International Journal of Nanomedicine

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REVIEW

Recombinase Polymerase Amplification-Based Biosensors for Rapid Zoonoses Screening

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Abstract: Recent, outbreaks of new emergency zoonotic diseases have prompted an urgent need to develop fast, accurate, and portable screening assays for pathogen infections. Recombinase polymerase amplification (RPA) is sensitive and specific and can be conducted at a constant low temperature with a short response time, making it especially suitable for on-site screening and making it a powerful tool for preventing or controlling the spread of zoonoses. This review summarizes the design principles of RPA-based biosensors as well as various signal output or readout technologies involved in fluorescence detection, lateral flow assays, enzymatic catalytic reactions, spectroscopic techniques, electrochemical techniques, chemiluminescence, nanopore sequencing technologies, microfluidic digital RPA, and clustered regularly interspaced short palindromic repeats/CRISPR-associated systems. The current status and prospects of the application of RPA-based biosensors in zoonoses screening are highlighted. RPA-based biosensors demonstrate the advantages of rapid response, easy-to-read result output, and easy implementation for on-site detection, enabling development toward greater portability, automation, and miniaturization. Although there are still problems such as high cost with unstable signal output, RPA-based biosensors are increasingly becoming one of the most important means of on-site pathogen screening in complex samples involving environmental, water, food, animal, and human samples for controlling the spread of zoonotic diseases.

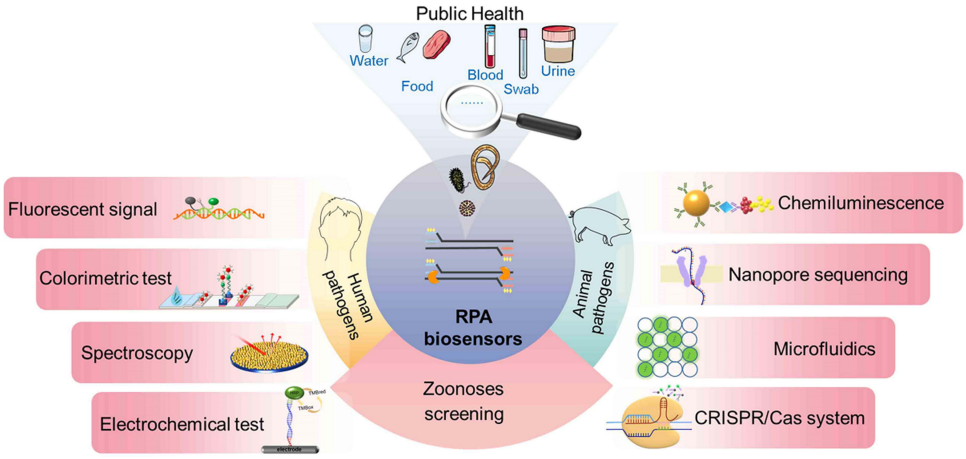
Keywords: recombinase polymerase amplification, biosensor, zoonoses, rapid detection, nanomaterials

Introduction

Since the emergence of severe acute respiratory *syndrome coronavirus-2 (SARS-CoV-2)* in 2019, this *coronavirus* has spread to more than 200 countries, cumulatively infecting 672 million people and causing 6.84 million deaths. Moreover, the emergence of numerous variant strains (*α, β, γ, δ,* and *Omicron*) presents a serious challenge for controlling the spread of the infectious disease.[1] Approximately 60% of new human infectious diseases are zoonoses.[2] The WHO proposed the "One Health" Initiative and multidisciplinary integration to address the growing risk of zoonotic diseases.[3] The rapid and sensitive detection of zoonotic pathogens from environmental, water, food, animal, and patient samples can effectively control the global spread of zoonotic diseases and reduce their adverse impact on public health. Isothermal amplification technology has attracted much attention from researchers because of its rapidity and simplicity with no need for complex temperature-changing instruments, such as strand displacement amplification (SDA), rolling circle amplification (RCA), nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), crossing priming amplification (CPA), single primer isothermal amplification (SPIA), multiple displacement amplification (MDA), exponential amplification reaction (EXPAR), and whole genome amplification (WGA) and recombinase polymerase amplification (RPA) (Table 1). Isothermal amplification simplifies amplification conditions, and the elimination of thermal cycling reduces the need for instrumentation or a laboratory environment. The warming and cooling process is omitted, effectively reducing the reaction time. Compare with these technologies,

Received: 2 September 2023
Accepted: 21 October 2023
Published: 6 November 2023

Graphical Abstract



RPA technology is highly valued for its advantages, including an undemanding primer design strategy, an easily achieved reaction temperature (25–42 °C), and a short reaction time (20 min). Recently, RPA-based biosensors combined with various nanomaterials have shown more rapid, sensitive, and accurate diagnostic performance than traditional RPA. This review summarizes the principle of RPA technology, the construction principles of various RPA biosensors, and their applications in zoonosis screening.

The Principle of RPA

The RPA reaction system includes three enzymes: recombinase, single-stranded DNA (ssDNA) binding protein, and strand-displacement DNA polymerase. Recombinant enzymes bind to primers, find the homologous sequence in the double-stranded DNA template, and initiate strand substitution by binding the primer to the homologous sequence. To stabilize the binding of the primer to the homologous sequence, a single-strand binding protein binds the displaced DNA strand. Subsequently, the recombinase dissociates, and the 3' end of the primer is exposed, recognized, and extended by strand-displacement DNA polymerase. Nucleic acid index amplification is achieved by cycling this process[15] (Figure 1). Although some studies recommend slightly longer RPA primers than those used in polymerase chain reaction (PCR),[16] RPA primers can initially be designed by the software (eg, Primer Premier 5) used for PCR primer design and experimentally screened according to the guidelines provided by the TwistDx Company.[17] The temperature of RPA is usually 25–42 °C,[18] but an increased reaction temperature can reduce nonspecific amplification. Because a crowding agent (polyethylene glycol) is added to promote the reaction, continuous shaking is needed for the distribution of components.[19] RPA showed good suitability in both DNA[20] and RNA[21] detection when reverse transcriptase and RNase H were added for one-step reverse transcription amplification.[22] With nucleic acid purification, sample types can be common specimens such as saliva, stool, blood, and urine.[23,24] The flexible applicability of RPA to a variety of samples shows its great potential for on-site screening.

RPA has several advantages: (1) No need for complex temperature-control instruments: A constant temperature of 25–42 °C is easy to achieve. (2) Short reaction time: RPA can be completed within 20 min. (3) High sensitivity and specificity: Its sensitivity and specificity are comparable to those of PCR. (4) Easy to operate: No professional technician is needed, and RPA can be implemented for home testing.[25] (5) The reagents are easy to store and transport: RPA reagents are used in lyophilized form with no need for refrigeration during transport.[26] (6) Multiplexing: Multiple detection of different pathogens or genes can be performed in one test.[27] (7) Robust adaptability: Excellent tolerance to common inhibitors with simple DNA extraction techniques.[28] (8) RPA tolerates primer mismatches with high fault

