

Development and Evaluation of a Rapid GII Norovirus Detection Method Based on CRISPR-Cas12a

XINYI HU^{1,2,#}, PEI HE^{1,2,#}, TONG JIANG^{1,2} and JILU SHEN^{1,2*}

¹The First Affiliated Hospital of Anhui Medical University, Hefei, China

²Anhui Public Health Clinical Center, Hefei, China

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Abstract

Norovirus is highly infectious and rapidly transmissible and represents a major pathogen of sporadic cases and outbreaks of acute gastroenteritis worldwide, causing a substantial disease burden. Recent years have witnessed a dramatic increase in norovirus outbreaks in China, significantly higher than in previous years, among which GII norovirus is the predominant prevalent strain. Therefore, rapid norovirus diagnosis is critical for clinical treatment and transmission control. Hence, we developed a molecular assay based on RPA combined with the CRISPR-CAS12a technique targeting the conserved region of the GII norovirus

genome, the results of which could be displayed by fluorescence curves and immunochromatographic lateral-flow test strips. The reaction only required approximately 50 min, and the results were visible by the naked eye with a sensitivity reaching 10² copies/μl. Also, our method does not cross-react with other common pathogens that cause intestinal diarrhea. Furthermore, this assay was easy to perform and inexpensive, which could be widely applied for detecting norovirus in settings including medical institutions at all levels, particularly township health centers in low-resource areas.

Key word: GII norovirus, gastroenteritis, RPA, CRISPR-Cas12a, diagnostic

Introduction

Norovirus was the first known as Norwalk virus, virus particles discovered in the feces of patients through immunoelectron microscopy by the American scholar Kapikian, during an outbreak of diarrhea in the town of Norwalk (Ohio, USA) in 1972 (Kapikian et al. 1972). Norovirus is one of the most predominant pathogens that lead to nonbacterial gastroenteritis in children and adults, which is associated with nearly 50% of acute gastroenteritis (AGE) outbreaks and represents the second largest burden of all infectious diseases (Lozano et al. 2012; Liu et al. 2023;). It is estimated by the World Health Organization (WHO) that norovirus causes over 600 million cases of diarrhea and 200 thousand deaths annually, resulting in an economic burden of approximately \$60 billion (Pires et al. 2015; Ludwig-Begall et al. 2021). However, these

numbers may underestimate the true incidence of norovirus-related gastroenteritis because this infection is self-limited, and not all infected patients will seek or require medical care.

Norovirus is a nonenveloped icosahedral small virus that belongs to the Norovirus genus of the Human Caliciviridae family, with a diameter of approximately 38 nm, and its genome consists of a single-stranded positive-sense RNA molecule with a base length of approximately 7.5 kb (Fernández and Gómez 2010). Currently, the genome of norovirus can be categorized into at least 10 genogroups (GI–GX) (Chhabra et al. 2019), among which GI, GII, GIV, GVIII, and GIX can infect humans. In China, GII norovirus infection accounts for the vast majority (over 80%) (Xue et al. 2018; Li et al. 2022; Qi et al. 2023). Norovirus is mainly transmitted via fecal-oral routes, encompassing human-to-human transmission or indirect

Xinyi Hu and Pei He contributed equally to this study.

* Corresponding author: J. Shen, The First Affiliated Hospital of Anhui Medical University, Hefei, China; Anhui Public Health Clinical Center, Hefei, China; e-mail: shenjilu@ahmu.edu.cn

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transmission via contaminated food or water (Vega et al. 2011; Shang et al. 2017), and can infect humans at a low dose with only 10–100 viral particles (Teunis et al. 2008). Norovirus is, therefore, highly contagious, which can give rise to large outbreaks and induce great harm to the population.

Studies have reported that norovirus can be cultured in human cells (Jones et al. 2014; Ettayebi et al. 2016), but it is slow-growing and unstable, so no clinical laboratories use culture to detect norovirus. There are some detection methods for norovirus infection, such as electron microscopy (Utagawa et al. 2002), RT-PCR (Kantarwar et al. 2018), and ELISA technique (Kele et al. 2011). Nucleic acid molecular testing based on real-time fluorescence quantitative PCR is the gold standard for norovirus but requires specialized laboratories for analysis (Rupprom et al. 2017). However, rather than relying on expensive equipment, these techniques require highly trained professionals, significantly limiting their use at the point of care or in low-resource regions. Therefore, there is a need for a simple, rapid, highly specific, and sensitive assay that does not require special equipment or instrumentation to detect norovirus.

