high. False negatives imply missed detection, which not only prevents suspected patients from being rapidly diagnosed but also causes those missed to become potential sources of viral transmission. Therefore, improving the detection rate of SARS-CoV-2 nucleic acid is extremely urgent. Current guidelines from the National Health Commission require that samples be placed at temperatures above 56°C to inactivate the virus before nucleic acid extraction. This inactivation process is undoubtedly necessary to protect clinical testing personnel from viral exposure, but it also compromises the integrity of viral nucleic acids, leading to some samples being undetectable and contributing to the high false negative rate. Recently, we used porcine PDEV coronavirus (vaccine) as a model to study the effect of high-temperature inactivation on viral nucleic acid integrity. The results showed that for samples stored in the commonly used isotonic salt solution Hank' s solution, incubation at 56°C for 30 minutes damaged half of the detectable coronavirus nucleic acid, while incubation at 92°C for 5 minutes resulted in a loss of over 96% of detectable coronavirus nucleic acid. When a commercially available R503 sample preservation solution was used to store PEDV, the amount of detectable viral nucleic acid after incubation at 56°C for 30 minutes was three times that of Hank' s solution, and if incubated at 92°C for 5 minutes, the detectable amount was 42 times that of Hank' s solution. These results suggest that using a sample preservation solution that can effectively protect sample RNA, particularly by preventing sample RNA damage during high-temperature inactivation, not only allows clinical testing personnel to continue using samples after high-temperature inactivation but also holds promise for improving the positive detection rate.

Full Text

Preamble

High-Temperature Inactivation Before Viral Nucleic Acid Extraction Significantly Reduces Detectable Viral Nucleic Acid Template Quantity

Qinxin Zhang, Qingshun Zhao*
Model Animal Research Institute, Nanjing University

*Corresponding author: Model Animal Research Institute, Nanjing University, Nanjing, 210061, qingshun@nju.edu.cn

Abstract: SARS-CoV-2 is the pathogen causing coronavirus disease 2019 (COVID-19). However, the false-negative rate of clinical SARS-CoV-2 nucleic acid detection is extremely high. False negatives imply missed diagnoses, which not only prevents suspected patients from being rapidly confirmed but also allows undetected carriers to become potential sources of viral transmission. Therefore, improving the detection rate of SARS-CoV-2 nucleic acid is urgently needed. Current guidelines from the National Health Commission require samples to be incubated at temperatures above 56°C to inactivate the virus before nucleic acid extraction. This inactivation process is undoubtedly necessary to protect clinical testing personnel from viral exposure, but it also compromises viral nucleic acid integrity, leading to undetectable samples and contributing to the high false-negative rate. Recently, we used porcine PEDV coronavirus (vaccine) as a model to investigate the effects of high-temperature inactivation on viral nucleic acid integrity. The results showed that samples stored in Hank' s solution, a commonly used isotonic salt solution, lost half of their detectable coronavirus nucleic acid after incubation at 56°C for 30 minutes, while incubation at 92°C for 5 minutes resulted in a loss of over 96% of detectable coronavirus nucleic acid. When a commercial sample preservation solution R503 was used to store PEDV, the detectable viral nucleic acid after 56°C incubation for 30 minutes was 3 times that of Hank' s solution, and after 92°C incubation for 5 minutes, it was 42 times that of Hank' s solution. These results suggest that using a sample preservation solution that effectively protects sample RNA, particularly from damage during high-temperature inactivation, can not only allow clinical testing personnel to continue using samples after inactivation but also potentially improve positive detection rates.

Keywords: coronavirus; 2019-nCoV; viral high-temperature inactivation; nucleic acid degradation; false negative; fluorescent quantitative RT-PCR

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1. Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is believed to be the pathogen causing coronavirus disease 2019 (COVID-19) [1]. Whether an

individual carries SARS-CoV-2 has become one of the key clinical indicators for infection, and two consecutive negative SARS-CoV-2 nucleic acid tests are also among the clinical criteria for recovery from COVID-19 pneumonia. However, numerous reports indicate that the false-negative rate of SARS-CoV-2 nucleic acid detection in clinical testing is very high, with a positive rate of only 30-50% [2]. False negatives imply missed diagnoses, which not only prevents rapid confirmation of suspected patients in clinical practice but also allows undetected virus carriers to become potential sources of viral transmission. Therefore, improving the detection rate of SARS-CoV-2 nucleic acid is extremely urgent.

Current SARS-CoV-2 nucleic acid detection primarily uses fluorescent quantitative RT-PCR technology. PCR is an extremely sensitive technique; theoretically, even a single template copy (one virus) can be detected, and when the template quantity exceeds 100 copies per 10 microliters, PCR amplification results are very stable. SARS-CoV-2 is a single-stranded positive-sense RNA virus. The viral gene template used for detection is obtained from patient samples through nucleic acid extraction. A teaching video on the nucleic acid testing process produced by Peking Union Medical College Hospital based on the "Novel Coronavirus Pneumonia Laboratory Testing Technical Guidelines (Third Edition)" issued by the National Health Commission (online version, from 3:08 to 3:40 in the video) shows that before template preparation, collected samples must be inactivated at 56°C for 30 minutes. The "Novel Coronavirus Pneumonia Laboratory Testing Technical Guidelines (Fourth Edition)" issued by the National Health Commission also addresses the operation of inactivated materials: "Nucleic acid detection, antigen detection, serological detection, biochemical analysis, and other operations on infectious materials or live virus after reliable inactivation should be conducted in a Biosafety Level 2 laboratory" [3]. The "Expert Consensus on Novel Coronavirus Pneumonia Virus Nucleic Acid Testing" published by the Chinese Medical Association Laboratory Medicine Branch clearly states that samples must be incubated at 56°C for at least 45 minutes or at higher temperatures for inactivation [4]. The purpose of this process is to inactivate the virus. Due to the extremely strong infectivity of SARS-CoV-2, inactivating collected samples at 56°C has become an essential step required by many frontline clinical testing units to protect the safety of testing personnel.

