Review

Detection of Parasites in the Field: The Ever-Innovating CRISPR-Cas12a

Xin LI , Zhisheng DANG , Wenqiang TANG , Haoji ZHANG , Jianwei SHAO , Rui JIANG , Xu ZHANG * , Fuqiang HUANG *

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Review

Detection of Parasites in the Field: The Ever-Innovating CRISPR-Cas12a

Xin Li [1,2], Zhisheng Dang [3], Wenqiang Tang [2,4], Haoji Zhang [1], Jianwei Shao [1], Rui Jiang [5], Xu Zhang [1,*], Fuqiang Huang [1,*]

[1] School of Life Science and Engineering, Foshan University, Foshan 528225, China; lixin990730@163.com (X.L.)
[2] State Key Laboratory of Hulless Barley and Yak Germplasm Resources and Genetic Improvement, Lhasa 850002, China
[3] National Institute of Parasitic Diseases, Chinese Center for Diseases Control and Prevention (Chinese Center for Tropical Diseases Research), Key Laboratory of Parasite and Vector Biology, National Health Commission of the People's Republic of China (NHC), World Health Organization (WHO) Collaborating Center for Tropical Diseases, National Center for International Research on Tropical Diseases, Shanghai 200025, China
[4] Tibet Academy of Agriculture and Animal Husbandry Sciences, Lhasa 850002, China
[5] College of Animal Science and Veterinary Medicine, Huazhong Agricultural University, Wuhan 430070, China
* Correspondence: qxhuangfuqiang@fosu.edu.cn (F.H.); fuzhangxu@fosu.edu.cn (X.Z.)

Abstract: The rapid and accurate detection of parasites is crucial for timely curative intervention in parasitosis and for epidemiological surveillance. To meet the needs of clinical diagnosis, it is imperative to develop a diagnostic tool based on nucleic acid that combines the sensitivity and specificity of established nucleic acid amplification tests with the speed, cost-effectiveness, and convenience of isothermal amplification methods. A new nucleic acid detection method, utilizing the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) nuclease, holds promise for point-of-care testing. Specifically, the CRISPR-Cas12a system has demonstrated numerous advantages for detecting parasites, with hopeful outcomes for identifying malaria, toxoplasmosis, and other parasitic ailments. This review provides an overview of how CRISPR-Cas12a can be utilized for parasite detection, evaluates its advantages and disadvantages, and suggests ways to improve the efficiency and sensitivity of CRISPR-Cas12a-based assays.

Keywords: detection; CRISPR; suboptimal crRNA; light-activated crRNA; tandem repeats; POCT

1. Introduction

Parasitosis, caused by parasitic infection, is a prevalent cause of morbidity among humans worldwide. Tropical zones, particularly those that are impoverished, conflicted, or unsanitary, serve as endemic foci for a range of parasitic diseases [1]. The World Health Organization (WHO) has reported that annually 48.4 million cases and 59,724 deaths are attributed to the prevalence of 14 significant parasites, representing a total burden of 8.78 million disability-adjusted life years (DALYs). Of these instances, 48% represent foodborne parasitic diseases, accounting for 76% of the DALYs [2]. Transmission through contaminated food is prevalent in low- and middle-income countries [2]. Approximately 241 million cases of malaria and 627,000 deaths resulting from malaria were reported across the world in 2020. Innumerable deaths were caused by other parasitic infections, most notably neglected tropical diseases (NTDs) [3,4].

Unlike the vast majority of bacterial and viral infections, which have an incubation period as short as hours to days, parasitic diseases tend to have an incubation period of weeks or even months [4]. The incubation period of specific parasitic diseases, like alveolar echinococcosis, can extend up to

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10 years [5]. Therefore, early and precise detection of parasitosis is imperative for timely curative interventions and the prevention of pandemics.

