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Nucleic acid detection of norovirus in food

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Abstract. Norovirus is a common foodborne virus that can cause diseases such as acute gastroenteritis, posing a threat to food safety and human health. As a non-enveloped, positive-sense, single-stranded RNA virus, norovirus cannot be detected using conventional virus isolation and culture methods because no stable and broadly applicable in vitro cell culture system has yet been established. Therefore, nucleic acid-based detection techniques offer a more suitable approach for norovirus detection. Among these techniques, reverse transcription quantitative Real-Time PCR (RT-qPCR), regarded as the gold standard for norovirus detection, warrants extensive study. This study reviews and analyzes norovirus enrichment methods, retrieves gene sequences of specific norovirus species, and designs PCR primers based on these sequences. It primarily examines an experimental report by Gao et al. on a norovirus contamination survey conducted between 2016 and 2017 in Heilongjiang Province, a major berry-producing region in China. The survey investigated norovirus contamination in commercially available fresh and frozen berries. Through independent analysis of the report, this study explores the experimental system of RT-qPCR. In addition, it summarizes existing norovirus detection methods, analyzes the prospects for future norovirus detection, and expresses an optimistic outlook on other emerging technologies.

Keywords: norovirus, Real-time RT-PCR, analysis of the experimental system

1. Introduction

Foodborne diseases are among the most prominent public health challenges worldwide. According to statistics from the World Health Organization (WHO), approximately 866 million people worldwide fall ill each year as a result of consuming contaminated food, and approximately 1.52 million of them die. Productivity losses caused by unsafe food amount to as much as USD 310 billion annually. Furthermore, the burden of foodborne diseases is unevenly distributed across regions and populations, with low-income countries and children experiencing more severe health consequences[1]. Biological hazards, including foodborne bacterial, viral, and parasitic infections, account for the vast majority of foodborne disease cases. Therefore, controlling biological hazards is essential to reducing the incidence of foodborne diseases.

[1] World Health Organization. Estimating the burden of foodborne diseases [EB/OL]. https://www.who.int/activities/estimating-the-burden-of-foodborne-diseases.

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Norovirus is a common food contaminant belonging to the family Caliciviridae. It is a non-enveloped, positive-sense, single-stranded RNA virus and has become the leading cause of acute gastroenteritis. Infection can cause symptoms such as vomiting, diarrhea, abdominal pain, and fever, and may even result in death. Norovirus is estimated to cause approximately 685 million cases and 200,000 deaths worldwide each year, imposing a total socioeconomic burden of more than USD 60 billion [1]. To date, with the exception of a limited number of reports related to antiviral treatment in humans, no human norovirus vaccine has been approved for marketing [2]. Therefore, rapid and early detection of norovirus infection is of great significance, as it facilitates early intervention, symptomatic management, and infection control.

Based on variations in the capsid protein VP1, encoded by ORF2, noroviruses are classified into several genogroups, including GI, GII, GIV, GVIII, and GIX. Among these, GI and GII are the predominant genogroups, accounting for more than 99% of all infection cases [3]. The norovirus genome contains three Open Reading Frames (ORFs). ORF1 encodes a large nonstructural polyprotein that is cleaved by a virus-encoded protease into six nonstructural proteins: p48, NTPase, p22, VPg, 3CL protease, and RNA-dependent RNA polymerase. These proteins participate in viral replication and transcription. ORF2 encodes the major capsid protein VP1, which forms the outer shell of the viral particle. VP1 contains a highly conserved shell (S) domain and a highly variable protruding (P) domain. The P domain is a key region for viral binding to host Histo-Blood Group Antigen (HBGA) receptors and is also a major target of neutralizing antibodies. ORF3 encodes the minor capsid protein VP2, which is associated with viral particle stability and genome packaging. Nucleotide sequences vary considerably among different norovirus genotypes, whereas the ORF1–ORF2 junction is the most conserved region. Consequently, designing primers targeting this region enables the simultaneous detection of GI and GII noroviruses while minimizing the risk of false-negative results.

