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Palm-sized CRISPR sensing platform for on-site *Mycoplasma pneumoniae* detection

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ABSTRACT

Pneumonia remains a prevalent childhood illness and is the foremost cause of mortality due to infectious diseases among children under the age of five. *Mycoplasma pneumoniae* (*M. pneumoniae*) causes the most frequent type of atypical pneumonia in this age group, has raised global health concern. Therefore, there is a pressing need for a rapid, low cost, and user-friendly method for the early diagnosis of *M. pneumoniae* pneumonia. Herein, we develop a CRISPR sensing platform for on-site *M. pneumoniae* detection, termed CRAFT (Crispr-based rapid assay device for field testing). The CRAFT provides "sample in-result out" functionality. It completed sample processing and nucleic acid extraction within 5 min at room temperature, with efficiency comparable to commercial kits. RPA and CRISPR/Cas12a reagents were isolated in a closed tube using a movable magnetic bead valve, and the RPA product was then mixed with the CRISPR reagent. The limit of detection for *M. pneumoniae* using CRAFT was 100 copies/μL, and the method exhibited no cross-reactivity with other respiratory pathogens. CRAFT was utilized to validate 50 clinical samples, and the results demonstrated 100 % consistency with those obtained by qPCR. This versatile platform holds significant potential for point-of-care testing of *M. pneumoniae*, particularly in resource-limited settings.

1. Introduction

Mycoplasma pneumoniae (*M. pneumoniae*) is a notable pathogen responsible for respiratory tract infections, significantly impacting public health, particularly in children and young adults(Yan et al., 2024; Zhang et al., 2024). The clinical manifestations of *M. pneumoniae* infections can vary from mild upper respiratory symptoms, such as pharyngitis and bronchitis, to severe lower respiratory conditions, including atypical pneumonia(Rodman Berlot et al., 2021). In severe cases, complications may extend beyond the respiratory system, resulting in conditions such as encephalitis, myocarditis, and hemolytic anemia, which can lead to significant morbidity and occasionally mortality (Liu et al., 2023a; Metsälä et al., 2021). Conventional diagnostic approaches for *M. pneumoniae* include culture techniques, serological assays, and polymerase chain reaction (PCR). The isolation of encompass from liquid cultures may require up to eight weeks, and the detection rates remain suboptimal (She et al., 2010). Serological assays frequently exhibit limited sensitivity, particularly in the early stages of infection, which can result in false-negative outcomes(Lin et al., 2020). Polymerase chain reaction (PCR) techniques have demonstrated reliability in diagnosing such pathogens. However, PCR require sophisticated instruments, well-equipped laboratories, and specially trained personnel, rendering them unsuitable for point-of-care (POC) applications, particularly in resource-limited settings.

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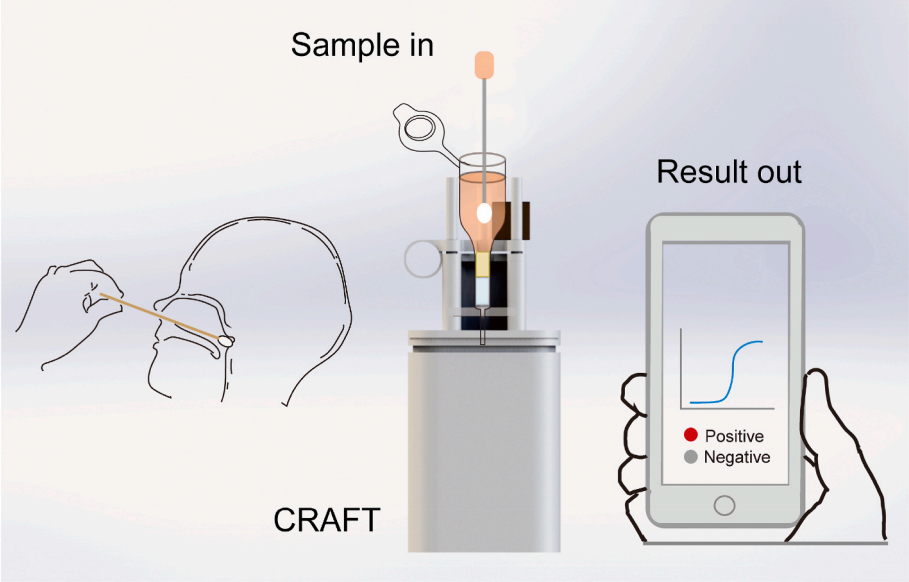


Fig. 1. Schematic illustration of CRAFT for *M. pneumoniae* detection. Nasopharyngeal swab samples were collected, and a one-step nucleic acid extraction and reaction process was conducted using a low-cost, multifunctional CRAFT device. The device facilitated incubation, real-time fluorescence signal acquisition, and the subsequent transmission of results to a user-friendly smartphone.