Recombinase polymerase amplification (RPA) is a new thermostatic amplification technology first proposed in 2006 by scholars Piepenburg et al. (2006), and the amplification can be completed within 10–20 min under a thermostatic condition of 37°C–42°C in a water or dry-bath incubator, independent of variable-temperature equipment such as a PCR instrument. CRISPR-Cas system, which is an adaptive immune system formed in prokaryotes during long-term evolution, can discriminate targets and non-targets based on PAM sites on nucleic acids of target sequences via the complementary pairing of guide RNAs (gRNAs) to nucleic acids of target sequences (Liu et al. 2022). Upon the activation of Cas12a by a specific target sequence, non-specific shear is available for arbitrary single-stranded DNA. Current isothermal amplification approaches such as RPA and LAMP can be applied in conjunction with CRISPR-Cas12a for diverse *in vitro* diagnostics and bioassays such as COVID-19 (Broughton et al. 2020), *Clostridioides difficile* toxins (Jiang et al. 2023), carbapenemase genes (Xu et al. 2022). This study combined the two techniques to establish a GII Norovirus detec-

tion method on the strength of RPA-Cas12a-lateral flow immunochromatographic strips. We believe this platform enables rapid, highly sensitive, and cost-effective detection of GII norovirus, with potential application in point-of-care testing (POCT).

Experimental

Materials and Methods

Reagents and chemicals. All primers, FAM-BHQ1 co-tagged single-stranded DNA probe, and FAM-Biotin co-tagged single-stranded DNA probe utilized in the present study were designed independently by our laboratory, and their synthesis was accomplished by Sangon Biotech (Shanghai) Co., Ltd. (China); crRNA was synthesized by GeneBioGist company (China). All sequences are listed in Table I. The RPA reagent was procured from TwistDx™ Ltd. (UK), while LbaCas12a and NEBuffer™ 3.1 were supplied by New England Biolabs (USA). The nucleic acid extraction kit (SPARKeasy Virus RNA Kit, Sparkjade Biotech, China), Norovirus positive control product (Norovirus full sequence pseudovirus standard, 10⁶ copies/μl), and Norovirus nucleic acid detection kit (PCR-fluorescent probe method) were purchased from Land Medical Co., Ltd. (China).

Clinical sample collection. Diarrheal fecal samples were obtained in February 2023 from 35 patients with clinically suspected norovirus infection at the First Affiliated Hospital of Anhui Medical University and Anhui Provincial Children’s Hospital. All patients met the diagnostic criteria for acute gastroenteritis and the inclusion criteria: (1) increased stool frequency (three or more bowel movements per day); (2) change in fecal character (including watery stools and loose stools); (3) routine fecal symptoms suggesting that both leukocyte and erythrocyte counts were ≤ 10/higher magnification field of view (+); and (4) duration of the disease was not more than 2 weeks.

Nucleic acid extraction. Nucleic acids were extracted from 35 diarrheal fecal samples in accordance with the specifications of a nucleic acid extraction kit and then preserved in a –80°C freezer. The following eight diarrheal pathogens DNA were extracted using

Table I
Primers and crRNA sequences.

Oligonucleotide	Sequence (5'–3')
RPA forward primers	CCTCTCTTCACGGACCCCTCTTTCTACAGC
RPA reverse primers	TTCATTACAAAATTGGGAGCCAGATTGC
crRNA	AGUGCCUGGGAGAAAGAUGUCGUUU
ssDNA reporters	FAM-TTATTATT-BHQ1
	FAM-TTATTATT-Biotin

Table II
Bacteria and viruses involved in this study.

Name	Source of strains
<i>Shigella flexneri</i>	CMCC(B)51572
<i>Escherichia coli</i> O157:H7	NCTC12900
<i>Vibrio parahaemolyticus</i>	ATCC® 17802™
<i>Salmonella</i> Typhimurium	Clinical isolates
<i>Campylobacter jejuni</i>	ATCC® 33291™
<i>Yersinia enterocolitica</i>	CMCC(B)50024
Human astrovirus	Clinical isolates
Rotavirus	ATCC® VR-2274™
Enteric adenovirus	ATCC® VR-930™

nucleic acid extraction kits (Table II). The standard strains were commercially available from Shanghai Shifeng Biological Co., Ltd. (China), and the *Salmonella* Typhimurium and human Astrovirus were from the Microbiology Lab of the Department of Clinical Laboratory, the First Affiliated Hospital of Anhui Medical University (China). *Rotavirus* and *Enteric Adenovirus* standards were purchased from Henan Engineering Research Center of Industrial Microbiology (China).

Design of GII norovirus RPA primers and crRNA. RPA primers and crRNA were designed on the strength of the nucleotide sequences in the RdRp region (GenBank accession number: OQ451905.1) on the GII norovirus ORF target gene fragment. RPA primers were designed using Primer Premier v5.0 primer design software (PREMIER Biosoft, USA). Finding protospacer adjacent motif (PAM) site of the original spacer sequence from the primer-amplified sequence was used for designing crRNA based on the PAM site.