Table 1 Nucleic Acid Amplification Technology

Amplification Technology	Target	NO. of Primer	Primer Design Strategy	Amplification Time (min)	LOD (Copies)	Multipl-Exing	Temperature (°C)	Temperature Gradient Amplification	Portability of the Device	Ref.
PCR	**DNA**	2	Undemanding	120–180	100	Yes	50–95	Yes	Bulky	[4]
Q-PCR	**DNA**	2	Undemanding	60–120	10	Yes	55–95	Yes	Bulky	[5]
SDA	**DNA**	4	Complex	60–120	10	Yes	30–55	No	Portable	[6]
RCA	**DNA/RNA**	2–3	Complex	60–240	10	No	30–65	No	Portable	[7]
NASBA	**RNA**	2	Undemanding	60–180	1	Yes	41	No	Portable	[4]
LAMP	**DNA**	4–6	Complex	60	10	Yes	60–65	No	Portable	[8]
HDA	**DNA**	2	Undemanding	30–120	1	Yes	65	No	Portable	[6]
CPA	**DNA**	5	Complex	60–120	10	Yes	60–65	No	Portable	[9]
SPIA	**DNA/RNA**	1	Undemanding	30–90	10	Yes	47	No	Portable	[10]
MDA	**DNA**	Random	Undemanding	120	1	Yes	30–37	No	Portable	[11]
EXPAR	**DNA**	Random	Undemanding	60–120	1	Yes	60	No	Portable	[12]
WGA	**DNA**	Random	Complex	60	10	Yes	37	No	Portable	[13]
RPA	**DNA**	2	Undemanding	20–40	1	Yes	25–42	No	Portable	[14]

https://doi.org/10.2147/IJN.S434197

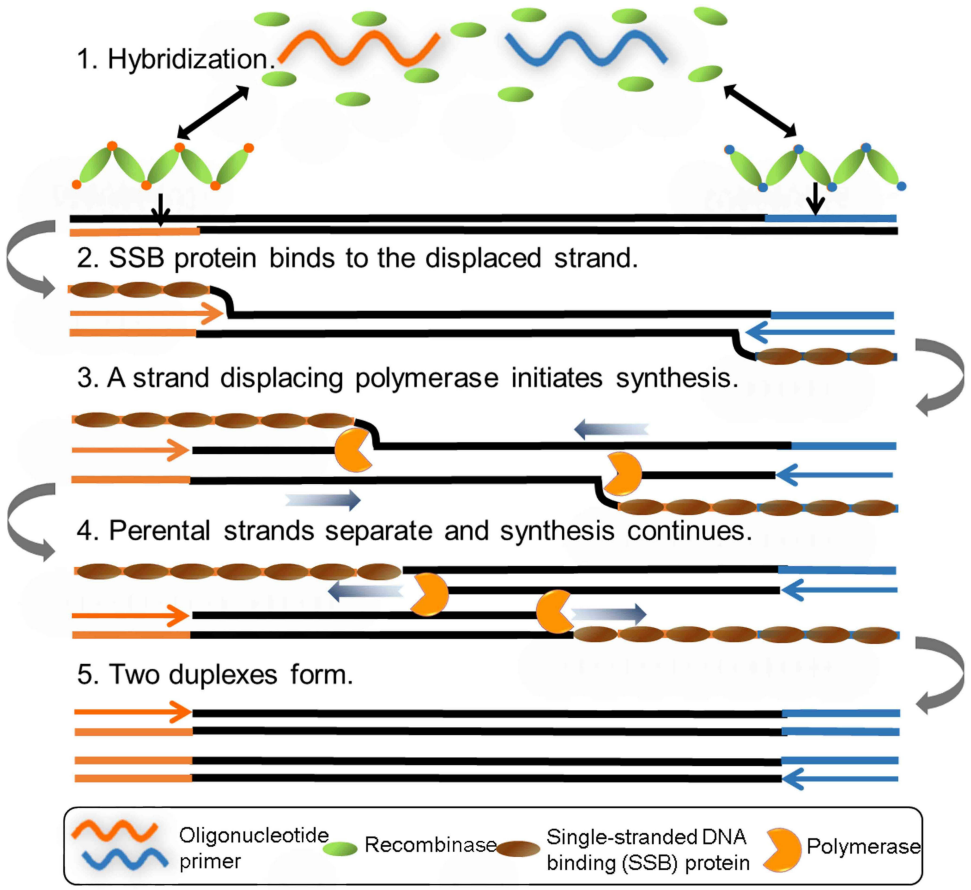


Figure 1 The RPA cycle.

tolerance.[29] However, this technology has some shortcomings: (1) There is no dedicated software for designing primers or probes. (2) It is easy to produce aerosol pollution. (3) Excessive template concentrations can inhibit RPA reactions.[30]

Application of the RPA Biosensor in the Detection of Zoonotic Pathogens

Zoonoses are diseases that undergo natural transmission between vertebrates and humans[31] via a variety of routes, such as respiratory, fecal-oral, skin contact, sexual, and arthropod routes. Climate and environmental change, agricultural intensification, frequent animal-human interactions, international trade in food, and the use of underfunded medical treatment systems have all exacerbated the prevalence of zoonoses.[32] To date, more than 200 zoonoses have been identified in humans.[33] Large-scale zoonotic outbreaks pose a serious threat to public health worldwide with negative impacts on the global economy and sustainable development, such as the *H1N1* influenza pandemic (2009), the West African *Ebola virus disease* (EVD) epidemic (2013–2016), the Congo EVD epidemic (2018–2020), and c*oronavirus disease* 2019 (*COVID-19*).[34] With the advantages of high sensitivity, rapid response, and on-site detection of RPA, accurate screening assays can be useful tools for rapidly controlling the spread of zoonotic epidemics. RPA-based biosensors combined with new nanomaterials provided methods for the early and rapid detection of zoonoses from complex samples. These biosensors involve a variety of signal conversions (Figure 2), including fluorescent signals, lateral flow assays (LFAs), enzymatic catalytic reactions, spectroscopic techniques, electrochemical techniques, chemiluminescence, nanopore sequencing techniques, microfluidic digital RPA, and clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas) systems.

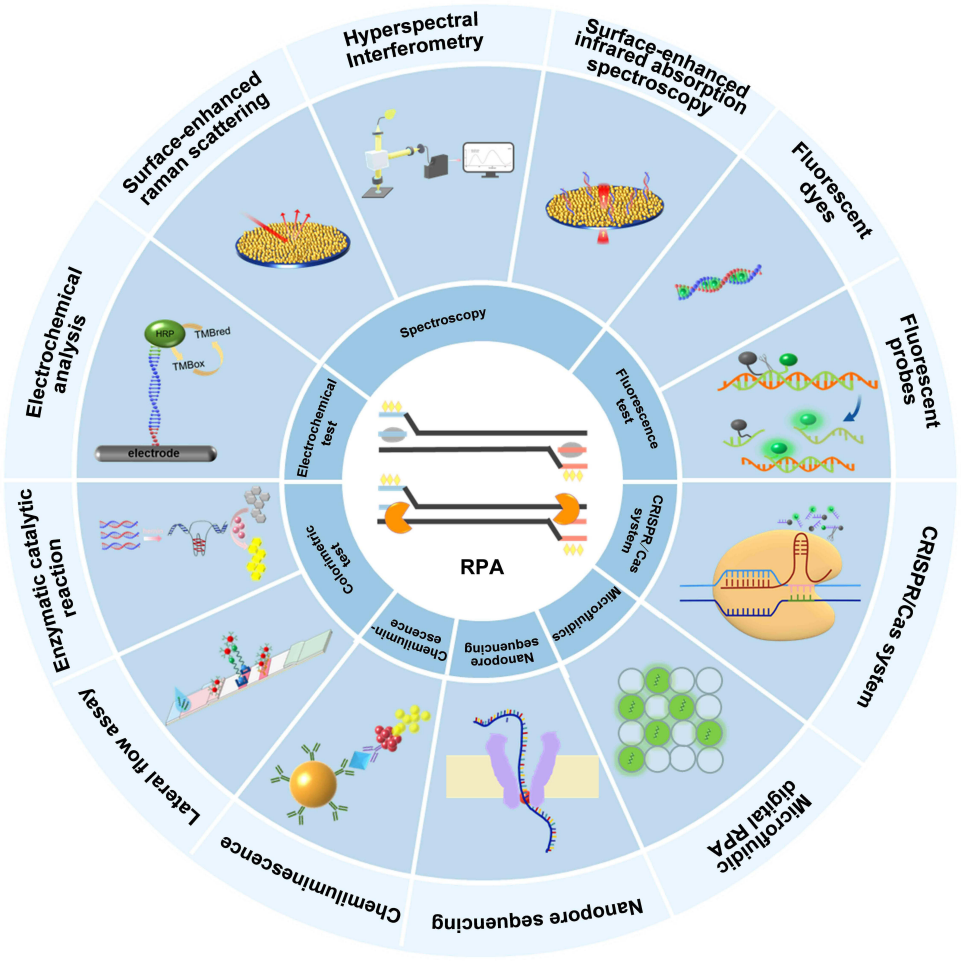


Figure 2 Principles of RPA biosensors for various signal conversions.