A promising new method for nucleic acid detection utilizes the CRISPR-associated (Cas) nuclease, which can overcome the limitations of instrument dependence and laborious operational processes. The present review compares the CRISPR-Cas12a system with alternative molecular methods for detecting parasitic diseases. Emphasis is placed on the enhancement of one-pot recombinase polymerase amplification (RPA) Cas12a and even the improvement of CRISPR-Cas12/Cas13 detection techniques.

2. Application of Nucleic Acids Amplification Tests in Parasite Detection

Currently, the diagnosis of parasitic diseases relies on various methods, including microscopy, epidemiology, pathophysiology, immunodiagnostics, and nucleic acid amplification tests (NAATs). Among these options, the microscopic detection of parasites remains the most reliable [6,7]. In underdeveloped regions with high rates of parasitosis, however, skilled operators are often scarce, making this technique challenging to implement [8]. Furthermore, this approach is unsuitable for conditions linked to parasites at developmental stages that are challenging to detect in blood or stool specimens.

Immunoassay-based diagnostic measures have been in use for decades and are widely utilized for detecting parasites. However, the application of immunoassays for parasitosis diagnosis has been limited by several drawbacks, including the possibility of false negatives and false positives [9,10].

Molecular detection of nucleic acids demonstrates superior sensitivity, specificity, and reproducibility compared to alternative methods (Table 1). As a result, NAATs are the preferred molecular detection tools due to their ability to amplify trace amounts of DNA and RNA, allowing for highly specific detection by complementary nucleotide pairing. Although these techniques have been used to establish dependable parasitosis diagnostic methods for malaria, filariasis, toxoplasmosis, and echinococcosis [11-14], such methods require long reaction times, complex handling, expensive laboratory equipment, and highly skilled technicians.

Table 1. Main strengths and weaknesses of different approaches for parasite detection.

Discipline	Strength	Weakness
Morphology	·Accuracy (gold standard) ·Can detect multiple species at the same time	·Lower sensitivity ·Difficulty distinguishing parasite-like egg ·High demand for professional skills
Immunology	·Strong specificity ·Strong sensitivity	·High cost and time consuming ·False positives for cross-reactivity ·False negatives in immunocompromised patients ·Inability to differentiate between ongoing and past infections
Molecular biology	·Strong specificity ·Strong sensitivity ·Strong repeatability	·High cost ·Limitations related to sample preparation and equipment ·Logistics systems requiring fresh sample analysis (e.g. cryogenic)

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Isothermal amplification technology compensates for these shortcomings. In contrast to PCR, isothermal amplification technology, exemplified by loop-mediated isothermal amplification (LAMP) and RPA, significantly reduces the reaction time and dependence on instruments. RPA is an efficient method for on-site detection due to its simple primer design, low temperature requirements, and easy storage [15,16]. However, because the reaction occurs at low temperatures, non-specific amplification by RPA can lead to low specificity. Isothermal amplification technology has been employed in diagnosing various parasitic diseases, including malaria, leishmaniasis, and schistosomiasis [15-17].

In addition to the selection of detection methods for NAATs, which has a significant impact on the accuracy and sensitivity of parasitic disease diagnosis, the selection of target genes is also a key point in the establishment of detection methods. Along with 18S ribosomal RNA (rRNA), Internal Transcribed Spacer (*ITS*), and mitochondrial genes, stable tandem repeats are beginning to come into focus. In most parasites, repetitive sequences make up a substantially greater proportion of the genome when compared to coding sequences, comprising an estimated 20% or even exceeding 30% [18,19]. Numerous tandem repeats have been utilized to detect multiple protozoans and worms, such as *Trypanosoma cruzi*, *Onchocerca volvulus*, and *Schistosoma mansoni* (Table 2).

Table 2. List of partial parasitic repeat sequences.

