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Jingyi Jiang, Jian Xu, Li Gong, Qiong Li, Ping Yao, Rong Wang, Jinyi Jiang, Lei Xu, Fengming Wang & Xujian Mao

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One-step detection of *Chlamydia psittaci* using recombinase polymerase amplification and CRISPR/Cas12b

Jingyi Jiang [1,4], Jian Xu[1,4], Li Gong[1,4], Qiong Li[1], Ping Yao[1], Rong Wang[2], Jinyi Jiang[1], Lei Xu[3]✉, Fengming Wang[1,2]✉, Xujian Mao[1]✉

[1]Pathogen Inspection Center, Changzhou Center for Disease Control and Prevention, 203 Taishan Road, Changzhou 213022, Jiangsu, China

[2]School of Public Health, Nanjing Medical University, Nanjing 211166, Jiangsu, China

[3]Key Laboratory for Pathogen Infection and Control of Jiangsu Province, Nanjing Medical University, Nanjing 211166, Jiangsu, China

[4]These authors contributed equally: Jingyi Jiang, Jian Xu, Li Gong

Corresponding authors e-mail: xulei_nj1989@163.com; wfm0519@163.com; m5575383@163.com

Abstract

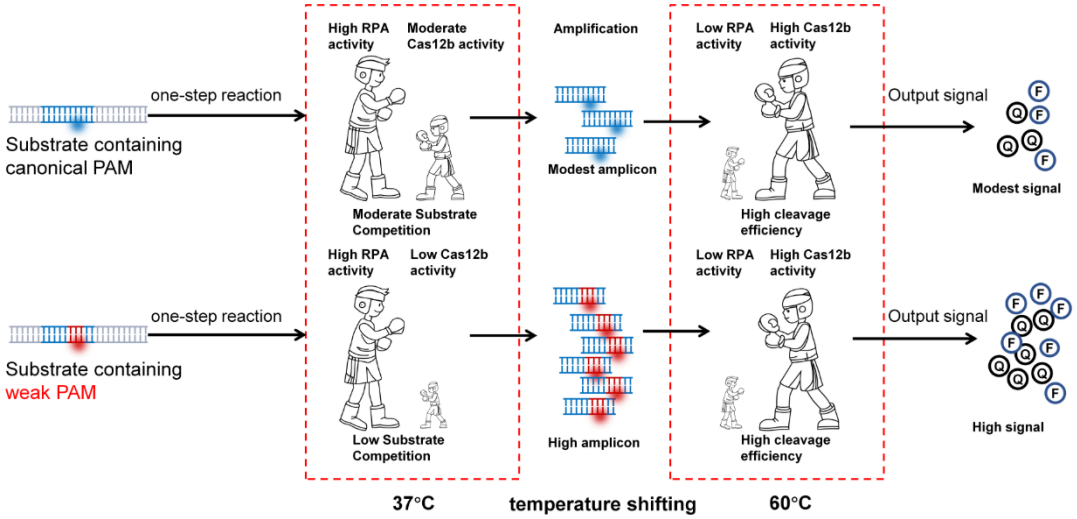
The development of rapid, sensitive, and user-friendly diagnostic methods for *Chlamydia psittaci* (*C. psittaci*) is critical for early diagnosis and effective control of infections. Current techniques, such as qPCR and next-generation sequencing, face limitations in accessibility and practicality for point-of-care testing (POCT). In this study, we present the WPTTS platform (one-step recombinase polymerase amplification (RPA)-CRISPR/Cas12b assay using weak protospacer adjacent motif (PAM) recognition with two-temperature shifting), which leverages the temperature-dependent PAM stringency of *Alicyclobacillus acidophilus* Cas12b (AapCas12b) to overcome the challenges of one-step CRISPR detection. By employing weak PAM sequences and a two-temperature protocol (37°C for amplification, 60°C for detection), WPTTS minimizes interference between RPA amplification and CRISPR cleavage, achieving a sensitivity of 10^2 copies/reaction—equivalent to two-step methods—while maintaining operational simplicity. The platform also demonstrates enhanced specificity, enabling single-base resolution at critical target positions. Clinical validation with 40 samples confirmed its superior performance, with 95% positive agreement and 100% negative agreement compared to qPCR. This study highlights the potential of WPTTS as a robust, rapid,

and highly sensitive tool for POCT applications in *C. psittaci* detection and broader infectious disease diagnostics.

Keywords

Recombinase polymerase amplification; CRISPR/Cas12b; *Chlamydia psittaci*; Nucleic acid detection; Protospacer adjacent motif

Graphical Abstract



Introduction

Chlamydia psittaci (*C. psittaci*) is a significant zoonotic pathogen capable of causing influenza-like symptoms and respiratory diseases(Zhang et al., 2022). Although macrolide antibiotics demonstrate good therapeutic efficacy against it, the high similarity of its clinical manifestations to influenza, coupled with the limitations of current detection technologies, often leads to misdiagnosis and delayed treatment in clinical practice(Dai et al., 2022; Nieuwenhuizen et al., 2018).

Currently, quantitative real-time PCR (qPCR) serves as the primary method for detecting this pathogen. However, its high requirements for laboratory equipment and technical expertise make it difficult to implement in primary healthcare institutions, emergency settings, or home testing scenarios. While next-generation sequencing enables accurate identification, its long turnaround time, high costs, and reliance on specialized bioinformatics analysis also render it unsuitable for routine detection needs(Nieuwenhuizen et al., 2018; Wang et al., 2024). Therefore, the development of a user-friendly, cost-effective, rapid, and highly sensitive

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detection technology adaptable for point-of-care testing (POCT) applications holds significant practical importance for achieving early diagnosis and precise prevention and control of *C. psittaci* infections.

In recent years, the CRISPR/Cas system, derived from prokaryotic adaptive immune mechanisms, has brought revolutionary breakthroughs to the field of molecular diagnostics due to its high programmability, simplicity, and efficiency(Chen et al., 2018; Yang et al., 2023). The system consists of CRISPR RNA and a Cas protein with nuclease activity guided by it(Koonin et al., 2017). To achieve highly sensitive molecular detection, CRISPR-based technology is often combined with isothermal nucleic acid amplification techniques(Mao et al., 2024). This integrated strategy enables nucleic acid amplification and detection without thermal cycling, a feature of significant value for developing simplified nucleic acid testing methods and devices(Kang et al., 2022). Leveraging these characteristics, detection platforms such as DETECTR, HOLMES, and CDetection have emerged(Chen et al., 2018; Li et al., 2018; Teng et al., 2019).