Target fragment amplification. With the harvested nucleic acids serving as templates, TwistAmp® basic kit (TwistDx™ Ltd., UK) was utilized for RPA amplification. Following the kit instructions, the reaction was performed with the reaction system well-mixed in the Eppendorf tube (1.5 ml), and equivalent paraffin oil was subsequently supplemented to the surface to avoid aerosol contamination.

RPA reaction system (50 µl): 2 µl forward (F) primer (10 µM), 2 µl reverse (R) primer (10 µM), 29.4 µl A buffer, 2 µl RNA template, 2.5 µl B buffer, and ddH₂O supplement to 50 µl. Reaction conditions: 30-min incubation at 42°C. Subsequently, the amplified products were purified using a PCR product purification kit, followed by 2% agarose gel electrophoresis.

Development of RPA-Cas12a-fluorescence assay. The Cas12a cleavage reaction system (20 µl): 2 µl NEBuffer™ 3.1, 2 µl crRNA (1 µM), 1 µl LbCas12a (1 µM), 1 µl fluorescein isothiocyanate- and quencher-labeled reporter (1 µM), 2 µl RPA amplified product, and 12 µl ddH₂O supplement. Incubation was performed at 37°C

for 20 min using an Applied Biosystems™ 7500 Real-Time PCR instrument (Thermo Fisher Scientific, Inc., USA), and fluorescence intensities were recorded at 1-min intervals. The results were visualized by fluorescence curves, wherein significantly increased fluorescence values signified positive while unchanged fluorescence values signified negative.

Development of RPA-Cas12a-immunochromatographic assay. The Cas12a cleavage reaction system (20 µl): 2 µl NEBuffer™ 3.1, 2 µl crRNA (1 µM), 1 µl LbCas12a (1 µM), 1 µl FAM-coupled biotin reporter (1 µM), 2 µl RPA amplified product, and 12 µl ddH₂O supplement. Following incubation for 20 min at 37°C, 20 µl ddH₂O was added into the reaction system and well-mixed, which was next added with immunochromatographic test strips, followed by observations within 5 min; the presence of test line color reaction (color reaction of the control line or not) was regarded as positive, while only the control line color reaction was considered negative. A schematic of both RPA-Cas12a-fluorescence and An RPA-Cas12a-immunochromatographic assay is presented in Fig. 1.

Assessment of sensitivity and specificity. Norovirus positive control product (10⁶ copies/µl) was diluted in 10-fold to 6 gradient concentrations (10⁶, 10⁵, 10⁴, 10³, 10², 10 copies/µl). Nucleic acids were extracted referring to a nucleic acid extraction kit, and the above-mentioned concentrations of controls were detected using RPA-Cas12a-fluorescence and RPA-Cas12a-immunochromatographic assays to ascertain the lowest detection limit. The specificity of our method was verified by RPA-Cas12a-fluorescence and RPA-Cas12a-immunochromatographic assays with pathogen nucleic acids described in Table II as templates.

Assessment of RPA-Cas12a-immunochromatography using clinical samples. Using the aforementioned RPA-Cas12a-fluorescence assay and RPA-Cas12a-immunochromatographic assay, 35 diarrheal fecal samples from patients with clinically suspected norovirus infection were tested. The Norovirus nucleic acid detection kit (PCR-fluorescent probe method) was adopted as the reference method. Data from the two tests were compared, and the Chi-square test was utilized for calculating positive and negative coincident rates.

Results

Results of primers amplification and crRNA. The RPA amplified fragments and design results of crRNA are depicted in Fig. 2. The results showed that our primers could successfully amplify the nucleic acids of the norovirus-positive control product and the nucleic acids of the two positive specimens. Meanwhile, the fluorescence value results showed that our designed crRNA