In this study, we used porcine epidemic diarrhea virus (PEDV), a coronavirus vaccine, as a model to investigate the effects of high-temperature inactivation on coronavirus integrity in Hank' s solution. The results showed that compared with the non-inactivated control, the inactivation process of incubation at 56°C for 30 minutes caused a loss of half of the detectable coronavirus template, while incubation at 92°C for 5 minutes left less than 3.2% of the detectable coronavirus template compared to the control. Interestingly, when R503 was used to preserve the samples, high-temperature inactivation did not affect the detectable viral template quantity. In summary, our findings indicate that high-temperature inactivation promotes degradation of viral nucleic acids in samples, leading to insufficient SARS-CoV-2 nucleic acid quantity and ultimately causing false negatives in some samples. Using an optimized preservation solution can

improve the accuracy of viral nucleic acid detection in samples while ensuring the safety of testing personnel through virus inactivation, thereby avoiding or reducing false negatives.

2.1 Total RNA in Zebrafish Embryo Cells Shows Significant Degradation After 56°C Treatment

Multiple SARS-CoV-2 nucleic acid testing guidelines mention the need for virus inactivation at temperatures above 56°C before nucleic acid extraction to reduce infection risk. However, SARS-CoV-2 is a single-stranded RNA virus, and RNA itself is susceptible to ribonuclease degradation, with the optimal activity temperature for ribonucleases being 60°C. Therefore, this high-temperature inactivation process theoretically damages RNA integrity in samples, improperly reducing viral template quantity and ultimately preventing detection of some samples. To verify this hypothesis, we first tested the effect of 56°C treatment on RNA stability in zebrafish 72 hpf embryo cells.

The experiment was divided into three groups, each with three replicates: Group 1 consisted of embryos treated with Trizol (a ribonuclease inhibitor) followed by incubation in a 56°C water bath for 30 minutes; Group 2 consisted of embryos treated with Trizol followed by static incubation in a 4°C refrigerator for 30 minutes; and Group 3 consisted of embryos treated with 1× PBS followed by incubation in a 56°C water bath for 30 minutes. Total RNA was then extracted from the embryo cells using conventional methods. The quality of the extracted nucleic acids was observed using 1% agarose gel electrophoresis, and the results showed [Figure 1: see original paper] that without protection from ribonuclease inhibitors (Trizol), the 56°C inactivation step caused obvious RNA degradation with significant smearing, and representative low-molecular-weight RNA bands were reduced more noticeably. When zebrafish embryo samples were preserved in Trizol, subjected to 56°C inactivation, and then total RNA was extracted, the results were even worse, with the representative 28S RNA band becoming significantly weaker, indicating that more (long-chain) RNA was degraded.

2.2 Total RNA and Genomic DNA in Human Cells in Hank' s Solution Show Significant Degradation After Treatment Above 56°C

Furthermore, we tested the effect of treatment above 56°C on RNA stability in human cell line cells. Using Hank' s solution as the cell preservation medium, we suspended cells (293T cells) to prepare six experimental sample groups. Each sample group also included porcine PEDV coronavirus (an RNA virus) and bacteriophage DNA (a DNA virus). Four groups were incubated at 56°C for

30, 45, and 60 minutes respectively, the fifth group was incubated at 92°C for 5 minutes, and the sixth group was kept at 4°C as a control.

After all incubations were completed, nucleic acids (including DNA and RNA) were extracted using conventional methods and subjected to 1.2% gel electrophoresis. The results showed [Figure 2: see original paper] that compared with samples stored at 4°C, nucleic acids in samples incubated at 56°C for 30-60 minutes showed obvious degradation, with both 28S and 18S RNA bands becoming blurred to the point of invisibility. After 92°C incubation for 5 minutes, almost no electrophoresis bands were visible.

2.3 Detectable Template Quantity of Porcine PEDV Coronavirus in Hank' s Solution Significantly Decreases After Treatment Above 56°C

To investigate whether treatment above 56°C also damages viral nucleic acids, we used fluorescent quantitative RT-PCR to detect the content of porcine PEDV coronavirus after treatment. The results [Figure 3: see original paper]-6 showed that compared with the Ct (threshold cycle value) of the 4°C control group (31.94 ± 0.10), the average Ct of the 56°C incubation for 30 minutes group (32.94 ± 0.18) increased by 0.996 ($p<0.01$), meaning that the 56°C incubation for 30 minutes treatment left only 50.11% (1/2^0.9967) of the detectable viral template compared to the non-inactivated treatment. For the 92°C inactivation treatment for 5 minutes group, the average Ct (36.84 ± 0.37) increased by 4.8947 ($p<0.0001$), meaning that the 92°C inactivation treatment for 5 minutes left only 3.36% (1/2^4.8947) of the detectable viral template compared to the non-inactivated treatment.