Recombinase Polymerase Amplification (RPA) is a nucleic acid detection technology that serves as an alternative to PCR, offering advantages such as high sensitivity, compatibility with multiplexing, isothermal reaction conditions, and the absence of the need for costly equipment (Lobato and O'Sullivan, 2018). However, the nonspecific amplification during the RPA assay can lead to false positive results (Patchsung et al., 2020). Detection methods named SHERLOCK (Gootenberg et al., 2017; Kellner et al., 2019) and DETECT(Mustafa and Makhawi, 2021) enhance the specificity of RPA, thereby facilitating precise target detection. However, this system necessitates an additional step of transferring the product to a different tube or strip, thereby elevating the risk of aerosol contamination. To address this issue, several studies have responded by integrating CRISPR/Cas12a or Cas13a with RPA in a tube through various methods (Liu et al., 2023b; Yao et al., 2024). In our prior research, we successfully tackled the challenge of performing RPA and CRISPR/Cas12a reactions within a single tube (Xu et al., 2023). However, the aforementioned research did not incorporate sample processing and nucleic acid extraction. Recently, a CRISPR-based diagnostic platform for the field-deployable detection of monkeypox virus was reported (Wang et al., 2024). However, the platform carried out nucleic acid extraction and RPA/CRISPR reaction in different tubes, respectively. The process of opening the lid still had the risk of aerosol contamination and failed to achieve the goal of "sample in-result out" detection.

To address the limitations of existing studies, we have developed a **C**rispr-based **r**apid **a**ssay device for **f**ield **t**esting, termed **CRAFT**. The CRAFT is capable of conducting one-step extraction of nucleic acid, amplification of the *M. pneumoniae* target gene, and digital readout. As illustrated in Fig. 1, a palm-sized and user-friendly homemade device named CRAFT is capable of completing the entire "sample in-result out" process without the need for advanced instrumentation. CRAFT demonstrates significant potential for application in resource-limited settings. In addition to substantially enhancing the diagnostic capabilities for *M. pneumoniae* pneumonia, CRAFT exhibits versatility and scalability by adapting different reaction systems to facilitate the field detection of various pathogens.

2. Materials and methods

2.1. Materials

All primers, crRNA, and the ssDNA reporter were synthesized by the Sangon Biotech Co. Ltd., Shanghai, China. The Recombinase Polymerase Amplification (RPA) kit was purchased from Lesun Biotech Co., Ltd., Wuxi, China, and the CRISPR kit was obtained from MagiGen Biotech Co. Ltd., Guangzhou, China. Additionally, nucleic acid extraction was performed using the TRIeasy LS Total RNA Extraction Reagent, which was acquired from Yi Sheng Co. Ltd., Shanghai, China.

2.2. Design of RPA primers and crRNAs

Three RPA primers and four crRNAs were manually designed and sequences were summarized in Supporting Information Table SI1.

2.3. crRNA screening

The Cas12a reaction for the crRNA screening was performed using the following condition: 2 μL 10 × reaction buffer, H_2O 9.5 μL, 2 μL ssRNA (80 nM), 2 μL crRNA (40 nM), 4 μL Cas12a (40 nM), 0.5 μL RNase Inhibitor (10U), 1 μL DNA target. The CRISPR reaction was performed at 37 °C for 15 min, and fluorescence signals were measured using the BioTek Synergy H1 fluorescence microplate reader. Excitation was set at 450 nm, and emission was recorded at 510 nm.

2.4. Optimization of the CRISPR/Cas12a detection system

The CRISPR/Cas ribonucleoprotein (RNP) ratios (Cas12a/crRNA) and RNP concentration were optimized with the optimal crRNA. The specific CRISPR/Cas12a detection procedure was the same as that in 2.3.

2.5. RPA primers screening

Nine pairs of primers were examined and a pair with the maximal amplification efficiency was chosen. In accordance with the kit instructions, minor modifications were carried out. The RPA mixture consisted of 12.5 μL of dry powder solution, 1 μL forward primer, 1 μL reverse primer, 1 μL DNA target, and 8 μL ddH_2O. After combining the

