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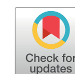
Synergetic performance of isothermal amplification techniques and lateral flow approach for nucleic acid diagnostics



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A B S T R A C T

The advancement in developing sensitive, rapid, and specific sensing tools is crucial in diagnostics and biotechnological applications. Although various isothermal amplification approaches exist for the detection and identification of nucleic acids, post-amplicon analysis is still based on traditional methods such as gel electrophoresis, colorimetry, turbidity, which could be non-specific and inconvenient. Thus, this review will first elaborate various isothermal amplification techniques (principle, merits, and demerits) and their potentials when combined with lateral flow approach for point-of-care nucleic acid diagnostics. Different methods for monitoring carryover contamination resulting from amplification product contamination will be discussed. Then, we will present recent advances in diagnostics with both target pre-amplification and CRISPR-Cas systems, which exhibit collateral cleavage of target nucleic acid and a reporter single strand nucleic acid within the vicinity. When the reporter is fluorophore-labeled, it provides a detectable signal by fluorescence or lateral flow biosensors. Lastly, we will discuss how CRISPR-Cas system based diagnostics could be more effective, affordable and portable for on-site detection.

1. Introduction

Nucleic acid detection techniques are rapidly growing owing to the critical importance in the diagnosis of infections, genetic diseases, and cancers [1]. PCR has been exploited for nucleic acids detection; however, it is limited for on-site application due to thermocyclers and skilled-personnel demands. Various isothermal amplification methods which amplify nucleic acids isothemally and allow for the detection of various nucleic acid targets have been in use. These include recombinase polymerase amplification (RPA) [2], rolling circle amplification (RCA) [3], loop-mediated isothermal amplification (LAMP) [4], cross priming amplification (CPA) [5], multiple cross displacement amplification (MCDA) [6], polymerase spiral reaction (PSR) [7], nucleic acid sequence-based amplification (NASBA) [8], and helicase dependent isothermal DNA amplification (HDA) [9] have been put employed and some their distinct features, advantages, and shortcomings are covered in Table 1. They are an alternative to PCR, which lacks on-site quantitation, fluorescence detection dependency, and is cross-contamination susceptible. Different methods including colorimetric [10–15], fluorescence [16–20], chemiluminescence [21,22], electrochemistry [23–28], and surface-enhanced Raman spectroscopy [29,30] have been developed for the analysis of isothermal nucleic acid amplification products. However, most of these methods are apparatus based, which could hinder their on-site application. So, the development of potential approaches for on-site deployment is therefore essential.

Lateral flow biosensor (LFB), one of the foremost commonly exploited methods, owing to its rapidity, simplicity, stability, low cost,

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Table 1
General remarks, advantages, and disadvantages of commonly used nucleic acid isothermal amplification techniques.

Technique	Merits	Demerits	General comments	Ref.
RPA	● Uses exponential amplification ● Operates at the low-temperature range (37 and 42 °C) compared with other isothermal amplification types, which make a positive attribute since there is no requirement for continual temperature control ● Does not require initial denaturation steps to generate ssDNA from the dsDNA target ● Provides a fast response compared to other isothermal amplification types (15–30 min) ● RPA reagents are supplied in stable lyophilized form and can last up to six months while other isothermal reagents require refrigeration	● RPA kits are costly ● Prior to amplification, RPA requires protein digestion, and purification to prevent the impairment of flow ● Real-time RPA amplification requires highly skilled personnel because it is not straightforward ● It has not been yet validated by FDA to be used for no-site testing applications	● RPA is used to amplify a wide variety of target nucleic acids (RNA, miRNA, ssDNA, and dsDNA) from diverse samples. It is highly selective and sensitive as it can reach as low as 1–10 copies of amplified target. More importantly, it does not require continuous temperature regulation. However, its future applications need to address on-site exploitation for commercial devices	[2,84]
LAMP	● Displays an amplification power close to that of PCR ● Exhibits high sensitivity and specificity due to its two sets of primers with the ability to compliment six different target sequences ● LAMP amplified target products can be indirectly detected by other methods such as turbidity or non-specific dyes ● Improved ability for multiplex detection ● Test kits available for both RNA and DNA detection	● Sometimes requires initial denaturation support to yield ssDNA from the dsDNA target ● Presents extreme challenges in designing multiple primers, which may also result in contamination ● Difficult to use for multiplex detection since designing primers requires skills ● Requires careful calibration and accurate temperature monitoring	● LAMP is a fascinating simple, rapid (30–60 min), cost-effective and sensitive (1–5 copies) isothermal nucleic acid amplification technique having close similarity and performance with PCR. However, LAMP is carried out isothermally at moderately low temperature through the strand displacing technique	[85–87]
RCA	● Works on circular DNA such as plasmids, bacteriophage, and circular RNA ● Suitable for amplification at high temperatures ● Can amplify as low as 10 copies/reaction	● Requires an initial denaturation step to generate ssDNA from the ds DNA target ● requires a thermal cycler and a thermostable DNA polymerase ● Restricted to amplification of circular nucleic acids ● Limited application since test kits are only available for virus detection	● RCA is an enzyme based isothermal amplification technique that uses circular DNA to amplify target nucleic acids (DNA or RNA). It has excellent amplification power as high as it was even used for the amplification of the whole viral genome	[88,89]
NASBA	● Amplification occurs at a constant temperature of 41 °C ● Does not require an initial denaturation step to generate ssDNA from the dsDNA target ● Does not need additional reverse transcription step like PCR, preventing contamination ● Can selectively amplify RNA even in the presence of genomic DNA	● Is designed for the specific detection of RNA targets ● Is not completely isothermal since some heat denaturation steps are required, enzymes are also separately added due to their differences in temperature stability ● Can only amplify only short nucleotides ranging from 120 to 250 base pairs	● NASBA is also known as self-sustained sequence replication (3SR), is a sensitive, and transcription-dependent isothermal amplification technique that is mostly applied for the identification of RNA targets. It is highly sensitive since it can even amplify RNA in the presence of genomic DNA	[90,91]
HDA	● Does not require an initial heat denaturation temperature to generate ssDNA from the dsDNA target ● The entire amplification reaction can take place at a constant temperature ● It is a good alternative to PCR because of its simplicity, procedure, and fair compatibility to various nucleic acids detection methods ● Can be easily miniaturized due to its simple protocols	● Requires optimization of primers which is mostly accomplished through PCR ● The efficiency of helicase is highly doubtful since it does not provide functional dissimilarities with other polymerases during the unwinding of dsDNA	● HDA amplification is similar to PCR procedure. However, HDA uses helicase to recognize and unwind the double structure of DNA. It also uses other specific strand-binding proteins to prevent reannealing of separated strands	[92,93]
MDA	● Less-prone to non-specific binding since it can be preferred to amplify the whole genome in preference to PCR ● Can rapidly amplify the minute amount of target nucleic acid without further nucleic acid isolation or purification.	● Requires an initial denaturation step to generate ssDNA from the dsDNA target ● When hexamer primers are used during amplification, they slow down the initial priming because of its high concentration	● MDA is an advanced version of SDA because both share the same amplification working principle. It presents an outstanding efficiency because it can even amplify the small concentration of the target	[94,95]
LCA	● Applies unique hybridization procedure by using a single DNA strand and its complementary synthetic oligonucleotide ● Uses highly thermostable DNA ligase ● Is highly sensitive, specific, easy to perform, and can be readily automated	● May generate false-positive results due to high background signal while free ligations occur in the absence of target DNA ● Presents difficulties in inactivating post-amplification products ● Contamination quickly arises when coupled with other methods	● LCA is an exponential isothermal amplification technique which is somehow similar to PCR. The generated LCA target products can be selectively detected by other techniques like fluorescent label detection, polyacrylamide gel electrophoresis, and auto-radiography.	[96]
NEAR	● Can be used to identify both DNA and RNA	● Generates many non-specific products that not only interfere with the progression of the forward reaction but also limit the sensitivity, which may require more specific and accurate amplification analysis techniques to prevent detection errors	● Is applied for the detection of small DNA or RNA fragments that are directly generated from the target nucleic acid. It relies on primers, nicking endonucleases, and a strand displacing DNA polymerase	[90]