Current norovirus detection methods can be classified into morphological observation, immunological assays, and molecular biological methods. Early detection relied primarily on direct observation of viral particles in fecal samples using electron microscopy. However, this method has low sensitivity, requiring a viral load of 106—107 particles/mL, and involves expensive equipment and cumbersome procedures, making it unsuitable for large-scale screening. Immunological detection methods are based on antigen–antibody reactions and include Enzyme-Linked Immunosorbent Assays (ELISA) and immunochromatographic assays. Although these methods offer operational simplicity and rapid results, their sensitivity is relatively low, and their performance is considerably affected by genotypic variation. Wu's study showed that the detection accuracy of an immunochromatographic test kit for norovirus genotypes GII.4 and GII.17 was 94.4% and 87.5%, respectively [4]. Positive results obtained using these methods generally require further confirmation by molecular techniques. Therefore, because immunological assays are prone to false-negative results, they are currently used primarily for preliminary screening rather than definitive diagnosis [2, 5].

Molecular biological methods can directly identify viral RNA sequences and offer greater specificity and sensitivity. Among these methods, reverse transcription quantitative Real-Time PCR (RT-qPCR) has gradually become the gold standard for norovirus detection [6]. The principle involves reverse-transcribing viral RNA into complementary DNA (cDNA), followed by PCR amplification in the presence of a fluorescent dye [7] or a specific TaqMan probe. Fluorescence intensity is monitored in real time during amplification, enabling quantification of the initial template. TaqMan probes hybridize specifically to the target amplicons, effectively reducing the risk of false-positive results and significantly improving detection accuracy. Kageyama et al. reported that by designing specific primers and probes targeting different genogroups, GI and GII noroviruses could be detected simultaneously in a single reaction system, and multiplex detection of GI, GII, and GIV could also be achieved [8]. In recent years, the development of ready-to-use, lyophilized multiplex RT-qPCR reagents has further improved detection stability and convenience. These reagents reduce dependence on the

cold chain and minimize operational errors, making them more suitable for primary-level laboratories and on-site testing [9].

2. Research project content

2.1. Overall project objectives

This project targets norovirus and employs RT-qPCR to enable rapid, high-throughput detection of norovirus in food samples at testing laboratories.

2.2. Selection of sample pretreatment methods

Based on a literature review, the currently used methods for norovirus enrichment are summarized in Table 1. Given the research objective of enabling rapid, high-throughput testing at testing laboratories, immunomagnetic bead enrichment can be used to rapidly concentrate viruses from large batches of samples. It can also be combined with automated equipment to achieve efficient sample processing. If further development of technologies suitable for on-site testing is pursued, ultrafiltration centrifugation can be selected because it does not require large-scale equipment.

Based on the procedures and characteristics of the enrichment methods described above, I consider the PEG–NaCl precipitation method more suitable for laboratory use, whereas the membrane adsorption–elution method is preferable for on-site testing because it saves time.

Table 1. Current research on norovirus enrichment methods

Enrichment method	Principle	Advantages	Disadvantages	Applicable samples
PEG–NaCl precipitation [10]	High concentrations of Polyethylene Glycol (PEG) and sodium chloride alter the osmotic conditions of the solution, causing intact viral particles to precipitate. The precipitate is then collected by centrifugation.	Low cost; readily available reagents; compatible with large-volume water samples and food eluates; recognized as a reference method in ISO standards.	Time-consuming, typically requiring overnight incubation at 4°C; co-precipitation of impurities from feces and wastewater results in substantial PCR inhibitors; relatively low viral recovery rate (6–12%).	Wastewater, surface water, fruit and vegetable eluates, and shellfish samples.
Ultrafiltration centrifugation [11]	Molecular Weight Cutoff (MWCO) centrifugal filters retain viral particles because they are larger than the effective pore size of the membrane, while smaller impurities pass through the membrane and are removed.	Rapid processing, typically completed within 20–40 minutes; limited carryover of inhibitors; relatively high recovery rate; no overnight incubation required.	Membranes are susceptible to clogging when processing highly turbid samples; limited sample processing volume; high consumable costs; potential nonspecific adsorption.	Water samples and low-turbidity environmental samples.

