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Rapid and sensitive Mycoplasma detection in antibody bioprocessing via RPA-CRISPR/Cas12a

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ABSTRACT

Mycoplasma species are prevalent microbial contaminants in the production of biological products, such as monoclonal antibodies, posing significant threats to the safety and efficacy of these products. Current regulatory guidelines as well as pharmacopoeias mandate the demonstration of the absence of *Mycoplasma* in the cell culture and further downstream processing to ensure product safety. Despite recent advancements in sensitive detection techniques for *Mycoplasma* in eucaryotic expression systems, these methods remain complex and time-consuming. There is a pressing need for a rapid, simple, and sensitive process analytical technology (PAT) for *Mycoplasma* detection. Here, we report the first development and application of a recombinase polymerase amplification (RPA)-assisted CRISPR-Cas12a (RPA-CRISPR/Cas12a) system spcifically tailored for *Mycoplasma* detection in biopharmaceutical production. This system combines the high-sensitivity isothermal nucleic acid amplification capabilities of RPA with the trans-cleavage activity of CRISPR-Cas12a reporter probes, enabling the rapid and accurate detection of *Mycoplasma*, accommodating various experimental requirements and application scenarios. By designing RPA universal primers and crRNA targeting the highly conserved sequences of *Mycoplasma* 16S rRNA and optimizing reaction conditions, we achieved dual-specific recognition with unprecedented efficiency in bioprocessing samples. All tested *Mycoplasma* specimens were detectable with limits between 10 and 0.1 copies/μL, with the whole process taking less than 1 hour. We further evaluated the feasibility of this method in detecting *Mycoplasma* in the cell culture of antibody products and further downstream processing samples. This method reduces the risk of false-positive signals due to non-specific amplification, enhancing detection sensitivity and specificity while significantly reducing analysis, representing the first PAT-compatible method for rapid *Mycoplasma* monitoring in antibody manufacturing, thereby providing robust assurance for the quality and safety of biological products.

Abbreviations: **PAT,**, process analytical technology; **RPA,**, recombinase polymerase amplification; **NAT,**, Nucleic Acid Amplification Techniques; **EP,**, European Pharmacopoeia; **FDA,**, Food and Drug Administration; **ssDNA,**, single-stranded DNA; **qPCR,**, Quantitative Real-time PCR; **crRNA,**, guide RNA; **crRNA,**, CRISPR RNA; **CHV,**, Cell harvest; **DF,**, depth filtration; **PAC-EP**, Protein A chromatographic elution peak; **ATCC,**, American type culture collection; **CHO,**, Chinese hamster ovary cells.

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1. Introduction

Mycoplasma species are pervasive unique class of microorganisms in the production of biological products, including monoclonal antibodies, where they pose substantial risks to the safety, efficacy, and quality of these products[1,2]. Characterized by the absence of a cell wall, *Mycoplasma* exhibit intrinsic resistance to commonly used antibiotics, which complicates their eradication from cell cultures[3]. Their diminutive size and pleomorphic nature enable them to pass through standard 0.22 μm filter membrane. The tiny structure of Mycoplasma species and their lack of a rigid cell wall make them difficult to visualize using traditional microscopy techniques, requiring specialized detection methods to identify their presence.

The impact of *Mycoplasma* contamination is multifaceted, affecting cell culture performance and product quality through various mechanisms, including competition for nutrients, invasion or fusion with host cells, and induction of abnormal cell growth and cytopathic effects[4]. Furthermore, *Mycoplasma* can alter gene expression profiles in host cells, thus impacting normal cellular functions and production processes[5]. Consequently, precise detection and control of *Mycoplasma* are imperative for maintaining the integrity of biopharmaceutical production[6]. Current regulatory guidelines and pharmacopoeias mandate rigorous testing to demonstrate the absence of *Mycoplasma* in cell culture systems and downstream processing steps[7]. Traditional detection methods, such as culture-based approaches, are considered the gold standard due to their high sensitivity. These methods involve inoculating cell culture samples onto both liquid and solid media, detecting *Mycoplasma* through metabolic activity-induced pH changes in liquid media or characteristic colony morphology on solid media[8,9]. However, these methods are time-consuming, often requiring up to 28 days, and may fail to detect "non-culturable" species[10]. Additionally, the necessity for sensitivity testing of each batch of culture medium and risk of microbial contamination during live culture further complicate this approach.

Alternative detection methods, such as cytological staining and Nucleic Acid Amplification Techniques (NAT), offer potential solutions to the limitations of culture-based methods. Cytological staining, which employs fluorescent DNA-binding dyes to visualize *Mycoplasma* under a fluorescence microscope[11], provides faster results but suffers from lower sensitivity and complex result interpretation. On the other hand, NAT methods[12], including qPCR, have been validated and accepted by regulatory authorities such as the European Pharmacopoeia (EP) and the U.S. Food and Drug Administration (FDA) as viable alternatives[13,14]. These methods offer enhanced sensitivity and specificity, reduced detection time, and lower risk of false negatives due to compromised *Mycoplasma* viability.

Recombinase Polymerase Amplification (RPA) is an isothermal nucleic acid amplification method that operates at constant temperature, facilitating rapid and specific DNA amplification without the need for complex thermal cycling equipment[15]. RPA's ability to amplify DNA under isothermal conditions simplifies the experimental procedure and is particularly suitable for high-background samples[15]. Additionally, RPA offers significant flexibility in primer design, with a high tolerance for single base mismatches. Longer RPA primers provide greater specificity than shorter PCR primers, ensuring more precise target amplification.

CRISPR/Cas12a (also known as Cpf1), a molecular tool known for its ability to recognize specific DNA sequences and exhibit robust trans-cleavage activity against single-stranded DNA (ssDNA), has revolutionized nucleic acid detection. The DETECTR[16], which combines RPA with CRISPR/Cas12a, leverages the specific target recognition and collateral cleavage activity of Cas12a to generate detectable fluorescent signals upon target DNA amplification. This combination significantly reduces the risk of false-positive signals due to non-specific amplification[17,18]. It is widely used in pathogen detection, clinical diagnosis, and visualization detection of large-scale epidemics combined with lateral flow strips[19,20].This study presents the first integration of RPA-CRISPR/Cas12a technology into biopharmaceutical *Mycoplasma* detection, addressing critical gaps in current PAT frameworks. Compared to qPCR methods, the RPA-CRISPR/Cas12a system not only offers comparable sensitivity but also enables aequipment-free platform for rapid detection, making it an ideal candidate for real-time quality control in biopharmaceutical production (Fig. 1). These characteristics align perfectly with the requirements of next-generation analytical platforms for biopharmaceutical production, especially for Process Analytical Technology (PAT)[21].