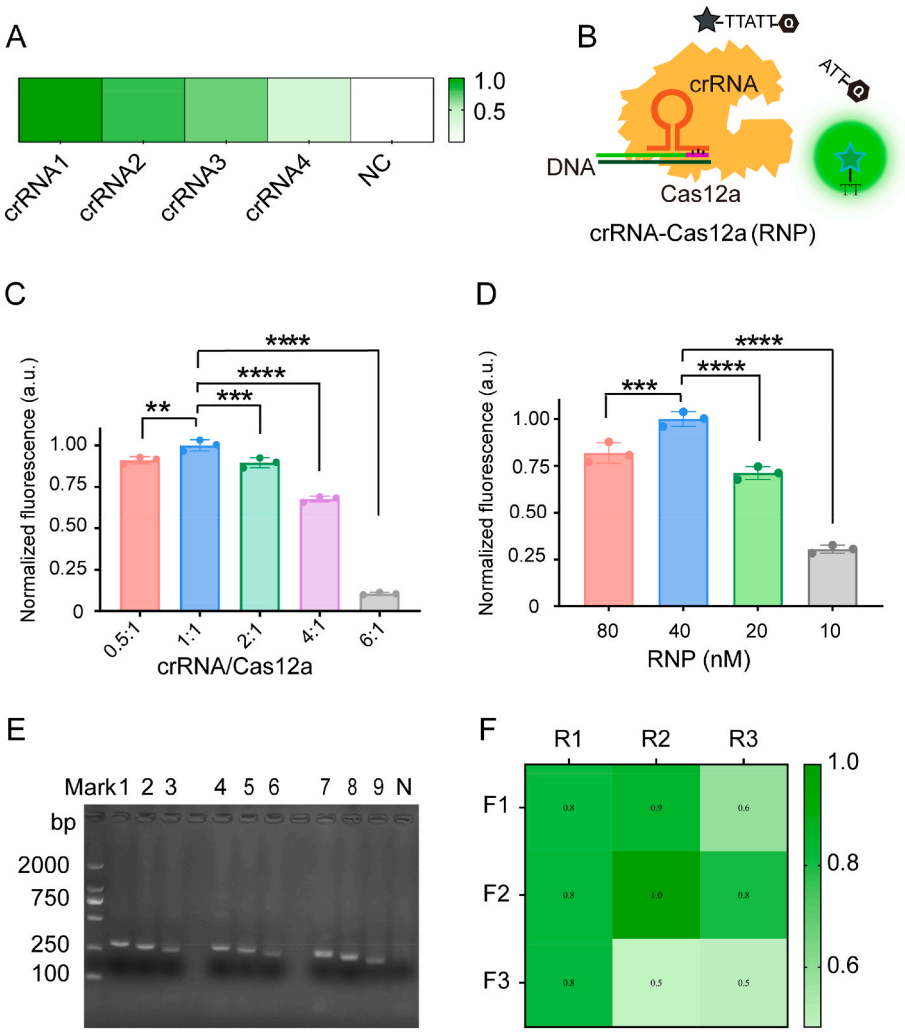


Fig. 2. Optimization of the CRISPR reaction system. A, heatmap of crRNA optimization. B, diagram of Cas12a RNP target detection. C, the Cas12a to crRNA concentration ratio optimization experiment is presented, with the result indicating that the optimal ratio for efficient performance is 1:1. D, optimization of RNP concentration, ranging from 10 nM to 80 nM (n = 3). E, results of agarose gel electrophoresis for RPA primers. F, heatmap of fluorescence signals for primer optimization. All bar graphs are shown as mean ± SD.

RPA mixture with the buffer solution, 1.5 μL of initiator was added. The mixture was then incubated at 37 °C for 15 min. Next, the RPA products were utilized as samples for CRISPR detection, respectively. The specific CRISPR/Cas12a detection procedure was the same as that in 2.3.

2.6. qPCR assay

The reaction system consisted of 10 μL TB Green, 1 μL forward primer, 1 μL reverse primer, 0.5 μL ROX, 2 μL teMplate, and 5.5 μL ddH_2O. The reaction program consisted of 95 °C for 3 min, 40 cycles of 95 °C for 5s and 60 °C for 34s.

2.7. Clinical samples

A total of 50 nasopharyngeal swab samples were collected from Renji Hospital. Nasopharyngeal swabs were stored in a normal saline solution and maintained at −20 °C. Based on the qPCR results, 21 nasopharyngeal swabs were *Mp* positive. Among the 29 *Mp* negative samples, 3 cases were SARS-CoV-2 positive, 3 cases were afflicted with influenza A, and 3 cases were afflicted with influenza B. All samples used in this study followed the informed consent and agreement of patients as well as the ethical regulations of Shanghai Jiaotong University School of Medicine Renji Hospital. Ethics (Approval number: KY2021-192).

2.8. Statistical analysis

Statistical significance was calculated by GraphPad Prism (Vision 9.0). The heat map was initially drafted by GraphPad Prism and fine-tuned by Adobe Illustrator. The two-tailed Mann–Whitney *U* test was employed to compare two groups, with a *P*-value <0.05 serving as a threshold for significance. Three technical replicates were carried out to enhance the statistics.

3. Results and discussion

3.1. Optimization of the RPA-CRISPR/Cas12a reaction system

In order to identify the most appropriate crRNA for the detection of *M. pneumoniae*, we made use of PCR products as sample. Four candidate crRNAs were initially selected based on their capacity to recognize the target sequences. As depicted in Fig. 2A, the crRNA1 that exhibited the highest fluorescence intensity was selected for subsequent experiments. Cas12a ribonucleoprotein (RNP) is a widely utilized RNA-guided CRISPR-associated nuclease in molecular diagnostics. The direct hybridization of target DNA to the crRNA triggers the activation of Cas12a′s ribonuclease activity. Once activated, Cas12a cleaves both the crRNA-bound target DNA and the unbound single-stranded DNA