RPA Biosensors Based on Fluorescent Signal Output Mode

Fluorescent Probes Used in RPA Biosensors

Similar to the TaqMan probe in PCR, the real-time RPA system contains a fluorophore, a tetrahydrofuran (THF) residue, a quencher, and a blocking structure (SpC3, phosphate) located at the 3' end of the primer,[35] in which fluorescence quenching occurs based on fluorescence resonance energy transfer (FRET), which depends on proximity. As nucleic acid exonuclease (Exo) III cleaves the THF site and separates the fluorophore and the quencher, the fluorescent signal accumulates synchronously with the amplification and can be used for real-time[35] or end-point detection.[36] There is no need to open the lid throughout the process, which reduces the risk of aerosol pollution as well as false positive. In addition, the fluorophore label on the primer effectively prevented false-positive signals caused by the primer dimer and improved the accuracy of detection.[37] Unlike fluorescence quantification in PCR, which requires complex and expensive instruments, RPA can be miniaturized for on-site detection due to its low reaction temperature and low energy input required.[38] Therefore, numerous portable fluorescence monitors in RPA-based biosensors have been developed for zoonosis screening (Table 2).

A porous membrane paper-based RPA correlates the number of fluorescence signal spots with the concentration of the input HIV template (Figure 3a). The quantitative detection of fluorescence nucleation sites using image analysis algorithms is suitable for field testing via mobile phone-based image capture systems. The proposed biosensor also reduces the impact of primer mismatches on amplification efficiency and time to threshold.[29] In addition, a 3D printer was reversibly converted into an automated RNA extraction device to extract nucleic acids from urine specimens within 15 min, and the extruder was used as a heat source to perform real-time reverse transcription RPA (RT-RPA).[30] Based on

Table 2 RPA Biosensors Developed Using Fluorescence Detection

Zoonotic Pathogen	Transduction	Probe	Signaling Mechanism	Amplification Time (min)	LOD	Selectivity	Sample	POCT	Ref.
Dengue virus	Q-RPA	Exo probes	FRET	6	-*	100%	Blood of children	Yes	[39]
Rabies virus	Q-RPA	Exo probes	FRET	10	10 molecules/reaction	90.91%	Spiked cerebro-spinal fluid	Yes	[40]
H9N2 avian influenza virus	Q-RPA	Exo probes	FRET	8	1 EID_{50}/mL	100%	RNA from clinical swabs	Yes	[41]
West Nile virus	Q-RPA	Exo probes	FRET	15	10 copies/reaction	100%	Human plasma and serum	No	[42]
SARS-CoV-2	Q-RPA	Exo probes	FRET	20	35 copies/reaction	100%	Human saliva and serum	Yes	[43]
Schistosoma haematobium	Q-RPA	Exo probes	FRET	20	10 copies/reaction	100%	Spiked ddH_2O samples	Yes	[44]
Vibrio vulnificus	Q-RPA	Exo probes	FRET	25	17 copies/reaction	100%	Seafood homogenate	No	[45]
Listeria monocytogenes	Q-RPA	Exo probes	FRET	10	2.8 pg/μL	90%	Milk and hard cheese	No	[46]
Clostridium tetani	Q-RPA	Exo probes	FRET	20	20 copies/reaction	100%	Spiked pus and serum	Yes	[47]
Yersinia enterocolitica	Fluorescent dyes	SYBR green I dye	SYBR green I inserts into dsDNA	15	10 CFU/μL	100%	Meat	Yes	[48]

Note: *No data.

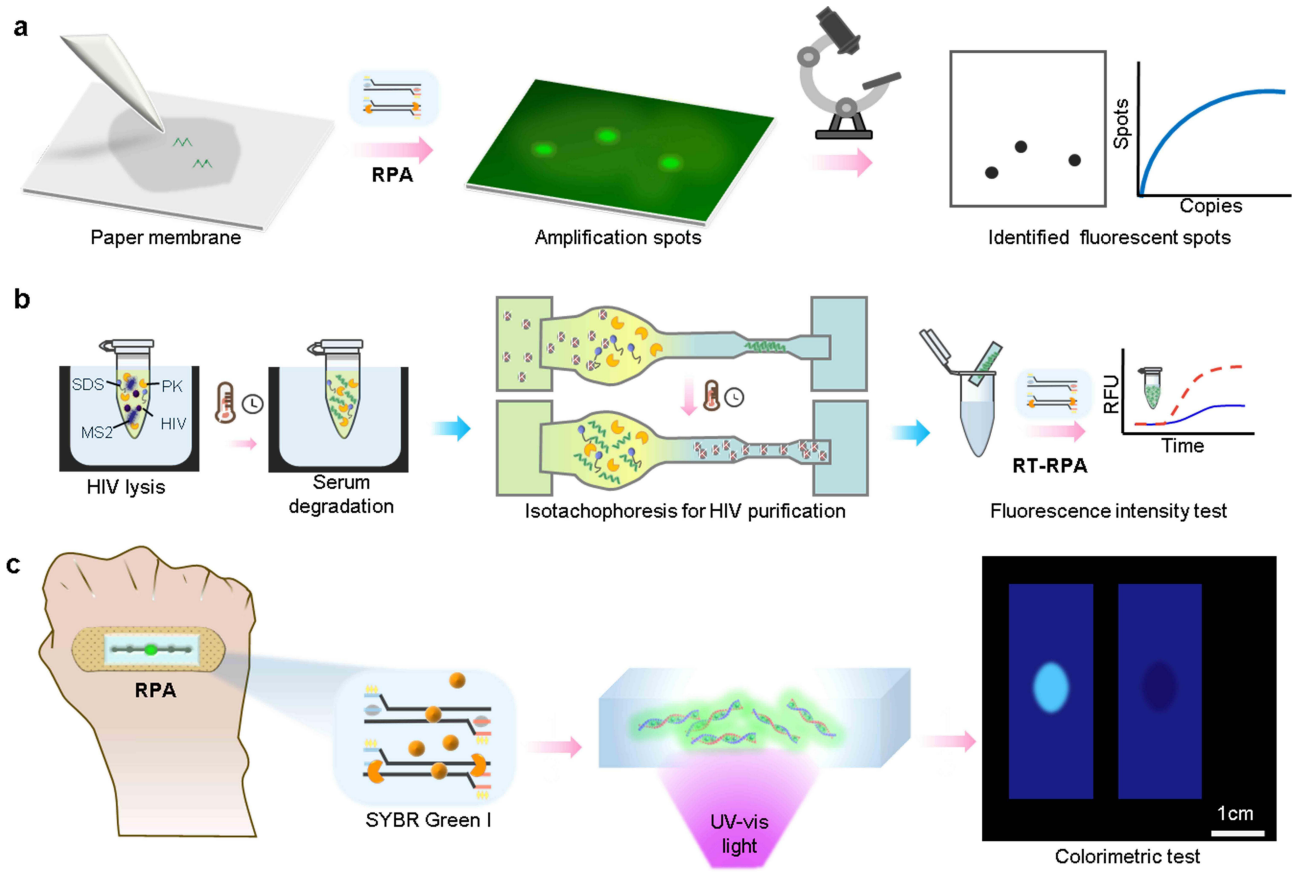


Figure 3 RPA biosensors based on fluorescent signal output mode. (**a**) Porous membrane paper-based RPA.[29] (**b**) Microfluidic paper-based sotachophoresis RPA.[23] (**c**) A wearable flexible microfluidic RPA device based on SYBR Green I.[50]

the electrophoretic mobility of the microfluidic paper-based isotachophoresis analysis device for nucleic acid extraction from blood samples, RT-RPA can be used to obtain results in 45 min with a limit of detection (LOD) of 5×10^3 copies/mL in HIV testing (Figure 3b).[23] Since RPA reaction reagents do not require cold chain transportation, a variety of RPA-enabled mobile laboratories have been successfully applied in the detection of zoonotic outbreaks.[26,49] The RPA mobile suitcase laboratory for parasite detection via real-time fluorescence quantitation (Q-RPA) allows the rapid extraction of nucleic acids by magnetic beads with a sensitivity similar to that of PCR.[49]

The combination of microfluidic technology and Q-RPA promoted the field detection of pathogens. *Mycobacterium tuberculosis* is detected by a centrifugal microfluidic chip with an LOD of 1×10^2 CFU/mL.[51] A microfluidic device using a single finger press as a driving force can be used for on-site testing without a centrifugation device.[52] Since the RPA reaction temperature is close to human body temperature, a watch-type microfluidic device made of polydimethylsiloxane can perform RPA by using hand temperature for heat transfer. The impact of ambient temperature is reduced through an insulated wristband attached to the wrist. A mobile phone detection system was adopted to implement the HIV test, and the LOD of the biosensor was 1×10^2 copies/mL.[53] However, the use of RPA-enabled fluorescent biosensors usually increases the cost of the assay.