In this study, we pioneered a detection system combining RPA with CRISPR/Cas12a specifically for *Mycoplasma* surveillance in biopharmaceutical workflows. We designed universal RPA primers and gRNA targeting highly conserved *Mycoplasma* 16S rRNA gene sequences (Table 1), optimized reaction conditions, increased sensitivity, accelerated detection time, and established its compatibility with industrial PAT requirements through minimal handling steps. This approach shows promise as a PAT tool for robust risk management and control in biopharmaceutical production, enhancing the quality and safety of biological products.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

The LbCas12a protein was obtained from New England Biolabs (Ipswich, MA, USA). The TwistAmp basic nucleic acid amplification kit was purchased from TwistDX (Cambridge, UK). The Venor®GeM Sample Preparation Kit was sourced from Minerva-Biolabs (Berlin, Germany). Power SYBR® Green PCR Master Mix was purchased from Applied Biosystems™ (Carlsbad, CA, USA). DNA primers, single-stranded DNA (ssDNA) probes, and crRNA were synthesized by Shanghai Shenggong Biological Engineering Co., Ltd. (Shanghai, China), Claricap™ M02 was obtained from Cobetter (Hangzhou, China).

2.1.2. Equipment

The biosafety cabinet was from AIRTECH (Suzhou, China), the PCR instrument was from BIO-RAD (Hercules, CA, USA), the qPCR instrument and the Microvolume Spectrophotometer was from Thermo Fisher Scientific (Waltham, MA, USA), the bio-electrophoresis image analysis system was from Shanghai Furi Technology (Shanghai, China), and the AKTA Avant chromatography system was from GE Healthcare (Chicago, IL, USA).

2.1.3. Strains

Mycoplasma pneumoniae (ATCC15531) and *Mycoplasma oralis* (ATCC23714) were sourced from the American Type Culture Collection (Gaithersburg, MD, USA). Plasmids harboring the 16S rRNA sequences of the following *Mycoplasma* species were synthesized by Beijing Tsingke Biotechnology Co., Ltd (Beijing, China): *Mycoplasma fermentans* (ATCC 19989), *Spiroplasma citri* (ATCC 27556), *Mycoplasma arginini* (ATCC 23838), *Acholeplasma laidlawii* (ATCC 23206), *Mycoplasma hominis* (ATCC 23114), *Mycoplasma salivarium* (ATCC 23064), and *Mycoplasma suis* (ATCC 17981).

2.1.4. Cell line

Vero, CHO, HEK293, NK92 cell lines were obtained from Shanghai Zhangjiang Biotechnology Co. (Shanghai, China)

2.2. Methods

2.2.1. Design and synthesis of RPA primers, crRNA, ssDNA probes and plasmids

The design of RPA universal primers and CRISPR RNA (crRNA) targeting *Mycoplasma* species was based on conserved sequences within the 16S rRNA genes of nine common *Mycoplasma* species[22]. The 16S

rRNA gene sequences of the nine *Mycoplasma* species were retrieved from the ATCC database (https://www.atcc.org). The sequences were aligned using MEGA11 software to identify conserved regions and to design crRNAs targeting protospacer adjacent motif (PAM) sequences (TTTN) critical for Cas12a activity. Corresponding RPA primers (RPA-F and RPA-R) were designed to amplify these conserved regions, with each primer approximately 30 bp in length and amplicon sizes kept under 500 bp. BLAST analysis was conducted to ensure primer specificity (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the primer sequences were verified for secondary structures (e.g., hairpins, self-dimers, and heterodimers) using the online software Oligo Analyzer (https://sg.idtdna.com/calc/analyzer). For the positive control plasmid, the pUC57 vector was used, and 16S rRNA sequence from the nine *Mycoplasma* species were cloned into the vector as target sequences[23].

2.2.2. Positive template preparation

A positive template was prepared by harvesting 1 ml of bacterial culture from the logarithmic growth phase. The culture was transferred to a centrifuge tube and subjected to heat treatment at 95 °C for 5 minutes to break the cell wall and release genetic material, the mixture was centrifugated at 10,000 g/min for 3 minutes. The supernatant was collected as the crude bacterial extract, which was used as the template for subsequent reactions.

Mycoplasma DNA was extracted from culture supernatant using the Venor®GeM Sample Preparation Kit according to the manufacturer's instructions for nucleic acid extraction.

2.2.3. Quantification of positive plasmid standard

To quantify the *Mycoplasma* positive plasmid, 2 μL of plasmid solution was measured using a Nanodrop spectrophotometer to determine the nucleic acid concentration. The number of plasmid copies per microliter was calculated based on this concentration. A 10-fold serial dilution of the plasmid was performed to generate a series of standards with decreasing concentrations of positive plasmid.

2.2.4. Construction of RPA-CRISPR detection system

The RPA system was prepared by combining the following components: 30 μL RPA rehydration buffer, 10 μL ddH2O, 2.5 μL RPA-F (10 μM), 2.5 μL RPA-R (10 μM) into RPA freeze-dried reaction microspheres. The mixture was vortexed briefly and divided into four parts, with one part used for each RPA premix (10 μL).

For the CRISPR system, the following reagents were mixed: 13 μL Cas12a Buffer (1 ×), 2 μL Cas12a (1 μM), 2 μL crRNA (1 μM), and 1μL F-Q reporter probe (10 μM), giving a final volume of 18 μL per CRISPR premix.

RPA-CRISPR system: 2 μL of sample and 0.5 μL MgOAc (280 mM) were added to the tube cap. The RPA premix (10 μL) was placed in the bottom of the tube, followed by brief centrifugation and mixing. The tube was incubated at 37 °C for 20 minutes to allow RPA pre-amplification. The pre-amplified product (2 μL) was then transferred into the CRISPR premix (18 μL) for further processing. The reaction mixture was quickly mixed and incubated at 37 °C for 20 minutes in a qPCR instrument, where the fluorescence signals were collected every 30 second.

2.2.5. Agarose gel electrophoresis

To confirm the RPA amplification products, the RPA products were first denatured at 95 °C for 3 minutes to release the DNA fragments. The samples were then immediately transferred to an ice bath for 1 minute to stabilize the DNA fragments and prevent re-annealing. After centrifugation at 10,000 g for 2 minutes, the supernatant was carefully removed to avoid disturbing any precipitates. The supernatant was then mixed with an appropriate loading buffer, and analyzed by 1 % agarose gel electrophoresis.

2.2.6. Preparation of CHO cell samples contaminated by Mycoplasma

Engineered CHO cells were cultured under simulated fermentation conditions using a shaker culture system, with a temperature of 37 °C, 5 % CO_2, 120 rpm shaking speed, and pH maintained between 6.9 and 7.0. *Mycoplasma* was inoculated into the culture at concentrations below 10 CFU/ml. Continuous sampling over a 6-day period was conducted to monitor *Mycoplasma* growth and its impact on the CHO cell fermentation process.