Furthermore, we used fluorescent quantitative PCR to detect viral DNA (phage) in the samples. The results [Figure 7: see original paper]-8 showed that compared with the Ct of the 4°C storage group (23.02 ± 0.15), the average Ct after 56°C incubation for 30 minutes (23.55 ± 0.20) increased by 0.5230 ($p<0.01$), meaning that samples stored in Hank' s solution retained only 69.59% (1/2^0.5230) of the detectable viral template after 56°C incubation for 30 minutes compared to the non-high-temperature treatment. For the 92°C inactivation incubation for 5 minutes group, the average Ct (25.58 ± 0.21) increased by 2.5572 ($p<0.0001$), meaning that samples stored in Hank' s solution retained only 16.99% (1/2^2.5572) of the detectable viral template after 92°C incubation for 5 minutes. These results demonstrate that high-temperature treatment significantly reduces the detectable quantity of both RNA and DNA viruses.

2.4 Sample Preservation Solution Containing SDS Can Partially Protect RNA in Human 293T Cells from Degradation Caused by High-Temperature Treatment

To improve the detectable quantity of viral nucleic acids in samples after high-temperature treatment, developing new sample preservation solutions is essential. To this end, we first prepared three preservation solutions (Solution 1, Solution 2, and Solution 3) to test whether any could protect viral samples from damage caused by high-temperature treatment.

The experiment was divided into three groups, with human cell line cells (293T) treated with Solution 1, Solution 2, and Solution 3 respectively. Each group had a 56°C treatment subgroup (incubated in a 56°C water bath for 30 minutes) and a 4°C storage subgroup (stored in a 4°C refrigerator for 30 minutes). After all treatments were completed, total RNA was extracted from the samples using conventional methods and subjected to 1.0% agarose gel electrophoresis. The results showed [Figure 9: see original paper] that high temperature had the greatest impact on samples in Solution 1, with the characteristic 28S and 18S bands completely disappearing. In contrast, RNA quality in Solution 2 (containing proteinase K) and Solution 3 was better, with 28S and 18S bands clearly visible in Solution 2 samples and almost no smearing observed that was seen in other groups. These results suggest that SDS (absent in Solution 1 but present in both Solution 2 and 3) may have a protective effect against RNase degradation, and the combination of proteinase K and SDS may provide even better protection (Solution 2 contains both substances, while Solution 3 contains only SDS). Interestingly, when samples stored in the three preservation solutions were kept at 4°C for half an hour, obvious RNA degradation still occurred, possibly because SDS solubility decreases at low temperatures.

2.5 R503 Significantly Improves Protection of RNA and Genomic DNA in Human 293T Cells

To further optimize protection of RNA from damage caused by high-temperature treatment, we tested R503 (Nanjing Vazyme Biotech Co., Ltd.) as a cell preservation solution. Similar to the Hank' s solution experiment, we prepared six experimental sample groups using R503 (each sample group also included porcine PEDV coronavirus and bacteriophage DNA). Four groups were incubated at 56°C for 30, 45, and 60 minutes respectively, the fifth group was incubated at 92°C for 5 minutes, and the sixth group was kept at 4°C as a control. After all incubations were completed, nucleic acids were extracted using conventional methods and subjected to 1.2% agarose gel electrophoresis. The results showed [Figure 10: see original paper] that compared with samples stored at 4°C, nucleic acids in samples incubated at 56°C for 30-60 minutes showed obvious degradation, with significant smearing near the 28S and 18S bands indicating human cell total RNA and reduced brightness of large fragment bands indicating genomic

DNA. After 92°C incubation for 5 minutes, almost no genomic DNA bands or 28S bands were visible. However, compared with Hank' s solution, nucleic acid degradation was less severe, suggesting that R503 provides significant protection for nucleic acids (both RNA and genomic DNA).

2.6 Detectable Viral Template Quantity of Porcine PEDV Coronavirus in R503 Preservation Solution Shows No Significant Change After Treatment Above 56°C

To investigate whether treatment above 56°C also damages viral nucleic acids preserved in R503, we used fluorescent quantitative RT-PCR to detect the content of porcine PEDV coronavirus after high-temperature treatment. The results [Figure 11: see original paper]-14 showed that compared with the Ct of the 4°C control group (31.64 ± 0.10), the average Ct of the 56°C incubation for 30 minutes group (31.31 ± 0.17) showed no significant change (difference less than 0.5). Similarly, the average Ct of the 92°C inactivation treatment for 5 minutes group (31.43 ± 0.08) showed no significant change (difference less than 0.5). These results indicate that treatment above 56°C did not significantly affect the detectable quantity of virus preserved in R503, suggesting that although viral nucleic acids were degraded, the remaining small RNA fragments could still be used as templates for fluorescent quantitative RT-PCR without significant change in template quantity.

Furthermore, we used fluorescent quantitative PCR to detect phage DNA content after high-temperature treatment. The results [Figure 15: see original paper]-16 showed that compared with the Ct of the 4°C control group (21.61 ± 0.06), the average Ct of the 56°C incubation for 30 minutes group (21.71 ± 0.04) showed no significant change (difference less than 0.5). However, the average Ct of the 92°C inactivation treatment for 5 minutes group (22.90 ± 0.26) increased by 1.2880, meaning that samples stored in R503 retained only 40.95% (1/2^1.2880) of the detectable viral template after 92°C incubation for 5 minutes. This suggests that even when stored in R503, 92°C treatment significantly reduces the detectable quantity of DNA viruses.