Table 1. Continued

Immunomagnetic bead enrichment [12]	Magnetic beads modified with antibodies or mucins—a class of highly O-glycosylated macromolecular glycoproteins widely present on animal mucosal surfaces—specifically capture intact norovirus particles, which are then recovered by magnetic separation.	High specificity and effective removal of impurities; high recovery rate (approximately 30%); ability to distinguish intact viral particles from free nucleic acids; suitable for low-concentration samples.	Expensive consumables; strong dependence on antibody titer; susceptibility to interference from sample impurities; limited throughput.	Food, shellfish, wastewater, and fecal suspensions; suitable for studies requiring assessment of infectious viruses.
Membrane adsorption–elution using positively charged filters [13]	The pH and magnesium ion concentration are adjusted to promote viral adsorption onto a charged membrane. The viruses are subsequently recovered by elution with a buffer solution.	Capable of processing very large volumes of water; suitable for on-site sampling; membranes are easy to store and transport; moderate cost.	Membranes are highly susceptible to clogging when processing highly turbid wastewater; variable recovery rates; requires a vacuum filtration apparatus; secondary concentration is often necessary.	Drinking water, surface water, and large-volume environmental water samples.

2.3. Primer design

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ORIGIN
        1 atgaagatgg cgtcgagtga cgccaaccca tctgatgggt cctcagccaa cctcgtgcca
       61 gaggtcaaca atgaggttat ggctttggag cccgttgttg gtgccgctat tgcggcacct
      121 gtagcgggcc aacaaaatgt aattgacccc tggattagaa acaattttgt acaagccgct
      181 ggtggggagt ttacagtgtc ccctagaaac gctccaggtg aaatactatg gagcgcgccc
      241 ctaggccctg acctaaatcc ctacctatcc catttggcca gaatgtacaa tggttatgca
      301 ggtggttttg aagtgcaggt aattctcgcg gggaacgcgt tcaccgccgg gaagattata
      361 tttgcagcag tcccaccaaa ttttccaact gaaggcttaa gtcccagcca ggtcactatg
      421 ttcccccata taatagtaga tgttagacaa ttagaacctg tgctgatccc cttacccgat
      481 gttaggaata atttctatca ttataatcag tcaaatgatt ccactattaa gttgatagca
      541 atgttgtata caccacttag ggctaataat gctggagatg atgttttcac agtttcgtgc
      601 cgagttctca cgagaccatc ccccgatttt gatttcatat ttttagtgcc acccacagtt
      661 gagtcaagaa ctaaaccatt ctctgtccca gttttgactg ttgaggagat gaccaattca
      721 agattcccca tccctttgga aaagttgttc acaggcccca gcagtgcctt tgttgtccag
      781 ccacaaaacg gcaggtgcac aactgatggc gtgctcctag gcaccaccca actttctcct
      841 gtcaacatct gcaccttcag aggggatgtc acccacatca caggtagtcg caactacaca
      901 atgaatttgg cttctcaaaa ttggaacaac tatgacccaa cagaagaaat cccagcccct
      961 ctaggaactc cagattttgt ggggaagatt caaggcatgc tcacccaaac cacaaggaca
     1021 gatggttcaa cacgcggcca caaagctaca gtgtacactg ggagcgccga ctttgctcca
     1081 aaactgggta gagttcaatt tgaaactgac acagaccatg attttgaagc taatcaaaac
     1141 acaaagttca ccccagtcgg tgtcatccaa gatggtagca ccacccaccg aaacgaaccc
     1201 caacagtggg tgctcccaag ttactcaggc agaaatactc acaatgtaca tttggccccc
     1261 gctgtagccc ccacctttcc gggtgagcaa cttctcttct tcagatccac catgcccgga
     1321 tgcagcgggt accccaacat ggatttggac tgtctgctcc cccaggaatg ggtgcagtac
     1381 ttctaccaag aggcagcccc agcacaatct gatgtggctc tgctaagatt tgtgaatcca
     1441 gacacaggta gggttttgtt tgagtgcaag cttcacaaat caggctatgt tacagtggct
     1501 cacactggcc aacatgattt ggttatcccc cccaatggtt actttaggtt tgattcctgg
     1561 gtcaaccagt tctacacgct tgcccccatg ggaaatggaa cggggcgtag acgtgcacta
     1621 taa
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Figure 1. Complete coding DNA sequence (CDS) of the capsid protein (VP1) gene of norovirus GII.4 isolate Hu/016P03ZS/GD/CHN/2016

The nucleotide sequence of the norovirus capsid protein (VP1) gene was retrieved from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) (Figure 1). A pair of primers (P1) for detection was independently designed using the Primer-BLAST tool and compared with the primers (P2) reported in the literature [14] (Table 2).