2.2.7. Medium culture method

The *Mycoplasma* culture was performed following the guidelines specified in the Chinese Pharmacopoeia (3301, Method 1) for *Mycoplasma* testing in biological products.

2.2.8. Real-time quantitative PCR analysis

qPCR Reaction System:10 μL Power SYBR Green PCR Master Mix (2 ×), 0.8 μL PCR F(10 μM), 0.8 μL PCR R(10 μM), 3.2 μL ddH2O, 5 μL sample.

The qPCR instrument settings were as follows: initial activation at 95

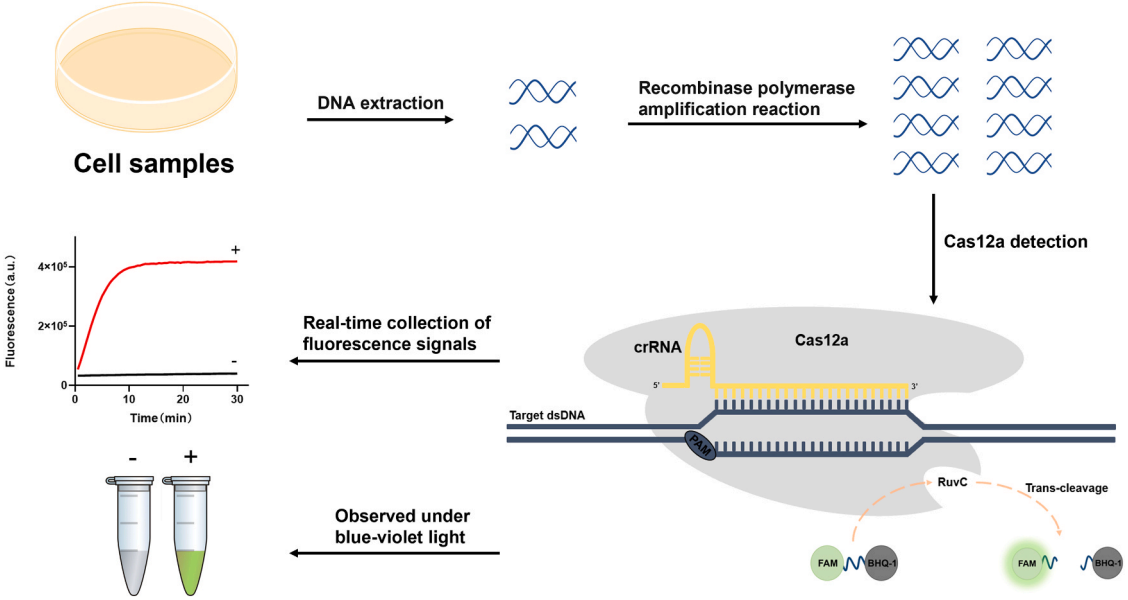


Fig. 1. Caption: Schematic of the RPA-CRISPR/Case12a detection system.

°C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 57 °C 30 seconds, and extension at 72 °C for 30 seconds. A melting curve analysis was performed from 95 °C for 15 seconds, 60 °C for 1 minutes, and finally 95 °C 15 seconds and 60 °C 15 seconds.

2.2.9. Downstream process

Engineered CHO cell cultures were subjected to a depth filtration step to remove cellular debris and other contaminants. Following filtration, antibody purification was carried out using Protein A affinity chromatography columns. The chromatography process was performed using the following buffers: 20 mM PB + 150 mM NaCl (pH 7.0) as the equilibration buffer and 20 mM citric acid (pH 3.0) as the elution buffer.

2.2.10. Data processing

Data were analyzed using Graphpad Prism 8 statistical software. The fluorescence values obtained from the experiments were analyzed using a *t*-test to determine statistical significance, with a threshold of $p < 0.05$ indicating significant differences.

3. Results

3.1. Feasibility analysis for RPA-CRISPR/Cas12a as a PAT tool

We amplified the target gene sequences of nine common *Mycoplasma* species using RPA to verify the generality and practicability of the designed primers. After RPA amplification of the nucleic acids of these *Mycoplasmas*, the amplified products were purified and subsequently identified by 1 % agarose gel electrophoresis. The results showed that all samples produced specific amplification products of approximately 470 bp, which was consistent with the expected length of the gene sequence (Fig. 2a). In addition, no non-specific bands were observed in the negative control group, further confirming the specificity and accuracy of the experimental method.

A series of controlled experiments were designed to investigate the effect of the absence of a certain component on the RPA/CRISPR-Cas12a reaction system. The experimental results show that the integrity of the RPA/CRISPR-Cas12a reaction system is crucial to the success of the experiment. As shown in the figure, no matter which component is missing, the whole reaction system cannot achieve the expected detection effect (Fig. 2b-c). Through these controlled experiments, we not only verified the importance of each component in the RPA/CRISPR-Cas12a reaction system, but also further optimized the experimental conditions to ensure the stability and repeatability of the experiment.

3.2. Optimization of the RPA-CRISPR/Cas12a system for enhanced detection

3.2.1. Temperature optimization for enhanced amplification efficiency

In order to optimize the performance of the RPA/CRISPR-Cas12a fluorescence system, we designed a series of experiments. The experimental data revealed that five different reaction temperatures (25 ℃, 30 ℃, 35 ℃, 40 ℃, 45 ℃) could produce similar fluorescence curves at a high concentration of template DNA (10^7 copies/μL), and the fluorescence intensity was similar(Fig. 3a-b). However, when the concentration of template DNA was reduced to 10^3 copies/μL, the reaction temperature of 35 ℃ and 40 ℃ significantly promoted the amplification efficiency, and the fluorescence signal was significantly enhanced (Fig. 3c-d). This finding makes it clear that RPA pre-amplification reactions work best in the temperature range of 35 ° C to 40 ° C.

3.2.2. Time optimization of RPA pre-amplification reaction

In this study, we optimized the time of RPA pre-amplification reaction. The experimental results showed that when the template DNA concentration reached a high level (10^7 copies/μL), the fluorescence curve showed a high consistency and the fluorescence intensity tended to be stable after more than 10 minutes of RPA pre-amplification reaction (Fig. 3e-f). When the template DNA concentration was further reduced to 10^3 copies/μL, we found that a significant fluorescence signal was successfully detected with a reaction time of 15 minutes (Fig. 3g-h). These optimizations provide an important experimental basis for the time optimization of RPA pre-amplification reaction, and ensure that rapid and sensitive fluorescence detection can be achieved at different template concentrations.