Fluorescent Dyes Used in RPA Biosensors

Commonly used fluorescent dyes can be combined with RPA to visualize the amplification results.[50] For SYBR Green I can embed into double-stranded DNA (dsDNA), the RPA amplified product binds to it to form a complex, and the single linear oxygen ($^1O_2$) produced by photosensitization of the complex is used to oxidize tetramethylbenzidine (TMB) to emit a distinctly visible fluorescence signal under ultraviolet-visible light. This biosensor is used for *Salmonella* test with an LOD of 10 copies/μL.[54] In addition, using SYBR Green I as an indicator combined with a wearable flexible

microfluidic device (Figure 3c), an RPA biosensor triggered by body temperature can detect 10 copies/μL *zika* virus in 10 min.[50] However, SYBR green I affects the background signal of visual analysis.[50] Moreover, detection with SYBR Green I or other fluorescent dyes is affected by recombinant enzymes, and the accuracy of this biosensor is not high.[55]

RPA Biosensors Based on Colorimetric Analysis

LFA-Based Visual RPA Biosensors

LFA is widely used in field tests for zoonoses because of its simplicity, convenience, and visualization of results (Table 3), which typically include sample pads, binding pads, nitrocellulose membranes, absorbent pads, plastic backing, and biological reagents.[56] RPA products are labeled with biotin and fluorescein (FAM, etc.), which are added to the sample pad and migrate to the binding pad driven by capillary force. The anti-fluorescein antibody labeled on gold nanoparticles (AuNPs) captures the products and continues to flow forward. Then, the complex is intercepted by streptavidin, and the test line appears red.[57] In this biosensor, AuNPs coated with antibodies are the key component of signal conversion in the process of converting the increase in nucleic acid amplification products to color changes caused by the aggregation of AuNPs. The color change based on the surface plasmon resonance (SPR) effect on the surface of AuNPs is easily observed by the naked eye. RPA detection by LFA requires amplification products to be diluted 10–50 times to reduce their viscosity and allow them to flow easily.[58] Despite the additional dilution step, LFA-based RPA biosensors are currently the most widely used endpoint assay in amplification product tests.[5]

To detect multiple pathogen targets simultaneously, a variety of different fluorescent groups (eg, FITC, digoxin) can be added to primers. However, this multiplex detection should have similar-sized targets to prevent biased amplification of dsDNA. The reaction balance can be maintained by changing the buffer concentration or the ratio of primers. Notably, multiple test lines must be placed in a specific order to prevent cross-reactivity.[65] Jauset-Rubio Miriam used double RPA in combination with LFA to detect *Yersinia pestis* and *Francisella tularensis* genomic DNA, obtaining LODs of 243 fg and 4 fg, respectively. The reaction involved a tailing primer, a fishing probe, and proteinase K. The products obtained with the tailing primer can be hybridized with the capture and reporter probes labeled on AuNPs for direct detection. The use of proteinase K and the fishing probe reduces the cost and eliminates the need for centrifugation.[66] In addition, LFA-based RPA allows the quantitative detection of nucleic acids after the introduction of a competitive internal amplification control of known copy numbers, which is used as the control in each reaction to ensure that negative results are not due to reaction inhibition.[46] By controlling for similar amplification efficiency between the nucleic acids of the control and the sample to be tested, this method can reliably distinguish the threshold of ≤600 or ≥1400 copies of HIV from 1000 copies.[67] However, opening the cap in this biosensor increases the risk of aerosol contamination when adding samples to the reaction. The enclosure of the LFA in a microfluidic chip can solve this problem (Figure 4a), reducing the possible exposure to aerosol contaminants in *SARS-CoV-2* testing.[22] Generally, LFA has slightly lower sensitivity than Q-RPA.[15] The use of soluble barriers between the binding pad, the test line, and the control line can effectively improve the sensitivity of LFA-based RPA.[68] In addition, two sets of graphene nanosheets of different sizes were used as reporter probes: the smaller one bound to the analyte, and the larger one underwent additional binding to achieve signal amplification of LFA-based RPA.[69]

Enzymatic Catalytic Reactions Involved in RPA Biosensors

The use of the enzyme-catalyzed color development of specific substrates allows the visualization of results with high specificity and low cost. Methemoglobin chloride induces the formation of a G-quadruplex deoxyribonuclease with similar activity to horseradish peroxidase from a "G"-rich sequence, which catalyzes substrates to produce a visible blue color with characteristic absorbance at 450 nm.[17] As a typical DNA nanomaterial with enzymatic catalytic activity, the application of G-quadruplex in RPA biosensors solves the problem of dsDNA-ssDNA conversion and color identification in amplification technology.[20] TiO_2 nanoparticles used in RPA biosensors achieved an LOD of 4 CFU/mL when detecting *Salmonella* in colorimetric analysis by adsorbing primers to aggregate into larger nanoclusters, thereby increasing the partial concentration to enhance RPA. The 5' end of the forward primer contains an endonuclease recognition region and a G-quadruplex sequence (attached to TiO_2 nanoparticles). After amplification, the bifunctional structural domain is embedded in dsDNA products. The endonuclease binds to the specific region for cleavage, and the polymerase induces

Table 3 RPA Biosensors Developed Using Colorimetric Analysis

Zoonotic Pathogen	Transduction	Probe	Signaling Mechanism	Amplification Time (min)	LOD	Selectivity	Sample	POCT	Ref.
Salmonella typhimurium	LFA	Peptide nucleic acid @AuNP	SPR	25	4 CFU/mL	-*	Spiked milk	Yes	[59]
Dengue virus	LFA	Anti-FAM antibody@AuNP	SPR	30	10 copies/reactions	100%	Spiked ddH_2O samples	Yes	[60]
Middle east respiratory syndrome coronavirus	LFA	Anti-FITC antibody@AuNP	SPR	25	1.2 copies /μL	100%	Human throat swabs; oral and nasal swabs of Camelidae	Yes	[61]
Ebola virus	LFA	Anti-FAM antibody@AuNP	SPR	30	134 copies / μL	-*	Cultured virus	Yes	[62]
Babesia	LFA	Anti-FAM antibody@AuNP	SPR	10	0.25 parasites/uL	100%	Human blood; mice blood	Yes	[63]
Listeria monocytogenes	LFA	Anti-digoxin antibody@AuNP	SPR	25	1.5 CFU/mL	100%	Spiked food	Yes	[64]

Note: *No data.