However, a critical gap remains between the promise of these platforms and the requirements for robust POCT. While one-step CRISPR assays (where isothermal amplification and CRISPR detection occur in a single tube with one-time sample loading) offer the ultimate simplicity for on-site use, their development has been hindered by a fundamental challenge in reaction chemistry: the competition for substrates between isothermal amplification and CRISPR cleavage(Chen et al., 2025; Lin et al., 2023; Tong et al., 2024).

To address this challenge, current mainstream technology platforms primarily adopt the following regulatory strategies: (1) Physical isolation, where reagents are compartmentalized in tube caps, tube walls, or microfluidic devices(Chen et al., 2025; Chen, Zong, et al., 2022); (2) Physical deceleration through constructing miscible multiphase systems(Lin et al., 2022; Yin et al., 2020), to delay the contact between CRISPR and amplification reactions; (3) Chemical isolation by detecting only amplification products without lysing the original target templates(Ding et al., 2020; Yang et al., 2024), or employing photo-regulation strategies to achieve "off-

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on" switching of guide RNA(Chen, Xu, et al., 2022; Hu et al., 2022; Liu et al., 2024) ; and (4) Chemical deceleration via selection of suboptimal PAM sequences(Lin et al., 2023; Lu et al., 2022) or modification of Cas enzymes(Tong et al., 2024) to reduce the initial cleavage efficiency. While these approaches represent significant advances, they often introduce additional complexity in reagent formulation or device engineering, and many still struggle to achieve the ideal combination of single-tube simplicity, rapid detection speed, and consistently high sensitivity required for practical POCT.

In our previous study(R. Wang et al., 2023) we developed a nucleic acid detection system based on *Alicyclobacillus acidophilus* Cas12b (AapCas12b) protein. Guided by single-guide RNA (sgRNA), AapCas12b specifically cleaves target double-stranded DNA when the protospacer adjacent motif (PAM, 5'-TTN-3') is present. The cleavage reaction demonstrates optimal activity at elevated temperatures ranging from 60 to 65°C(Teng et al., 2018). Upon activation, AapCas12b exhibits trans-cleavage activity, enabling nonspecific degradation of single-stranded DNA (ssDNA) reporter probes, thereby converting detection results into fluorescent signals (Joung et al., 2020). Leveraging this mechanism, we established two-step and one-step LAMP-CRISPR/Cas12b assays for detecting *C. psittaci*. While the two-step method showed high consistency with qPCR results, it involved cumbersome operations and carried risks of aerosol contamination. The one-step method, although operationally simpler, demonstrated significantly reduced sensitivity, limiting its practical application. These limitations have driven us to develop a more sensitive, specific, and rapid one-step CRISPR detection system.

In this study, investigation of the PAM recognition characteristics of AapCas12b revealed a temperature-dependent stringency pattern: PAM recognition stringency significantly decreases at elevated temperatures but intensifies at lower temperatures. Based on this finding, we developed the WPTTS (one-step RPA-CRISPR/Cas12b assay using weak PAM recognition with two-temperature shifting) platform. This platform innovatively employs a strategic temperature modulation

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to decouple the RPA amplification and CRISPR detection stages in a single tube: the initial low-temperature phase suppresses AapCas12b cleavage via stringent PAM recognition to allow uninterrupted RPA amplification, while the subsequent high-temperature phase activates robust cleavage via relaxed PAM recognition to ensure maximal detection sensitivity.

This platform not only offers straightforward operation and rapid detection capabilities, but more importantly demonstrates exceptional detection performance, making it particularly valuable for clinical diagnosis and field detection of C. psittaci infections.

Methods

Materials

All RPA primers, templates, and the ssDNA-FQ reporter were synthesized by GENEWIZ (Suzhou, China), sequences are provided in the Table S1 and Table S2. AapCas12b was obtained from Tolo Biotech Co., Ltd (Shanghai, China; Catalog: 32118-03). The TwistAmp® Basic Kit was purchased from TwistDX, Inc (UK; Product Code: TABAS03KIT). A commercial *C. psittaci* nucleic acid detection kit was acquired from Yonglang Biotechnology Co., Ltd (Nanjing, China; Catalog:250827). A magnetic bead-based nucleic acid rapid extraction kit was sourced from Zybio Inc (Chongqing, China; Catalog: 2501005). Nuclease-free water was obtained from Thermo Scientific™ (USA; Catalog: R0582). Target sequences corresponding to the *CPSIT_*0429 region of the *C. psittaci* genome (GenBank accession: CP002549.1) were selected for the assay.

Sample Preparation

Sputum or bronchoalveolar lavage fluids were used as the source of pathogen templates and were obtained from the Surveillance Program for *C. psittaci* in Jiangsu Province. The analytical specificity of the assay was evaluated using a panel of reference strains and clinical isolates preserved in our laboratory, including the reference strains *Chlamydophila pneumoniae* (ATCC 53592), *Streptococcus pneumoniae* (ATCC 49619), *Mycoplasma pneumoniae* (ATCC 15531), and *Staphylococcus aureus* (ATCC 25923), as well as clinical isolates of SARS-CoV-

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2 (JN.1 strain), Influenza A virus (H1N1 strain), Influenza B virus (Victoria lineage strain), and Respiratory Syncytial Virus (subtype A). Pathogen templates were extracted by an automatic nucleic acid extraction instrument and nucleic acid rapid extraction kit (magnetic bead method).

CRISPR/Cas12b detection assay

The CRISPR/Cas12b nucleic acid detection system consisted of 0.5 μL of AapCas12b (10 μM), 2 μL of sgRNA (2.5 μM), 2 μL of the ssDNA-FQ reporter (10 μM), and 2 μL of HOLMES Buffer for Cas12b (10×) in each reaction tube. Then, an appropriate volume of template DNA was added, followed by nuclease-free water to reach a final volume of 20 μL. After shaking and centrifugation, the reaction tube was immediately incubated in an Applied Biosystems® QuantStudio Q5 (ABI Q5) instrument at the set temperature for 1 hour, after which the fluorescence signal was recorded every minute.