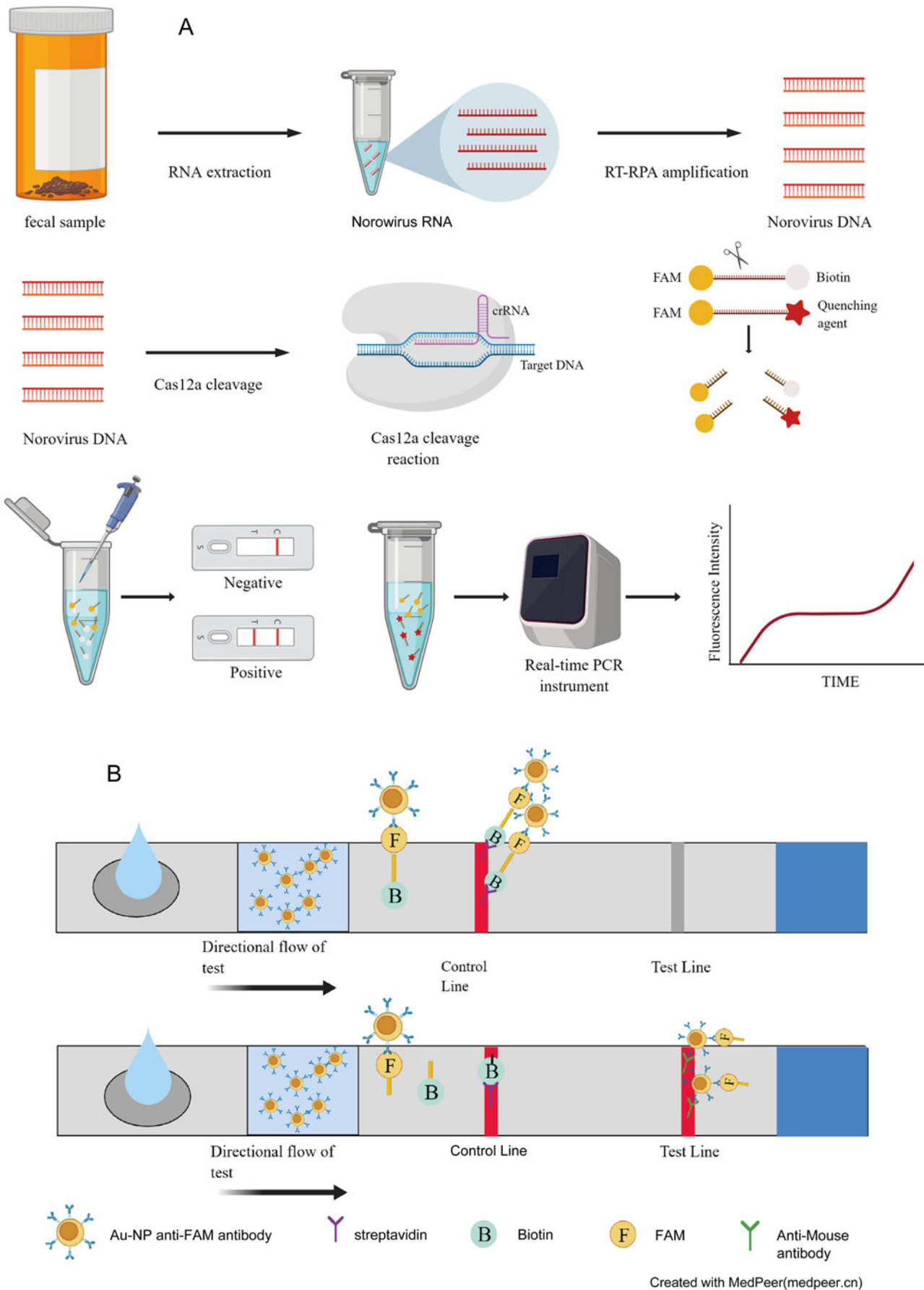


Fig. 1. Detection principle of RPA-Cas12a-fluorescence analysis and RPA-Cas12a-immunochromatography detection method. A) RPA-Cas12a method detection process; B) principle of Immunochromatography Flow Strip.