2.7 RNA Coronavirus Template Quantity Detectable in R503-Preserved Samples After 56°C Treatment Is Significantly Higher Than in Hank' s Solution-Preserved Samples

To compare the performance of R503 versus common isotonic solution in protecting detectable quantities of trace RNA coronavirus, we first examined whether there was any difference in detectable RNA coronavirus quantity between the two preservation solutions under 4°C storage conditions. The results showed [Figure 17: see original paper] that virus detection rates were similar under

both preservation solutions, with the difference in average Ct values being less than 0.5 (3.64 ± 0.10 vs 31.95 ± 0.10), suggesting no significant difference in detectable viral template quantity between the two preservation solutions at 4°C.

However, after 56°C incubation for 30 minutes, the virus detection Ct values were 31.31 ± 0.17 (R503) and 32.94 ± 0.18 (Hank' s). The average Ct of Hank' s-preserved samples increased by 1.6305 compared to R503 ($p<0.0001$; [Figure 18: see original paper]), meaning that after 56°C inactivation treatment for 30 minutes, Hank' s solution-preserved samples retained only 32.30% (1/2^1.6305) of the detectable viral template compared to R503-preserved samples. Furthermore, after 92°C inactivation treatment for 5 minutes, the virus detection Ct values were 31.43 ± 0.08 (R503) and 36.84 ± 0.37 (Hank' s). The average Ct of Hank' s solution-preserved samples increased by 5.4125 compared to R503 ($p<0.0001$; [Figure 19: see original paper]), meaning that after 92°C inactivation treatment for 5 minutes, Hank' s solution-preserved samples retained only 2.35% (1/2^5.4125) of the detectable viral template compared to R503-preserved samples.

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3. Discussion

RNA integrity is susceptible to destruction by ribonucleases, and strict requirements for proper handling of RNA samples are emphasized in basic molecular biology training. Trainees are always required to operate under RNase-free conditions, including wearing masks during experimental operations and not speaking directly toward samples; wearing gloves and not touching samples directly with hands; using RNase-free pipette tips (filter tips may be necessary to prevent contamination from pipettors) and RNase-free reagents; and cleaning work surfaces, with RNase decontamination reagents used when necessary. Before samples are protected by RNase inhibitors, they must be kept in ice boxes or 4°C refrigerators. In short, RNA degradation by ribonucleases must be prevented throughout the entire operation process.

In clinical testing, particularly when detecting nucleic acids from highly infectious viruses, testing personnel are constantly threatened by exposure due to incomplete hardware conditions. Therefore, high-temperature inactivation before "opening" samples has become a consensus among experts to protect clinical testing personnel. However, theoretically, this high-temperature inactivation treatment damages sample RNA, including the integrity of any viral RNA present, reducing the detectable quantity of viral template.

In this study, we treated zebrafish embryo cells and human cell line cells at temperatures above 56°C and found significant degradation of total cellular RNA. Using swine fever virus as a coronavirus model, we found that samples stored in common isotonic solution lost half of their detectable coronavirus template

after 56°C incubation for 30 minutes, and over 96% of detectable coronavirus template after 92°C incubation for 5 minutes.

SARS-CoV-2 is threatening the health and safety of people nationwide and worldwide with its extremely strong infectivity. However, the positive rate of SARS-CoV-2 detection using fluorescent quantitative RT-PCR is currently only 30-50%, resulting in an extraordinarily high false-negative rate. Many factors contribute to this high false-negative rate, and template quality should be considered an important factor.

A recently published study by Chen Peisong et al. stated that treating oropharyngeal swab specimens at 56°C for 30 minutes had no significant effect on subsequent 2019 novel coronavirus nucleic acid qPCR detection [5]. However, the authors also mentioned limitations in their discussion: "1) Due to the small number of positive oropharyngeal swab specimens, verification was only possible with two positive specimens; 2) For the two positive specimens tested, both CT values were relatively high (19 and 24 before dilution), and whether the same correlation exists for specimens with lower CT values requires further investigation; 3) The experimental results were completed relatively early (finished on January 24), when single-channel reagents without internal references were commonly used—combining internal reference genes, N gene, and ORF gene in the analysis would provide more detailed verification information; 4) This study only compared reagents from one manufacturer, and whether reagents from other manufacturers show the same results requires more data support." Considering these limitations and combined with experimental evidence of high-temperature inactivation damaging porcine coronavirus nucleic acid integrity, we recommend immediately launching systematic research on the effects of high-temperature inactivation on SARS-CoV-2 nucleic acid integrity and developing sample preservation solutions that protect SARS-CoV-2 nucleic acid from high-temperature inactivation damage:

1) Research should confirm whether high-temperature inactivation indeed affects SARS-CoV-2 nucleic acid integrity, leading to a significant decrease in detectable viral nucleic acid quantity. Theoretically, when RNase activity is not inhibited, high-temperature inactivation treatment causes nucleic acid degradation, significantly reducing viral nucleic acid detection rates. Our experiments using porcine PEDV coronavirus as a model support this theoretical speculation. However, our research was conducted only on porcine PEDV coronavirus, and direct evidence is still lacking on whether the detectable quantity of SARS-CoV-2 decreases after high-temperature inactivation (we do not have SARS-CoV-2 materials and are not yet qualified to conduct direct SARS-CoV-2 research). Therefore, it is extremely urgent to immediately initiate research on the impact of high-temperature inactivation on SARS-CoV-2 nucleic acid detectability.