Two-step system of Recombinase Polymerase Amplification (RPA) and CRISPR/Cas12b

RPA reaction: The primer sequences were designed based on the instructions of the TwistAmp™ Reaction Kit. The RPA reaction was performed according to the instructions of the TwistAmp® Basic Kit (TwistDx). The reaction mixture was as follows: 29.5 μL of primer-free rehydration buffer, 13.7 μL of nuclease-free water, 2.4 μL of each of the forward and reverse primers (10 μM) and 2 μL template DNA were mixed in an Eppendorf tube and added to the reaction tube containing the lyophilized RPA enzyme. The mixture was vortexed until the lyophilized powder of the RPA enzyme was completely dissolved, followed by brief centrifugation. Draw 14 μL from 50 μL of the mixed reaction solution and add 1 μL of magnesium acetate (280 mM), making a total volume of 15 μL as one reaction unit. The reaction tube was then incubated at 37°C in a metal thermocycler for 20 minutes. CRISPR/Cas12b reaction: The reaction mixture was as follows: 1.3 μL of AapCas12b (10 μM), 5 μL of sgRNA (2.5 μM), 5 μL of the ssDNA-FQ reporter (10 μM), 5 μL of HOLMES Buffer for Cas12b (10×) and 33.7 μL of nuclease-free water were mixed in an Eppendorf tube. Pipette 15 μL from it and add to 15 μL of the RPA reaction system. After mixing

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thoroughly to a total volume of 30 μL, incubate in the ABI Q5 instrument at 60°C for 40 minutes.

RPA coupled with CRISPR/Cas12b in a one-step reaction

RPA and CRISPR/Cas12b one-step reaction is a slight modification based on the above two-step reaction. First, 15 μL of the CRISPR/Cas12b reaction mixture is added to the Eppendorf tube, then 15 μL of the RPA reaction mixture (the same as the above two-step reaction) is added, and the total 30 μL reaction system is mixed. The reaction tube was covered and placed in a centrifuge for slow and brief centrifugation and then incubated in an ABI Q5 instrument at 37°C for 20min and 60°C for 40min. Fluorescence values were collected at 60°C and recorded every minute.

Quantitative polymerase chain reaction

The qPCR assay was performed according to the instructions provided in the *C. psittaci* nucleic acid detection kit. Each reaction consisted of 16 μL reaction liquid I, 1 μL reaction liquid II, and 3 μL sample nucleic acid, making a total volume of 20 μL. The reaction was performed as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15s for annealing, and extension at 60°C for 40 s. The fluorescence signal from the PCR was monitored at each extension step using an ABI Q5 instrument. Samples with a Ct value ≤ 35 were considered positive, while those with a Ct value > 35 were considered negative.

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Results

AapCas12b exhibits temperature-dependent PAM stringency characteristics

Previous study has demonstrated that Cas12a can effectively modulate its cleavage kinetics by adopting suboptimal PAM strategy, thereby significantly improving the sensitivity of one-step detection(Lu et al., 2022). To explore the applicability of this strategy in other Cas12 orthologs and further optimize our previously developed *C. psittaci* detection method based on the combination of LAMP and AapCas12b, we systematically compared the recognition efficiency of AapCas12b for the canonical PAM (TTC) and five suboptimal PAMs under low-copy

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template conditions. The result showed that in the one-step LAMP-CRISPR/Cas12b detection system, the use of suboptimal PAM did not exhibit superior detection performance compared to the canonical PAM (Fig. S1). This finding is also consistent with conclusions from other studies (Tong et al., 2024).

To investigate whether temperature could influence PAM recognition characteristics and thereby enhance detection performance, we constructed a target mutant library encompassing all 64 possible 3-base PAM sequences (NNN) based on our previously designed sgRNA targeting the *CPSIT*_0429 gene of *C. psittaci*(R. Wang et al., 2023). This library was systematically evaluated for trans-cleavage activity across six temperature gradients (35, 40, 45, 50, 55, and 60°C). The results indicated that the canonical PAM (TTN) exhibited significant activity across this temperature range (Fig. S2). Notably, as the temperature increased to 60°C, AapCas12b displayed a pronounced relaxation in PAM recognition, manifested as a reduced difference in endpoint fluorescence signals between canonical and non-canonical PAM. This phenomenon can be mechanistically explained by thermally-induced dsDNA destabilization, where elevated energy promotes localized strand separation to form transient single-stranded regions that facilitate Cas12b/sgRNA complex invasion regardless of PAM type. In contrast, under lower temperatures, AapCas12b demonstrated stricter PAM selectivity, with a significant disparity in endpoint fluorescence signals between the two PAM types (Fig. 1), as the maintained dsDNA stability reinforces the system's dependence on canonical PAM sequences for activation. These findings reveal that AapCas12b’s PAM recognition exhibits distinct temperature-dependent characteristics, aligning with previously reported phenomena where elevated temperatures lead to reduced PAM restriction in Cas proteins(Chen et al., 2023), representing a thermodynamic modulation of the energy barrier for PAM recognition.

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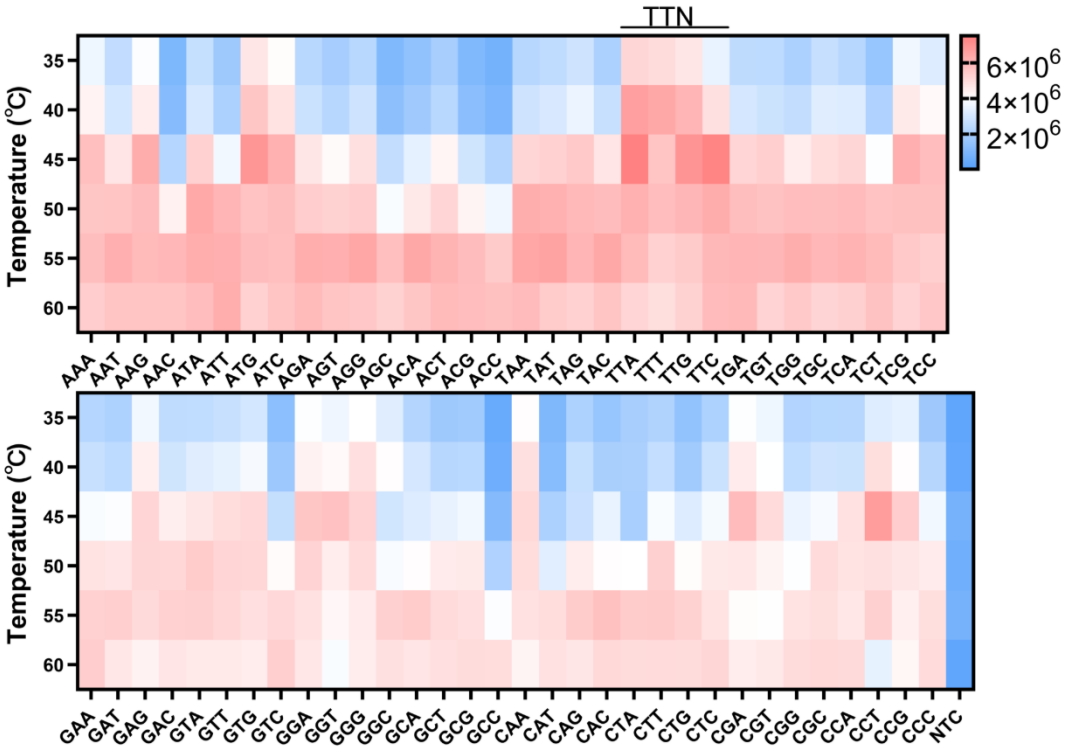