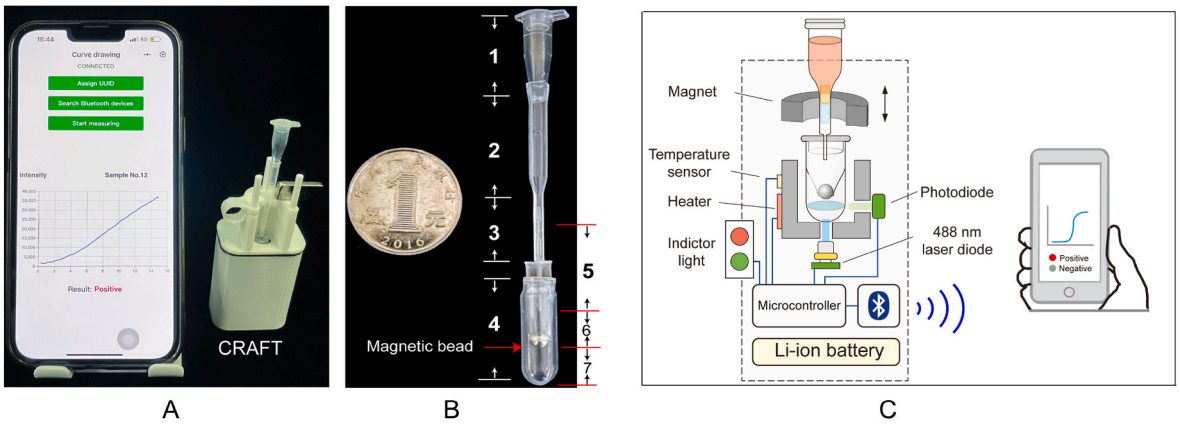


Fig. 3. Fabrication of the CRAFT analysis device. A, CRAFT photograph. B, the integrated tube photograph. 1. sample processing zone, 2. mineral oil storage zone, 3. sterile water storage zone, 4. amplification and detection zone, 5. glass capillary, 6. RPA reaction zone, 7. CRISPR/Cas12a reaction zone. C, the schematic of the CRAFT working principle.

(ssDNA) reporter (Fig. 2B). Next, we tested different RNP ratios between Cas12a and crRNAs, and a 1:1 ratio of Cas12a to crRNA showed the highest fluorescence intensity (Fig. 2C). Furthermore, RNP concentrations of 40 nM showed significant differences (Fig. 2D). Taken together, we determined the optimal condition for the *M. pneumoniae* detection assay by having an equal amount of Cas12a and crRNA at a concentration of 40 nM. The optimized condition was subsequently applied to the following experiments. Next, the RPA detection system was optimized. Based on the principle of RPA primer design, 3 front primers and 3 reverse primers were designed. As shown in Fig. 2E, all 9 primers were capable of achieving amplification reactions. Subsequently, RPA amplification and CRISPR/Cas12a detection were carried out using 1000 copies/μL plasmid samples and 9 pairs of primers, respectively. As illustrated in Fig. 2F, the F2R2 exhibited the highest fluorescence intensity and was thus employed as the optimal primer for subsequent experiments.

3.2. Fabrication of the CRAFT

The CRAFT was developed to accommodate the above CRISPR-based detection assay. As illustrated in Fig. 3A, the CRAFT primarily features an integrated tube for sample manipulation, nucleic acid extraction, and target gene amplification as well as a device for fluorescence detection, digital readout, and result transmission. As shown in Fig. 3B, the tube has a total length of 80 mm and include a sample processing zone (1, with a length of 20 mm), a mineral oil storage zone (2, having a length of 20 mm and a diameter of 4 mm), a sterile water storage zone (3, featuring a length of 15 mm and a diameter of 2 mm), and an isothermal