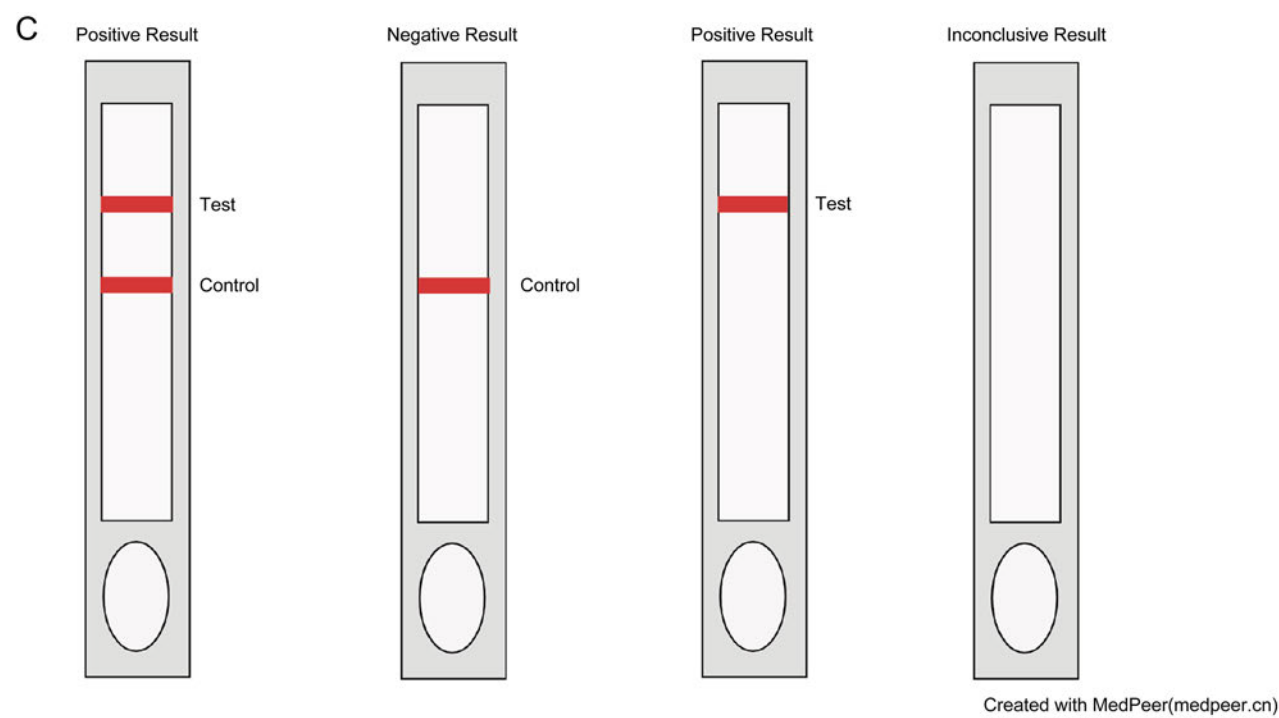


Fig. 1. Detection principle of RPA-Cas12a-fluorescence analysis and RPA-Cas12a-immunochromatography detection method.
C) the schematic diagram for judging the results of immunochromatography flow strips

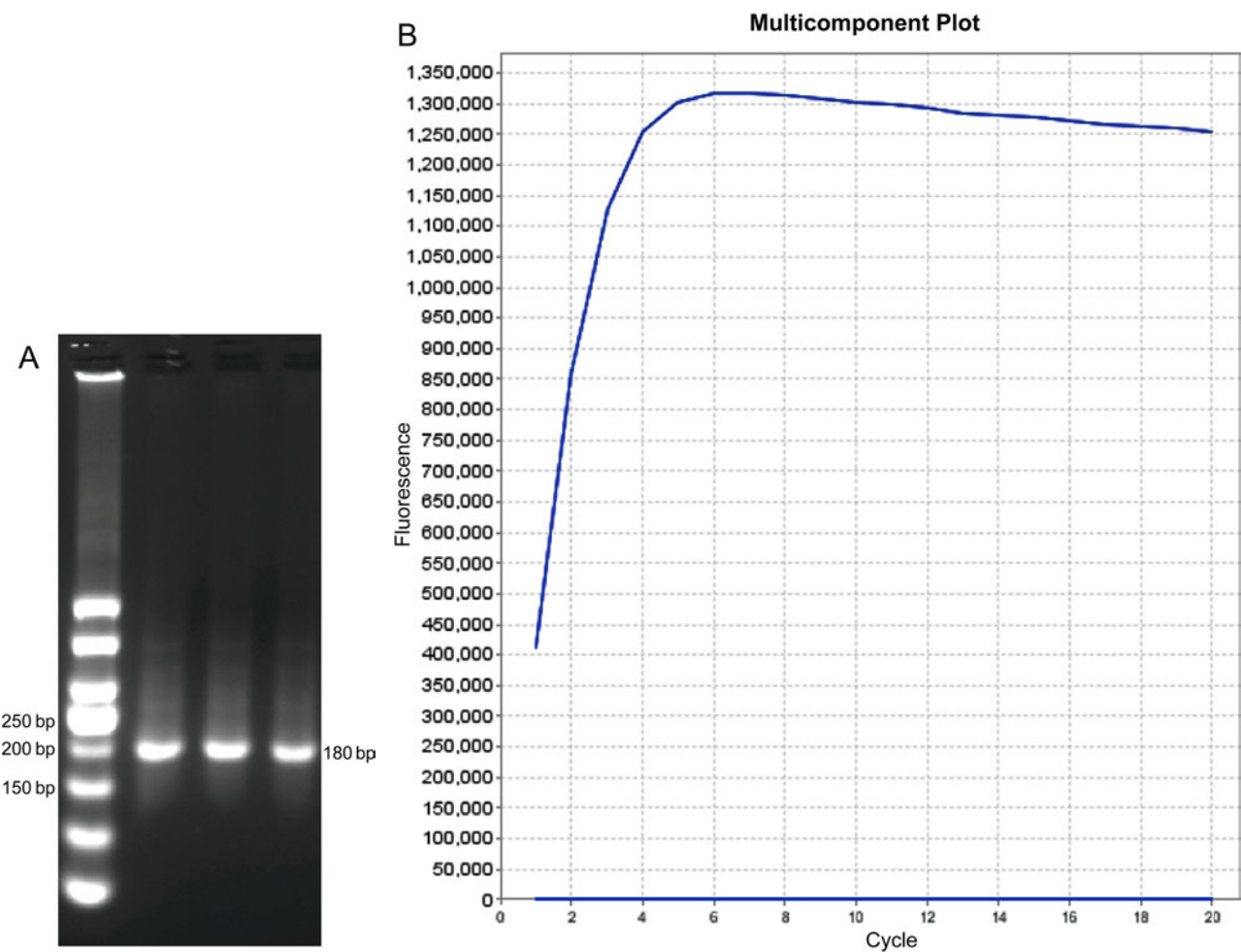


Fig. 2. RPA amplified fragments and design results of crRNA.