Parasite	Repeat Sequence Name	Length(bp)	Quantity	GenBank Accession	Refs
Protozoa					
Trypanosoma cruzi	TCNRE	195	12% of the total genome	K01772	[20]
Toxoplasma gondii	/	529	200-300 copies per genome	AF146527	[21]
Plasmodium falciparum	Pfr364	716	41 copies per genome	/	[22,23]
Plasmodium vivax	Pvr47	333	14 copies per genome	/	[22-24]
Cestodes					
Echinococcus granulosus	EgG1 Hae III repeat	269	6900 copies per haploid genome (1% of *E. granulosus* genomic DNA)	DQ157697	[25,26]
Taenia solium	Tsol-9	158	None Related Description	U45987	[27]
Taenia saginata	HDP1	1272	0.4% of the T. saginata DNA	AJ133764	[28]
Trematodes					
Schistosoma mansoni	Sml-7（DraI）	121	12% of the total genome	M61098	[29-31]
Schistosoma haematobium	DraI	121	over 15% of the *S. haematobium* genome	DQ157698	[32]

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Trichobilharzia ocellata	ToSau3A	396	10,000 copies per haploid genome（1.5% of the T. ocellata genome）	AF442689	[33]
Nematodes					
Strongyloides stercoralis	/	765	None Related Description	AY028262	[34]
Brugia malayi	HhaI repeat	320	several thousand copies per haploid genome（about 12% of the genome）	M12691	[35,36]
Wuchereria bancrofti	SspI	195	300 copies per haploid genome	L20344	[37]
	LDR	1674	None Related Description	AY297458	[38]
Onchocerca volvulus	O-150	149	4500 copies per haploid genome	J04659	[39-41]
Parafilaroides decorus	Pd65	689	None Related Description	MT053285	[42]

Specific information on tandem repeats that have been used for parasite detection, including GenBank accession numbers and references, is provided.

Point-of-care testing (POCT), a priority for strategies relying on mass drug administration to control many neglected tropical diseases (NTDs), is a medical diagnostic tool that can be used near or at the point-of-care, allowing for on-site testing [43]. Therefore, it is imperative to develop nucleic acid-based diagnostic tools that combine the sensitivity and specificity of established NAAT with the convenience, cost-effectiveness, and speed of isothermal amplification methods. CRISPR-based diagnostics have the potential to fulfill all these requirements (Table 3).

Table 3. Comparison of CRISPR-Cas12a and commonly used detection technologies in molecular biology.

Technology	Device dependency	Specificity	Reaction time (min)	Number of primers	Quantification	Cost	Results View Method	POCT potential
PCR	Moderate	Strong	60-180	2	No	High	Gel electrophoresis	Moderate
q-PCR（qRT-PCR）	High	Strong	>60	2	Yes	Extremely high	Fluorescent and computer system	LOW

d-PCR	High	Strong	>60	2	Yes	Extremely high	Fluorescent and computer system	LOW
LAMP	Low	Strong	<60	4-6	No	Low	·Gel electrophoresis ·Color ·Turbidity	High
RPA	Low	Moderate	20-60	2	No	Low	·Gel electrophoresis ·Fluorescent ·Lateral flow	High
Cas12a	Low	Strong	20-60	2	No	Low	·fluorescent ·Lateral flow	High

3. CRISPR-Cas12a for POCT

3.1. Discovery of CRISPR

The CRISPR-Cas system was initially identified by Ishino in 1987 [44] and named in 2002 [45]. Extensive research has since focused on identifying and characterizing the proteins and molecules involved in the CRISPR-Cas system [46]. CRISPR-Cas systems are composed of Cas genes organized in operons and a CRISPR array, which consists of unique genome-targeting sequences (called spacers) interspersed with identical repeats [47]. Jennifer Doudna and Emmanuelle Charpentier were the first to illustrate the potential of the CRISPR-Cas9 system as a means of gene editing [47]. Subsequently, Janice Chen and Feng Zhang led the primary studies of CRISPR-Cas12a [48] and CRISPR-Cas13a [49] for the purpose of detection.