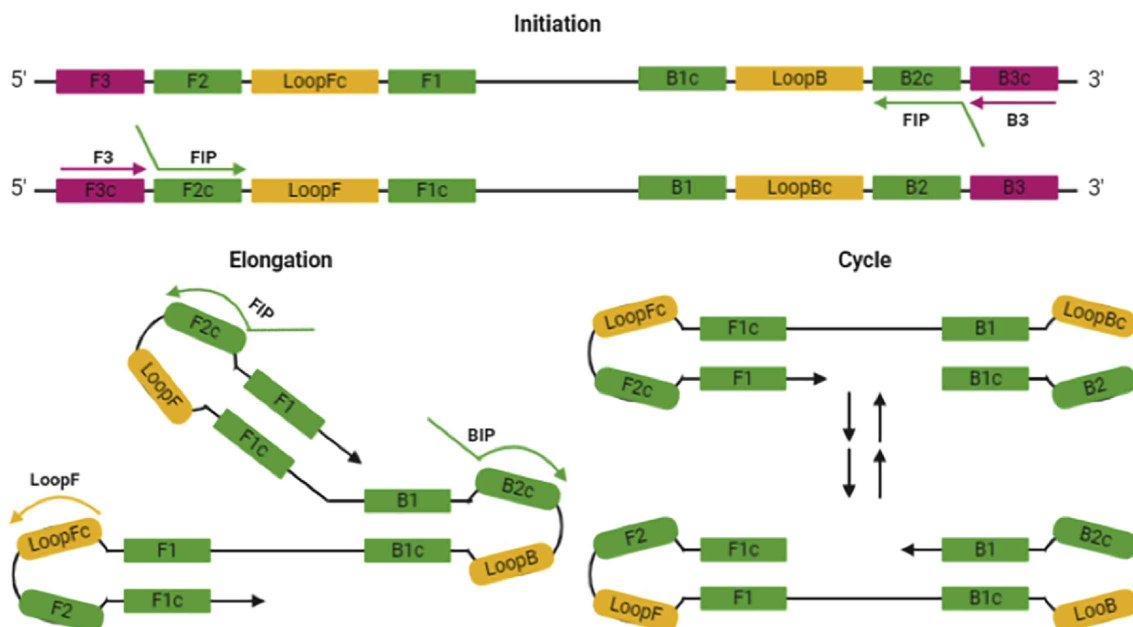

Fig. 1. Schematic representation of LAMP.

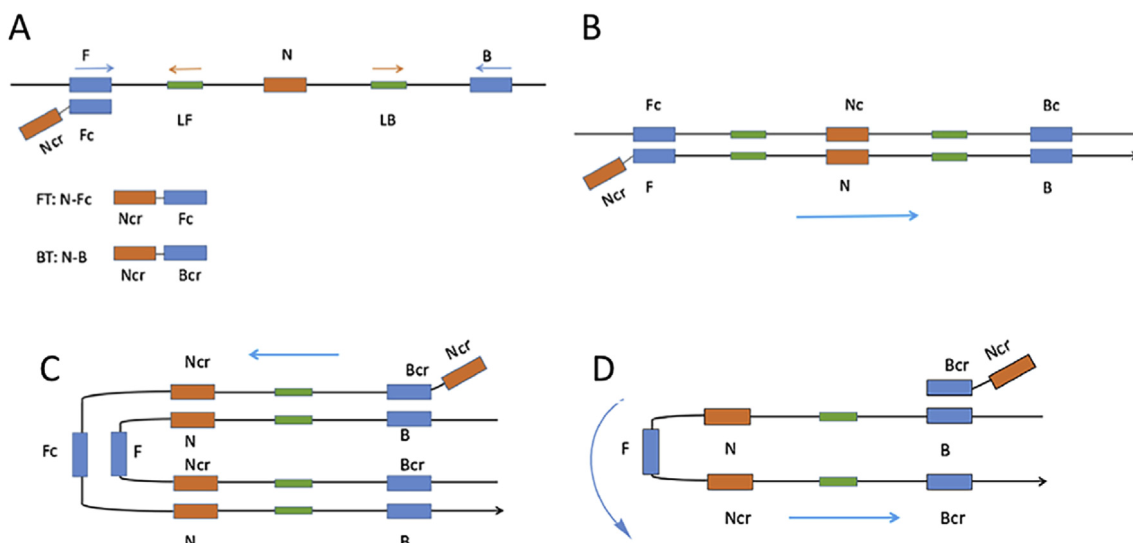

Fig. 2. Schematic diagram of PSR amplification. Reprinted with permission from Ref. [53] Copyright 2020, Springer.

visual, and user-friendly characteristics [31], has been widely used in the designing of detection assays. When integrated with one or more of the previous isothermal amplification techniques, they offer a high effective sensing and read-out tool, apparatus-free, affordable in multitude, and user-friendly, that enables the visual detection of nucleic acids by the nake eye of the operator. Nanoparticles such as quantum dots [32], silver nanoparticles [33], and carbon nano-materials [34] have been employed for development of lateral flow biosensor. A huge number of reported LFBs has focused on gold-nanoparticles (AuNPs) as common colorimetric markers due to their easily modified surface and macroscopic optical properties [35]. LFB consists of five main sections: a sample pad, a section on which the sample is introduced; conjugate pad, a section on which labeled tags reacts with the biological element; nitrocellulose membrane which houses a test line and a control line to capture a target; an absorbent pad to retain wastes; and lastly a backing pad which works as a protective support of the whole system [36] (Fig. 2). It is easy to construct, and the results are observable by naked eye (see Fig. 3).

Most recently, CRISPR-Cas system, a genome editing tool [37], has been applied in biosensing. Cas12 and Cas13 effectors have shown to exhibit collateral cleavage of pre-amplified target nucleic acids and non-specific single-stranded sequences [38–43]. When the latter are fluorophore-quencher labeled and in presence of the target sequence (s), they can provided readable signal after trans-cleavage. In this review, we present a wide range of isothermal amplification methods as well as post-amplification product analysis using LFB. Finally, we will emphasize on the usage of currently developed methods that combine isothermal amplification, CRISPR-Cas system, and LFB towards the on-site deployment.

2. LAMP based lateral flow biosensor

LAMP is a fascinating simple, rapid and cost-effective isothermal nucleic acid amplification technique having close similarity, power, and performance with PCR. Due to its use of two primer sets complementary to six different sequences of the target, the LAMP also realizes high