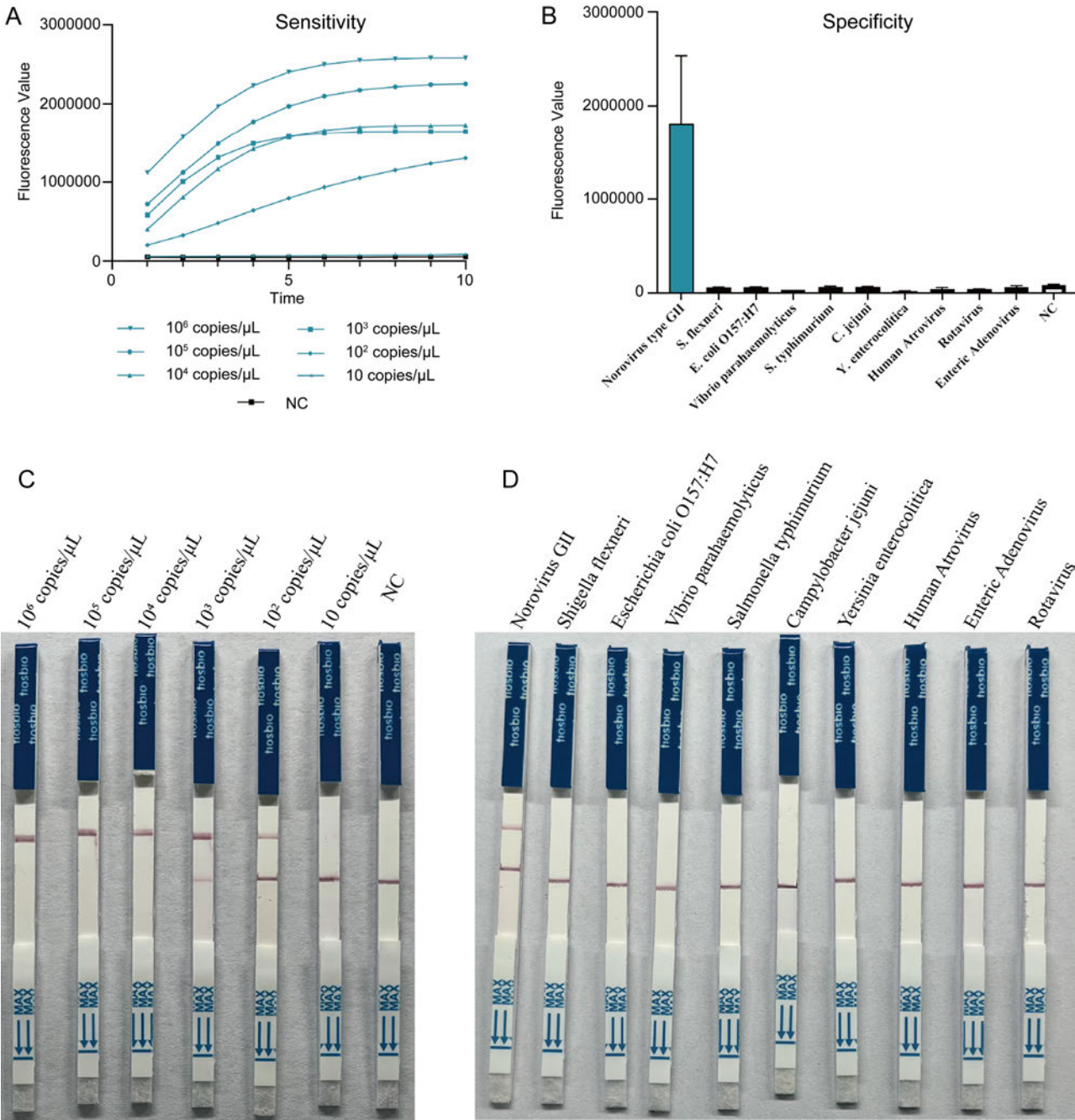


Fig. 3. Verification of sensitivity and specificity of the RPA-Cas12a-fluorometric and RPA-Cas12a-immunochromatographic assays. A) Sensitivity curves of different concentrations of norovirus positive quality control products amplified by RPA; B) specificity of RPA-Cas12a-fluorometric assay; C) RPA-Cas12a-immunochromatographic results of different concentrations of Norovirus; D) specificity of RPA-Cas12a-immunochromatographic assay

activated CRISPR-Cas12a, allowing CRISPR-Cas12a to cleave the reporter molecules double-labeled with fluorescein isothiocyanate and a bursting agent in the system.