The results showed that P1 corresponds to nucleotides 761–1296 of the capsid protein (VP1) gene, producing an amplicon of 536 bp. The melting temperature of P1F was 59.97°C, with no 3′ complementarity. The melting temperature of P1R was also 59.97°C, with no 3′ complementarity. These characteristics comply with the principles of primer design and are consistent with those of the primers reported in the literature.

Table 2. Comparison of independently designed primers with those reported in the literature [14]

Primer name	Sequence (5′–3′)	Length (nt)	Melting temperature (°C)	GC content (%)	Amplicon length (bp)
P1F	GCAGTGCCTTTGTTGTCCAG	20	59.97	55.00	536
P1R	GAGAAGTTGCTCACCCGGAA	20	59.97	55.00	
P2FA [15]	GTGGGATGGACTTTTACGTGCCAAG	25	64.26	52.00	129
P2R [15]	CGTCAYTCGACGCCATCTTCATTCAC	26	66.00	53.85	

2.4. Design of the RT-qPCR reaction system

Based on the literature review [15] and the requirements of the intended detection setting, the reverse transcription and PCR reaction systems were designed as presented in Tables 3 and 4, respectively. The PCR buffer contained 500 mmol/L KCl, 15 mmol/L $MgCl_2$, 1% Triton X-100, and 100 mmol/L Tris-HCl at pH 8.8. The reverse transcription reaction was performed at 37°C for 1 h. The qPCR mix contained DNA polymerase, dNTPs, and SYBR Green. The qPCR cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s, with a final extension at 60°C for 1 min.

Table 3. Reverse transcription reaction system[2]

Reagent	Final concentration	Stock concentration	Volume (μL)
10×PCR buffer	10×	1×	4
25× dNTP Mix	4 mM (each dNTP)	100 mM (each dNTP)	1.6
Oligo $(dT)_{18}$	1×	10×	4.0
RNA template (containing 1 μg of total RNA)	Total RNA: 25 ng/μL		20
MultiScribe™ Reverse Transcriptase	2.5 U/μL	50 U/μL	2
RNase Inhibitor	1 U/μL	20 U/μL	2
Nuclease-free H_2O			6.4
Total volume (μL)		40	

[2] https://documents.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017977_highcap_cDNA_RT_UG.pdf

Table 4. qPCR reaction system (designed with reference to [16])

Reagent	Final concentration	Stock concentration	Volume (μL)
PCR buffer	1×	10×	2.5
dNTP mixture	Each 0.2 mmol/L	Each 2.5mmol/ L	2
cDNA template			5
High-fidelity Taq DNA polymerase	0.1 U/μL	5 U/μL	0.5
Deionized water			13.8
Primer pair	0.4 mmol/L	10 mmol/L	1
Probe	0.08 μmol/L	10 μmol/L	0.2
Total volume		25	

2.5. Simulated practice in result analysis

A simulated analysis exercise was conducted using data from the literature [15]. Figure 2 presents a set of standard curve data. Based on the amplification curves in Figures 2B and 2C, the standard curve in Figure 2A and the linear relationship between viral genome copy number and Ct value can be obtained. The linear equations are $y = -3.435x + 37.48 (R2 = 0.995)$ for genogroup GI and $y = -3.399x + 35.56$ for genogroup GII.