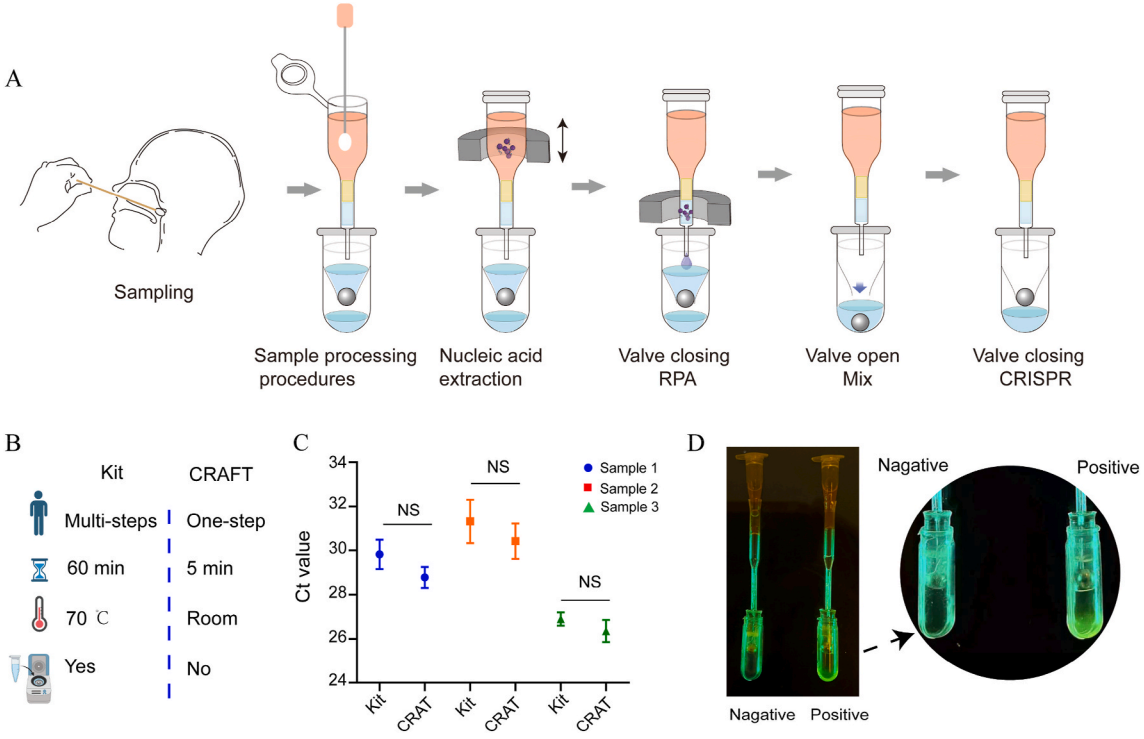


Fig. 4. The operation process and function verification of the CRAFT device (A). Operational procedure of the CRAFT. (B). Comparison of nucleic acid extraction from clinical samples using CRAFT and kits. (C) The efficiency of nucleic acid extraction from clinical samples using CRAT was compared, and no significant difference was identified in PCR Ct values. (D) The results could read by naked-eye just through the blue colors of indicator lights. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

amplification and detection zone (4, having a length of 25 mm and a diameter of 7.5 mm). Among them, 2, 3, and 4 are hoses, a glass capillary (5, have a length of 20 mm and an internal diameter of 0.5 mm) is incorporated into 3 and linked to 4 by a PDMS stopper and a magnetic bead with a diameter of 4 mm can be placed in 4 to serve as a moving valve. The bottom of PCR tube 6 which was placed inside tube 4, has been carefully cut open to allow the insertion of the magnetic bead. The RPA reaction is conducted in tube 6, while the CRISPR reaction takes place at the bottom of tube 4 (7). The magnetic bead functions as a mobile valve to partition the reaction space. Following a 15-min RPA reaction in tube 6, the magnetic bead is displaced downward by a magnet, facilitating the transfer of the RPA reaction mixture to position 7 and ensuring thorough mixing with the CRISPR reaction solution. Subsequently, the magnetic bead is repositioned to the bottom of tube 6, thereby initiating the CRISPR reaction.

The device is equipped with a stand having a height of 8 mm for the magnet to move. The overall system contains the functions including: temperature control, optical sensing, data communication and result display (Fig. 3C). The schematic diagram and the photograph illustrating the internal component architecture of the CRAFT device are presented in Supplementary information (Fig. SI1). The temperature control is achieved by using a thin-film heater and a NTC temperature sensor. A self-adaptive PID algorithm is implemented for the temperature control to provide a stable and accurate reaction environment. Fluorescence intensity of the FAM-labeled reporter is measured by a light-to-digital converter (TSL25711FN, ams-OSRAM, USA) with a laser diode (488 nm, Sharp, Japan) as the exciting light. The measured results are acquired by the microcontroller (STM32L432, ST, Italy) and transmitted to the mobile phone via the Bluetooth module. The light emitting diodes (LEDs) are used to indicate the result of assay. A mobile phone APP is developed to receive and visualize the results. The smartphone interface we described appears to serve as a bridge between users and the CRAFT system, with two core functions, connection with CRAFT system and results display (Supplementary information Fig. SI2). The schematic diagram of the integrated electronic device was presented in Fig. SI3.The mini-program continuously records fluorescence intensity values for subsequent analysis. At the end of the 15-min incubation period, an algorithm calculates the mean fluorescence intensity collected during the final segment (e.g., the last 30 s) and compares this average to a predefined threshold, established through extensive validation using clinical data. If the calculated intensity exceeds this threshold, the interface automatically displays a "positive" result; otherwise, it indicates a "negative" outcome. A lithium battery is used to supply the system, enabling fully wireless detection. The whole size of the device is only 40 mm × 25 mm × 55 mm.

The printed circuit board (PCB) of the CRAFT comprises the following components: power supply, temperature control module, light-emitting diode (LED), optical sensor, Bluetooth module, and microcontroller (Supplementary information Fig.SI4). The tube is disposable, while the device is reusable. The device is user-friendly, enabling single-handed operation for rapid nucleic acid extraction and can independently perform signal acquisition and result interpretation, and do so wirelessly through a smartphone application. This CRAFT requires no external equipment and serves as a flexible and versatile technology platform capable of addressing the increasing demand for molecular diagnostics. The miniaturized, cost-effective, and user-friendly nature of this equipment makes it highly promising for application in primary healthcare settings.