https://doi.org/10.2147/IJN.S434197

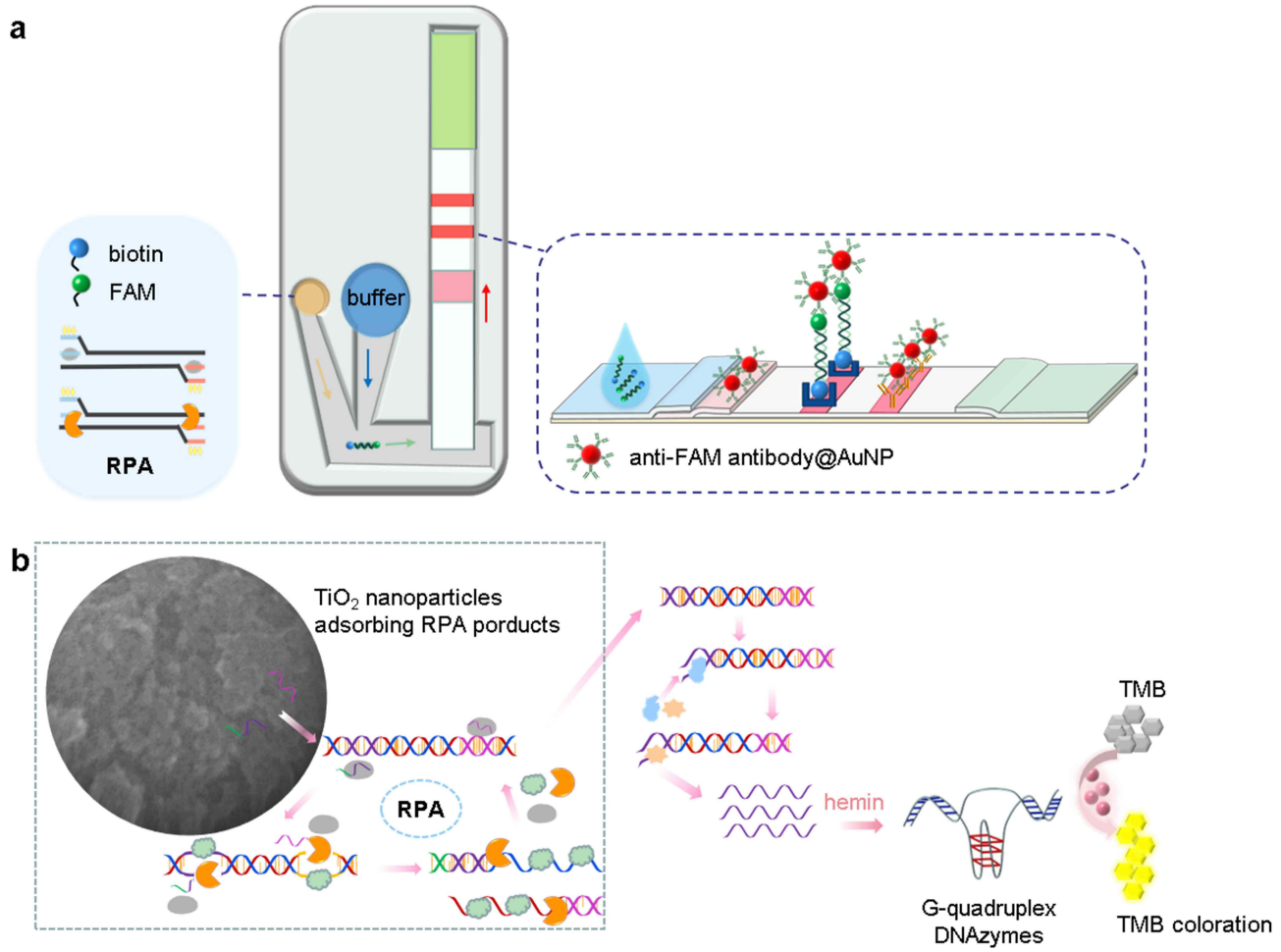


Figure 4 RPA biosensors based on colorimetric analysis. (**a**) RPA and LFA enclosed in a microfluidic chip.[21] (**b**) TiO_2 nanoparticle-enhanced RPA biosensor for colorimetric analysis.[20]

strand shift and extension, generating a single-stranded G-quadruplex DNA. Permethemoglobin chloride induces the formation of G-quadruplex deoxyribonuclease with enzymatic catalytic activity to promote the color development of TMB (Figure 4b). Moreover, a label-free visual RPA biosensor enhanced by a double-strand specific enzyme and terminal deoxynucleotidyl transferase can detect *Salmonella* at 6 CFU/mL.[17] In addition, RPA products recognized by CRISPR-dCas9 and amplified by RCA ultimately produced G-quadruplets, which catalyzed the color development of 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid).[70] This biosensor was used for the detection of *Leishmania* with a sensitivity of 1 zeptomole at 23 °C. However, the variety of substrates currently used for enzymatic reactions is limited, so multiplex assays are challenging.

RPA Biosensors Based on Spectroscopic Analysis

Surface-Enhanced Raman Scattering (SERS) Used in RPA Biosensors

Recently, SERS has become a commonly used technology for inelastic light scattering sensing, and two mechanisms are generally agreed upon: electromagnetic field enhancement (local electric field enhancement on metal surfaces) and chemical enhancement (electron transfer between metals and molecules).[71] When molecules are adsorbed on corrugated metal surfaces, such as silver or AuNPs, the signal in SERS can be greatly enhanced by a factor of 10^8 or more, allowing even single-molecule detection.[72] An RPA-integrated microfluidic paper analysis device was designed using SERS and CRISPR/Cas12a, which was applied in *Salmonella typhimurium* testing with a sensitivity of 1 CFU/mL.[73] A conjugated gold nanopillar-4-mercaptobenzoic acid-gold nanoshell probe was designed as a signal conversion element, which linked

ssDNA hybridized to the complexes with surface-adsorbed DNA 1 and DNA 2. In the presence of the target gene, the target DNA activates the trans cleavage of CRISPR/Cas12a, resulting in a dispersed colloidal solution of the nanoprobe due to the breakage of ssDNA. In contrast, the nanoprobe is aggregated due to cross-linking via ssDNA in the absence of the target DNA. The change from dispersion to aggregation was determined by SERS, and the quantitative measurement of pathogens was achieved within 45 min.[57] However, SERS requires special optical instruments, and the characteristic peaks of labeled amplicons need to be determined experimentally beforehand.[74] At present, substrates for SERS are mainly precious metals, which limits the application of SERS in biosensors. A study showed that a conductive glass/Ag/zeolite imidazole skeleton (FTO/Ag/ZIF-8) sandwich structure may serve as a substitute for SERS substrates.[75]

Surface-Enhanced Infrared Absorption Spectroscopy (SEIRA) Used in RPA Biosensors

SEIRA is an extended infrared spectroscopy technology using molecular vibrations coupled with surface equivalent excitation resonance to enhance the infrared signal of the molecule to be measured by 10^3–10^6 times.[76] Gold and silver nanomaterials are widely used in SEIRA due to their high stability and proper dielectric properties.[77–80] An infrared spectroscopic biosensor based on RPA was proposed to detect *SARS-CoV-2* using the SEIRA effect on AuNPs.[81] The hybrid was formed by using the complementary DNA probe to capture the target nucleotide, and the SEIRA signal of the chemical environment change of the functional group was detected. When this method is combined with RPA, 2.98 copies/μL *SARS-CoV-2* can be detected within 30 min. Moreover, a sufficient distance from the gold surface to the first base of the DNA probe is important to facilitate the hybridization of the probe with the target. However, a disadvantage of this method is the need to purify the RPA product, which adds an additional step. SEIRA suffers from the same problem as SERS in that the substrates are primarily precious metals, although other nanomaterials such as indium tin oxide nanoparticles have been proposed as alternatives.[82]

Hyperspectral Interferometry Used in RPA Biosensors

Hyperspectral interferometry detects the material with nanometer-scale spectral images based on the fundamental principle of the Fourier transform of spectroscopy, each pixel of which contains a continuous spectral curve that allows identification of the substance corresponding to the object.[83] The 3D image data generated by satellite push scans of hyperspectral imaging extend beyond the visible spectrum at very high resolution, allowing finer spectral structures to be measured.[84] The solid-phase self-interference RPA chip (1.8 mm × 1.8 mm) was combined with hyperspectral interference. After RPA, the solid-phase primer attached to the chip captured the target fragment, causing a redshift of the hyperspectral interference signal. The increased optical length of the RPA chip was used to demonstrate the existence of the target. The system can detect 6 copies/reaction of *Plasmodium falciparum* DNA in 20 min, and the cost is only 1/50 of that using Q-RPA. Since the chip is disposable, the cost of a single reaction can be further reduced through a multiplex RPA chip.[85] The optical components used in imagers for hyperspectral interferometry are expensive, and the imagers rely on precise scanning to generate 3D data, which is not conducive to field inspection in resource-poor settings, although the spectral reconstruction algorithm to extract spectral information from RGB images may be a new alternative method.[83]