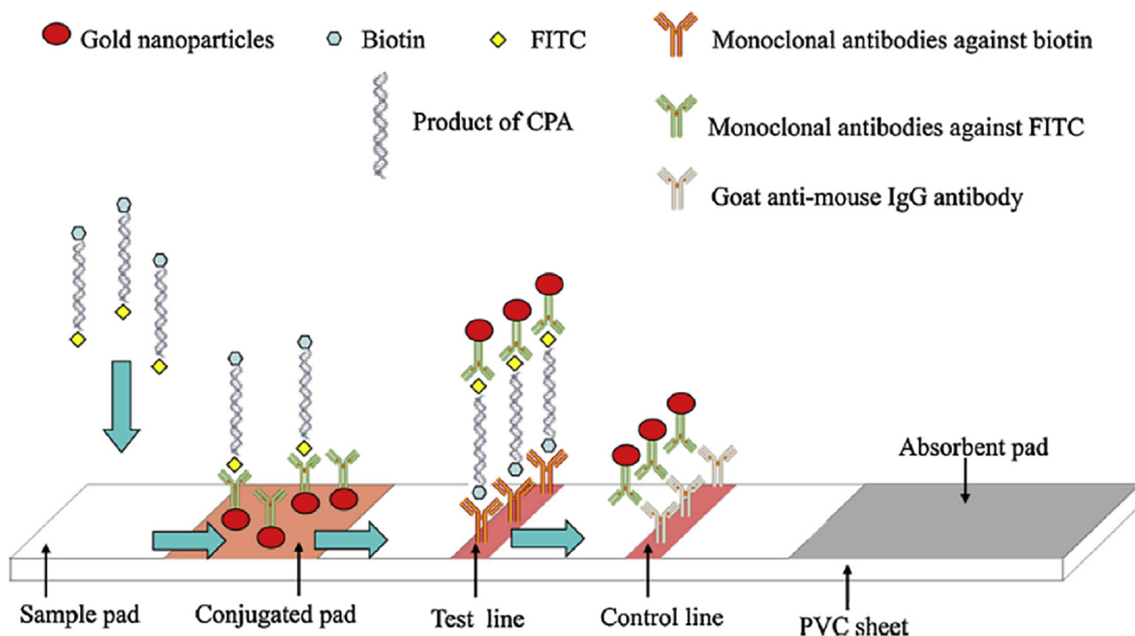

Fig. 3. Schematic diagram of the CPA-LF assay. Reprinted from Ref. [56] with permission Copyright 2018, Elsevier.

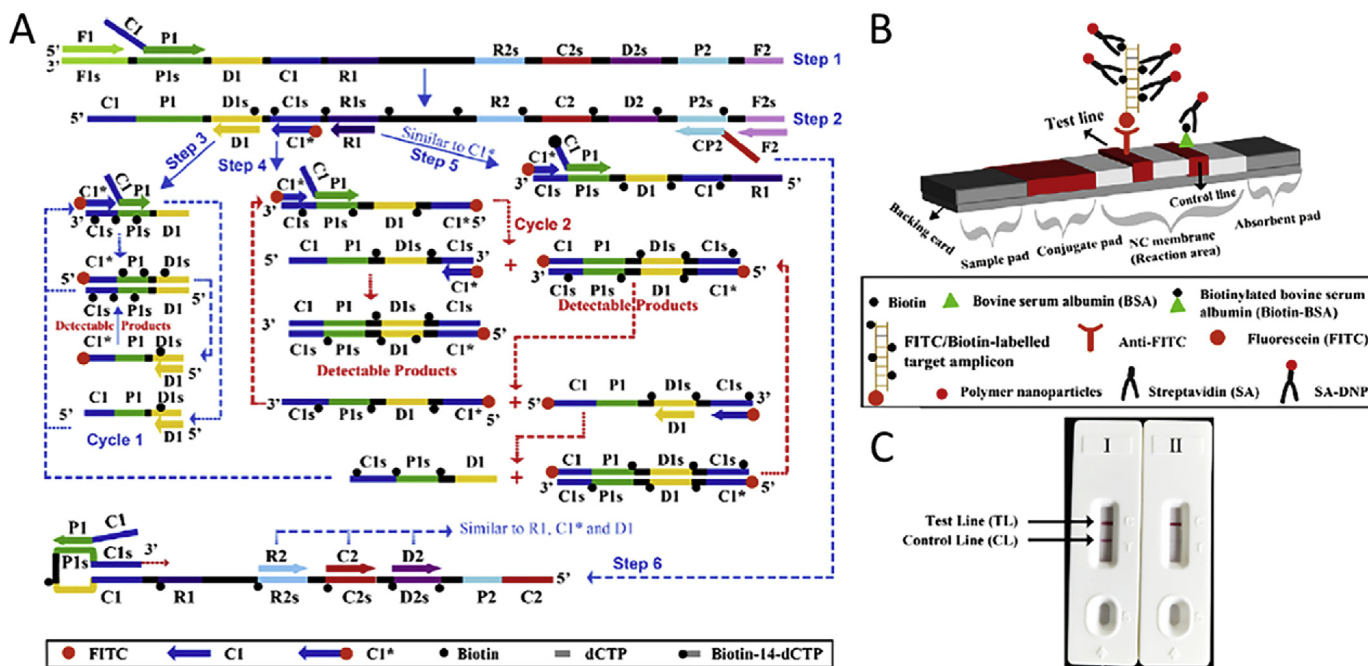

Fig. 4. The schematic showing the mechanism of MCDA (A) coupled with LFB (B). Reprinted from Ref. [60] with permission Copyright 2017, Elsevier.

target specificity. The principle of LAMP is illustrated in Fig. 5. In the forward primer set, a couple of primers are named inner primer (FIP, F1c + F2) and out primer (F3). At 60–65 °C, the F2 region of FIP first binds to the target and is extended by a DNA polymerase. After the completion of F3 annealing to F3c target strand, a new strand is synthesized by the extension of F3 using a specific enzyme polymerase. The newly formed strand shapes into a stem-loop structure at the 5′ end as a result of the interaction between F1c and F1 site. By the action of polymerase, the reverse outer primer (B3) and inner primers (BIP, B1c + B2) anneals to the 3' end and generates a new strand having a stem-loop structure. The dumbbell structured DNA enters the exponential amplification cycle, and strands with several inverted repeats of the target DNA can be made by repeated extension and strand displacement. Different from PCR, there are multiple primer pairs (4–6 pairs) used to amplify a target gene, and numerous amplification products of various lengths are produced. LAMP can generate about 10^9 target DNAs and a large number of by-products within 60 min [44–46], even when large amounts of non-target DNA are presented [46]. The resulting products of LAMP consists of numerous distinctive sizes of stem-loop DNAs with several altered repeats of the target sequence and cauliflower-like structures with several loops. The major disadvantage of LAMP resides in complications for designing primers since six regions of the target are covered, even though 4 primers can also work. Moreover, LAMP spurious amplification has been reported, which is highly confusing false positive result as seen in either electrophoresis or colorimetric analysis. To resolve this drawback, and owing to its trending favorable advantages and applications, different types of biosensors were developed using various lateral flow approaches. For