Fig. 1 Comparison of AapCas12b trans-cleavage activity at different temperatures using 64 types of PAM (NNN), TTN represents canonical PAM. Heatmap showing the mean increase in fluorescence signal within 1 hour after target DNA addition. NTC non-target control, $n = 3$.

Temperature-dependent regulation of weak PAM activity demonstrates universality

To further investigate the universality of temperature-dependent PAM activity regulation, we conducted quantitative assessment and ranking of the cleavage efficiency for all tested PAM sequences. Based on this, we identified five PAM sequences (GCC, ACC, AAC, AGC, CAT) with the lowest cleavage efficiency under low-temperature conditions, which were defined as "weak PAM" (Fig. S3). We extended these weak PAM from 3-base to 4-base sequences and validated them using three targets with varying GC contents. Experimental results demonstrated that: (1) The extension from 3-base to 4-base PAM did not significantly alter cleavage efficiency across three targets with different GC contents, regardless of the extended base type, suggesting that the core recognition motif of weak PAM may be limited to three bases; (2) Under low-temperature conditions, weak PAM consistently exhibited reduced activity compared to canonical PAM across all tested

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targets with different GC contents, indicating that their recognition pattern shows no significant correlation with the GC content of the spacer region, which may represent a universal characteristic; (3) When temperature was elevated to 60°C, weak PAM regained cleavage capacity, achieving efficiency comparable to canonical PAM (Fig. 2).This temperature-induced alleviation of PAM restriction is highly consistent with our previous observations.

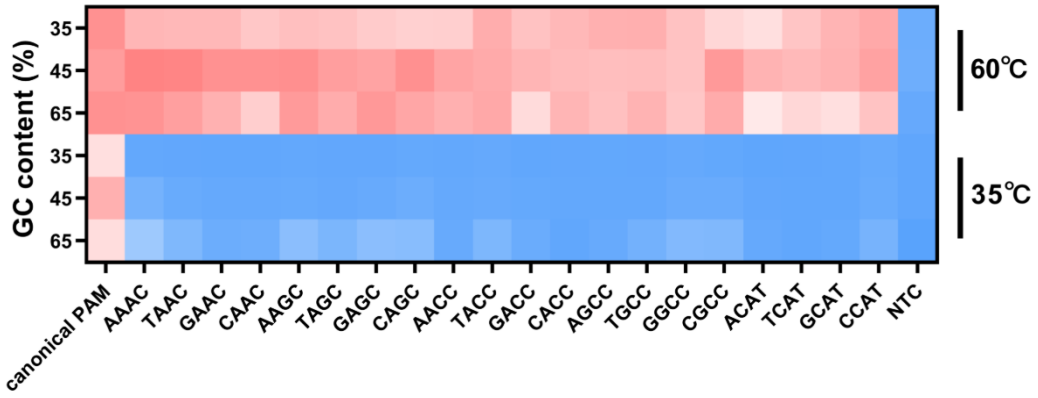


Fig. 2 Comparison of AapCas12b trans-cleavage activity across two temperatures (35°C and 60°C) for 20 extended PAM variants (4-nt) derived from 5 weak PAM variants (3-nt) and canonical PAM. Heatmap showing the mean increase in fluorescence signal within 1 hour after target DNA addition. NTC non-target control, $n = 3$.

Development of a temperature-shifted one-tube detection system based on weak PAM

Building on these findings regarding the temperature-dependent activity of PAM, we have developed the WPTTS detection (one-step RPA-CRISPR/Cas12b assay using weak PAM recognition with two-temperature shifting) platform. To establish an optimal temperature regime that balances RPA amplification efficiency and CRISPR-Cas12b detection activity, we performed a comprehensive orthogonal optimization of a two-stage temperature-shift protocol. We tested all combinations of three RPA temperatures (37°C, 42°C, 45°C) with three Cas12b detection temperatures (37°C, 45°C, 60°C). Among the nine combinations evaluated, only the protocols employing 60°C for Cas12b detection—specifically 37°C/60°C and 42°C/60°C—generated significant fluorescence signals (Fig. S4). We selected the

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37°C to 60°C temperature-shift protocol as it provided optimal signal-to-noise ratio and amplification kinetics.

The core innovation of this platform lies in its precise temperature-programmed regulation of AapCas12b cleavage activity to achieve optimal reaction conditions at different stages (Fig. 3a). Specifically, during the low-temperature phase (RPA amplification-dominant stage), we employ weak PAM to maximally suppress AapCas12b cleavage activity, thereby preventing interference with RPA amplification. In the subsequent high-temperature phase (detection-dominant stage), we fully activate AapCas12b's cleavage capability to ensure highly efficient signal detection. This strategy effectively resolves the challenge of mutual interference between amplification and detection in conventional single-tube methods, providing a novel approach for developing highly sensitive integrated molecular diagnostic systems.

To evaluate the platform performance, we conducted comparative studies between weak PAM (ACC) and canonical PAM (TTC) using a two-temperature protocol (37°C for 20 min → 60°C for 40 min), with parallel assessments of both two-step (Fig. S5) and one-step (Fig. 3b,c) RPA-CRISPR/Cas detection systems. The results demonstrate that in two-step detection, both canonical and weak PAM achieved comparable sensitivity of 10^2 copies/reaction. However, in one-step detection, while canonical PAM showed a sensitivity of 10^3 copies/reaction, the weak PAM exhibited significantly enhanced performance, improving sensitivity to 10^2 copies/reaction- matching the performance of two-step detection. To verify system stability, we conducted 10 replicate tests at the detection limit of 10^2 copies/reaction, with confirming the system's exceptional robustness (Fig. 3d).