2) Research and development of sample preservation solutions that protect SARS-CoV-2 nucleic acid from high-temperature inactivation damage are needed to improve detectable SARS-CoV-2 nucleic acid quantity. High-

temperature inactivation is a necessary measure to protect frontline testing personnel and is therefore widely used in clinical SARS-CoV-2 nucleic acid testing. Theoretically, if RNase activity can be inhibited, the high-temperature inactivation process will have less impact on viral nucleic acid integrity. In this study, when porcine PEDV was stored in R503 preservation solution, the detectable viral nucleic acid after 56°C incubation for 30 minutes was 3 times that of samples stored in Hank' s solution; after 92°C incubation for 5 minutes, the detectable coronavirus quantity was 42 times that of Hank' s solution-preserved samples. These results suggest that a preservation solution exists that can effectively protect viral nucleic acid integrity and thereby improve its detectable quantity. However, this conclusion still lacks direct evidence from SARS-CoV-2. Using SARS-CoV-2 as a research subject requires first answering whether virus preserved in the new preservation solution remains infectious after high-temperature inactivation, and second, whether this RNase-inhibiting preservation solution can indeed improve SARS-CoV-2 nucleic acid detectable quantity after high-temperature inactivation.

High-temperature inactivation also damages DNA integrity, reducing the detectable template quantity of DNA viruses. This will not be discussed in detail here.

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Appendix 1: Figures and Legends

Figure 1. Electrophoresis results of total RNA quality from zebrafish 72 hpf embryo cells after 56°C incubation. From left to right: Lane 1 is DNA Marker (from bottom to top: 100 bp, 250 bp, 500 bp, 750 bp, 1000 bp, 2000 bp, 3000 bp, 5000 bp); Lanes 2-4 are total RNA extracted after 56°C incubation for 30 minutes following TriZol addition; Lanes 5-7 are total RNA extracted after 4°C storage following TriZol addition; Lanes 8-10 are total RNA extracted after 56°C incubation for 30 minutes following PBS addition.

Figure 2. Agarose gel electrophoresis results of nucleic acids extracted from samples stored in Hank' s solution after different inactivation treatments. From left to right: Lane 1 is DNA Marker (Vazyme #MD103); Lanes 2-3 are total RNA and DNA extracted after 56°C incubation for 30 minutes; Lanes 4-5 are total RNA and DNA extracted after 56°C incubation for 45 minutes; Lanes 6-7 are total RNA and DNA extracted after 56°C incubation for 60 minutes; Lanes 8-9 are the control stored at 4°C; Lanes 10-11 are total RNA and DNA extracted after 92°C incubation for 5 minutes.

Figure 3. Fluorescent quantitative RT-PCR results (Ct values) of porcine PEDV coronavirus nucleic acids extracted from samples stored in Hank' s solution after high-temperature treatment above 56°C. H-4-0: control sample stored at 4°C; H-56-30: sample incubated at 56°C for 30 minutes; H-56-45: sample incubated at 56°C for 45 minutes; H-56-60: sample incubated at 56°C for 60 minutes; H-92-5: sample incubated at 92°C for 5 minutes. Each incubation treatment had one replicate, and each sample had one duplicate well in quantitative PCR. NTC: negative control.

Figure 4 [Figure 4: see original paper]. Typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples stored in Hank' s solution after different high-temperature treatments. Different colors represent different treatments (see Figure 3).

Figure 5 [Figure 5: see original paper]. Comparative analysis of typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples stored in Hank' s solution after 56°C incubation for 30 minutes versus 4°C storage control samples. Different colors represent treatment types shown on the right.

Figure 6 [Figure 6: see original paper]. Comparative analysis of typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples stored in Hank' s solution after 92°C incubation for 5 minutes versus 4°C storage control samples. Different colors represent treatment types shown on the right.

Figure 7. Fluorescent quantitative PCR results (Ct values) of phage virus DNA extracted from samples stored in Hank' s solution after high-temperature

treatment above 56°C. H-4-0: control sample stored at 4°C; H-56-30: sample incubated at 56°C for 30 minutes; H-56-45: sample incubated at 56°C for 45 minutes; H-56-60: sample incubated at 56°C for 60 minutes; H-92-5: sample incubated at 92°C for 5 minutes. Each incubation treatment had one replicate, and each sample had one duplicate well in quantitative PCR. NTC: negative control.

Figure 8 [Figure 8: see original paper]. Typical amplification curves of fluorescent quantitative PCR for phage virus DNA extracted from samples stored in Hank' s solution after different high-temperature treatments. Different colors represent different treatments (see Figure 7).

Figure 9. Electrophoresis results of total RNA quality from human 293T cells after 56°C incubation for 30 minutes in different preservation solutions. From left to right: Lane 1 is DNA Marker (from bottom to top: 100 bp, 250 bp, 500 bp, 750 bp, 1000 bp, 2000 bp, 3000 bp, 5000 bp); Lanes 2-3 are total RNA extracted with RNA preservation Solution 1, with loading order of 56°C and 4°C; Lanes 4-5 and 6-7 are total RNA extracted with RNA preservation Solution 2 and Solution 3 respectively, with the same loading order as Solution 1.

Figure 10. Agarose gel electrophoresis results of nucleic acids extracted from samples preserved in R503 after different high-temperature treatments. From left to right: Lane 1 is DNA Marker (Vazyme #MD103); Lanes 2-3 are total RNA and DNA extracted after 56°C incubation for 30 minutes; Lanes 4-5 are total RNA and DNA extracted after 56°C incubation for 45 minutes; Lanes 6-7 are total RNA and DNA extracted after 56°C incubation for 60 minutes; Lanes 8-9 are the control stored at 4°C; Lanes 10-11 are total RNA and DNA extracted after 92°C incubation for 5 minutes.