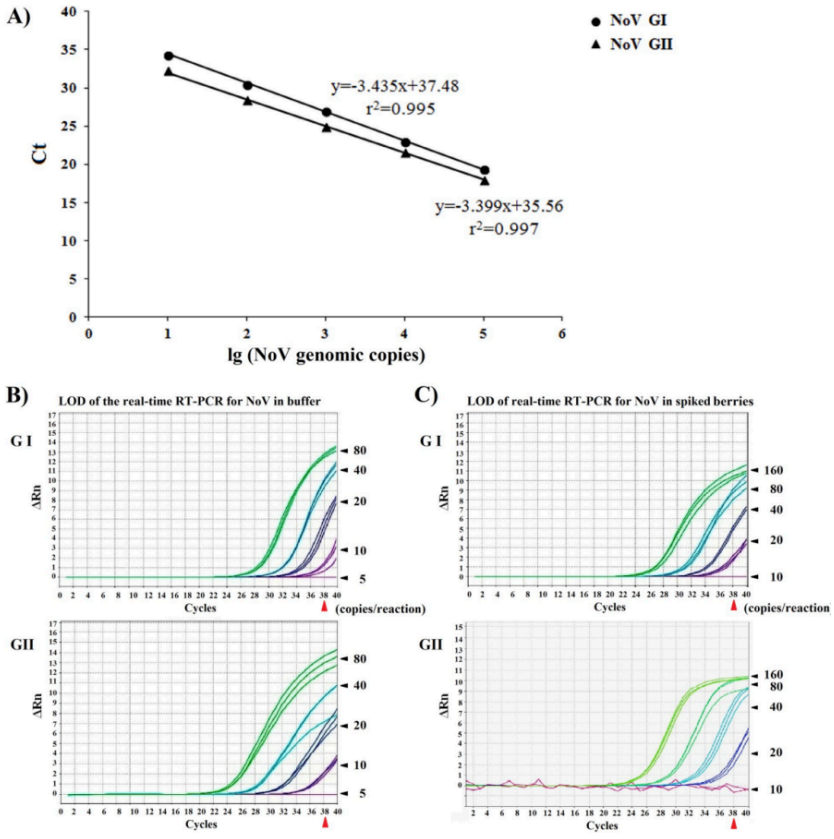

Figure 2. RT-qPCR standard curve data (source: [16])