3.2. CRISPR-Cas12a Is More Suitable for Rapid On-Site Detection

The Cas12a effector protein, also referred to as the Cpf1 effector protein, is a programmable RNA-guided DNA nuclease that was identified as part of the type-V class Ⅱ CRISPR-Cas system [50]. This protein may have come from a distinct TnpB transposase gene family [51]. Compared to Cas9, the design of Cas12a is simpler and more cost-effective since it only needs one CRISPR RNA (crRNA) and no trans-activating crRNA (tracrRNA).

Cas12a accurately identifies target sequences and double-stranded DNA, creating gaps by recognizing T-rich protospacer adjacent motif (PAM) sequences and catalyzing its own crRNA maturation [52]. It was later discovered that Cas12a exhibits collateral activity and can cleavage single-stranded DNA without the presence of a complementary crRNA sequence [48]. The non-target strand and RuvC domains are highly flexible, with the target strand being particularly flexible when located at the nuclease active site. As a result, the RuvC domain becomes significantly active during R-loop formation, enabling the entry of single-stranded DNA into the enzyme's active center for degradation [53].

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Therefore, the target DNA has the potential to act as an activator, triggering both cis- and trans-cleavage events of the Cas12a nuclease. The FQ reporter in the system is cleaved, releasing fluorescence signal that is then measured to detect pathogen. The CRISPR-Cas12a system has found extensive application in detecting COVID-19, with studies demonstrating a detection limit as low as 5-10 copies utilizing the CRISPR-Cas12a system and isothermal amplification technology [54,55]. The sensitivity of the method was comparable to that of qPCR, and results were obtained via naked-eye observation within 45 minutes. These advantages enable the detection of parasites through the CRISPR-Cas12a system and offers benefits beyond those of other nucleic acid detection methods (Table 2).

3.3. CRISPR-Cas12a Has Been Applied to Parasite Detection

The technique's application to diagnose parasitic diseases, like malaria, has undergone a thorough evaluation. Asymptomatic carriers considerably hinder controlling and eradicating the parasite. Achieving malaria eradication mandates a hyper-sensitive diagnosis of infections with a low parasitic load [4]. Unfortunately, resource-limited areas experience frequent malaria outbreaks, posing a challenge to screening parasite carriers. Lee and colleagues developed a nucleic acid diagnostic method to detect Plasmodium falciparum by combining CRISPR-Cas12a with RT-RPA [56].

The method involves heating human serum, whole blood, or dried blood spots in buffer at 95 °C for 10 minutes, followed by the transfer of the suspended sample to a pre-mixed Cas12a-RPA system. The mixture is then incubated at 40 °C for 30 minutes. The reaction outcomes can be observed using a plate reader or a handheld fluorometer, facilitating on-site detection. This technique significantly lowers the LOD to 0.36 parasites per microliter, which is well under the WHO's rapid diagnostic test threshold of 200 parasites per microliter [57].

Additionally, the CRISPR-Cas12a combination with RPA has been successfully utilized for detecting Toxoplasma gondii [58]. The detection system achieved a sensitivity of 3.3 genome copies per microliter, surpassing real-time fluorescent RPA (33 genome copies per microliter) and other comparable methods [59]. Furthermore, this system was utilized to examine a range of parasites, including Cryptosporidium parvum [60,61], Enterocytozoon hepatopenaei [62], and Heterodera schachtii [63] (Table 4).

Table 4. Application of the CRISPR/CAS12 system to parasite detection.

Species	Method	Time (min)	LOD	Specificity	Refs
Plasmodium falciparum	Cas12a-RPA	30 (+10) [a]	0.36 parasites/μL	100%	[56]
Plasmodium vivax	Cas12a-RPA	30 (+10) [a]	1.2 parasites/μL	100%	
Toxoplasma gondii	Cas12a-RPA	35 (+20) [a]	99~115 copies/μL [b]	100%	[58]
Cryptosporidium parvum	Cas12a-RPA (two steps)	30 + 60 (+20) [a]	10 oocysts	100%	[60]
	Cas12a-RPA	90	1 oocyst	100%	[61]
Enterocytozoon hepatopenaei	Cas12a-RPA	60	50 copies/μL [b]	100%	[62]

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Heterodera schachtii	Cas12a-RPA	60	10^{-4} single cysts	100%	[63]

a, The time in parentheses is the time required for sample preparation or DNA extraction. b, Copy numbers indicate the copies of target genes rather than the genomes.