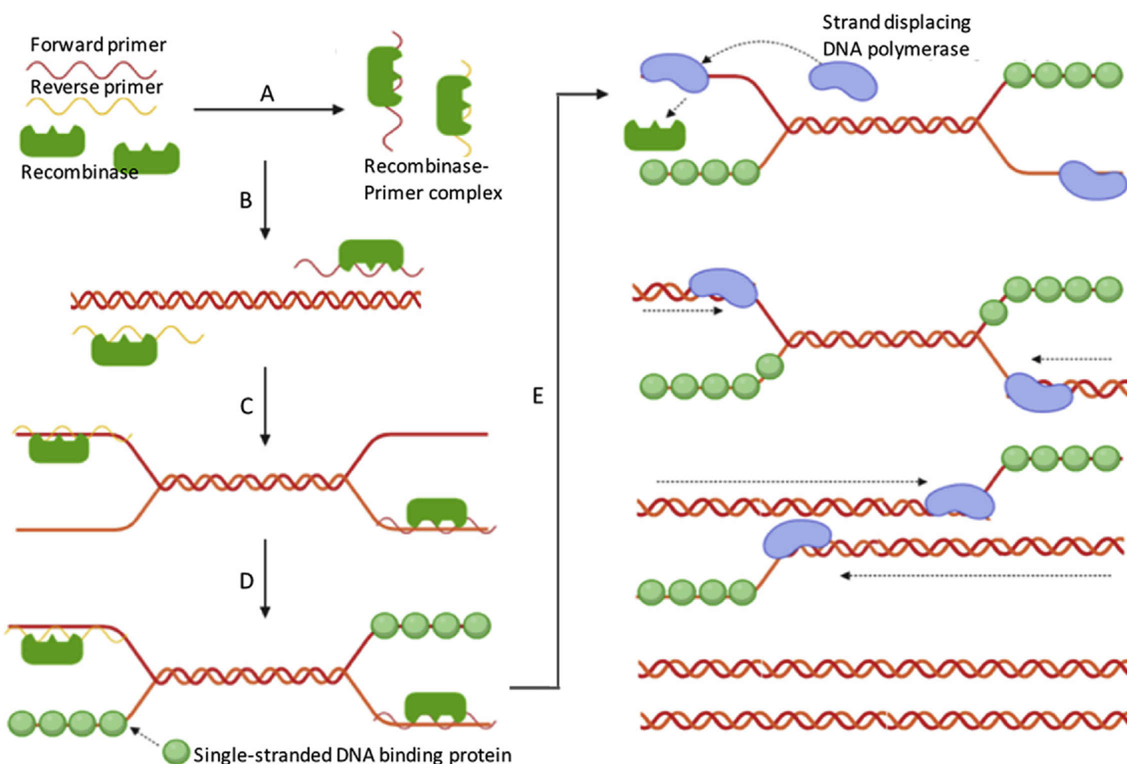

Fig. 5. RPA principle. (A) The forward and reverse primers bind to the recombinase to form primers-recombinase complexe, which recognize only for homologous sequences (B). (C) Strand invasion by the complex at the cognate site. (D) Stabilization of the displaced DNA sequence by single-stranded DNA binding proteins. The recombinase opens the target sequence and a DNA polymerase, which has a strand displacement activity, binds the 3′ end of the primer and elongate it (E). The cyclic continuity of this process leads to the accomplishment of exponential amplification.

example, an integrated rotary microfluidic system was proposed by Park et al. [47], which is capable of DNA extraction, LAMP, and lateral flow strip based detection for *Salmonella Typhimurium*. It achieved a high specificity and a detection limit (LOD) of 50 CFU within 80 min. Another LAMP-LFD approach has been developed to detect *Salmonella* strains within food samples (LOD: 13.5 fg/μl of genomic DNA and 6.7 CFU/ml of cell) [48]. In this study, all 52 strains of *Salmonella* were positively detected, and no cross-contamination was observed within 37 tested non-*Salmonella* strains. This assay showed demonstrated a sensitivity of 100–1000 times compared to the conventional PCR and real-time PCR assays, respectively. The accuracy of this assay was 100% when compared to the standard culture based method (ISO 6579:2002) after an enrichment step at 37 °C for 6 h.

In another study, LAMP coupling with a lateral flow was applied for the rapid detection of genetically modified crops (soybean and maize with a limit of detection of 0.5%) [49]. In retrospect, pointing a huge need for developing multiple targets detection based apparatus, LAMP showed multiplexing potentials due to the ability of primers to target different sequences. LAMP has also improved ability for multiplexing detection when coupled with lateral flow assay. For instance, Yin H.Y. et al. [50], proposed a LAMP based lateral flow assay for the simultaneous detection of the sea and seb genes of enterotoxin *Staphylococcus aureus*. Within this study, the sea and seb genes were labeled with biotin and separately labeled by digoxigenin and fluorescein isothiocyanate, respectively. The detection of the two amplicons (sea and seb genes) was achieved by incorporating NeutrAvidin-tagged gold nanoparticles with LFD, allowing the quantification and visual detection through competitive sandwich approach. The LOD of 10-fold lower than of a multiplex PCR (102 CFU/ml) was achieved. This assay established the assay's ease-of-use characteristics, high sensitivity and specificity, making it applicable for P–O–C testing.

3. PSR based lateral flow biosensor

PSR, one of the recently discovered isothermal amplification technique with high efficiency, rapidity and specificity, was first reported by Liu Wei in 2015 [51]. It operates at a constant temperature ranging from 61 to 65 °C within less than 1 h by using betaine and Bst DNA polymerase, as a double strand destabilizing agent and unwound single strand extending enzyme, respectively. It is highly effective owing to the simplicity of primers designing using the conventional PCR software. PSR has the advantages of both PCR and LAMP. PSR primers differ from PCR primers in the exogenous sequences (Nr and N) that are extended on the 5′ end, while, the remaining bases at the 3' end correspond to the target region. Reports demonstrate that PSR is highly sensitive 100 times more than PCR, cost-effective because it uses a water bath for constant temperature monitoring, and convenient with a simple color change to yield results. In addition, the effects of the reaction include a high amount of byproducts of the pyrophosphate ion, which can be clearly visualized by adding an effective pH marker. Increasing numbers of studies have indicated that the PSR method offers a promising isothermal DNA amplification approach that can be employed for fast and minimal resource diagnosis, including the detection of certain clinical pathogens [52,53]. Unfortunately, PSR based LFB have not been explored at this time. A number of PSR publications have been reported on chromogenic substrate SYBR Green I, which employs fluorescence as a color changing visual detection signal, however, PSR should be considered for future prospects as a potential isothermal amplification method for nucleic acid diagnosis owing to its extremely high sensitivity.

4. CPA based lateral flow biosensor

Cross-priming amplification (CPA) is an isothermal amplification process of DNA invented by Ustar Biotechnologies (Hangzhou, China)

[54]. CPA has proven to be highly specific and sensitive, producing amplicons from as low as four bacteria. This approach uses several cross-linking primers (six to eight primers) to amplify a target DNA sequence at a constant temperature producing hairpin-shaped products with various amounts. The amplification process can be split into three paths: (1) products that are extended with the cross primer and displaced with displacement primers, (2) multiple extensions and displacements with detection probes, and (3) extensions with hairpin-like structure-specific probes.

Xin Huang et al. [55], proposed a CPA based LF strip for nucleic acid detection. Typically, for the principle of this assay, the priming configuration included: a pair of displacement primers detaching single strand sequences at their 3 ends, one or more cross primers adding an additional priming site at each amplification cycle, a biotinylated primer and a FITC-labeled probe. Dual-labeled CPA products with biotin and FITC could be read easily on a LFB, where the template sequence was immobilized by fixed FITC antibodies to the test line by the help of streptavidin-coated latex particles. This assay had a good detection limit as low as 30 copies, small than that of conventional PCR.

In another study, for on-site detection of African swine fever virus, Yao Gao et al. [56], designed a CPA assay to target the p54 gene in combination with immunochromatographic strip (CPA-strip), First, the p54 gene was amplified by CPA using the specified primers and probes, and at the 5-ends, the products were labeled with biotin and FITC to facilitate subsequent interaction and immobilization on the LF strip. The complex of probes and target was allowed to interact with the antibodies fixed at the test and control lines to yield results that could be visualized by the naked eye. It showed a limit of detection of 200 copies with good recoveries. To sum up, the CPA-LFB assay is accurate, responsive, convenient and low cost, and possesses the potential for effective diseases monitoring.

5. MCDA based lateral flow biosensor

MCDA, a recently established assay by Yi Wang et al. [57], has shown potential to achieve extreme sensitivity compared to the previously reported nucleic acid amplification techniques such as PCR, LAMP, and CPA. This method which do not require thermal –regulating equipment, operates at a temperature ranging from 61 to 65 °C and uses a set of 10 specifically designed primers covering ten different target sequence sections, which are known as cross primers (CP1 and CP2), displacement primers (F1 and F2), and amplification primers (D1, C1, R1, D2, C2 and R2) with corresponding different target sites for binding namely P1s, P2s, F1s, F2s, D1s, C1s, R1s, D2s, C2s and R2s, respectively (Fig. 4).

During the reaction, the 4 double-stranded DNAs is fixed at primary-template hybrid dynamic reaction stage, hence the high proportion of primers attach to the target strands without a heat denaturing step to trigger the amplification by the action of *Bst* polymerase. A set of primary binding and extension processes generate multiple single -stranded DNAs and single-stranded single sterm-loop DNA structures for further isothermal amplification stage. Then, these DNA products permit the exponential amplification of the strand-displacement reaction to begin.