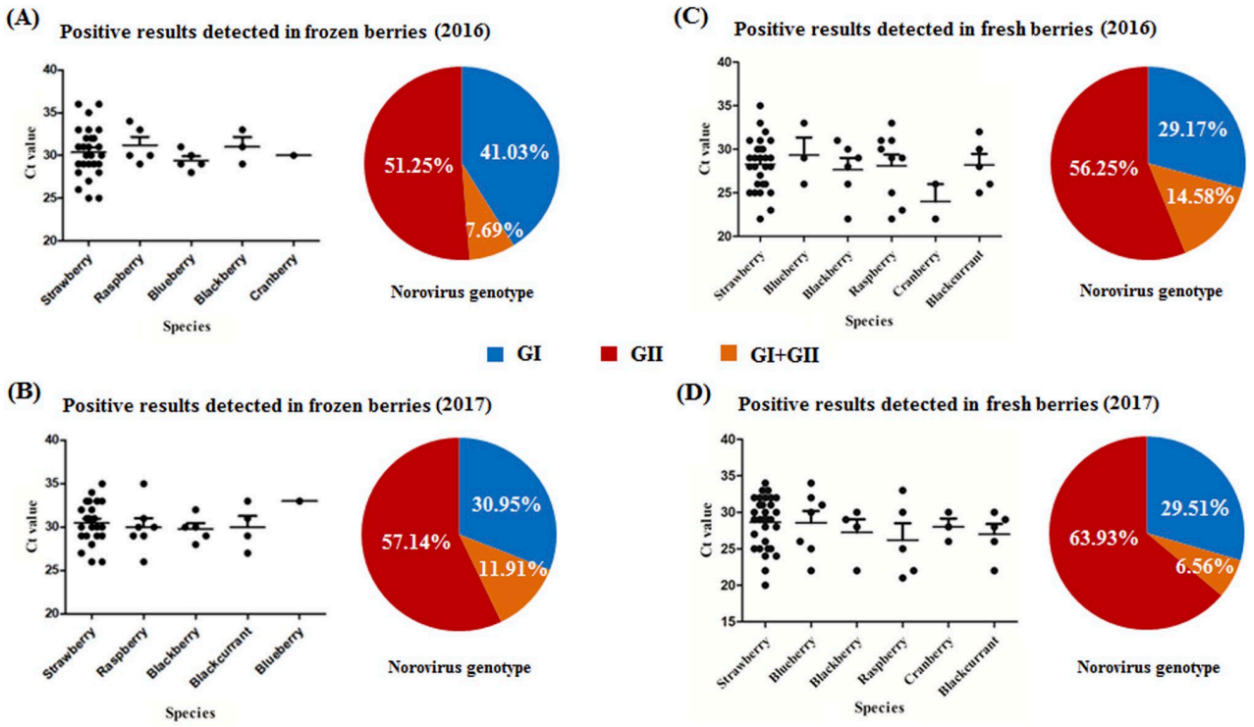

Figure 3. Detection results for the samples under investigation (Source: [15])

Figure 3 presents the detection results for the samples under investigation. The Ct value for the frozen strawberry sample collected in 2017 was 31. Substituting this value into the linear equation yielded an initial viral copy number of 70 in the sample. The Ct value for the frozen blueberry sample collected in the same year was 33, corresponding to an initial viral copy number of 76.607. These results indicate that the norovirus content in blueberries was higher than that in strawberries.

2.6. Prospects for the application of nucleic acid detection technologies in norovirus detection

As the gold standard for norovirus detection in food, RT-qPCR offers several advantages, including high specificity, high sensitivity, rapid detection, and high throughput. However, it requires instruments capable of precise thermal cycling and relies on fluorescent reagents or probes for result interpretation. Consequently, it is more suitable for use in testing institutions and laboratories. In addition, this method provides only relative quantification of the initial target concentration and is susceptible to false-positive results caused by nonspecific amplification, which limits further improvements in detection accuracy.

In recent years, various emerging technologies have been developed to address these limitations. On the one hand, combining isothermal nucleic acid amplification with visual detection has enabled rapid on-site testing without the need for precision instruments. For example, isothermal amplification techniques such as Recombinase Polymerase Amplification (RPA) [16] and Loop-Mediated Isothermal Amplification (LAMP) [17] can amplify nucleic acids at a constant temperature without requiring sophisticated thermal cyclers. When combined with fluorescent probes or lateral flow test strips, these techniques enable visual interpretation of results and offer considerable potential for primary-level testing and rapid on-site diagnosis. On the other hand, the emergence of digital PCR (dPCR) has provided a solution for the absolute quantification of target concentrations. This technique partitions the reaction mixture into tens of thousands of independent microreaction compartments. Following endpoint PCR amplification, the proportion of positive droplets is

determined, and the absolute copy number of the initial template is calculated based on the Poisson distribution. Compared with RT-qPCR, digital PCR enables absolute quantification without relying on a standard curve and exhibits greater tolerance to PCR inhibitors commonly found in food and environmental samples. It therefore offers distinct advantages in detecting samples with low viral loads and analyzing complex matrices. However, the cost of digital PCR instruments and reagents remains high, and the technology has not yet been widely adopted for routine testing [18]. Nevertheless, it has the potential to replace RT-qPCR in certain applications in the future.

In conclusion, nucleic acid detection technologies, owing to their specificity, sensitivity, and high degree of design flexibility, are expected to become one of the mainstream approaches for detecting norovirus and other viruses. Research and development of novel detection methods are of considerable significance to food safety, public health, environmental monitoring, and related fields.

3. Conclusion

In summary, this study aims to investigate the use of RT-qPCR for rapid, high-throughput detection of norovirus in food samples at testing laboratories. As the gold standard method, RT-qPCR enables effective detection of norovirus in food, thereby helping to ensure food safety. First, this study compares the currently available norovirus enrichment methods. It then designs RT-qPCR primers based on viral gene sequences and analyzes experimental data reported by Gao et al. to explore how the viral content in food samples can be estimated from the linear relationship between viral genome copy number and Ct value. Finally, it examines the prospects for the application of nucleic acid detection technologies. This study has several limitations. The analysis and learning exercises rely exclusively on experimental data reported by Gao et al. More comprehensive and in-depth investigations are needed to obtain further insights. Considerable work remains to be done to improve the accuracy of nucleic acid detection. Such efforts should not be limited to RT-qPCR, as RPA, LAMP, and digital PCR also hold considerable potential for further development and application.

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