It is estimated that 47% of the worldwide population lacks adequate access to medical diagnostic tools, particularly in underdeveloped areas [64]. Cas12a and RPA-based diagnostic technologies are anticipated to resolve this problem since the method satisfies almost all of the POCT requirements [65]. Nevertheless, many concerns still need to be addressed because of the brief time of research and limited large-scale clinical trials. The sensitivity of these assays for POCT, particularly concerning specific sample preparation, ought to be enhanced. Furthermore, the time required to attain results should be decreased.

4. Optimization of the CRISPR-Cas12a One-Pot Detection Assay

Due to the low initial concentration of the target gene in a sample and the kinetic rates that result in an amplification-free LOD in the picomolar range under standard assay conditions [66], Cas12a detection often requires an amplification process prior to application. That means that signal amplification usually involves two processes. Target genes were initially amplified using RPA or LAMP techniques. The resulting amplified products were subsequently transferred to the Cas12a system for cleavage, followed by fluorescence signal generation. To streamline operations and prevent cross-contamination during field tests, the one-pot method is now predominantly utilized. This assay allows the amplification and cleavage of Cas12a to occur simultaneously. However, this leads to the cis-cleavage of Cas12a, which reduces the concentration of the target genes while RPA enhances it. Therefore, it is crucial to optimize amplification in the initial phase of the reaction.

4.1. One-Pot and One Step

4.1.1. Determinants of Cas12a Enzyme Kinetics

Several studies have reported rapid single-turnaround, cis-cleavage reactions at low target concentrations, with typical reaction times of approximately 100 seconds [67]. A Michaelis-Menten model for Cas12a trans-cleavage activity was established and validated by a team from Stanford University. This was achieved through the utilization of varying concentrations of substrates, targets, and crRNAs [66,67]. The authors suggest that the concentration of trans-cleavage product formed over time can be described using the following scaling equation:

$$\frac{[P](t)}{S_0} \approx \left(1 - \exp\left(-\frac{t}{\tau}\right)\right) \text{ [67]}$$

The production efficiency of trans-cleavage product P is influenced by both reaction time and τ. To refer to the target-activated Cas12-crRNA-target DNA complex, use E, and subsequently, [E] represents the concentration of this complex. The characteristic time to complete trans-cleavage is governed by the time scale τ, which is proportional to K_M and inversely proportional to kcat and [E] [67]. The rate constant k_{cat}/K_M of enzymatic reactions is affected by the Cas type, crRNA, incubation time, pH, and temperature [66]. During the early stages of the reaction, [E] equals the concentration of the target molecule (c), which depends on c_0, amplification, and cis-cleavage. Therefore, one could use a suboptimal crRNA to weaken cis-cleavage or employ other methods to ensure that amplification dominates the pre-reaction period, resulting in a rapid increase of [E] (Figure 1).

4.1.2. Reduced crRNA Efficiency by PAM

In the CRISPR-Cas system, the effector nuclease must identify the PAM neighboring the target site to initiate target recognition [68]. Studies of the crystal structure of the LbCas12a-crRNA binary complex [69] and the AsCas12a-crRNA-target DNA ternary complex [70,71] disclose the mechanisms involved in Cas12a and crRNA recognition, as well as the operations of crRNA-directed DNA targeting and PAM recognition. These findings suggest that the PAM-binding channel of Cas12a is

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flexible in conformation, allowing for the identification of both canonical and non-canonical PAMs [72]. LbCas12a and AsCas12a identify TTTV and CTTV/TCTV/TTCV as canonical and non-canonical PAM, respectively [72,73].