RPA Biosensors Based on Electrochemical Analysis

The principle of electrochemical technologies is to use solid-based electrodes incorporating biosensitive molecules to immobilize one or more reaction components onto the electrode surface, specifically recognizing and trapping target molecules on the electrode surface to initiate redox reactions.[86] The electrode acts as a signal transducer to convert the reaction signal into an electrical signal that can be measured. A wash-free and rapid electrochemical method is described to detect RPA-amplified dsDNAs using a zinc finger protein (Figure 5a). Electrochemical detection is achieved using proximity-dependent electron mediation of ferrocenemethanol between a glucose-oxidase label and an electrode, which differentiates the specifically electrode-bound or -unbound labels without a washing or purification step. The LOD of the biosensor was 300 copies (13.2 μL) for the detection of *Rickettsia salmonis*, with the entire assay completed in 17 min. However, a certain degree of nonspecific amplification is generated at the affinity-modified electrode, with centrifugal filtration required to remove the electroactive dithiothreitol, which adds a reaction step.[87] In addition, a reagent-free DNA sensor enhanced by RPA was developed using a combination of ferrocene and reporter probe-modified AuNPs and

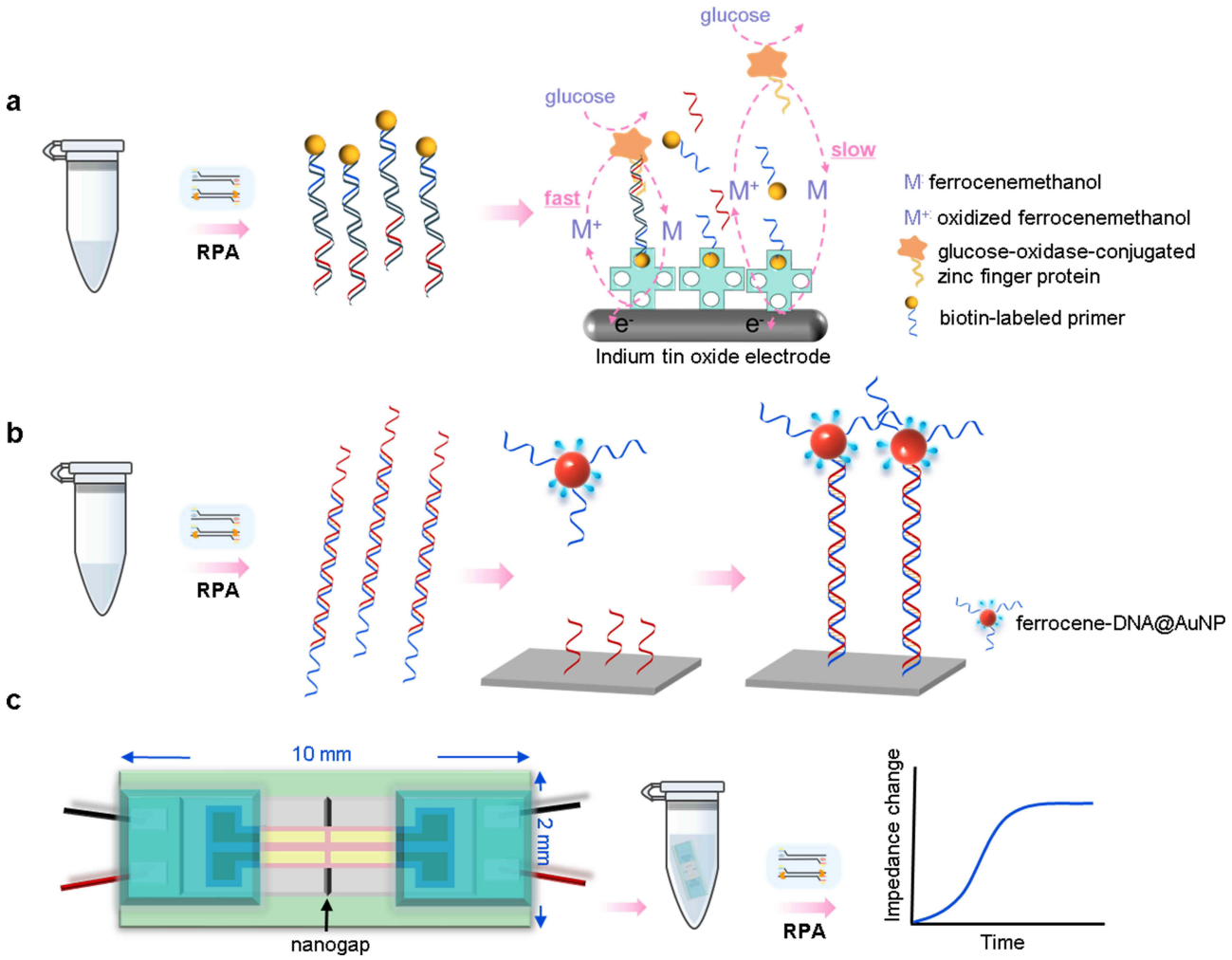


Figure 5 RPA biosensors based on electrochemical analysis. (**a**) RPA-based washing-free electrochemical biosensor using a zinc finger protein.[87] (**b**) A reagent-free DNA sensor enhanced by RPA based on ferrocene and primer decorated AuNPs.[88] (**c**) RPA-based nanogap impedimetric sensor for real-time DNA monitoring.[89]

modified primers (Figure 5b). RPA was performed using primer binding sites and C3 spacer-modified primers to produce amplicons with single-stranded tails at both ends. These tails were designed to be complementary to the capture probe immobilized on the gold electrode and the reporter probe on AuNPs. The demonstrated generic electrochemical genosensor can be exploited for the detection of any DNA sequence.[88] In addition, a nanosensor of size 2 mm × 10 mm was immersed in a 150 µL RPA system to analyze *E. coli* O157:H7 through the change in solution impedance during the reaction (Figure 5c), showing that one copy of gDNA can be detected within 5 min. However, the large reaction system increases the cost of this biosensor.[89] RPA is combined with microbead-mediated electrophoresis, and the number of amplicons is detected by dielectrophoretic impedance measurement.[90] Biotinylated probes are recognized and captured by streptavidin-labeled magnetic microbeads, with amplicons generated by RPA attached to the beads. Electrical measurements are carried out to obtain amplification results when the beads are added to the microelectrodes. The LOD of this biosensor was 2 copies/reaction with a total detection time of 26 min for antimicrobial-resistant *E. coli* testing. In addition, using the reaction conditions of RPA close to body temperature, a wearable device based on an electrochemical biosensor with a multimicroelectrode array was established for the detection of *SARS-CoV-2*, and 0.972 fg/µL (RdRP gene) and 3.925 fg/µL (N gene) genomes can be detected within 40 min.[91] The main problems of electrochemical technologies are poor stability and limitations in field applications. The use of micro- and nanoprocessing techniques may be an opportunity to reduce the size of electrochemical biosensors to make them applicable for on-site testing.[92]

Chemiluminescence-Based RPA Biosensors

The chemiluminescence (CL) method uses radioluminescence emitted during the conversion of electrons from the excited state to the ground state during a chemical reaction, with low interference, high specificity, and a higher signal-to-noise ratio than fluorescence.[93] A CL microfluidic chip combined with heterogeneous asymmetric RPA achieved highly sensitive detection of *Legionella* with an LOD of 87 genomic units/μL.[94] First, the samples are pretreated with photoactivated propidium monoazide, and this step allows us to distinguish viable and nonviable *Legionella*. Subsequently, the assay is performed on a microarray, and quantitative detection can be achieved by point amplification. The reverse primer is immobilized on the microarray, and the forward primer is encapsulated with biotin, which is linked to horseradish peroxidase by streptavidin and finally catalyzes the color development of the substrate. However, light emission is completed in a few seconds in most CL systems, so data acquisition and imaging time are limited. In recent years, continuous luminescence emitted for a long time has become a new optical imaging modality. For CL requires high instrumentation, camera equipment with high resolution, high sensitivity, and high interference immunity is necessary for CL imaging applications.[95]

RPA Biosensors Based on Nanopore Sequencing Technology

Nanopore sequencing technology is a third-generation sequencing method that allows the sequencing of individual DNA or RNA molecules. The principle of this technology is to sequence different nucleotide bases based on changes in electrical signals generated when they pass through a nanopore, which can enable on-site testing by portable nanopore sequencing devices.[96] Combining RPA with nanopore sequencing technology for *Mycobacterium tuberculosis* detection is comparable in accuracy and specificity to PCR technology combined with Illumina sequencing methods. This RPA biosensor is rapid, low-cost and can be automated for resource-poor settings.[97] The nanopore sequencing technology R9.4.1 has relatively low processing power for homopolymers, so the sequence accuracy is low. Although the latest Oxford Nanopore R10.4 sequence accuracy is up to 99%, its cost is high.[98] These drawbacks limit the wide application of nanopore sequencing technology in RPA-based biosensors.