3.3. The operation of the CRAFT device

The detection process of *M. pneumoniae* was depicted in Fig. 4A, the 30 μL CRISPR/Cas12a reaction mixture was introduced to the CRISPR reaction zone of the tube (7), and the magnetic beads were positioned at the bottom of tube 6. Subsequently, 10 μL of the RPA reaction mixture was introduced into the RPA reaction zone. Next, 50 μL of sterilized deionized water (containing magnesium ions) and 100 μL of mineral oil were added to zones 3 and 2 of the tube, respectively. The tube (1 zone −3 zone) was inserted into 4 zone, and 300 μL of lysate (1M GuSCN, 0.2 % SDS, 1M Tris-HCl, pH 7.4) was added to tube 1 zone.

In this study, a one-step method was employed for nucleic acid extraction. Upon acquiring the patient sample, the nasopharyngeal swab was inserted into the lysate in zone 1 of the tube, gently rotated, and subsequently withdrawn. Thereafter, 50 μL of magnetic nanoparticles were added and incubated for 3 min. The nucleic acids released from the lysed sample were adsorbed onto the magnetic nanoparticles. By manipulating the magnet on the scaffold, the magnetic nanoparticles, which had adsorbed the nucleic acids, traversed through zone 2 (oil phase) and entered zone 3 (liquid phase) under the influence of the magnetic field. Due to the polarity of nucleic acids and intermolecular interactions, they are insoluble in oil but soluble in water. Consequently, rapid nucleic acid extraction from clinical samples can be achieved without the need for waste liquid transfer by utilizing the movement of magnetic nanoparticles. Following the release of nucleic acids in the liquid phase, 10 μL of the solution from zone 3 was manually transferred to the RPA zone using a pipette.

Open the CRAFT temperature control module, and after 15 min at 37 °C, take out the integrated tube, transfer the magnetic bead to the bottom of 4 zone under the action of the magnetic field, and fully mix the RPA amplification product with the CRISPR/Cas12a reaction solution. Then, the tube was inserted into the CRAFT again, and the magnetic bead was transferred to the RPA reaction zone. Open the CRAFT fluorescence detection and result transmission mode, and react at 37 °C for 15 min.

Compared with the multi-step and time-consuming (approximately 60 min) operation of nucleic acid extraction using commercial kits, the one-step extraction method of the CRAFT we developed takes only 5 min and can be carried out at room temperature, having better potential for field application (Fig. 4B). To evaluate the comparative performance of the CRAFT device versus a commercial kit for nucleic acid extraction from clinical samples, PCR analysis was performed on *Mycoplasma pneumoniae* DNA obtained from three clinical specimens using both methodologies. As demonstrated in Fig. 4C, statistical analysis revealed no significant difference in PCR Ct values between DNA extracted via the CRAFT device and that obtained through the commercial kit. Compared to the kit, CRAFT requires no additional auxiliary equipment for nucleic acid extraction, is simple and rapid, and can be performed at room temperature, making it suitable for field detection of *M. pneumoniae* in resource-limited settings. The results could be readout with naked-eyes using a blue light. As shown, in contrast to the negative results, the positive results of *M. pneumoniae* exhibited green fluorescence (Fig, 4D).

The operational protocols of the CRAFT system and qPCR are comprehensively outlined in Table SI2 of the Supporting Information. The CRAFT system demonstrates a turnaround time (TAT) of 35 min for *Mycoplasma pneumoniae* detection, representing a 72 % reduction compared to the 125-min duration mandated by conventional qPCR. This integrated diagnostic platform executes the complete analytical workflow within one tube, achieving sample-to-answer functionality. Distinct from qPCR-based approaches, the CRAFT technology eliminates dependency on specialized PCR facilities, obviates the requirement for advanced technical personnel, and enables decentralized point-of-care testing applications.

The CRISPR/Cas12a cleavage reaction and nucleic acid amplification are incompatible. When the isothermal amplification system is integrated with the CRISPR/Cas12a reaction in a single tube, the CRISPR/Cas12a cleavage can degrade the template, thereby reducing amplification efficiency and detection sensitivity. To address the aforementioned issues, a number of improvements have been developed, including the isolation of reagent physical space in closed tubes (Chen et al., 2020; Wang et al., 2021), use of phase separation solutions (Lin et al., 2022) or using photochemically activated caged crRNA(Hu et al., 2022; Sun et al., 2024). The above methods still reduce the efficiency of