3.2.3. Optimization of LbCas12a and crRNA concentration for rapid reaction saturation

In order to achieve rapid saturation of the reaction, different concentrations of LbCas12a protein and crRNA (0 nM, 10 nM, 25 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM) were systematically screened. The concentration ratio of LbCas12a protein to crRNA is maintained at 1:1. The experimental results revealed the significant effect of concentration on the reaction rate: at the concentration of 10 nM, the reaction progressed slowly, the fluorescence curve showed a relatively gentle growth trend, and the complete reaction was not achieved in the observation period of 20 minutes. In contrast, when the concentration of LbCas12a and crRNA was increased to 100 nM, the fluorescence curve rose rapidly, almost identical to the curve at higher concentrations, indicating that this concentration was sufficient for the reaction to reach saturation quickly, and the fluorescence intensity was sufficient to be observed by the naked eye (Fig. 3i-k). Based on these findings, we determined that 100 nM is the optimal LbCas12a and crRNA concentration for the RPA/CRISPR-Cas12a fluorescence system, which not only ensures rapid saturation of the reaction, but also achieves high fluorescence intensity output in a short time.

3.2.4. Fluorescence probe concentration adjustment for cost-effective detection

In the process of optimizing the fluorescence reporting probe concentration of the RPA-CRISPR/Cas12a system, we systematically evaluated different concentrations of F-Q probes. Experimental data show that compared with 1000 nM high concentration probe, when the F-Q probe concentration is adjusted to 500 nM, the reaction can quickly reach an equilibrium state and achieve saturation in just 10 minutes. In contrast, low-concentration probes at 125 nM and 250 nM, especially those at concentrations below 250 nM, have fluorescence intensities that are too weak to be discerned by the naked eye. For the sake of cost-effectiveness, we recommend choosing the probe concentration in the 500 nM concentration range. Not only did this concentration produce a fluorescence signal strong enough to be viewed by the naked eye, but the effect was comparable to that of the 1000 nM high concentration probe, while significantly exceeding the fluorescence performance of the negative control group (Fig. 3l-n). Based on this optimization, we conclude that 500 nM single-strand DNA fluorescence reporter probes

Table 1
Primers for experiments.

Name	Sequence(5′-3′)	Base number	Use
RPA-F	GACTACCAGGGTATCTAATCCTGTTTGCTC	30	RPA primer
RPA-R	AAACTCCTACGGGAGGCAGCAGTAGGGAAT	30	
crRNA	UAAUUUCUACUAAGUGUAGAUCGCCCAAUAAUUCCGGAUAACGC	44	crRNA of Cas12a
ssDNA probe	5'6-FAM-CTCTCATTTTTTTTTTAGAGAG−3′-BHQ1	22	Fluorescence reporter probe

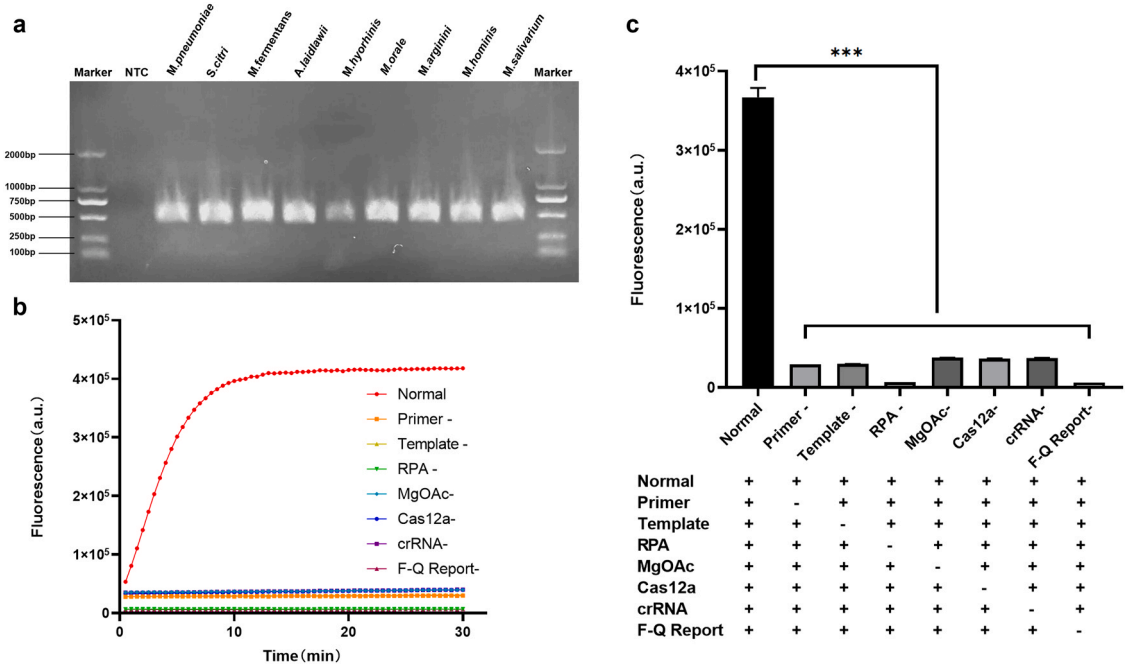


Fig. 2. Caption: System feasibility analysis. (a) Agarose gel electrophoresis demonstrating the amplification of *Mycoplasma* DNA using RPA primers, confirming the feasibility of RPA primers rom amplification. (b, c) Reaction component missing experiments assessing the effect of missing components on the system's ability to detect *Mycoplasma* DNA, highlighting the critical role of each component in the assay.

are the optimal concentration choice for RPA-CRISPR/Cas12a systems, which ensure efficient fluorescence signals while also being economical.

3.3. Validation of universality, specificity and sensitivity

3.3.1. Detection universality and mismatch tolerance for Mycoplasma species

In a study that followed pharmacopeia guidance for the detection of nine *Mycoplasmas*, we implemented a precise and efficient detection strategy that successfully identified all target *Mycoplasmas*. In particular, for *A. laidlawii*, although there was A two-base mismatch between the amplified target sequence and crRNA, the Cas12a enzyme slowed down the release of the fluorescence signal during the process of recognizing this mismatch target and triggering the trans-cutting reporting probe, but this delay did not affect the accuracy of the detection (Table 2). In fact, after about 20 minutes, the intensity of the emitted fluorescence signal was high enough to be clearly identified by the naked eye. At 30 minutes, the intensity of the fluorescence signal has stabilized, indicating that the reaction has entered a plateau (Fig. 4a-b). These results confirm the high efficiency and reliability of our designed RPA primer and crRNA combination for the detection of nine common *Mycoplasma* species, including *A. laidlawii*.