In 2022, a Chinese team conducted a one-pot test called sPAMC, which refers to a suboptimal PAM of Cas12a-based test [74]. In comparing collateral activity, crRNAs utilizing suboptimal PAMs demonstrated lower potency and slower kinetics in comparison to those utilizing canonical PAMs. Nevertheless, over 80% of 120 suboptimal PAMs displayed quicker reactions than those of canonical PAMs in the one-pot reactions.

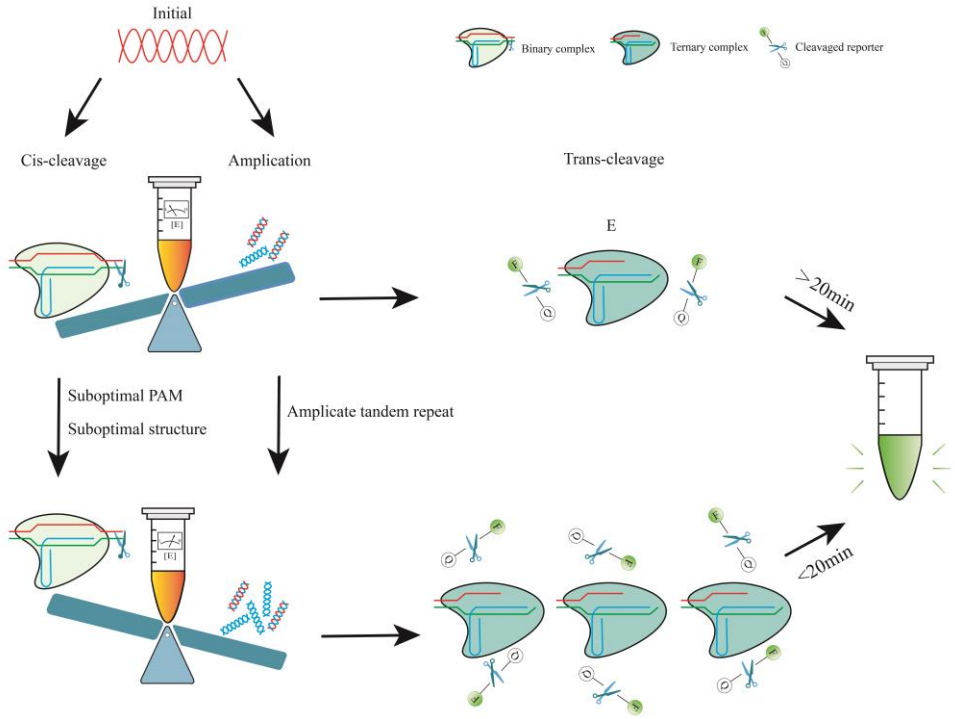

Figure 1. Effect of amplification and cleavage on detection efficiency. E is the target-activated Cas12-crRNA-target DNA complex. During the initial stage of the one-pot procedure, cis-cleavage is immediate, and the enzyme concentration is much greater than the target concentration. As amplification becomes more dominant, such as when amplification efficiency is increased and cis-cleavage speed is reduced, the amount of [E] is substantially larger and can be quickly increased, resulting in a significant improvement in reaction efficiency. On the other hand, if the target is quickly depleted in the initial stage, the emission of the fluorescent signal will decrease.

The emergence of the target amplicon occurred 2 minutes after the one-pot reaction utilizing suboptimal PAM, as opposed to 8–10 minutes for canonical PAM. Using a suboptimal PAM with varied concentrations of Cas12a/crRNA ribonucleoprotein produced steady kinetic curves, unlike with traditional PAMs. In one-pot reactions, numerous uncommon PAMs (such as VTTV, TCTV, and TTVV) and some TRTV, TTNT, and YYYN PAMs (excluding TTTV) outperformed canonical PAMs. The SARS-CoV-2 diagnostic method established using suboptimal PAM yields a sensitivity comparable to that of qPCR. The reaction time is only 15 minutes, and the variation among samples is less than 30% [74].