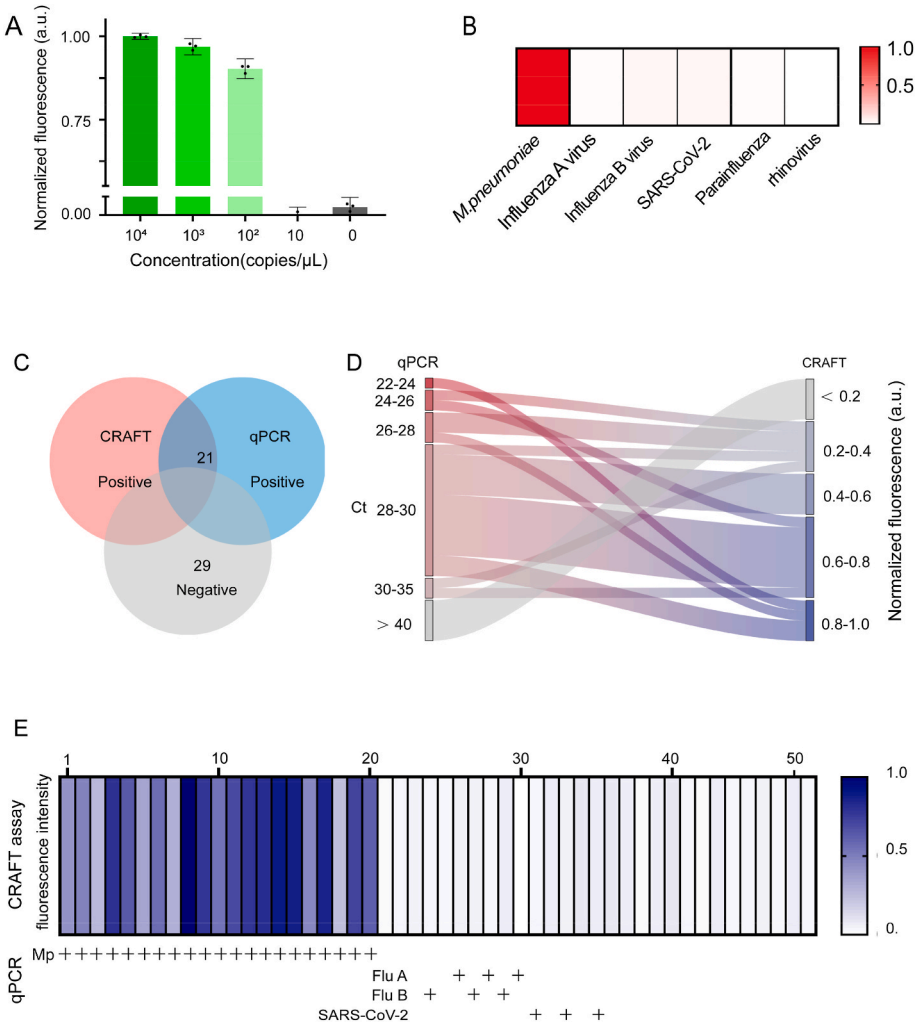


Fig. 5. Clinical validation of the CRAFT. (A). Sensitivity analysis of the CRISPR/Cas12a-RPA detection system. All the experiments shown above were replicated three times, and bars represent mean ± 95 % CI. (B) Validation of the cross-reactivity between M. pneumoniae detection and other viruses with similar symptoms detection including influenza A, influenza B, parainfluenza, SARS-CoV-2, and rhinovirus using CRAFT. (C) Comparison of the performance of qPCR and the CRAFT assay on 50 nasopharyngeal swab samples. (D) The Sankey diagram showing the correlation between the Ct values and the fluorescent intensity. (E) The fluorescence intensity of 50 clinical samples detection results.

CRISPR/Cas12a reactions, so the incompatibility problem is still not well solved. In this study, a magnetic bead was utilized as a mobile valve to isolate the reaction reagents. By facilitating the mixing of RPA amplification products and CRISPR/Cas12a reagents through the movement of magnetic bead, the efficiency of the CRISPR reaction was enhanced.

3.4. Clinical samples analysis

The sensitivity of the CRAFT was subsequently tested by using a gradient of the corresponding *M. pneumoniae* plasmid target at concentrations of 10000, 1000, 100, 10, and 0 copies/μL. As shown, the sensitivity of the system for the detection of *M. pneumoniae* was 100 copies/μL (Fig. 5A). The limit of detection of the CRAFT system is lower than that of qPCR, likely attributable to distinct reaction mechanisms. PCR utilizes thermal cycling to amplify DNA through three sequential phases: denaturation, annealing, and elongation. This process ensures efficient amplification of target DNA fragments. In contrast, the CRAFT system employs isothermal conditions, significantly simplifying experimental protocols. Nevertheless, such operational simplicity typically compromises amplification efficiency when compared with qPCR.

To validate the specificity of the system, we carried out experiments using standard samples of influenza A, influenza B, parainfluenza, SARS-CoV-2, *M. pneumoniae,* and rhinovirus. As depicted in Fig. 5B, the system successfully discriminated *M. pneumoniae* from other pathogens. These results indicate that the developed assay showed high sensitivity and specificity for detecting *M. pneumoniae*.

Next, to validate the feasibility of the CRAFT platform, the assay was employed to detect real samples from patients. The study was carried out in an unblinded manner. The DNA extracts of 50 clinical nasopharyngeal swab samples, which were previously identified by qPCR (including 21 *M. pneumoniae*, 3 FluA, 3 FluB, and 3 SARS-CoV-2 positive samples), were tested through the CRAFT assay. The results indicated that the detection results of CRAFT were in line with those of qPCR (Fig. 5C). The correlation between the range of Ct values of 23S rRNA of *M. pneumoniae* and the relative fluorescence values was visualized as a Sankey. As shown, the Ct values of 23S rRNA showed a negative correlation with the fluorescent intensity values (Fig. 5D). The fluorescence intensity of the CRAFT detection results was depicted in Fig. 5E. Among the 50 samples, 21 were positive for *M. pneumoniae*. Among the 29 negative samples, there were 3 cases each for Flu A, Flu B, and SARS-CoV-2. The results of CRAFT demonstrated 100 % concordance with qPCR across all clinical samples, with an area under the curve (AUC) of 1.0 (Supplementary information Fig.SI5). The results indicating that the

Table 1
Comparison of CRAFT with other point-of-care *M. pneumoniae* detection methods.