To evaluate the analytical specificity of our WPTTS platform, we challenged the system with a panel of clinically relevant respiratory pathogens. Our platform generated strong positive signals only for *C. psittaci* targets, while showing no detectable cross-reactivity with any of the non-target pathogens (Fig. S6). These results confirm the high interspecies specificity of the WPTTS platform.

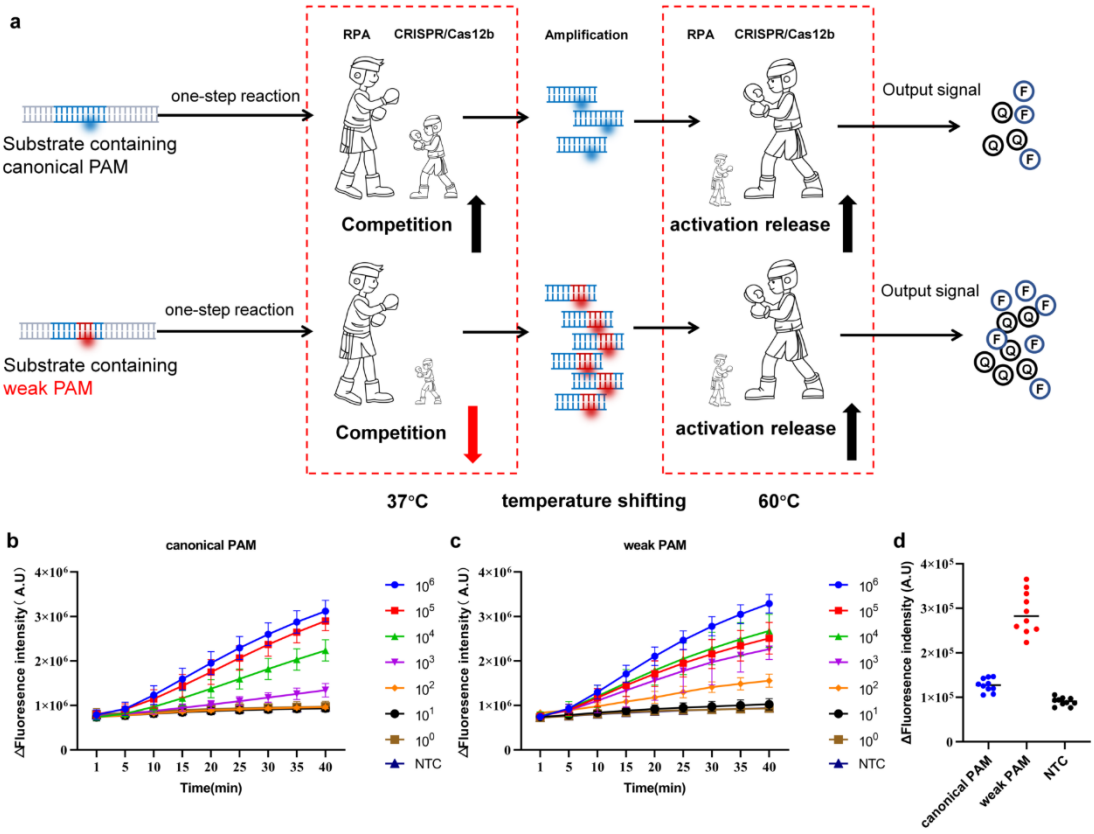


Fig. 3 (a) The core strategy of the WPTTS detection platform. During the low-temperature phase, substrates containing canonical PAM activate a certain level of AapCas12b cleavage activity. This activity competitively consumes substrates required for RPA amplification, thereby limiting amplification efficiency. In contrast, substrates containing weak PAM fail to effectively activate AapCas12b's cleavage function, allowing the RPA amplification reaction to proceed without interference and rapidly accumulate a large amount of amplification products. When the temperature increases to 60°C, the trans-cleavage activity of AapCas12b in both reaction systems is fully activated. The canonical PAM can only generate minimal signal relying on the limited initial amplification products, whereas the weak PAM, leveraging the abundant amplification products accumulated in the earlier stage, achieves superior detection performance. (b,c) The sensitivity of one-pot reaction using canonical PAM (b) and weak PAM (c) were compared. The target substrate was tenfold serially diluted to concentrations ranging from 10^6 to 10^0 copies per reaction. NTC non-target control. Error bars represent mean ±SD, $n = 3$. (d) The reliability of one-step reaction was systematically evaluated by comparing canonical PAM and weak PAM recognition efficiencies at a template concentration of 10^2 copies/reaction, with data derived from 10 independent experimental replicates.

Weak PAM demonstrates superior specificity compared to canonical PAM

To evaluate the specificity of weak PAM variants, we designed a series of targets containing single-base mismatches in the 20-bp spacer region downstream of the PAM site. The results demonstrated that compared to canonical PAM, weak PAM exhibited significantly reduced mismatch tolerance and more stringent specificity at most positions. Notably, at positions 10 and 11, single-base resolution was achieved. This characteristic provides unique advantages for molecular diagnostic applications requiring high specificity (Fig. 4).