When compared to other amplification techniques (like PCR, LAMP, and CPA), MCDA was 16-and 32- times more sensitive than the LAMP and CPA techniques, respectively, and results could be achieved in less than 15 min. Recent studies shows that MCDA provides a highly touted approach for nucleic acid amplification that can be used to achieve a desired sensitivity with relatively low diagnosis cost. Shoukui Hu et al. [58], proposed a duplex MCDA assay that employs disposable LFB for the detection of MCDA amplicons. This assay was successfully applied for the analysis of *Acinetobacter baumannii, a pathogen that cause* nosocomial infections*, by targeting* the *pgaD* and *blaOXA-23-like* genes. The assay also reports a specificity of 100%, and the practical feasibility in clinical settings. Similarly, Yi Wang et al. [59], designed a LFB coupled with MCDA (Fig. 4) for the analysis of nucleic acid and cross-contamination prevention. This method uses the enzyme, uracil-DNA glycosylase (AUDG) and self-avoiding molecular recognition system (SAMRS) components to eliminate cross-contamination, and false-positive results, and enhance MCDA sensitivity, respectively. To demonstrate the functionality for target analysis, *Mycobacterium tuberculosis* complex (MTC), a condition which causes of human tuberculosis (TB), has been identified by the proposed MCDA assay. MCDA reliability was assessed successfully in detecting MTC from pure culture and clinical specimens.

6. RPA based lateral flow biosensor

RPA is a sensitive and specific technique for isothermal nucleic acid amplification that relies on the formation of a complex mainly comprising a recombinase and target-specific primers [61]. The recombinase first binds to primers, and form a recombinase-primer complex. The complex allows the dsDNA to find the complementary homologous sequence, and thus influence the strand invasion by the primer at the cognate site. Then, the single-stranded DNA (ssDNA) binding protein binds to the displaced DNA to avoid the discharge of the embedded primer. Finally, a strand displacing DNA polymerase binds the 3' end of the primer and elongates the primer to form a newly synthesized strand. The latter serve as template for cyclic or exponential amplification (Fig. 1).

The characteristics, focal points, and drawbacks of RPA and other isothermal amplification teachniques have been broadly reviewed in Table 1. Most importantly, it is an effective approach in terms of sensitivity, specificity, and multiplexing. It operates at the constant and low-temperature range when compared with other isothermal amplification types, which make a positive attribute since there is no requirement for continual temperature control within its operating temperature range. Generally, RPA has proved favorable for widespread use when integrated with LFB to generate robust sensing tools that are affordable, steady, user-friendly, cost-effective, and with low-resource settings for P–O–C (Point-of-Care) testing. To date, RPA combined with LFBs are widely applied in the detection and identification of pathogenic bacteria [62,63], viruses [64], in food safety, environmental monitoring, and other biomedical fields [65–67]. For example, an RPA-LFB assay for the rapid authentication of mutton products has been developed [66]. In this assay, a pair of specific RPA primers (5′ end digoxigenin-modified forward primer and a 5′ end biotin-modified reverse primer) against the mutton cytochrome *b* (Cyt *b*) gene. As depicted in Fig. 2, the RPA amplicons, and AuNP-mouse *anti*-digoxigenin conjugates are mixed and then diluted prior to loading on the LFB sample pad. The RPA amplicons are captured by streptavidin immobilized on the test line, hence forming a complex of streptavidin avidin-RPA-antibody conjugated with AuNPs. Excess AuNP-mouse *anti*-digoxigenin complex is captured by goat anti-mouse antibodies attached to the control line. In the absence of mutton products, there is no RPA amplicons, thus no detactable test line [66]. In another study, a similar system named duplex RPA–LFD (RPA lateral flow dipstick) assay was introduced to detect influenza viruses [64]. Three different types of antibodies (Anti-fluorescein isothiocyanate, *anti*-digoxigenin, and biotinylated bovine serum albumin) were immobilized on the two test lines, and a control line, respectively; to capture dual-labeled products (influenza A and B viruses) and SA-Au modified by biotin, respectively. The assay achieved visible test lines with a LOD of 50 and 500 copies for both influenza A and B viruses, respectively [64]. Recently, Xu Yet al. [67], also developed a promising all-in-one platform based on RPA–LFD combining the strategy of a technique named universal blocking linker recombinase polymerase amplification (UBLRPA) and a peptide nucleic acid (PNA) based lateral flow device (PLFD) for the visual detection of pathogens in food and environmental samples. The DNA products were obtained using the working principle of UBLRPA amplification. Then, the DNA products were converted to a

single strand using the UBLRPA amplification system. Therefore, through dual hybridization of the AuNP modified PNA probe and the universal linker, UBLRPA products could detected, producing a characteristic visual red band on the LFB. This approach exhibited high sensitivity and specificity as a result of the strong binding affinity between PNA and DNA. More interestingly, this approach can be used to identify other types of pathogens by only changing the primers.

7. RCA based lateral flow biosensor

RCA is an enzyme based isothermal amplification technique that uses circular DNA to amplify target nucleic acids (DNA or RNA) with the help of DNA or RNA polymerase. It has the amplification power as high as PCR, with potential to amplify even a whole viral genome. The product of RCA contains tens to hundreds of repeated segments that are complementary to the circular mother template, which is usually an ssDNA or RNA (polyplex). RCA does not require complicated steps such as continuous temperature regulation using thermal cyclers and thermal stable DNA polymerase. RCA is direct, robust, and versatile for the amplification of circular DNA templates such as plasmids, bacteriophage, and circular RNA using the strand displacement capabilities of polymerases and it does so with both in pure and complex biological samples [68]. As described in Fig. 6, RCA primer anneals to the circular template and the nucleic acid begins to replicate, and simultaneously, the polymerases displace the circular template continuously by adding the nucleotides until it reaches the terminal end of the target, resulting in a complete loop. The polymerase with strand displacement activity then displaces the newly synthesized strand while starting the synthesis of the next loop. The same amplification procedure continues until the nucleotides are depleted, or the polymerase enzyme loses activity. Therefore, this results in the production of a long ssDNA strand having 10 to 100 of tandem repeats. This technique is very potent and can be used to amplify DNA strands up to 10^5 bp in length [69].

In a recent study reporting for the development of biosensor based on RCA technique, Yao and co-workers first developed a simultaneous, fast, sensitive, specific, and selective method based on RCA coupling with LFB strip for sensing miRNA 21 and miRNA let-7a, which achieved the LOD of 20 pM and 40 pM, respectively [70]. The padlock probe was designed specifically, whose 5′ and 3′ end were complementary to the sequence of target miRNA. After the padlock probe binding to the miRNAs and cyclizing in the presence of Splint R ligase, the amplification began under the function of DNA polymerase, and repeated ssDNAs were produced. The amplified products were then detected by AuNPs based lateral flow strip. In the presence of RCA products, the sandwich format of AuNPs-probe complexes and RCA products ran along the nitrocellulose membrane (NC) and captured by two specific probes. A positive signal as a red test line was observed. In contrast, when the RCA products were not present, the AuNPs-probe complexes were not recognized on the test line, leading to the absence of the test line. Moreover, strategies named HRCA-LFD [71], and E-RCA-LFD [72] were designed for the detection of *Karenia mikimotoi* and *Karlodinium veneficum,* respectively. The HRCA-LFD was specific and 100-fold more sensitive than PCR [71], and the E-RCA-LFD showed excellent sensitivity than the conventional PCR and reached a LOD of 0.01 cell/ml [72]. RCA-LFD is playing a vital role in increased sensitivity and reduced cost of experimentation, which may contribute to saving detection time and could be selected as a potential tool for the detection and identification of target nucleic acids.