Microfluidic Digital RPA Biosensors

Digital RPA (dRPA) achieves absolute quantification of nucleic acids by uniformly dividing them into multiple individual reaction units (eg, chambers or droplets), with the advantages of low equipment needs, short elapsed time, and high sensitivity and specificity.[6] Combining dRPA with a microfluidic chip can build a low-cost, high-throughput quantifiable thermostatic detection platform. An integrated multidigital RPA microfluidic chip combines DNA extraction, multidigital RPA, and fluorescence detection in a system for quantitative detection with no need to create a standard curve.[99] Nucleic acid extraction by magnetic beads can be performed in 15 min without any instrumentation. Reaction components are prepositioned in different areas of the chamber by means of a spiral valve, and the reagents are passively driven to the dRPA zone for detection using a fluorometer. The LOD for *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* is 10 bacterial cells.[99] A fully automated digital centrifugation microfluidic platform has been created to automate the entire process from swabbing nucleic acids to reading results in 55 min.[100] The difficulty of microfluidic dRPA is accurate initiation, and the additional time of magnesium acetate can seriously affect the amplification reaction. By coupling a microfluidic microinjector with a droplet generator, the addition time of magnesium acetate can be controlled to achieve absolute quantification of nucleic acids.[101] The second drawback is the narrow dynamic range. The lower detection limit of microfluidic dRPA is determined mainly by the total number of chambers or droplets and the volume of individual chambers or droplets, and the upper detection limit is determined mainly by the total volume of chambers or droplets. Increasing the number and total volume of chambers in a limited space can help to improve the dynamic range and sensitivity.[6]

CRISPR/Cas-Enhanced RPA Biosensors

RPA can rapidly amplify DNA exponentially with high sensitivity but is susceptible to nonspecific amplification. Coupling RPA and CRISPR Cas provides a new approach to solve this problem.[102] CRISPR is a microbial adaptive

immune system in most bacteria and all archaea. CRISPR RNA (crRNA) directs CRISPR-associated Cas proteins to recognize and cleave the target nucleic acid, ultimately separating the fluorescent and bursting moieties and emitting fluorescence.[103] The commonly used Cas proteins include Cas9, Cas12a, Cas12b, Cas13a, Cas13b, and Cas14, which exhibit side strand cleavage activity upon binding to their specific targets. After target recognition, the activated Cas nuclease cleaves nearby nucleic acids nonspecifically. By introducing nucleic acid reporters labeled with fluorophores into the reaction, cleavage can be detected with fluorescence intensity or AuNP-based LFA.[104] Based on their side chain cleavage activity, universal RNA reporter probes can be designed for the specific detection of target molecules to reduce reaction costs.[105] Cas9 specifically binds and cleaves nearby DNA through a guided RNA recognizing the protospacer adjacent motif (PAM) in the target DNA. Cas12a and Cas12b are RNA-directed DNases that recognize T-rich PAM sites and can directly use DNA as a substrate.[106] However, Cas12a can only recognize T-rich conventional PAMs, such as TTTV sequences, limiting its application. The Cas12a-based suboptimal PAM approach greatly expands the available options for crRNA.[107] Cas13a and Cas13b are RNA-directed RNA enzymes that require transcription by T7 RNA polymerase to convert amplified DNA into RNA for detection and do not require a specific PAM to recognize the target RNA.[108] After completing cleavage, they can remain active and cleave other nontarget RNAs. Cas14 is an RNA-primed targeted ssDNA nuclease that has a small molecular weight and excellent specific recognition of ssDNA and is not restricted by the PAM site. When activated, it can be associated with the nonspecific cleavage activity of ssDNA.

Currently, the CRISPR/Cas system is widely used in RPA biosensors (Table 4). In the one-step detection system of the biobarcode assay in conjunction with RPA and CRISPR-Cas12a, the signal is triple-amplified to achieve a single-colony limit of detection of *Salmonella*.[109] Although the antigen–antibody reaction in this biosensor is highly specific, the CRISPR/Cas reaction requires repeated lid opening, increasing the risk of aerosol generation. Integrating RPA and the CRISPR/Cas system into a single pot can effectively reduce the problems of nonspecific amplification and cross-contamination. A one-step detection platform (iSCAN-V2) was designed to visualize the easily observed signal of light-emitting diodes through the side chain cleavage of HEX-labeled ssDNA reporter genes.[110] CRISPR/Cas12a-assisted one-step RPA in *SARS-CoV-2* detection provides approximately 4–5 times higher sensitivity than digital RT-PCR, showing significant advantages in detection time and isothermal reaction conditions.[111] In addition, a paper-based multiplex RPA based on a sucrose valve was established to control the opening of the valve between the amplification chamber and the CRISPR detection chamber through the dissolution of sucrose (Figure 6a). By controlling the rate of sucrose dissolution, the valve opening time can be controlled, which has great potential for the rapid, sensitive, and reliable multimolecular diagnosis of infectious diseases in resource-limited environments.[102]

Due to the lack of a specific probe cleavage mechanism for CRISPR/Cas12a and CRISPR/Cas13a, their respective multiplex detection capabilities are limited. Combining Cas12a analysis and T7 transcription-mediated Cas13a analysis allows dual analysis in a single tube (Figure 6b).[121] However, when detecting RNA, the hybridization of cDNA-RNA results in slow initiation, the crRNA-Cas12a ribonucleoprotein is gradually inactivated, and nonspecific amplification consumes RPA reagents, resulting in low sensitivity.[122] Overcoming this problem requires the rapid production of DNA products from trace RNA templates and the rapid initiation of RPA. The removal of RNA from cDNA-RNA hybrids using the endocytosis function of RNase H solves this problem without compromising the transcriptional activity of Cas12a.[123] However, in the one-pot reaction, CRISPR detection competes with RPA, but the final detection signal is dependent on target amplification to generate sufficient substrate for CRISPR detection. Therefore, it is crucial to balance the processes of both.[107] In addition, increasing the Mg^{2+} concentration can improve the sensitivity of the test, as the increase in Mg^{2+} concentration enhances the hybridization between primers and DNA templates. The statistical design of experiments can also be used to optimize the one-step-based assay.[108] Mostly, the detection of CRISPR/Cas systems is based on fluorescence monitoring of the target. However, incomplete quenching of fluorescent moieties and unstable output signals are challenges for the application of this system. The development of CRISPR/Cas-enhanced RPA biosensors combining spectroscopic techniques, chemiluminescence, and electrochemical signals is an effective way to solve these problems.[124]