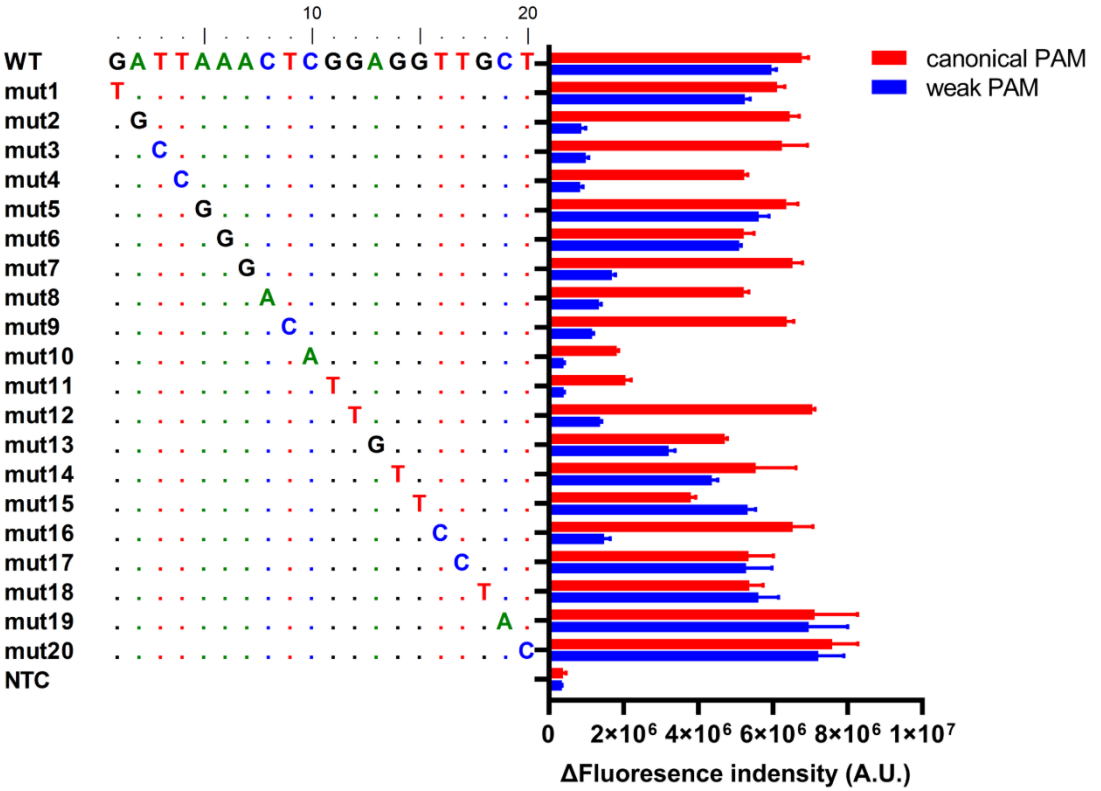


Fig. 4 The mismatch tolerance of canonical PAM and weak PAM with matched or mismatched *CPSIT*_0429 activators was evaluated. mut1-20 contained one mismatch relative to the on-target site, respectively. WT, wild type. NTC, non-target control. Error bars represent mean ±SD, n= 3.

Validation of the detection platform using clinical samples

Finally, to evaluate the clinical performance of the WPTTS detection platform, we applied it to the detection of *C. psittaci* in clinical samples (Fig. 5a). This study included 20 qPCR-confirmed positive samples and 20 negative control samples for validation. In terms of detection system design, we not only developed one-step and two-step assays targeting canonical PAM sequences, but also designed a weak-

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PAM target based on the *CPSIT*_0429 gene region. Through comparison of three detection strategies (one-step assay with weak-PAM, one-step assay with canonical PAM, and two-step assay with canonical PAM), we comprehensively evaluated the detection efficacy of the weak-PAM strategy. The detection threshold was set at 3 times the average fluorescence increase of non-target control. Results demonstrated that the one-step assay with weak PAM strategy significantly outperformed the one-step assay with canonical PAM, particularly showing higher detection rates in low viral load samples (Ct ≤ 30), with performance comparable to the two-step assay with canonical PAM (Fig. 5b). Compared with the gold standard qPCR results, all three methods achieved 100% negative agreement (20/20). Notably, the weak-PAM one-step method reached 95% positive agreement (19/20), surpassing the 75% (15/20) of the canonical two-step method while maintaining consistency with the canonical two-step results (Fig. 5c), thereby robustly validating its superior diagnostic performance and potential clinical value.

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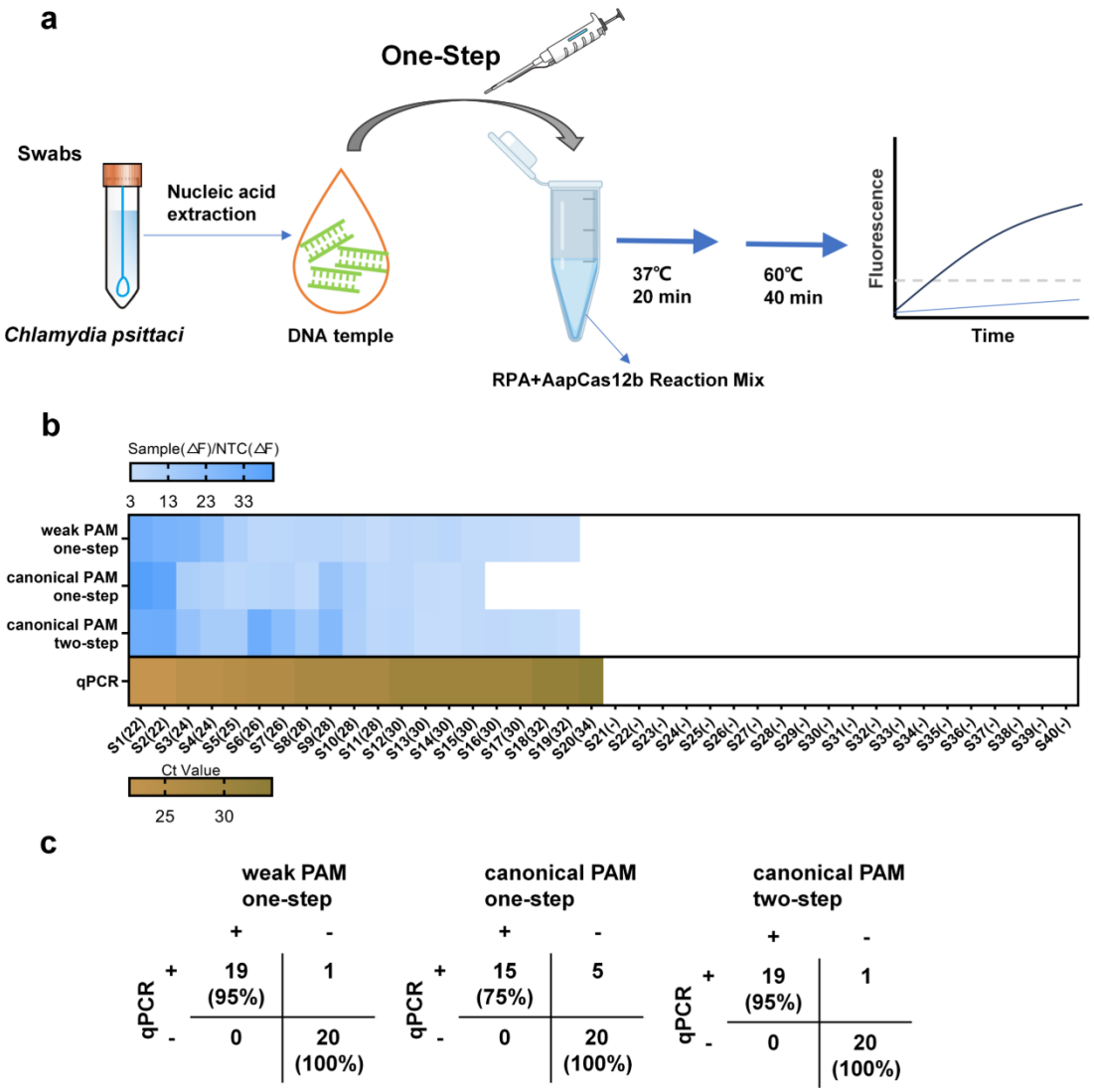