8. NASBA based lateral flow biosensor

NASBA was first reported by Clampton in 1991 [8] and later on, Gene-Probe successfully launched a NASBA based product, capable of doing both reverse transcription and amplification for the detection of RNA [73]. A typical NASBA reaction mixture consists of an avian myeloblastosis virus reverse transcriptase (AMV-RT), T7 RNA polymerase, RNase H and a couple of oligonucleotide primers (P1 and P2) (Fig. 7). NASBA can also produce false positives caused by genomic dsDNA, as it is the case with RT-PCR. In addition, NASBA is still limited by complex reaction mixture such as two primers and three enzymes – AMV-RT, T7 RNA polymerase, and RNase H - are involved in NASBA (Fig. 5). Moreover, high temperature treatment (denaturation) can deactivate them. Thus, NASBA is not suitable for amplifying dsDNA targets because there is no initial denaturation step unless cooling and amplification steps are involved [73]. Other examples includes FRET [74] and colorimetry [75] based NASBA to detect 18S rRNA of *Aspergillus* and *Leishmania*, respectively. They achieved ultrasensitive detection of NASBA products [74,75]. Although all those techniques have been developed, a more rapid and straightforward NASBA assay using lateral flow strip is not yet available.

9. HDA based lateral flow biosensor

The working principle of HDA is very similar to the standard PCR procedure [76], except that for isothermal amplification, HDA does not use DNA denaturation at high temperatures as in PCR, but uses helicase to separate DNA strand (Fig. 8), using single-stranded binding proteins to prevent the separation of separate DNA strands. It has close similarity to the cellular DNA replication as helicases untie dsDNA during the replication of DNA. The involvement of the helicase helps to eliminate the thermal cycler and allows the reaction to occur at room temperature while maintaining exponential amplification. As shown in Fig. 8, a

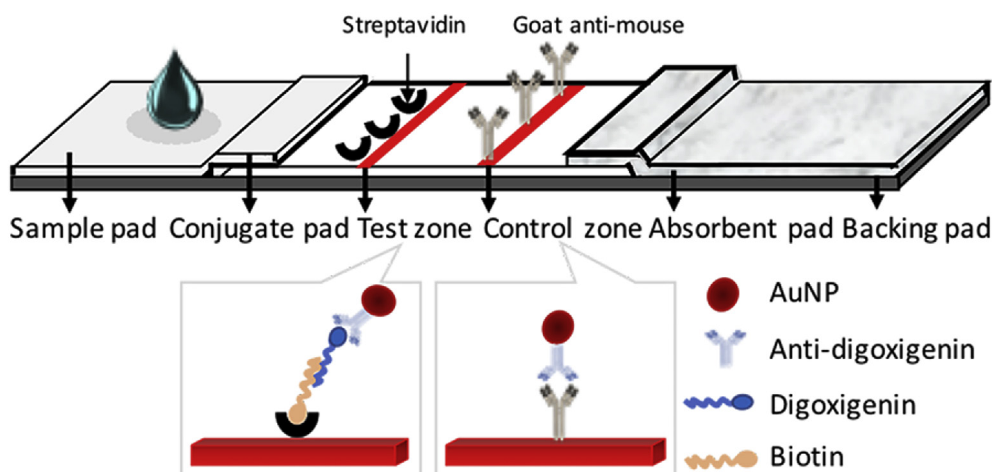

Fig. 6. Schematic illustration of RPA-LFD approach.

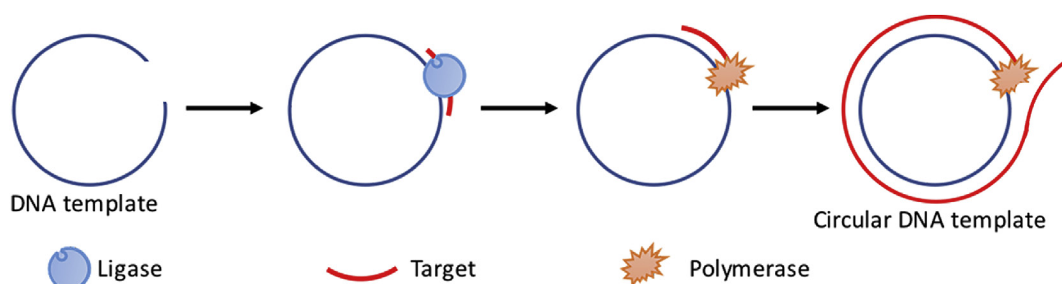

Fig. 7. Schematic illustration of RCA.

dsDNA is first expanded by helicase, then anneals with primer, and is extended by polymerase (exo^{-}Klenow fragment). The critical trait for HDA resides in no requirement for an initial heat denaturation step to generate ssDNA from the dsDNA target while many other isothermal amplification methods require an initial specific denaturation temperature before the target gets amplified at lower temperatures [76]. Nevertheless, before performing HDA, it is often desirable to optimize experimental conditions, including buffer compositions and primer sequences. Unfavorably, the optimization process usually involves the use of PCR, which causes a significant amount of extra cost to HDA. Consequently, additional work is required to attain the potentiality of HDA including identification of the rate-limiting steps. One possible rate-limiting step is the interaction between the ssDNA binding protein and the target DNA strand since this interaction considerably prevents re-hybridization of the DNA strands separated by helicase. The efficiency of the helicase-catalyzed target dsDNA separation is another possible rate-limiting step as tests for many polymerases have not shown any significant differences between them [76]. HDA is a fascinating alternative method to PCR because of its simplicity, limited complicated steps, and its excellent compatibility with various nucleic acid detection. Claudia and co-workers introduced a BacR HDA-strip assay for detecting ruminant faecal pollution sources, which can yield qualitative results paving the way for future simple-to-use microbial source tracking (MST) screening tools [77]. The entire process only took 2 h, and no extensive practical training was required.

Alternatively, a more advanced HDA named thermophilic HDA (tHDA), was put in place for improved performance of the conventional HDA. The tHDA uses a thermostable UvrD enzyme to separate the dsDNA and produce ssDNA templates used for subsequent amplification by the DNA polymerase. The dsDNA unwinding and amplification is conducted at isothermal temperature, between 60 and 65 °C that renders this method better suited for the construction of microbial point-of-need detection systems, because a thermocycler is not mandated for DNA denaturation at 95 °C to initiate the amplification. When combined with LFB, tHDA provides potentials for the visualization of amplified products. For example, Xin-jun Du et al. established a lateral flow read-out assay that could visualized the positive control of tHDA amplification products without the use of any sophisiticated equipment. Amplicons-conjugated and non-conjugated gold nanoparticles were used for signal read-out, as they could be captured by the streptavidin and antibody-antimouse immobilized at the test line and control lines, respectively. This assay was able to detect salmonella within real sample such as milk, chicken, and infant nutritional cereals, in limited time, with high sensitivity, and specificity and could be suited for the application in limited-resource area.