3.3.2. Specificity assessment in diverse cell line environments

The pharmacopeia definition of "specificity" emphasizes the importance of precise identification and evaluation of target nucleic acids among potentially complex biological components. In this study, we carried out specific detection of *Mycoplasma* for Vero, Chinese Hamster Ovary (CHO), HEK293, NK92 and other cell lines, aiming to verify the detection efficiency of our detection system for target nucleic acids in a diverse biological matrix environment, so as to establish its high specificity. Our results show that there is no cross-reaction between the detection system and the substrate components (Fig. 4c-d). The core advantage of this system is the highly specific amplification ability of RPA primers and the precise recognition mechanism of Cas12a enzyme, which together constitute a strong double specificity defense line, significantly improving the anti-interference ability of the system against non-target genes, thus ensuring the accuracy and reliability of detection results[23].

3.3.3. Sensitivity evaluation and detection limit determination

The definition of "detection limit" in the pharmacopoeia refers to the lowest concentration of the target nucleic acid that can be accurately and reliably detected in a sample. In this study, we selected highly quantitative positive plasmid standards as the test samples to evaluate the sensitivity of the RPA-CRISPR detection system. The experimental results showed that the RPA-CRISPR detection system could detect the target nucleic acid at a concentration as low as 4.42 copies/μL with sensitivity (Fig. 4e-g). In addition, we used the DETECTR system to analyze the sensitivity of different subtypes of *Mycoplasma* (Table 3, Figure S1). The experimental results revealed that the system had a significant detection sensitivity for different subtypes of *Mycoplasma*, with the detection limit ranging from 10^{1} to 10^{-1} copies/μL.

3.4. Performance in simulated bioprocessing environments

3.4.1. Application testing in antibody cell culture samples

To evaluate the practical applicability of the RPA-CRISPR/Cas12a system in real-world bioprocessing scenarios, we simulated the process of CHO cell culture, which is commonly used for antibody drug production. The experimental setup involved daily sampling from Day 0 to Day 6, with the DETECTR system serving as the primary monitoring tool for *Mycoplasma* contamination. Over the course of 24–72 hours, the system detected a weak *Mycoplasma* signal, and the 48–72-hour period corresponded to the logarithmic phase of *Mycoplasma* growth, as shown in Fig. 5a-b. Notably, the the early detection of *Mycoplasma* highlights the sensitivity of the system and its potential for early-stage intervention in bioprocessing. The ability to identify contamination at such an early stage is critical for minimizing the risk of widespread contamination and ensuring the quality of the final product.

3.4.2. Comparison with established detection techniques

To verify the effectiveness of the RPA-CRISPR/Cas12a system in detecting *Mycoplasma* in real samples, we collected 67 CHO cell samples

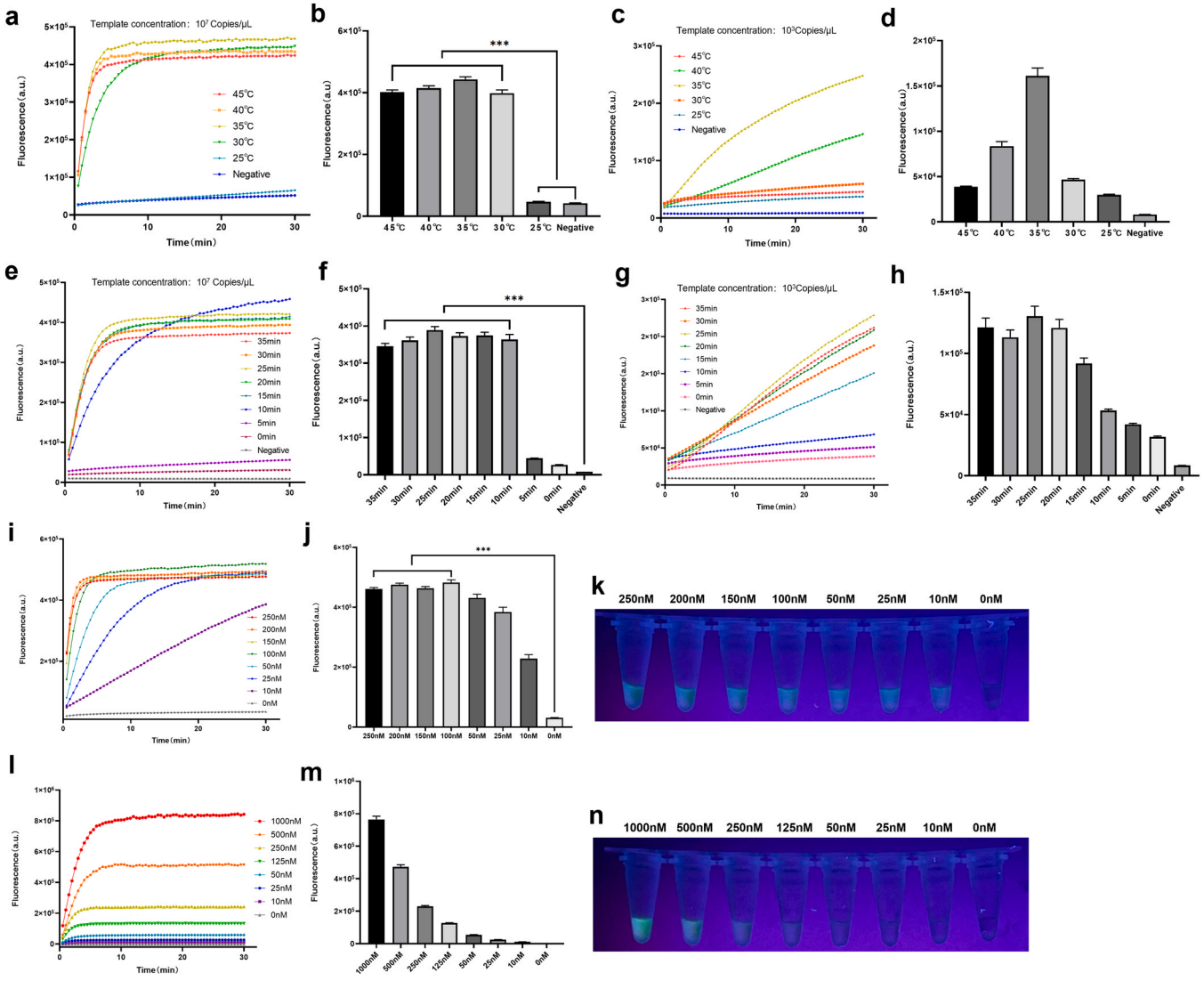


Fig. 3. Caption: Optimization of the RPA/CRISPR-Cas12a system. (a-d) Effect of pre-amplification temperature on RPA/CRISPR-Cas12a fluorescence signal. (e-h) Effect of the pre-amplification time on RPA/CRISPR-Cas12a fluorescence signal. (i-k) Effects of different Cas12a and crRNA concentrations on the fluorescence output of the RPA/CRISPR-Cas12a systems. (l-n) Optimization of ssDNA fluorescent probe concentration.