Fig. 5 (a)Schematic diagram of the WPTTS platform for *C. psittaci*. (b) The heatmap displays the detection results of the one-step assays tested against weak PAM or canonical PAM target, compared with two-step assays tested against canonical PAM target and qPCR. For the one-step and two-step assays, the color gradient represents the fold-change ratio of fluorescence signal increase relative to the non-target control during the 40-minute reaction period. In the qPCR results, the color gradient corresponds to the measured Ct values. White areas in the heatmap indicate negative detection results. The x-axis labels denote sample names and their corresponding Ct values

Discussion

Building upon our previously established two-step isothermal amplification-coupled CRISPR/Cas12b detection system for *C. psittaci*(R. Wang et al., 2023), this study successfully achieved conversion to a one-step reaction format, significantly enhancing its applicability for POCT.

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Here we report the temperature-dependent PAM recognition in AapCas12b: under low-temperature conditions, stringent PAM restriction was observed with significant activity differences between canonical and non-canonical PAM. We defined a subset of PAM types with minimal activity as "weak PAM". Remarkably, these differences were virtually abolished at elevated temperatures. This finding inspired a novel one-step assay design: by employing weak PAM, we minimized Cas protein interference with RPA amplification during the low-temperature phase while maintaining full detection activity during the high-temperature phase, thereby significantly improving overall reaction efficiency. The optimized system features remarkable operational simplicity, requiring only a single temperature transition (37°C→60°C) without additional reagents or handling steps, while delivering rapid detection (<60 minutes) and enhanced sensitivity (10^2 copies/reaction with weak PAM, equivalent to two-step performance). The assay demonstrates equally excellent performance in clinical sample testing, showing significant application value for rapid *C. psittaci* detection.

Among numerous Cas proteins, Cas12b is uniquely suited for one-step integration with isothermal amplification(Aman et al., 2021; Joung et al., 2020). Numerous one-step Cas12b-based detection strategies have been reported (Table 1). Most use a compromise temperature to balance both enzymatic activities(Aman et al., 2021; Cao et al., 2023; Cheng et al., 2025; Hu et al., 2025; Joung et al., 2020; Li et al., 2019; Li et al., 2021; Tong et al., 2024; Yang et al., 2024) (Table 1a-i), but this has key limitations: suboptimal temperatures weaken both enzymes, and Cas12b-mediated cleavage of amplification products remains unavoidable. This temperature compromise is a double-edged sword—simplifying operation at the expense of performance. A few studies employ temperature switching to reduce interference(Feng et al., 2025; X. Wang et al., 2023; Yu et al., 2025) (Table 1j-m). However, both previous work(Joung et al., 2020) and our study show that Cas12b with canonical PAM retains activity at low temperatures. This reveals a critical flaw: temperature regulation alone cannot fully prevent Cas12b interference during amplification. In contrast, our innovative use of a weak PAM resolves this issue and

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achieves superior sensitivity and specificity.

It should be noted that this study has limitations requiring further exploration: the clinical validation scale needs expansion for greater reliability; the method's applicability to other pathogens requires systematic assessment; and crucially, the molecular mechanism underlying weak PAM-mediated AapCas12b's temperature-dependent activity remains to be elucidated. Resolving these will be key to translational advancement.

With deeper mechanistic understanding and broader clinical validation, this innovative one-step RPA-CRISPR/Cas platform holds great promise for transformative applications in rapid infectious disease diagnosis and resource-limited screening scenarios.

Table1 Comparison among Cas12b-based one-step detection methods

No.	Name	Amplification method	Subtype of Cas12b	Limit of Detection	Temperature and time	One-step Design Strategy	PAM	Refs
a	iSCAN-V2	RT-RPA	AapCas12b	8 copies/μl (200 copies/ reaction)	42°C 60 min	optimized the reaction conditions	canonical PAMs	(Aman et al., 2021)
b	STOPCovid	LAMP	AapCas12b	100 copies per reaction	60°C 45 min	addition of taurine	canonical PAMs	(Joung et al., 2020)
c	CoLAMP	LAMP	AapCas12b	0.5 copies/μL	60°C 40 min	sgRNA is designed to recognize the activator sequence sited in the loop region of the LAMP product	canonical PAMs	(Cao et al., 2023)
d	AapCas12b mutant-	LAMP	AapCas12b	0.5 copies/μL	60°C 15 min	engineering PAM-interacting domains of	canonical PAMs	

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	mediated one-pot testing					Cas12b		(Tong et al., 2024)
e	CRISPR-top	RT-LAMP	AapCas12b	10 copies per reaction	59°C 40 min	a PAM site into the linker region of the core primer	canonical PAMs	(Li et al., 2021)
f	SURVEY	RPA	AapCas12b	0.6 copies/μL	37°C 20 min	addition of heparin sodium	canonical and suboptimal PAMs	(Cheng et al., 2025)
g	HOLMESv2	LAMP	AacCas12b	10^{-5} nM	55°C 120 min	Optimizing the reaction temperature	canonical PAMs	(Li et al., 2019)
h	OAR-CRISPR Assay	RPA	AacCas12b	30 copies/μL	40°C 12 min	introducing of asymmetric RPA process and optimizing the reaction system	PAM-free	(Yang et al., 2024)
i	PC&PS DNA-mediated regulation of DNACas	LAMP	BrCas12b	2 copies/μL	60°C 90 min	light regulation photocleavable phosphorothioate DNA	canonical PAMs	(Hu et al., 2025)
j	CRATS	RAA	AapCas12b	10 copies per reaction	37°C 20 min 60°C 20 min	introduction of temperature-shifting process and optimized the reaction condition	canonical PAMs	(Yu et al., 2025)