10. Carryover contamination monitoring

Inhibition of carryover contamination has been a subject since two decades ago within all the fields concerned with nucleic acids manipulation. The amplification of by-products, handling, and exposure of reaction tubes, including sampling, capping, and uncapping operations are the main sources that are linked with cross-contamination. A number of strategies were recommended to eliminate and monitor carryover contamination. For subsequent amplification, carryover of previously amplified amplicons is the most influencial source of contamination. Many approaches were used to prevent contamination of the carryover, such as physical or mechanical, and chemical treatment. Physical or mechanical treatment to avoid amplification products carryover requires strict isolation of laboratory zones where samples,

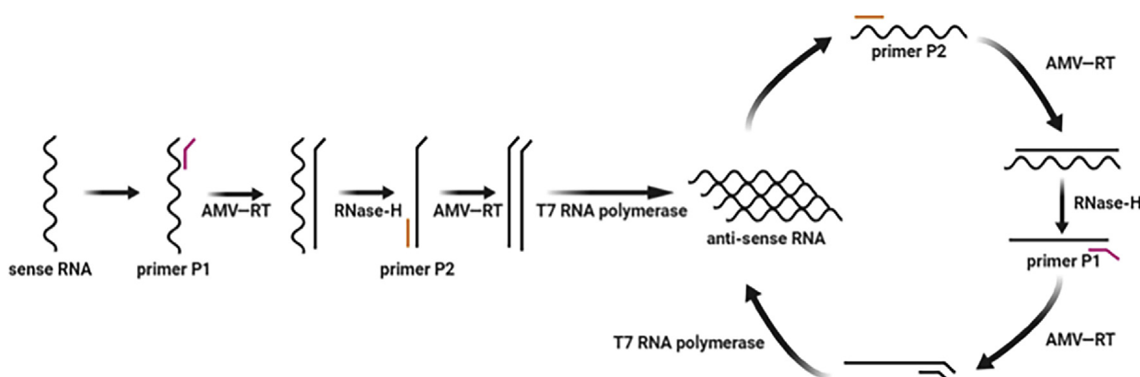

Fig. 8. Schematic illustration of NASBA. P1 is made of a 3′-end target recognition sequence and a 5′ end T7 RNA polymerase promoter sequence. Upon addition of the target RNA, P1 anneals to the complementary site at the 3′-end of the target, followed by the initiation of the synthesis of a complementary DNA strand catalyzed by AMV-RT to produce a DNA-RNA hetero-duplex. The RNase H then hydrolyzes the target RNA of the DNA-RNA hetero-duplex, leaving a ssDNA. P2 attaches to the 5′ end of the DNA strand, and AMV-RT begins to synthesize another DNA strand starting from P2 using the DNA strand as a template, thereby producing a DNA homologous duplex (dsDNA) containing the T7 RNA polymerase promoter sequence. T7 RNA polymerase continuously transcribes the complementary RNA strand of the dsDNA template. The new formed RNA is used as a secondary RNA target, and the new cycle begins with the binding of P2 to the synthesized RNA, thereby entering the cycle of amplification. Therefore, NASBA starts amplifying the target RNA exponentially and produces an ssRNA product, which amplifies the target by up to > 1000 times within 120 min at 41 °C.

materials, and reagents, are handled from the environments where amplification is conducted and amplification products are tested. Each laboratory zone must be fitted with the appropriate equipment, disposable tools, lab clothing, aerosol-free tubes and pipettes, and ventilation systems. All materials and disposables used for each zone must all be delivered directly to that area [78,79]. Chemical treatment involves the usage of techniques such as Ultra-violet light irradiation, Inactivation of nucleic acids with furocoumarins (which is based on amplicon sterilization and the prevention and blockage of Taq polymerase to extend or amplify primers during amplification reaction), primer hydrolysis (post PCR hydrolysis of RNA residues of the amplicons by NaOH), Hydroxylamine chemically modifies C interfere C + G pairing), and enzymatic inactivation with uracil-N-glycosylase [80–82].

The latter one is most efficient techniques that include dUTP and uracil DNA glycosylase (UDG) in amplification reaction. Cheng Qian et al. [83], developed UDG-mediated CRIPSPR/Cas12a combined with LAMP for visual detection and carryover contamination monitoring. In the amplification experiment, dUTP is used in this method to substitute TTP, so that all amplicons contain dUTP. Until starting each amplification, fully loaded reactions are treated using UDG, that cleaves the uracil base in dUTP carrying DNA (amplicon) but will not interact with dUTP and has no effect on original DNA templates comprising thymidine groups or RNA. UDG could be inactivated after initial treatment by thermal denaturation before the PCR. New PCR product, amplified from genuine DNA template, is not destroyed, although it contains dUTP. The destroyed DNA is not appropriate to be used as a guide for hybridization or as a template for DNA polymerases, thus in subsequent reactions, it can not be reamplified or used as contaminant.

11. CRISPR/cas-isothermal amplification based lateral flow biosensor

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) system endow bacteria and archaea adaptive immunity against foreign nucleic acids [97,98]. For effective targeting, this system utilizes a guide-RNA and Cas proteins to target an invading DNA, generating a protospacer sequence that integrates into the genome near PAM (protospacer adjacent motif) region for recognition and cleavage of future similar invader [37]. The CRISPR-Cas9 has been extensively used for gene editing, typing and mutant detection. It requires a CRISPR RNA (crRNA) and trans-activating or a chimeric single guide RNA [37,99–101] for targeting and cleavage. The RNA-guided cleavage is mediated by RuvC (a member of RNase H family) and HNH catalytic domains at the gRNA-target base-pairing site. When Cas9-gRNA complex recognizes a G-rich PAM region of the target sequence, it becomes activated followed by blunt-end cleavage [102]. Similar to Type II CRISPR-Cas9, Type V CRISPR-Cas effectors such as Cas12 and Cas13 have also been used for gene editing [38,103–105]. However, these enzymes require a single mature crRNA for self-assembly and processing, and ribonucleoprotein surveillance-dependent nuclease for interference activity [106,107] but not a dual functional crRNA-tracrRNA as for Cas9 [108,109]. Unlike Cas9 and Cas13, CRISPR-Cas12a system uses only a RuvC catalytic domain to guide gRNA and cut the cognate dsDNA by recognizing a T-rich PAM region that leaves a staggered 5′ and 3’ ends [110,111].

Based on this targeting, it has been recently discovered that Cas12a/b and Cas13a/b enzymes exhibit collateral cleavage of target nucleic acids and non-specific single-stranded nucleic acids [42,43,112] (Fig. 9). For example, an RNA detection method called Specific High Enzymatic Reporter UnLOCKing (SHERLOCK) harnessed Cas13 enzymes for the diagnosis of bacteria and single nucleotide polymorphisms (SNPs) [42], while DNA Endonuclease Targeted CRISPR *Trans* Reporter (DETECTR) uses LbCas12a for human papillomavirus detection [112]. These methods depend on several critical reaction components such as ssDNA activators, crRNAs, buffer and signalling single-strand nucleic acids (hereafter referred as reporters), DNA amplification mixture, and reverse transcription system (for SHERLOCK) to provide fluorescence signal readout. To enhance the sensitivity and fluorescence readout, these diagnostics and another method called One-Hour-Low cost Multipurpose highly Efficient System (HOLMES) [40] required pre-amplification of target nucleic acids using recombinase polymerase amplification (RPA) and PCR, respectively. RPA has currently shown potential when combined with CRISPR-Cas system [39,41,42]. Although RPA is formidable isothermal amplification of nucleic acids, it is still limited by target sequence length, complex reaction premix, low sensitivity and specificity, and cost compared to Loop-mediated isothermal amplification (LAMP) [4,113]; while PCR depends on expensive machinery such as thermocyclers. LAMP amplification allows the entire amplification at a single temperature, making it rapid, cost and time-effective but with difficulty in recognition of target bands and is more prone to cross-contamination. Moreover, HOMESv2 combined LAMP and RT-LAMP with Cas12b for the detection of DNA and RNA, respectively [43]. Although the approach has improved sensitivity, overall, the fluorescence-based result readout remains a concern (see Fig. 10).