Figure 11. Fluorescent quantitative RT-PCR results (Ct values) of porcine PEDV coronavirus nucleic acids extracted from samples preserved in R503 after high-temperature treatment above 56°C. V-4-0: control sample stored at 4°C; V-56-30: sample incubated at 56°C for 30 minutes; V-56-45: sample incubated at 56°C for 45 minutes; V-56-60: sample incubated at 56°C for 60 minutes; V-92-5: sample incubated at 92°C for 5 minutes. Each incubation treatment had one replicate, and each sample had one duplicate well in quantitative PCR. NTC: negative control.

Figure 12 [Figure 12: see original paper]. Typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples preserved in R503 solution after different high-temperature treatments. Different colors represent different treatments (see Figure 11).

Figure 13 [Figure 13: see original paper]. Comparative analysis of typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples preserved in R503 solution after 56°C incubation for 30 minutes versus 4°C storage control samples. Different colors represent treatment types shown on the right.

Figure 14 [Figure 14: see original paper]. Comparative analysis of typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples preserved in Hank's solution after 92°C incubation for 5 minutes versus 4°C storage control samples. Different colors represent treatment types shown on the right.

Figure 15. Fluorescent quantitative PCR results (Ct values) of phage virus DNA extracted from samples preserved in R503 after high-temperature treatment above 56°C. V-4-0: control sample stored at 4°C; V-56-30: sample incubated at 56°C for 30 minutes; V-56-45: sample incubated at 56°C for 45 minutes; V-56-60: sample incubated at 56°C for 60 minutes; V-92-5: sample incubated at 92°C for 5 minutes. Each incubation treatment had one replicate, and each sample had one duplicate well in fluorescent quantitative PCR. NTC: negative control.

Figure 16 [Figure 16: see original paper]. Typical amplification curves of fluorescent quantitative PCR for phage virus DNA extracted from samples preserved in R503 after different high-temperature treatments. Different colors represent different treatments (see Figure 15).

Figure 17. Comparative analysis of typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples preserved in R503 solution and Hank' s solution after 4°C storage. Different colors represent treatment types shown on the right.

Figure 18. Comparative analysis of typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples preserved in R503 solution and Hank' s solution after 56°C incubation for 30 minutes. Different colors represent treatment types shown on the right.

Figure 19. Comparative analysis of typical amplification curves of fluorescent quantitative RT-PCR for viral RNA extracted from porcine PEDV coronavirus samples preserved in R503 solution and Hank' s solution after 92°C incubation for 5 minutes. Different colors represent treatment types shown on the right.

Appendix 2: Materials and Methods

S2.1 Experiment on Effect of 56°C Incubation on Total RNA Quality in Zebrafish 72 hpf Embryo Cells

The zebrafish experimental protocol complied with the relevant experimental animal care and management regulations of Nanjing Yaoshunyu Biotechnology Co., Ltd. and was approved by the IACUC. For the experiment, 72 hpf zebrafish embryos were collected for total RNA extraction, with 15 embryos per group. After removing water, total RNA was obtained using the TriZol method.

1) The experiment was divided into three groups with three replicates each: Group 1 had 500 μl Trizol added to each replicate, placed in a 56°C wa-

ter bath for 30 minutes, then homogenized (optimally until no chunks remained); Group 2 had 500 µl Trizol added to each replicate, placed in a 4°C refrigerator for 30 minutes, then homogenized; Group 3 had 500 µl 1× PBS added to each replicate, placed in a 56°C water bath for 30 minutes, then the PBS solution was removed, 500 µl Trizol was added, and samples were homogenized.

2) 100 µl chloroform was added to the lysate. The centrifuge tube cap was tightly closed, vigorously shaken for 15 seconds, and left at room temperature for 3 minutes.

3) Centrifuged at 12,000 g for 15 minutes at 4°C.

4) The upper aqueous phase was carefully transferred to a new centrifuge tube, and 250 µl isopropanol was added. After mixing by inversion, the sample was left at room temperature for 10 minutes.

5) Centrifuged at 12,000 g for 10 minutes at 4°C.

6) The supernatant was carefully discarded, and 500 µl 75% ethanol (prepared with DEPC water) was added. Vortexed thoroughly to wash, and the tube bottom was gently flicked to suspend the pellet.

7) Centrifuged at 7,500 g for 5 minutes at 4°C, supernatant discarded, taking care not to lose the RNA pellet.

8) Steps 6-7 were repeated.

9) Dried in a fume hood for 10 minutes. 15 µl DEPC water was added to dissolve the RNA. After complete dissolution, a small amount was taken.

10) 1 µl was used for agarose gel electrophoresis.

S2.2 Experiment on Effect of Different Preservation Solutions on Total RNA Quality After 56°C Incubation for 30 Minutes

Human cell suspension was collected at 1,800 µl per tube, centrifuged at 1,500 g for 10 minutes, the supernatant was discarded, and the pellet was retained. A total of 6 tubes were collected, and total RNA was obtained using the TriZol method.