Table 4 RPA Biosensors Developed Using the CRISPR/Cas System

Zoonotic Pathogen	Transduction	Cas Type	Probe	Signaling Mechanism	Amplification Time (min)	LOD	Selectivity	Sample	POCT	Ref.
SARS-CoV-2	Colorimetric analysis	dCas9	Anti-FAM antibody@AuNP	SPR	15	4 copies/μL	100%	Human nasopharyngeal clinical samples	Yes	[112]
Hepatitis B virus	Fluorescence signal	Cas13a	FAM-Exo probe	FRET	30	1 aM	100%	Human blood	Yes	[113]
Duck tambusu virus	Fluorescence and Colorimetric analysis	Cas13a	FAM-N6-BHQ1 probe; Anti-FAM antibody@AuNP	FRET and SPR	20	1 copy/mL	100%	Duck	Yes	[114]
Influenza A virus	Colorimetric analysis	Cas12a	Anti-FAM antibody@AuNP	SPR	40	1 PFU	-*	Spiked human saliva	Yes	[115]
Legionella pneumophila	Fluorescence signal	Cas12a	TTAPE dye	TTAPE dye inserts into DNA	15	3 aM	100%	Environmental water	Yes	[116]
Yersinia pestis	Fluorescence signal	Cas12a	Anti-FAM antibody@up-converting phosphor nanoparticle	Up-converting phosphor technology	18	3 aM	90.63%	Mice blood	Yes	[117]
Staphylococcus aureus	Fluorescence and Colorimetric analysis	Cas12a	HEX-sequence-BHQ1 probe; Anti-FITC antibody@AuNP	FRET and SPR	20	5 copies/ reaction	100%	Environmental water	Yes	[118]
Francisella tularensis	Fluorescence signal	Cas12a	FAM-sequence-BHQ1 probe	FRET	30	0.5 copies/ reaction	100%	Human blood and sewage	Yes	[119]
Leptospira	Fluorescence signal	Cas12a	FAM-sequence-BHQ1 probe; Anti-FAM antibody@AuNP	FRET and SPR	40	100 cells/ mL	100%	Human blood	Yes	[120]

Note: *No data.

https://doi.org/10.2147/IJN.S434197

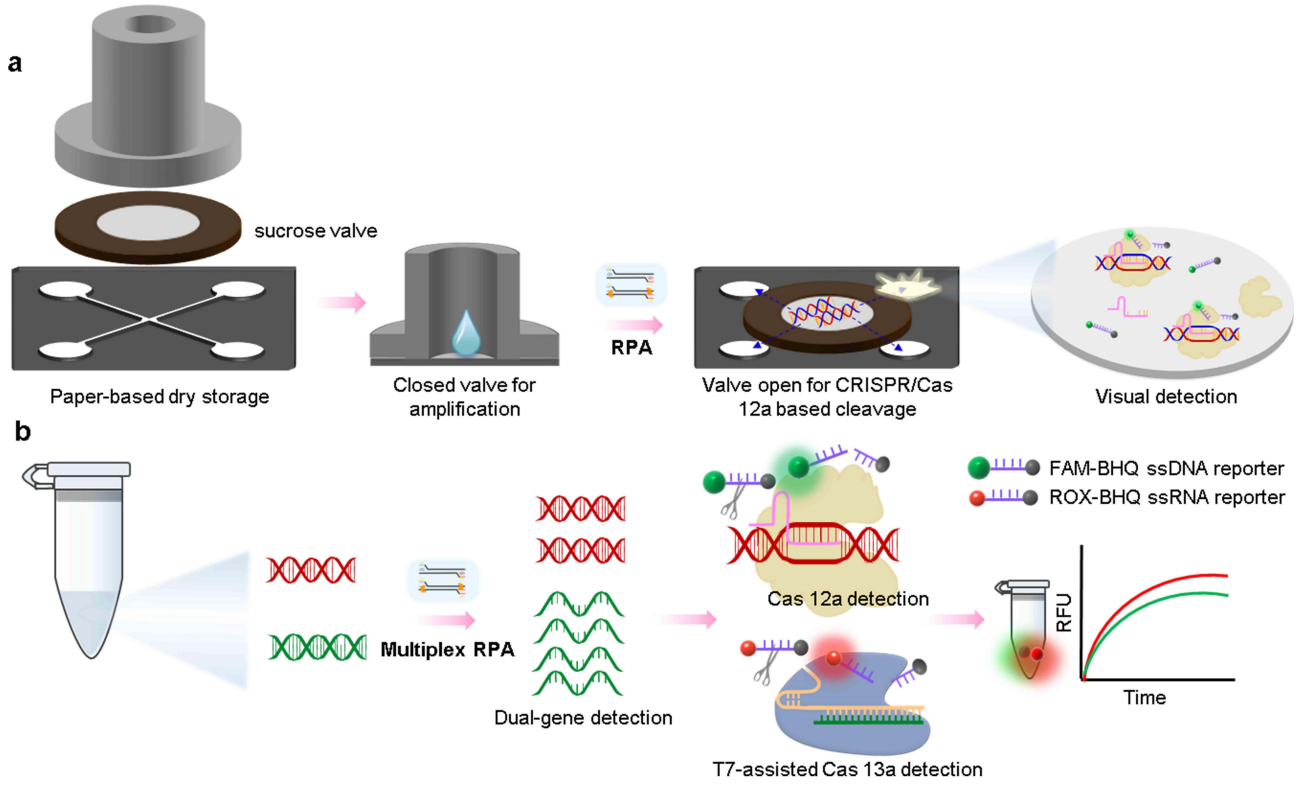


Figure 6 CRISPR/Cas-enhanced RPA biosensors. (**a**) CRISPR/Cas12a-enabled autonomous paper-based laboratory platform.[102] (**b**) Single-tube orthogonal Cas12a/Cas13a assay.[121]

Future Perspectives and Conclusions

RPA has gradually become a substitute for PCR and stands out among various nucleic acid technologies because it involves a rapid, compatible, constant temperature reaction (25–42 °C) with a short reaction time (20 min). Due to its natural suitability for field testing, RPA has been extensively studied in the context of zoonoses, although there are still some drawbacks. First, RPA suffers from a certain false-positive rate. An appropriate increase in temperature can reduce this rate. The combination of CRISPR/Cas may also improve the specificity. In addition, there is no universal primer design software, although traditional PCR primers have been shown to work in many studies. Finally, the complex process of nucleic acid extraction from samples limits its application in the field. As RPA has a good tolerance to inhibitors, nucleic acids can be extracted by thermal lysis to simplify the pretreatment step. It is worth noting that the rapid development of new nanomaterials has provided support for the development of integrated detection devices for RPA signal sensitivity and nucleic acid extraction, including functional DNA nanomaterials, precious metal nanomaterials, and composite nanomaterials. By combining fluorescence detection, optical discoloration, spectral analysis, electrochemical technology, chemiluminescence, nanopore sequencing technologies, digital microfluidics or the CRISPR/Cas system, traditional DNA amplification signals in RPA are transformed into fluorescent, optical or electrical signals for rapid, sensitive and accurate detection. The application of novel nanomaterials and multiple signal output modes aims to reduce the nonspecificity, enable multiplexed detection in a one-step reaction, and further enable the portability of detection devices.

Periodic outbreaks of zoonotic diseases pose a serious threat to the global environment, food safety, and animal and human health. The rapid and sensitive screening of zoonoses is essential to control the spread of pathogens from the environment, water, food, and animals to humans. The pretreatment methods of samples from different sources are inconsistent, and the background compositions are complex, including sludge, water, meat, milk, feces, throat swabs, whole blood, and serum. Compared with limited clinical assays for human samples, the detection of zoonotic pathogens faces greater challenges, which puts forward higher requirements for the stability and accuracy of detection methods in

zoonosis screening. At present, there are few relevant studies on different sample sources, and the tolerance of RPA biosensors to different types of sample processing needs to be further verified. In the future, research on RPA-based biosensors will mainly focus on the development of wearable sensors, multiplex high-throughput biosensors, one-step biosensors, visualization biosensors, and point-of-care detection to adapt to the increasingly complex zoonosis prevention and control situation. RPA is another leap forward in nucleic acid technology, which is expected to play an important role in the rapid detection of human-animal diseases in the near future, and is widely used in field screening to quickly and accurately identify pathogens and prevent the occurrence of disease pandemics.

Author Contributions

All authors made significant contributions to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

Funding

This study was supported by National College Students' innovation and entrepreneurship training program (S202213706035; S202313706015); the Foundation of Science and Technology Department of Jilin Province (20200404175YY); the Foundation of Ph.D. Research Project of the Jilin Medical University of Jilin Province (2022JYBS011KJ).

Disclosure

The authors have no conflicts of interest to declare for this work.

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