To overcome machinery limitations towards on-site deployment, an RNA-targeting method called SHERLOCK applied fluorescence reader and lateral flow biosensor, and combined RPA with Cas13, Cas12a, and Csm6 to detect nucleic acids [42]. The principle of this lateral flow is that cleaved FAM-biotin labeled reporters are not accumulated by the Anti-FAM antibody-gold nanoparticle immobilized at the test line [42,114]. The technique is advantageous compared to assays such as DETECTR [112] and HOLMES, which employe fluorescence readouts only [39,42]. However, SHERLOCK lateral flow biosensor is likely to be expensive owing that it requires complicated reaction with an *anti*-FAM antibody, streptavidin (SA), protein A, and fluorescence based detection of bi-labeled reporters. Our recent paper in press reported a low-cost lateral flow biosensor, which consists of a test line DNA probe that can not bind trans-cleaved biotinylated ssDNA reporter upon target recognition, paving the way towards developing sensitive, inexpensive devices for monitoring CRISPR/Cas reporter trans-cleavage [115].

12. Conclusion and future perspective

We reviewed the most effective isothermal amplification methods for nucleic acids detection such as, LAMP, RPA, MCDA, PSA, etc. However, they require combination with several techniques for result readout. This is due to the fact that isothermal amplification methods exhibit short-comings of result readout and stringent experimentation. Moreover, they have hardly shown the multiplexing abilities [4,116,117] and have also shown to depend on the dye for colorimetric based detection and quantification experiments, which could be prone to cross-contamination. To resolve this issue, we presented the use of CRISPR/Cas based enzymes, which have gained application in diagnostics owing to their collateral cleavage of target and non-target nucleic acids. Thus, it is likely that carryover contamination can be substantially reduced since the Cas effectors cleavage results in reduced amplicons. CRISPR-Cas based analysis of isothermal amplified products is rapid, sensitive and specific, and can be integrated into colourimetric or fluorescence-based portable devices, allowing their on-site implementation. They can also be multiplexed when specific reporter nucleic acids are labeled with different fluorescence molecules. However, the use of multiple enzymes and long reaction and steps complicates the methods thus requires rigorous optimization. Moreover, fluorescence-based techniques generate backgrounds; thus, it is still of great importance to developing robust techniques. The use of miniature Cas enzymes such as Cas14 could speed up the reactions. Besides, the discovery of new polymerases possessing both strand displacement and reverse transcription propensity -as in the case of RT-LAMP- could reduce the complexity of the trans-cleavage reaction in both DNA/RNA detection. Notwithstanding that CRISPR-based techniques have shown some promiscuous trans-cleavage; thus, thorough buffer and activator

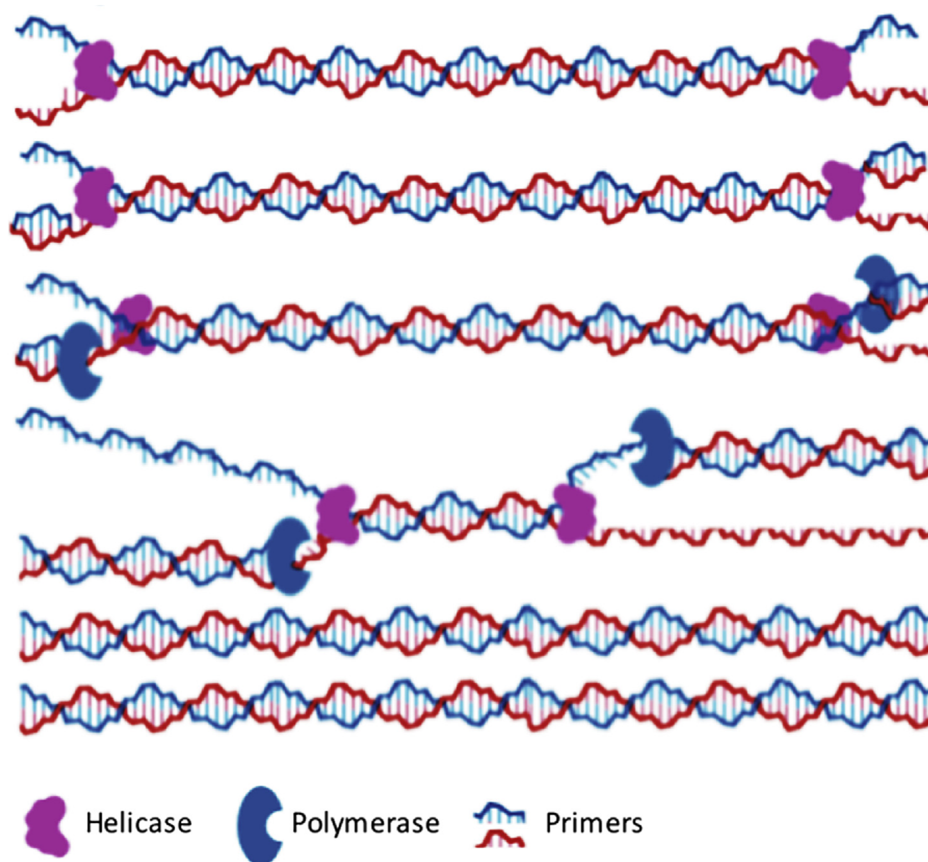

Fig. 9. Schematic illustration of HDA.

optimization are of great importance. Furthermore, one can replace antibodies with DNA probe-based lateral flow biosensor, which can likely boost up specificity, sensitivity and improve cost for its use in low-resource settings P–O–C testing.

Declaration of competing interest

None.

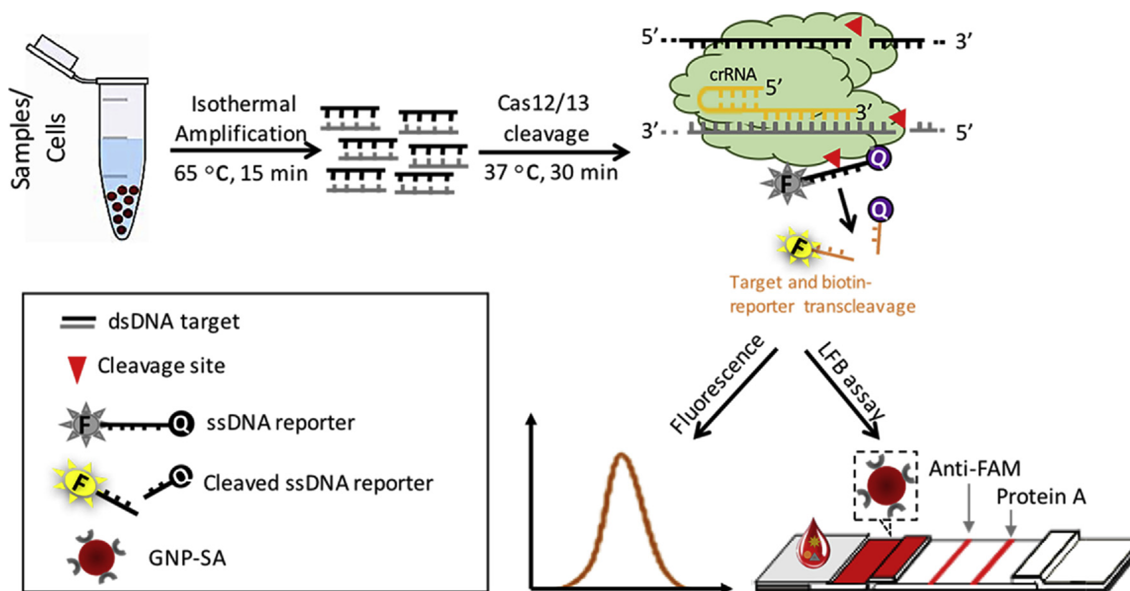

Fig. 10. Illustration of CRISPR/Cas-isothermal amplification detection principle. Clinical samples are subjected to nucleic acid extraction (heat treatment, genomic DNA extraction) for isothermal amplification such as RPA (37 °C, 15 min) or applied to LAMP amplification (65 °C, 15 min). The product of amplification is applied to Cas12 (for DNA target) or Cas13 (for RNA target) cleavage system, which collaterally cleaves the amplicons and the FAM-labeled reporter. The product of reporter targeting can be detected using fluorescence or lateral flow biosensor.

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