Table 2
RPA upstream and downstream primers and crRNA mismatch sites.

Strain	RPA F (5′-3′)	RPA R (5′-3′)	crRNA (5′-3′)
M.fermentans	GACTACCAGGGTATCTAATCCTGTTTGCTC	AGACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAAUAAUUCCGGAUAACGC
M.pneumoniae	GACTACTAGGGTATCTAATCCTATTTGCTC	ATACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAAUAAAUCCGGAUAACGC
S.citri	GACTACTAGGGTATCTAATCCTATTTGCTC	GAACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAAUAAAUCCGGAUAACGC
M.arginini	GACTACCAGGGTATCTAATCCTGTTTGCTC	AAACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAAUAAUUCCGGAUAACGC
M.orale	GACTACCAGGGTATCTAATCCTGTTTGCTC	AAACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAAUAAUUCCGGAUAACGC
A.laidlawii	GACTACCAGGGTATCTAATCCTGTTTGCTC	AAACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAGUAAAUCCGGAUAACGC
M.hominis	GACTACCAGGGTATCTAATCCTGTTTGCTC	AAACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAAUAAUUCCGGAUAACGC
M.salivarium	GACTACCAGGGTATCTAATCCTGTTTGCTC	AAACTCCTACGGGAGGCAGCAGTAGGGAAT	CGCCCAAUAAUUCCGGAUAACGC
M.hyorhinis	GACTACCAGGGTATCTAATCCTGTTTGCTC	AGACTCCTACGGGAGGCAGCAGTAAGGAAT	CGCCCAAUAAUUCCGGAUAACGC

Mismatch base sites are shown in bold red font.

(Table S1), including unknown, suspected, and positive cases. The results obtained with the RPA-CRISPR/Cas12a system were then compared with those from traditional *Mycoplasma* culture methods and the commonly used qPCR approach (Table 4, Figure S2). The results demonstrated a high degree of consistency across all three detection methods, with similar coincidence rates in detecting *Mycoplasma* contamination, as illustrated in Fig. 5c-d and Table 4. These findings underscore the reliability and accuracy of the RPA-CRISPR/Cas12a system, making it a viable alternative to more traditional methods. Furthermore, this consistency is particularly valuable when rapid and accurate detection is crucial, as is often the case in biopharmaceutical manufacturing where timely intervention is required to prevent production delays or safety concerns.

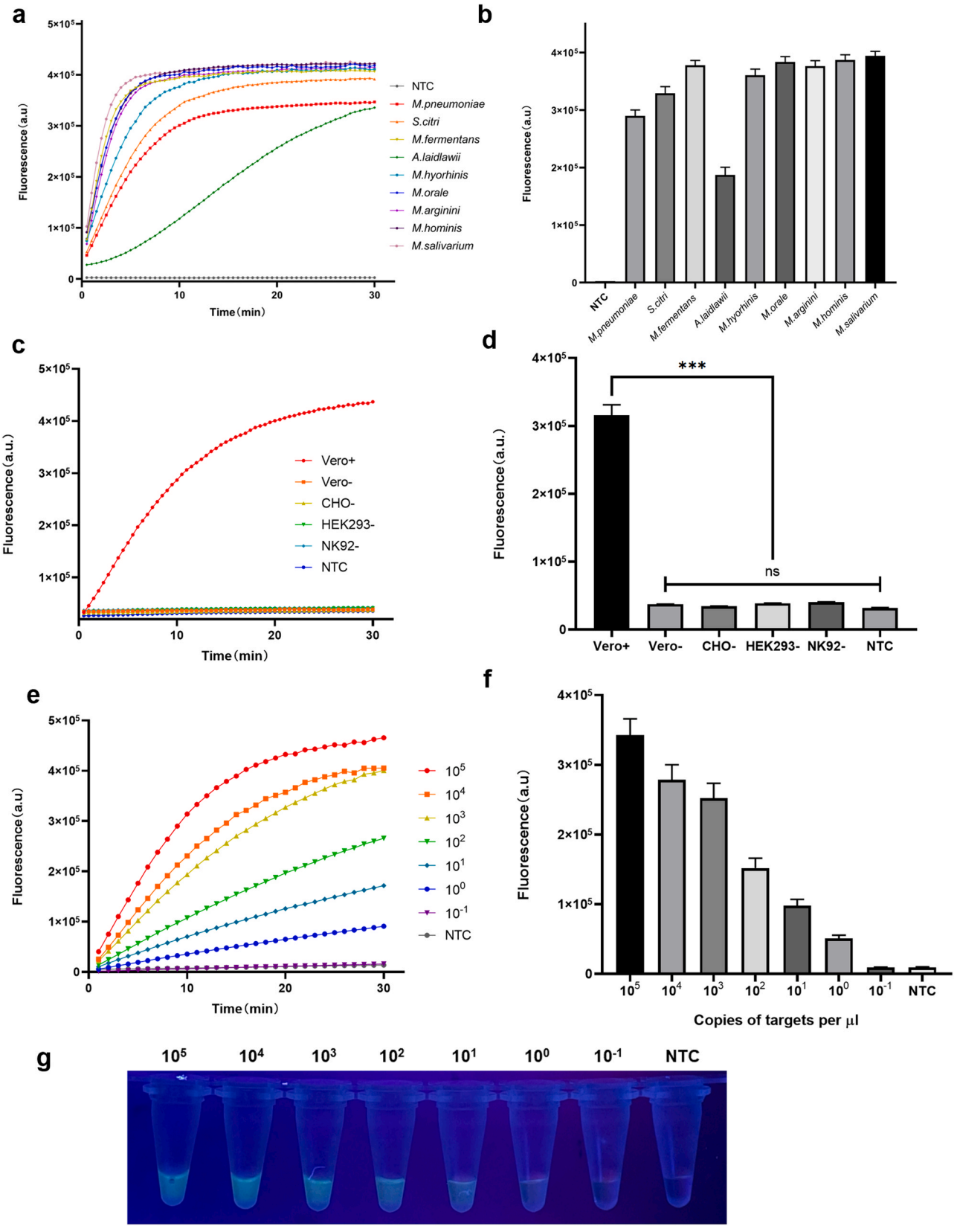


Fig. 4. Caption: System performance testing. (a-b) System commonality test. (c-d) System specific test. (e-g) System sensitivity test.

Table 3
Detection limits for different *Mycoplasma* species.