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k	CDetection.v2	RT-RAA	AapCas12b	5 copies/μL	42°C 7 min 52°C 23 min	introduction of temperature-shifting process and optimized the reaction condition	canonical PAMs	(X. Wang et al., 2023)
m	TRACER	RPA	BrCas12b	1 copy/μL	39°C 25 min 66°C 15 min	introduction of temperature-shifting process, leveraging the thermophilic properties of BrCas12b, and optimizing the reaction conditions.	canonical PAMs	(Feng et al., 2025)
n	WPTTS (This work)	RPA	AapCas12b	10^2 copies per reaction	37°C 20 min 60°C 40 min	introduction of temperature-shifting process and recognition of weak PAM	weak PAMs	

Conclusion

The WPTTS platform represents a significant advancement in one-step CRISPR-based diagnostics by addressing the key challenge of amplification-detection interference through innovative temperature-dependent PAM regulation. By harnessing the unique properties of weak PAM sequences and a two-temperature shifting strategy, our method achieves sensitivity comparable to conventional two-step assays while simplifying operational workflows. The successful clinical validation highlights its practical value in applications. Furthermore, the enhanced specificity of weak PAM variants offers additional advantages for discriminating single-nucleotide polymorphisms. Future studies should focus on expanding clinical validation, exploring the platform's adaptability to other pathogens, and elucidating the molecular mechanisms underlying weak PAM activity. With its rapid detection time, minimal equipment requirements, and robust performance, WPTTS shows broad application prospects in achieving POCT for pathogenic bacteria.

Author's contributions

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Jingyi Jiang: Conceptualisation, Methodology, Investigation, Data curation, Formal Analysis. **Jian Xu**: Conceptualisation, Methodology, Investigation, Formal Analysis. **Li Gong**Conceptualisation, Methodology, Investigation, Formal Analysis. **Qiong Li**: Methodology, Investigation, Validation. **Ping Yao:** Validation, Investigation. **Rong Wang:** Validation, Investigation. **Jinyi Jiang**: Validation, Investigation. **Lei Xu:** Supervision, Writing – Original Draft, Project administration. **Fengming Wang**: Conceptualization, Supervision, Writing – Original Draft, Project administration. **Xujian Mao:** Conceptualization, Supervision, Data curation, Writing – Original Draft, Writing – review & editing, Project administration.

Declaration of competing interest

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

Data availability

No datasets were generated or analysed during the current study.

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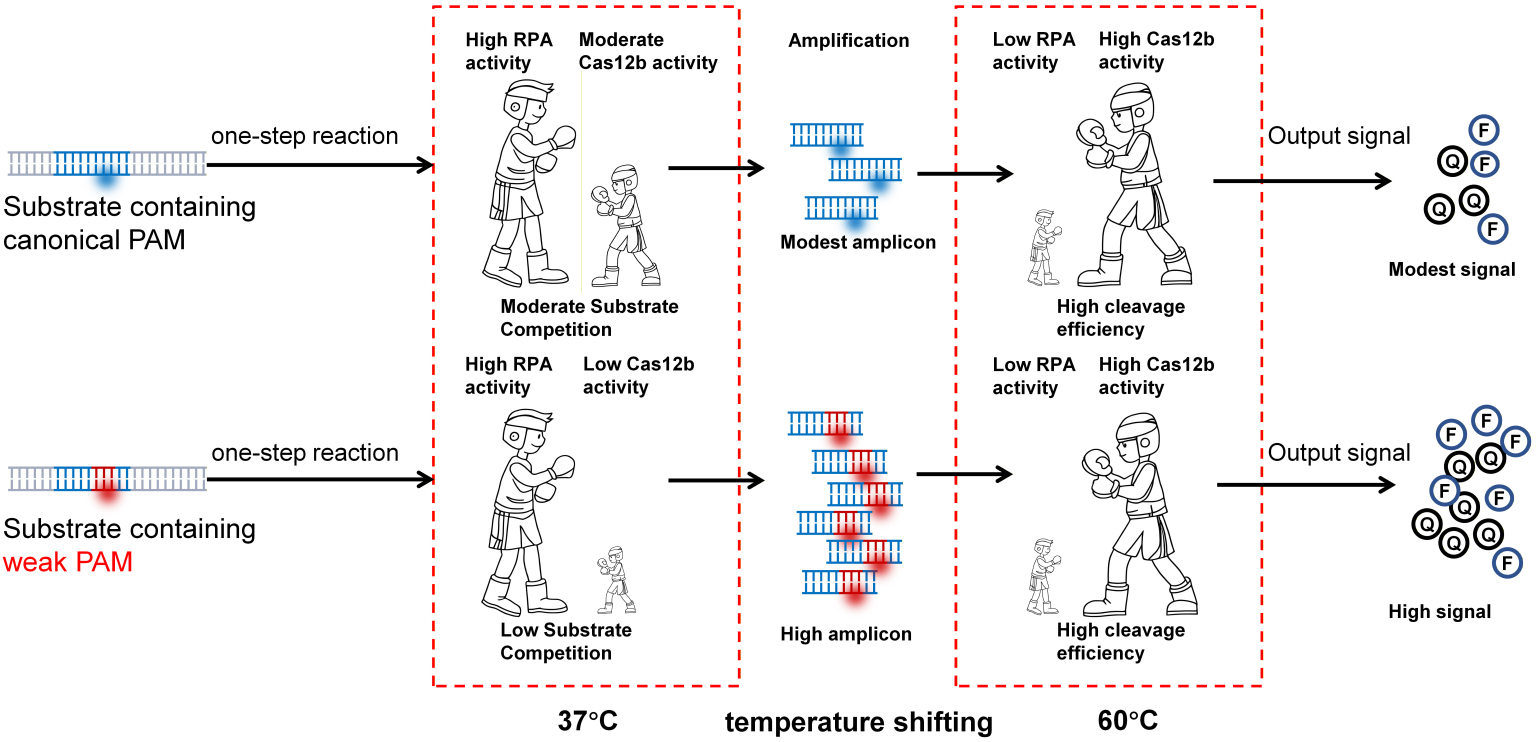
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one-step reaction
Substrate containing canonical PAM
High RPA activity
Moderate Cas12b activity
Moderate Substrate Competition
Amplification
Modest amplicon
Low RPA activity
High Cas12b activity
High cleavage efficiency
Output signal
Modest signal
one-step reaction
Substrate containing weak PAM
High RPA activity
Low Cas12b activity
Low Substrate Competition
High amplicon
Low RPA activity
High Cas12b activity
High cleavage efficiency
Output signal
High signal
37°C
temperature shifting
60°C