1) The 6 tubes were divided into 3 experimental groups: Group 1 had 500 µl RNA preservation Solution 1 (100 mM Tris (pH7.5), 12.5 mM EDTA, 150 mM NaCl, 250 µg/ml proteinase K) added, with one tube placed in a 56°C water bath for 30 minutes and another tube placed in a 4°C refrigerator for 30 minutes; Group 2 had 500 µl RNA preservation Solution 2 (0.5% SDS, 0.1 M NaCl, 0.01 M Tris-HCl (pH8.0), 0.05 M EDTA, 100 µg/ml proteinase K) added, with one tube placed in a 56°C water bath for 30 minutes and another tube placed in a 4°C refrigerator for 30 minutes; Group 3 had 500 µl RNA preservation Solution 3 (100 mM EDTA (pH8.0), 100 mM NaCl, 50 mM Tris-HCl (pH8.0), 0.5% SDS) added, with one tube placed in a 56°C

water bath for 30 minutes and another tube placed in a 4°C refrigerator for 30 minutes. After 30 minutes, all samples were centrifuged at 12,000 g for 5 minutes at 4°C, the supernatant was carefully discarded, and 2 ml TriZol solution was added.

2) 400 µl chloroform was added to the lysate. The centrifuge tube cap was tightly closed, vigorously shaken for 15 seconds, and left at room temperature for 3 minutes.
3) Centrifuged at 12,000 g for 15 minutes at 4°C.
4) The upper aqueous phase was carefully transferred to a new centrifuge tube, and 1,000 µl isopropanol was added. After mixing by inversion, the sample was left at room temperature for 10 minutes.
5) Centrifuged at 12,000 g for 10 minutes at 4°C.
6) The supernatant was carefully discarded, and 1,000 µl 75% ethanol (prepared with DEPC water) was added. Vortexed thoroughly to wash, and the tube bottom was gently flicked to suspend the pellet.
7) Centrifuged at 7,500 g for 5 minutes at 4°C, supernatant discarded, taking care not to lose the RNA pellet.
8) Steps 6-7 were repeated.
9) Dried in a fume hood for 10 minutes. 20 µl DEPC water was added to dissolve the RNA. After complete dissolution, a small amount was taken.
10) 1 µl was used for agarose gel electrophoresis.

S2.3 Experiment on Effect of Different High-Temperature Treatments on Total RNA, Genomic DNA, RNA Virus RNA, and DNA Virus DNA Quality in Samples Preserved with Hank' s and R503

S2.3.1 Materials

S2.3.1.1 Experimental Model Samples

1) HEK-293T cells (self-cultured)
2) Porcine transmissible gastroenteritis and porcine epidemic diarrhea combined live vaccine (WH-1R strain + AJ1102-R strain) (Wuhan Keqian Biology Co., Ltd.) (Note: Although it is a combined vaccine, the primer design only targets PEDV, so PEDV is detected)
3) DNA (Takara #3010)

S2.3.1.2 Experimental Reagents

1) Virus sample preservation solution (Vazyme #R503)
2) Hank' s solution (self-prepared)
3) Virus DNA/RNA extraction kit (Vazyme #RC311-C1)

4) HiScript II U+ One Step qRT-PCR Probe Kit (Vazyme #Q222-CN)
5) Equalbit 1× dsDNA HS Assay Kit (Vazyme #EQ121)
6) Equalbit RNA HS Assay Kit (Vazyme #EQ211)
7) DL 15000 DNA Marker (Vazyme #MD103)
8) 10× DNA Loading buffer (Vazyme #P022)
9) PEDV detection primers and probe (5' -3')
   - PEDV-F1: CGTGAGCCTGGCTTAGTCTTG
   - PEDV-R1: CATACGTCGCGATGAAACAAA
   - Probe (5'-3'): PEDV-P1: FAM-CGCATGAACTTCAAAATCATACTGCGACG-BHQ-1
   - Primer dilution method: Dilute to 10 µM with RNase-free water
10) DNA detection primers and probe (5' -3')
   - lambda DNA-QF4: TTTGCTGCGGTTGCAGAA
   - lambda DNA-QR4: ATGATTCGGTTTTCAGGAACATC
   - Probe (5'-3'): lambda DNA-QP4: FAM-TTACCGTCACCGCCAGTTAATCCGG-BHQ-1
   - Primer dilution method: Dilute to 10 µM with RNase-free water

S2.3.2 Experimental Methods

S2.3.2.1 Experimental Model Preparation Method

1) Model preparation:
   - Vazyme virus sample preservation solution R503 (1.5 ml): 1.36×10
   - Hank' s solution (1.5 ml): 1.36×10
   - HEK-293T: 1.5 µL
   - DNA: 1.5 µL
2) Treatment protocol: Mixed tubes were prepared according to the table above, aliquoted at 1.5 mL per tube, and then placed in a 4°C refrigerator, 56°C water bath, or 92°C water bath for different time treatments.

S2.3.2.2 Extraction Method (Vazyme #RC311-C1)

1) Vazyme virus sample preservation solution R503: 200 µL anhydrous ethanol was pre-aliquoted into 1.5 mL RNase-free centrifuge tubes (self-provided), then 500 µL virus sample preservation solution sample was added and vortexed to mix. Hank' s solution: 500 µL lysis solution was added to 1.5 mL RNase-free centrifuge tubes (self-provided, multiple samples could be pre-aliquoted), then 200 µL Hank' s solution sample was added and vortexed to mix.
2) The adsorption column was placed in a 2 mL collection tube, the above mixture was transferred to the adsorption column, and centrifuged at 12,000×g for 1 minute.
3) The filtrate was discarded, the adsorption column was placed back in the 2 mL collection tube, 600 µL wash solution was added, centrifuged at

12,000×g for 30 seconds, and the filtrate was discarded.

4) Step 3 was repeated once.

5) The adsorption column was placed back in the collection tube and centrifuged at 12,000×g for 2 minutes without any solution.