4.1.3. Reduced crRNA Efficiency by Structure

Suboptimal crRNAs can be selected based on their structure while ensuring specificity. If CRISPR-Cas9 cleavage is an energy-driven process, its efficiency relies heavily on nucleotide

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hybridization and changes in folding free energy [75]. The stability of gRNA-DNA for gRNAs exhibiting different efficiencies significantly varies. When local sliding is examined, an energy model accurately predicts the efficiency of gRNAs. Similar studies for Cas12a are currently unavailable. However, it is important to consider the use of optimal crRNAs when developing a one-pot detection method. Initial support for the feasibility of this approach is demonstrated by our recent experimental findings.

4.2. One-Pot but Two Steps

4.2.1. Light-Activated crRNA to Initiate Cleavage

Controlling chemical reactions through photocontrolled techniques can be achieved in a non-contact manner within seconds. This technology has been extensively used in both CRISPR-Cas9 research and practice [76-78], and it has also been progressively refined for CRISPR-Cas12a detection [79]. Initially, the CRISPR-Cas12a system was blocked by a photo-cleaved linker containing crRNA to ensure optimal RPA performance. After amplification, the Cas12a detection system was activated via light to initiate trans-cleavage and produce fluorescence signals [79]. However, this approach requires ongoing optimization of the ratio of the photo-cleaved linker to crRNA, and the lack of pre-binding of crRNA to Cas protein negatively impacts the stability of the Cas12a-crRNA complex.

The same group then developed a novel CRISPR-Cas12a detection assay that uses 6-nitropiperonyloxymethyl-caged thymidine (NPOM-dt) to modify crRNA [80]. This method involves caging crRNA to prevent base pairing between it and the target, rather than binding it to the Cas enzyme. The rapid activation can be attained by photoinduced decaying, which makes this approach simpler, faster, and more stable. It should be noted that optimizing the irradiation time and the number and position of NPOM may need to be reconsidered for different pathogens. In regards to POCT, challenges persist with reagent storage conditions, actual amplification time, and the portability of illumination devices.

4.2.2. Physically Separate the Two Processes

In addition to performing two reactions simultaneously in one tube, it is also possible to physically separate the two reaction systems in one tube to allow for sequential progression [43,81-83]. The CRISPR/Cas12a reagents were spun down for cleavage after DNA amplification by leveraging the physical property of the protein-containing liquid's enhanced surface tension [84]. Initially, the RPA reaction takes place at the bottom of the tube, while the CRISPR/Cas12a reaction is located at the lid, separate from the reaction. After amplifying for 20 minutes, briefly spin the CRISPR/Cas12a reagent into the reaction mixture without opening the tube. The reaction will continue, and the RPA amplicon will activate the Cas12a nuclease to trans-cleave the fluorescent ssDNA-FQ molecule, resulting in a fluorescent signal. However, this method can be cumbersome, particularly in large-scale POCT.

In brief, cis-cleavage plays a crucial role as the rate-determining step for overall performance in one-pot reactions [67,74]. During the initial stage, low-concentration targets are diminished due to cis-cleavage, which results in a slow and unstable accumulation of amplicons. Consequently, the growth of the signal decreases or may even disappear altogether (Figure 2). The kcat/K_M of the enzyme can be reduced by utilizing a suboptimal PAM or structure, which slows cis-cleavage. This results in a balance between the two signal amplification processes of RPA and trans-cleavage. Through careful engineering of primer design, crRNA design [74,85], reaction system [86,87], reporter selection [88,89], and reaction conditions, isothermal amplification and CRISPR detection can be effectively combined in a one-pot reaction. The light-activated CRISPR RNA and spatially isolated reagents may enhance the efficiency of the one-pot (but two steps) process. The resulting method provides a streamlined and efficient approach to detecting specific nucleic acid sequences with high sensitivity and specificity.