Mollicutes species	Detection limit (Copies/μL)
M.fermentans	7.93×10^{-1}
M.pneumoniae	4.35×10^{0}
S.citri	8.92×10^{0}
M.arginini	8.43×10^{0}
M.orale	4.42×10^{0}
A.laidlawii	1.73×10^{1}
M.hominis	5.98×10^{0}
M.salivarium	2.19×10^{0}
M.hyorhinis	6.24×10^{0}

3.4.3. Downstream process integration feasibility

In addition to its application in cell culture monitoring, we also explored the feasibility of incorporating the RPA-CRISPR/Cas12a detection system into the downstream processing steps of biopharmaceutical production. *Mycoplasma* contamination during downstream processing also presents a significant challenge, as conventional filtration methods may not fully eliminate the contaminant. To simulate this, we used engineered CHO cells expressing antibodies, intentionally contaminated with *Mycoplasma,* and subjected the samples to depth filtration and affinity chromatography process commonly employed in antibody purification[24,25]. Our results demonstrated that a depth filter membrane with a pore size of 0.02–0.2 microns effectively trapped *Mycoplasma*, although trace amounts of *Mycoplasma* nucleic acid remained detectable using the RPA-CRISPR/Cas12a system. In contrast, depth filter membrane with a pore size of 4–18 microns failed to effectively trap *Mycoplasma.* Moreover, virus removal filtration steps commonly used in downstream processing may offer improved removal efficiency. Additionally, the affinity chromatography process significantly reduced residual *Mycoplasma* nucleic acids, as shown in Fig. 5e-f.

These findings suggest that while depth filtration can effectively reduce *Mycoplasma* contamination, it may not entirely remove residual nucleic acids. The RPA-CRISPR/Cas12a system, however, is capable of detecting even trace amounts of *Mycoplasma* nucleic acids that persist after filtration, providing a crucial safety checkpoint. Integrating this detection system into downstream processes facilitates additional quality control measures, ensuring that *Mycoplasma* contamination is minimized throughout the entire production cycle. By incorporating the RPA-CRISPR/Cas12a system into routine production protocols, biopharmaceutical manufacturers can greatly enhance the safety, efficiency, and quality of antibody products, ultimately safeguarding both product integrity and patient safety.

Table 4
Comparison of three methods of testing for *Mycoplasma.*

Assay	Positive	Negative	Total	PPA (95% CI)	NPA (95% CI)
Traditional culture method	31	0	31	100%	100%
	0	37	37		
qPCR	31	0	31	100%	100%
	0	37	37		
RPA-CRISPR/Cas12a	31	0	31	100%	100%
	0	37	37		

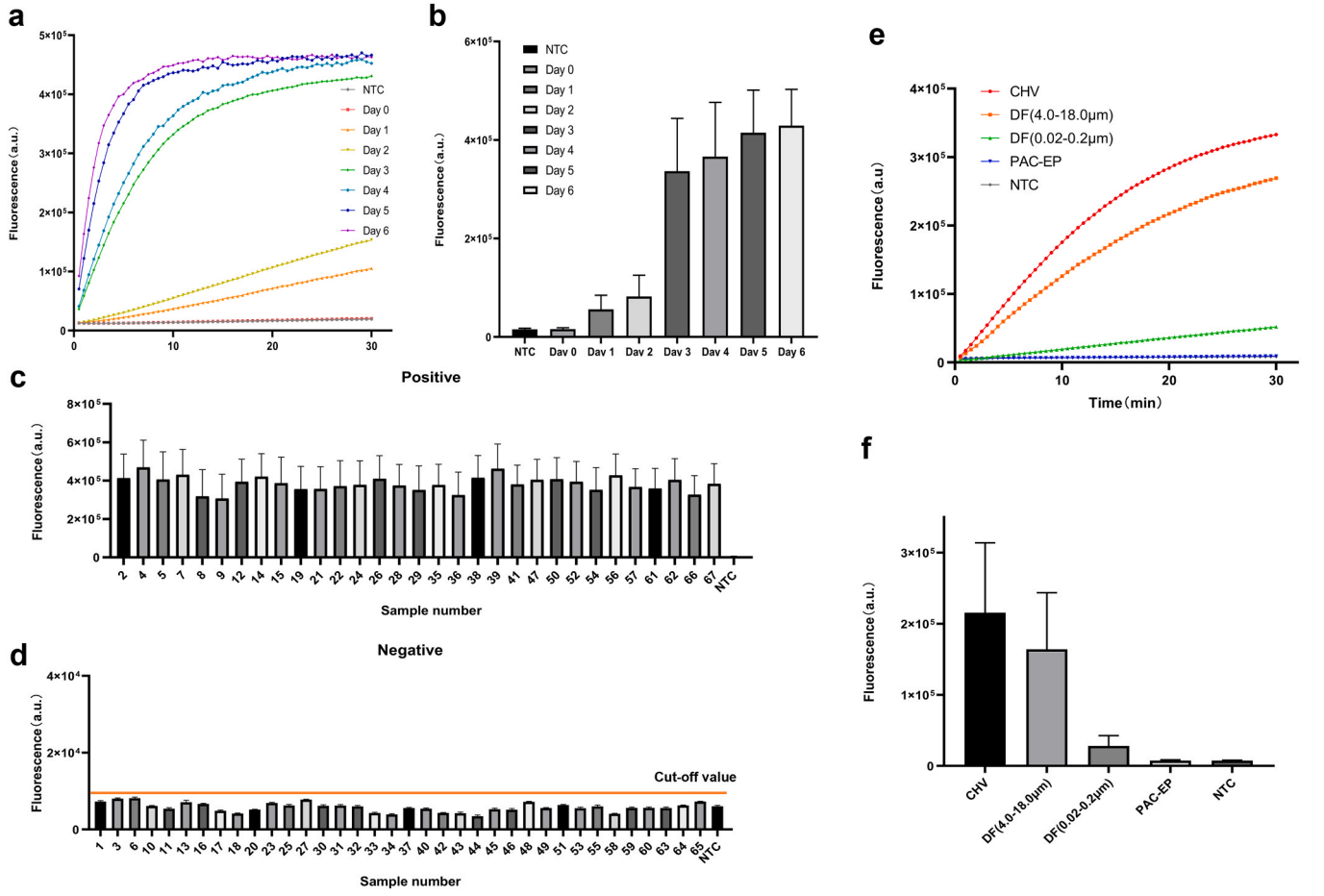


Fig. 5. Caption: Performance in simulated bioprocessing environments. (a-b) Application testing in antibody cell culture samples. (c-d) Comparison with established detection techniques. (e-f) Downstream process integration feasibility.