6) The adsorption column was transferred to a new 1.5 mL collection tube (provided with the kit). For Vazyme virus sample preservation solution: 100 µL elution solution was added to the center of the adsorption column membrane, left at room temperature for 1 minute, and centrifuged at 12,000×g for 1 minute. For Hank' s solution: 40 µL elution solution was added to the center of the adsorption column membrane, left at room temperature for 1 minute, and centrifuged at 12,000×g for 1 minute.

7) The adsorption column was discarded, and the obtained DNA/RNA could be used directly for subsequent detection or stored short-term at -30 to -15°C or long-term at -70°C or below.

S2.3.2.3 Detection Methods

1) Extracted cell sample gDNA/RNA detection 1.1) Cell gDNA concentration Qubit detection (Vazyme #EQ121) 1.1.1) Before use, all components in the kit were equilibrated to room temperature. 1.1.2) A sufficient number of 0.5 mL PCR tubes were taken for sample and standard detection and labeled on the tube caps. Note: Only 0.5 mL PCR tubes can be used. Qubit assay tubes (Cat. No. Q32856) or Axygen PCR-05-C tubes (VWR, part no. 10011-830) are recommended. 1.1.3) Detection standards were prepared. 190 µL Equalbit 1× dsDNA HS Working Solution from the kit was added to standard PCR tubes, then 10 µL Equalbit 1× dsDNA HS Standard #1 and Standard #2 were added to the corresponding standard PCR tubes, gently vortexed for 2-3 seconds, and bubbles were avoided. Ensure accurate pipetting in this step. 1.1.4) Detection samples were prepared. 199 µL Equalbit 1× dsDNA HS Working Solution was added to sample PCR tubes, then 1 µL of the dsDNA sample to be tested was added, making the final volume in each detection sample PCR tube 200 µL, gently vortexed for 2-3 seconds, and bubbles were avoided. Ensure accurate pipetting in this step. 1.1.5) All detection PCR tubes were incubated at room temperature protected from light for 2 minutes. 1.1.6) Concentration was measured according to the Qubit fluorometer operation instructions by selecting the dsDNA high-sensitivity detection program.

   1.2) Cell RNA concentration Qubit detection (Vazyme #EQ211) 1.2.1) Before use, all components in the kit were equilibrated to room temperature, and the extraction product was diluted 1:10 with RNase-free H2O. 1.2.2) A sufficient number of 0.5 mL PCR tubes were taken for sample and standard detection and labeled on the tube caps. Note: Only 0.5 mL PCR tubes can be used. Qubit assay tubes (Cat. No. Q32856) or Axygen

PCR-05-C tubes (VWR, part no. 10011-830) are recommended. 1.2.3) Detection working solution was prepared. Equalbit RNA HS Reagent from the kit was diluted with Equalbit RNA HS Buffer at a 1:200 ratio to prepare the detection working solution, which was prepared fresh. Detection standards were prepared. 190 µL detection working solution was added to standard PCR tubes, then 10 µL Standard #1 and Standard #2 were added to the corresponding standard PCR tubes, gently vortexed for 2-3 seconds, and bubbles were avoided. Ensure accurate pipetting in this step. 1.2.4) Detection samples were prepared. 199 µL detection working solution was added to sample PCR tubes, then 1 µL of the RNA sample to be tested was added, making the final volume in each detection sample PCR tube 200 µL, gently vortexed for 2-3 seconds, and bubbles were avoided. Ensure accurate pipetting in this step. 1.2.5) All detection PCR tubes were incubated at room temperature protected from light for 2 minutes. 1.2.6) Concentration was measured according to the Qubit fluorometer operation instructions by selecting the RNA high-sensitivity detection program.

1.3) Cell RNA concentration and purity OneDropt detection 1.3.1) The detection probe was cleaned and calibrated with elution solution, then 1 µL of sample was taken for RNA concentration and purity detection according to the OneDropt instrument operation instructions.

1.4) Cell gDNA and RNA integrity detection (Vazyme #MD103, P022) 1.4.1) 1.2% agarose gel was prepared. 1.4.2) 5 µL of extraction product was taken, 1 µL 10× DNA Loading buffer and 4 µL RNase-free H2O were added, vortexed to mix, then denatured in a PCR instrument at 72°C for 2 minutes. 1.4.3) 5 µL DL15000 Marker was loaded together. 1.4.4) Agarose gel electrophoresis was performed on an electrophoresis apparatus at 220V on ice for 7-10 minutes. 1.4.5) The gel was photographed with a gel imaging system.

2) Extracted trace sample DNA/RNA detection (Vazyme #Q222-CN) 2.1) The following mixture was prepared in RNase-free centrifuge tubes (using ABI StepOnePlus™ as the testing instrument): - RNase-free ddH2O: to 25 µL - 2× One Step U+ Mix: 12.5 µL - One Step U+ Enzyme Mix: 1.25 µL - 50× ROX Reference Dye 1: 0.5 µL - Gene Specific Primer Forward (10 µM): 0.5 µL - Gene Specific Primer Reverse (10 µM): 0.5 µL - TaqMan Probe (10 µM): 0.25 µL

2.2) One Step qRT-PCR reaction was performed under the following conditions: - Stage 1: Reps: 1, 55°C, 15 min - Stage 2: Reps: 1, 95°C, 30 sec - Stage 3: Reps: 45, 95°C, 10 sec; 60°C, 30 sec

S2.4 Statistical Tests

Group t-tests were used. $p<0.05$ indicates significant difference, $p<0.01$ indicates extremely significant difference.