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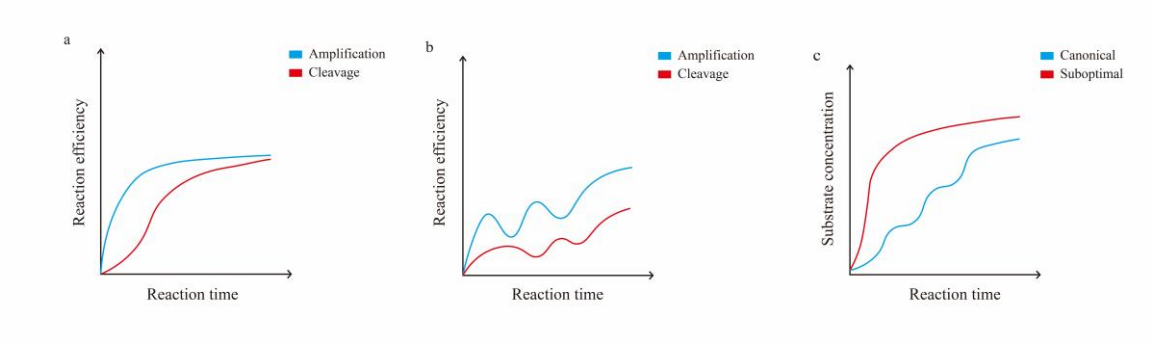

Figure 2. A schematic diagram of the relationship between amplification and cleavage in the one-pot method. a. Comparison of amplification and cleavage efficiency over time when using suboptimal protospacer or suboptimal crRNA to attenuate the early cleavage efficiency of Cas12a in the one-pot method. b. Comparison of amplification and cleavage efficiency over time using optimal crRNA in the one-pot method. c. Variation in substrate concentrations over time when using suboptimal and optimal crRNA in the one-pot method.

5. Conclusions

Parasites are prevalent in the natural world, particularly in underdeveloped regions, and result in high disability-adjusted life years and substantial economic losses annually. The emergence of CRISPR, and specifically recent examinations of Cas12a, compensates for the limitations of isothermal amplification and presents a fresh approach for POCT. With the collateral-activity of Cas12a, results can be evaluated intuitively via the inclusion of fluorophores. Combined with RPA, samples with even small amounts of pathogens can be quickly and accurately tested at the point of detection.

For POCT, the one-pot method is without a doubt the best option due to its ability to prevent cross-contamination and significantly simplify the procedure. Nonetheless, current one-pot detection techniques come with several limitations, including extended reaction times, low sensitivity, complicated operation, and reliance on sample pretreatment. Additionally, the utilization of RPA has restricted the advancement of CRISPR assays somewhat. As the most commonly used partner of CRISPR one-pot method, RPA kits are only sold by few companies, with high price and unstable supply [90].

By balancing the two processes of amplification and cleavage with a suboptimal PAM or structure, the detection performance of the one-pot method can be improved. With suboptimal conditions, PAM or crRNA's limitation on target genes may be weakened, thereby expanding the pools of target genes. In addition, light-activated crRNA and spatial isolation enable two reactions to proceed in one-pot, one after the other, without opening the lid. Furthermore, incorporating tandem repeats as targets can significantly enhance the detection's amplification efficiency and sensitivity, regardless of sample preparation methods. One-pot RPA-Cas12a is significantly improved with the use of these methods. These ways can enhance not only RPA-Cas12a but also all Cas12a detection methods involving amplification. Furthermore, it is important to assess these concepts not only in Cas12a, but also in other CRISPR systems, including Cas12b and even Cas13.

In the future, for the application of CRISPR-Cas12a to POCT, it is necessary to continually optimize one-pot method detection efficiency and identify a more compatible isothermal amplification technology. Also, the sample preparation for testing is crucial; otherwise, CRISPR detection would remain limited to the laboratory.

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Abbreviations

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; crRNA, CRISPR RNA; DALY, disability adjusted life year; NAAT, nucleic acid amplification test; NTD, neglected tropical disease; PAM, protospacer adjacent motif; POCT, Point-of-care testing.

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