Sensitivity and specificity of RPA-Cas12a-fluorescence assay. Different concentrations of norovirus nucleic acids extracted as above and pathogens' nucleic acids described in Table II were employed for RPA amplification, and the amplified products were mixed with the crRNA above for Cas12a-fluorescence assay. The detection limit of this approach was as low

as 10^2 copies/ μ L, and no fluorescence, i.e., no cross-reactivity, was detected in the nine common pathogens (Fig. 3). The results of the RPA-Cas12a-immunochromatographic assay were consistent with the RPA-Cas12a-fluorescence assay.

Validation of the GII norovirus Cas12a detection method in clinical samples. This study examined 35 diarrheal fecal samples of clinically suspected norovirus infection using RPA-Cas12a-fluorescence and RPA-Cas12a-immunochromatographic assays. These

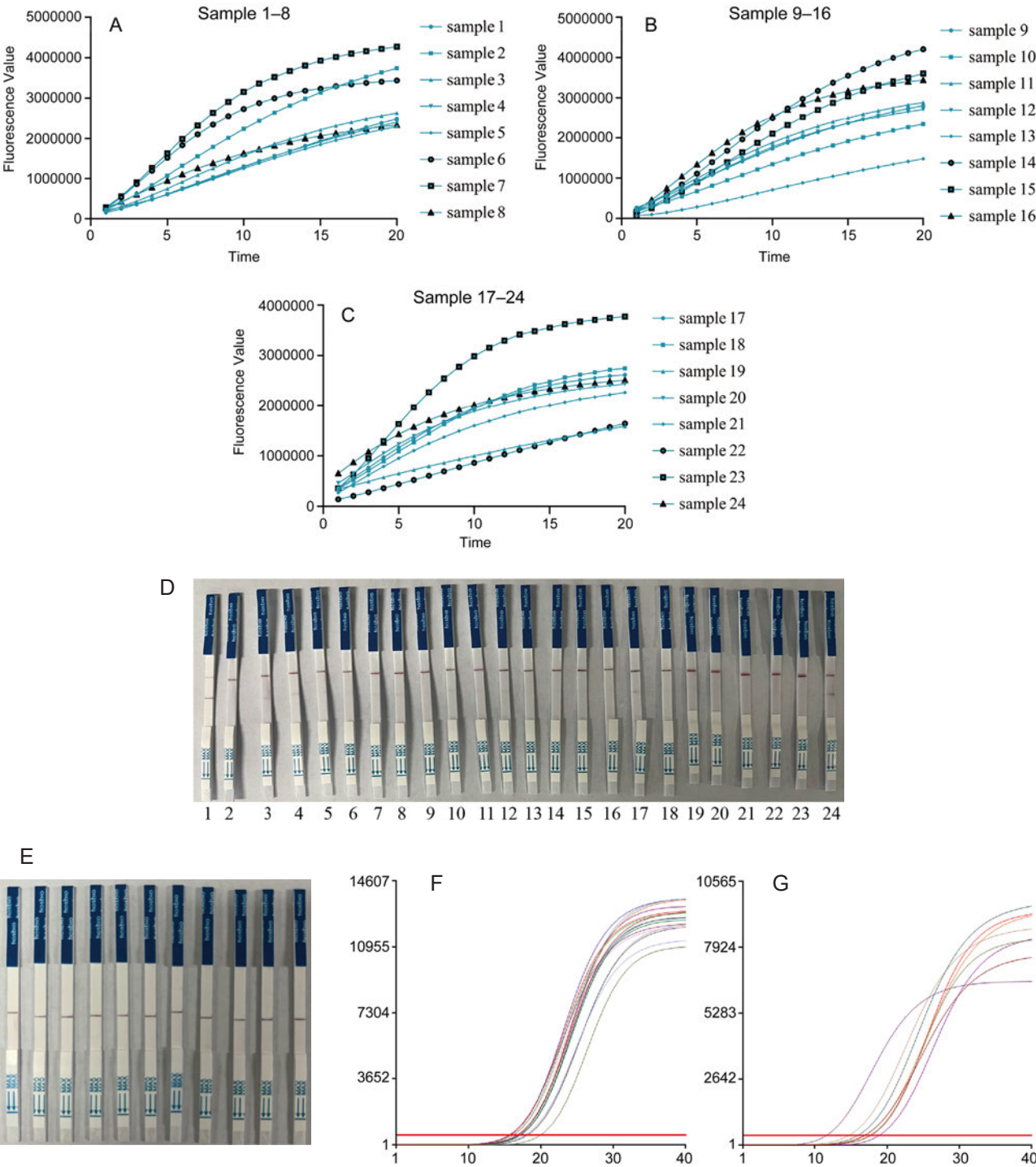


Fig. 4. Detection of GII norovirus in clinical samples based on CRISPR-Cas12a.