Methods	Target	Sensitivity (%)	Specificity (%)	Time-to-result (min)	Cost (USD)
CRAFT	DNA	100(21/21)	100	35	3
EasyNAT® MP (Chen et al., 2022)	DNA	100(80/80)	97.56	55	12
BioFire FilmArray respiratory panel (Jordana-Lluch et al., 2015)	DNA	98(208/212)	100	60	341
QIAstat-Dx Respiratory Panel (Leber et al., 2020)	DNA	100(25/25)	99.7	69	273
Capilia™Mycoplasma (Lin et al., 2020)	Antigen	44.6(25/56)	100	15	11

proposed CRAFT for *M. pneumoniae* detection had competitive detection performance compared with the qPCR assay, and with faster reaction speed, less user operation, and stronger field deployable ability.

As shown in Table 1, CRAFT outperformed existing methods for detecting *Mycoplasma pneumoniae*. Compared to EasyNAT® MP (Chen et al., 2022), BioFire FilmArray Respiratory Panel (Jordana-Lluch et al., 2015), QIAstat-Dx Respiratory Panel (Leber et al., 2020), and Capilia™ Mycoplasma (Lin et al., 2020), CRAFT provided results in just 35 min and cost only $3. In contrast, the Capilia *Mycoplasma* method completes antigen detection in 15 min but has the lowest sensitivity (44.6 %). Its cost is lower than some methods but still significantly higher than CRAFT. Compared to existing methods, CRAFT offers distinct advantages in sensitivity, specificity, detection time, and cost, positioning it as a promising platform for *Mycoplasma pneumoniae* detection.

CRAFT demonstrates notable versatility, allowing the systematic design and optimization of RPA primers and crRNAs for various respiratory pathogens. This capability enables precise detection of specific pathogens, including influenza A virus, influenza B virus, and respiratory syncytial virus. Despite the aforementioned advantages of CRAFT, several technical limitations should be noted. The CRAFT system currently employs a monochromatic fluorescent probe for the detection of Mycoplasma pneumoniae. The device is currently confined to single-pathogen detection and lacks multiplexing capabilities. Subsequent research may explore the development of multiplexed fluorescent probes precisely engineered to align with the distinct base-cleavage specificities of diverse Cas13 protein subtypes (Gootenberg et al., 2018), enabling simultaneous detection of multiplexed gene detection in a single-tube, one-pot format. Currently, the CRAFT system's detection reagents require cryogenic storage at −20 °C prior to deployment. To enhance field applicability, we are advancing lyophilization protocols to convert these reagents into stabilized powder formulations, which will be packaged into lyophilized reagent tubes compatible with ambient temperature storage. This technological refinement will ultimately facilitate point-of-care pathogen detection using the CRAFT platform.

4. Conclusion

In the present study, we developed a palm-sized CRISPR sensing platform, CRAFT, for point-of-care detection of *M. pneumoniae*. The CRAFT analysis device accomplishes the entire process, including sample pretreatment, pathogen lysis, DNA extraction, RPA, CRISPR/Cas12a reaction, signal acquisition, and result interpretation. We validated the developed assay in 50 clinical samples, and the testing results were consistent with qPCR. CRAFT has further facilitated the application of CRISPR technology in clinical research, and the platform can also serve as a general platform for pathogen detection in resource-limited settings.

CRediT authorship contribution statement

Jiajun Li: Writing – original draft, Software, Data curation. **Haiqian Huang:** Investigation, Formal analysis, Data curation. **Zerui Song:** Validation, Software, Investigation, Formal analysis. **Shiying Chen:** Writing – original draft, Investigation, Formal analysis, Data curation. **Jingsong Xu:** Software, Methodology, Investigation, Formal analysis, Data curation. **Jun Yang:** Validation, Supervision, Software. **Chenyue Zheng:** Software, Formal analysis, Data curation. **Yu Liu:** Investigation, Formal analysis, Data curation. **Junheng Zhang:** Writing – original draft, Software, Data curation. **Li Cao:** Visualization, Validation, Formal analysis, Data curation. **Qian Liu:** Resources, Methodology, Formal analysis. **Qiong Li:** Validation, Project administration, Investigation, Formal analysis. **Min Li:** Writing – review & editing, Funding acquisition, Conceptualization. **Zhen Gu:** Writing – review & editing, Resources, Methodology, Conceptualization. **Hua Wang:** Writing – review & editing, Validation, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

We declare that the manuscript "Palm-sized CRISPR sensing platform for on-site *Mycoplasma pneumoniae* detection" has not been previously published, is not currently submitted for review to any other journal. All authors of this paper have read and approved the final version submitted.

we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2025.117458.

Data availability

The data that has been used is confidential.

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