4. Discussion

The effects of *Mycoplasma* contamination on cultured cells are multifaceted, and they may not only change the physiological and growth characteristics of cells, but also affect the morphological, biochemical, immunological and genetic characteristics of cells. The presence of such contamination calls into question the accuracy of the results of biological tests performed on these cells, which in turn may lead to incorrect interpretations. To ensure the quality and safety of biologicals prepared in animal cell cultures, the U.S. Food and Drug Administration (FDA) strongly recommends the use of cells that have been rigorously tested and confirmed to be free of *Mycoplasma* contamination. Different studies have reported *Mycoplasma* contamination in cell lines ranging from 5 percent to 87 percent, a wide range of data that underscores the magnitude of the problem. Of the more than 200 known *Mycoplasma* species, 20 have been isolated from cell cultures. In particular, *M. arginini*, *M. fermentans*, *M. hominis*, *M. orale*, *M. hyorhinis*, *M. salivarium*, *M. pirum*, and *A. laidlawii*. These eight species account for more than 95 % of all cell culture pollution[26]. In terms of detection methods, a rapid and accurate strategy for antibody drug quality management promises to accelerate advancements in technological frameworks and intellectual paradigms, thereby strengthening product development and quality control capabilities[27]. To achieve this, the highly conserved sequence of 16srRNA was chosen as the basis for designing universal primers, which can not only enable the detection of *Mycoplasma*, but also serve as a warning system for other potential bacterial contaminants, which is crucial for maintaining the purity of cell culture[28].

To further enhance the flexibility and efficiency of the CRISPR-Cas12a system, recent advancements in protein engineering have expanded range of PAM sequences it can recognize[29]. This modification not only broadens the design space for crRNA but also offers significant advantages for the development of universal primers. In *Mycoplasma* detection, this extended PAM recognition enables the design and testing a wider variety of crRNA sequences, which in turn allows for more accurate and comprehensive detection across different *Mycoplasma* subspecies. As a result, these advancements, when combined with our method, could significantly improve both the coverage and accuracy of the detection.

In the process of practical application testing, the one-pot method and the traditional two-step method have their own advantages. Among them, the one-pot method reduces the risk of contamination by simplifying the operation process, reducing the operation time, and completing the amplification and detection in a single reaction tube. For example, the compatibility of RPA and CRISPR detection in a one-pot reaction was achieved through the technique of single-strand DNA modification of crRNA[30]. The sensitivity of the one-pot method is enhanced by the use of light-controlled cleavable single-stranded DNA modified crRNA[31]. In contrast, the two-step method provides greater flexibility, allowing the amplification and detection steps to be independently optimized to improve the specificity and sensitivity of the assay[18,20]. Although the two-step method is more complex, requires more time, and increases the risk of contamination, it allows for fine control of each step, which is valuable for some application scenarios that require a high degree of specificity and sensitivity[32] , especially in the biopharmaceutical industry.

In the European Pharmacopoeia, Section 2.6.7, the limit of detection (LOD) for NAT-based methods is defined as 10 CFU/ml. This LOD is typically expressed as the minimum number of genomic copies (GC) of the target organism that can be detected in a sample, whereas the LOD for traditional methods is expressed as the minimum number of viable microorganisms, quantified as colony-forming units (CFU), that can form colonies on the surface of solid culture media. It is important to note that the ratio of GC to CFU in *Mycoplasma* samples can fluctuate significantly, as the GC/CFU ratio is in a relatively dynamic state[33]. In this study, the copy number of a positive control plasmid was used for quantification to analyze the relative LOD sensitivity. This approach allows for a more accurate assessment of the sensitivity of *Mycoplasma* detection and provides an essential reference for subsequent experimental design and clinical applications. Overall, our method offers several notable advantages compared to conventional methods: Rapidity: The entire detection process is completed within 1 hour, which is significantly faster than culture-based methods and is comparable to or even faster than qPCR-based approaches. Ultrahigh sensitivity: Our method achieves detection limits as low as 0.1–10 copies/μL, surpassing the sensitivity of conventional methods, thus enabling detection at much lower concentrations. Dual specificity: By combining RPA's sequence-specific amplification with CRISPR/Cas12a's precise recognition, we minimize non-specific signals, ensuring a higher level of reliability compared to traditional NAT methods. Equipment-free simplicity: The isothermal nature of the RPA-CRISPR workflow eliminates the need for thermocyclers, thus enabling on-site integration with PAT. This is in contrast to qPCR, which requires specialized instrumentation. Broad applicability: The use of universal primers and crRNA targeting conserved 16S rRNA regions allows for the detection of nine pharmacopeia-relevant *Mycoplasma* species across various cell matrices, without cross-reactivity. Downstream compatibility: Successful validation in simulated bioprocessing environments demonstrates the practical utility of our method for real-time quality control in antibody production, which can be directly implemented in biomanufacturing settings. These advantages address critical gaps in current PAT frameworks, offering a rapid, sensitive, and field-deployable solution for *Mycoplasma* monitoring. Our approach holds the potential to significantly improve the detection and management of *Mycoplasma* contamination in biopharmaceutical manufacturing processes.

5. Conclusion

In this study, we successfully constructed a *Mycoplasma* detection platform based on RPA-CRISPR-Cas12a. The operation process of this platform is extremely simple: First, RPA reaction is performed in a water bath for 20 minutes, and then the amplified product is quickly transferred to the CRISPR-Cas12a detection system for another 20 minutes, the entire detection process takes only 40 minutes, and the final result can be directly viewed by the naked eye under blue-violet light. The method is extremely sensitive and can detect samples as low as 0.1–10 copies/μL, ensuring that cell samples can be effectively detected at the early stages of contamination. In terms of the broad spectrum of the universal primers, our method was able to successfully detect nine common *Mycoplasma* species prescribed by the pharmacopoeia, while showing excellent specificity in a variety of common cells and stroma, and no cross-reactivity was observed. The combination of RPA's efficient amplification ability and CRISPR's accurate recognition ability not only greatly improves the convenience of detection, but also significantly enhances the specificity of detection.

CRediT authorship contribution statement

Hou Sheng: Project administration, Funding acquisition. **Zhu Weifan:** Resources. **Li Jun:** Resources. **Ren Yule:** Resources. **Qian Weizhu:** Supervision, Resources, Project administration, Funding acquisition. **Guo Qingcheng:** Resources, Project administration, Funding acquisition. **Gao Tianyu:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zhang Dapeng:** Writing – review & editing, Supervision, Resources, Conceptualization. **Ji Lusha:** Resources. **Wang Chenguang:** Resources. **Liu Tao:** Resources. **Guo Menghui:** Resources. **Chen Yi:** Resources. **Guo Huaizu:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Xu Jin:** Supervision, Resources, Project administration. **Wang Fugui:** Resources. **Wang Xuekun:** Resources. **Pan Zhiyuan:** Resources. **Sun Ziqiao:** Resources.

Zhuang Huangzhen: Resources.

Declaration of Competing Interest

The authors declare they have no conflicts of interest with the contents of this article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2025.116904.

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