A, B, C) Positive results of RPA-Cas12a-fluorescence assay of 24 samples; D) positive results of RPA-Cas12a-immunochromatographic assay of 24 samples; E) negative results of RPA-Cas12a-immunochromatographic assay of 11 samples; F, G) qPCR results of 24 positive samples

Table III
Comparison between RPA-cas12a method and qPCR.

RPA-Cas12a	Quantitative Real-Time PCR		Total
	+	–	
+	24	0	24
–	0	11	11
Total	24	11	35

samples were simultaneously detected using a Norovirus nucleic acid detection kit (PCR-fluorescent probe), a currently commonly used clinical method, and the results are presented in Fig. 4. The positive and negative results of the GII Norovirus Cas12a assays were 100% coincident with the positive and negative results of the Norovirus nucleic acid detection kit, respectively (Table III).

Discussion

Recently, the prevailing trend of norovirus has been increasingly severe in China (Lian et al. 2019), in which GII norovirus has been the predominant epidemic strain; infected individuals in rural areas are more than those in urban areas, and asymptomatic individuals are more than symptomatic individuals in the infants (Chen et al. 2023). These factors undoubtedly increase the difficulty of a positive diagnosis of norovirus.

At present, the traditional norovirus detection methods include electron microscopy and immunoassay. However, improper electron microscopy analysis can lead to false negatives or false positives, and this method is time-consuming, laborious, and difficult to use for rapid diagnosis (Chin et al. 2022).

In contrast, immunochromatography in immunoassay has a low sensitivity in detecting norovirus antigens (Vinjé 2015), which cannot yield satisfactory results. Nowadays, RT-qPCR is believed to be a reliable norovirus detection method. However, this method relies on a real-time PCR machine and is inapplicable in low-resource areas such as primary township health centers in China.

Based on the limitations of the aforementioned methods, we developed a rapid, simple, and accurate method for norovirus detection. We designed a new CRISPR-Cas12a-based technique combined with RPA, which could achieve rapid and accurate detection and vastly reduce reliance on large-scale advanced instruments. Its reaction required only one water or metal bath, and fluorescence intensities could be read by a fluorescence quantitative PCR instrument after 30-min RPA amplification and 20-min CRISPR-Cas12a trans-cleavage, suitable for large-scale detection of samples in developed regions. The product could also be read by immunochromatographic lateral-flow test strips, suitable for point-of-care testing and applicable in vast resource-poor areas. To address the uncapping-caused aerosol contamination issue post-RPA reaction, we supplemented equivalent paraffin oil immediately after mixing with the activators of the RPA reaction to sequester air. We collected diarrheal fecal samples from 35 patients with suspected norovirus infection for clinical validation, and the results obtained by RPA-Cas12a-fluorescence and RPA-Cas12a-immunochromatography assays showed 100% positive and negative coincident rates with the results of the Norovirus nucleic acid detection kit (PCR-fluorescent probe method). Besides, we tested diluted norovirus-positive controls at a series of gradient concentrations using the Cas12a method with the a detection limit as low as 10^2 copies/ μ l, which was consistent with that of RT-qPCR currently on the market. We also tested eight common diarrheal pathogens using the Cas12a method and detected no cross-

reactivity. All the above data confirmed that our RPA-Cas12a detection method had relatively good specificity and sensitivity, which could be comparable with the currently accepted and reliable RT-qPCR.

Despite the above advantages of the RPA-Cas12a detection method, we must acknowledge that our method also had several limitations. For instance, after RPA amplification, an uncapping operation was required to transfer the amplicons, which undoubtedly increased aerosol contamination and false-positive probability. In addition, this study only detected GII Norovirus and did not detect a few prevalent strains. However, this detection method, independent of an instrument, is more conducive to the universal detection of various pathogens, including norovirus, providing an effective tool for on-site detection.

Conclusions

In summary, our CRISPR-based norovirus detection platform has the advantages of high sensitivity, high specificity, simple operation, and low cost, which makes it very suitable for point-of-care detection.

Author contributions

Xinyi Hu, Pei He and contributed equally to this work. All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Xinyi Hu, Pei He, Tong Jiang and Jilu Shen. The first draft of the manuscript was written by Xinyi Hu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Xinyi Hu, Pei He and Jilu Shen are participated in the study design and study management. Xinyi Hu, Pei He, Tong Jiang participated in the collection of bacterial strains and the extraction of DNA. Xinyi Hu and Jilu Shen participated in data interpretation and writing of the manuscript. All authors have agreed on the publication of this manuscript and agreed to be responsible for all their research work.

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Ethical statement

This study involved the use of excess or discarded feces samples from anonymous patients in our hospital. This research will not affect the health and privacy of the patient. All procedures performed in this study involving human participants were in compliance with the ethical standards of the Medical Ethics Committee of Anhui Medical University (Reference number: LISC20210802) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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