



A review of reaction enhancement strategies for isothermal nucleic acid amplification reactions

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ABSTRACT

Isothermal nucleic acid amplification techniques are used to detect a variety of molecules such as RNAs, DNAs and proteins. When compared to the gold standard polymerase chain reaction, isothermal methods are rapid and simple but often have lower specificity and sensitivity. In this review, we provide a summary of reaction additives and techniques that enhance the sensitivity, specificity, stability, robustness, or speed of isothermal nucleic acid amplification reactions used for sensing applications. We also provide the mechanism and purpose of each enhancement technique, which can provide guidance when selecting reaction additives to optimize novel isothermal amplification systems and assays.

1. Introduction

Isothermal nucleic acid amplification reactions are biochemical techniques that create DNA and RNA in the presence of a target molecule at a single temperature. These reactions have gained popularity as molecular detection tools due to their simplicity, speed, and ability to detect a wide variety of molecules [1,2]: isothermal reactions are used to detect DNA, RNA (viral RNA, mRNA, miRNA), proteins, and bacteria [3–5]. These molecular detection techniques have recently been in the spotlight due to the COVID-19 pandemic, during which rapid viral RNA diagnostics for the point of care has become an important tool for curbing disease spread [6–8]. A simple, rapid, and robust reaction is particularly advantageous for sensing in point of care and limited resource settings, which are common applications of isothermal amplification reactions. These applications have more stringent requirements for reaction stability and robustness than those run in a laboratory, however, and therefore will often require enhancement and optimization before use.

Currently, polymerase chain reaction (PCR) and derivative technologies are the gold-standard nucleic acid amplification techniques for sensing applications, as they typically have superior sensitivity and specificity [9–14]. PCR products can theoretically only double once during every thermal cycling event; this can provide a more controlled reaction with better specificity, but also limits the reaction speed and product yield. PCR also requires precise temperature control and rapid temperature cycling to denature DNA and subsequently anneal and extend short oligonucleotide primers. As this temperature cycling

requires additional equipment and time, isothermal nucleic acid amplification technologies fulfill the need for fast and inexpensive molecular detection methods that are highly applicable for point of care sensing applications. For example, heating of isothermal amplification reactions can proceed without electricity by using exothermic chemical reactions and a phase change material [15], and reactions typically proceed in less than one hour.

Many variants of isothermal nucleic acid amplification reactions have been created, each with a different strategy to overcome the need for the thermal DNA denaturation. Loop-mediated isothermal amplification (LAMP) uses primers that produce hairpin structures within DNA products, which as a result contain both single stranded and self-priming regions [16]. Rolling circle amplification (RCA) acts upon a circular template using a strand-displacing polymerase, which continuously produces a long, single stranded DNA product [17]. Other techniques rely on the synergistic activity of multiple enzymes to create product nucleic acids: Nucleic acid sequence-based amplification (NASBA) uses two primers, one of which contains a recognition sequence for a DNA-dependent RNA polymerase, along with a reverse transcriptase, RNaseH, and a DNA-dependent RNA polymerase. During NASBA, target RNA can create single stranded RNA products that can readily bind more ssDNA primers [18]. Helicase-dependent amplification (HDA) relies on the enzyme helicase to unwind double stranded DNA and single-stranded DNA binding proteins to prevent reannealing of the DNA target, which allows for primer binding and extension by a strand-displacing polymerase [19]. Strand displacement amplification (SDA) [20], exponential

Abbreviations: dsDNA, Double-stranded DNA; ssDNA, Single-stranded DNA; miRNA, microRNA; SSB-Single, Stranded DNA-Binding protein; PCR, Polymerase Chain Reaction; LAMP, Loop-Mediated isothermal Amplification; HDA, Helicase-Dependent Amplification; EXPAR, Exponential Amplification Reaction; RCA, Rolling Circle Amplification; RPA, Recombinase Polymerase Amplification; NASBA, Nucleic Acid Sequence-Based Amplification; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; Cas, CRISPR associated; NSA, Nonspecific Amplification

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amplification reaction (EXPAR) [21], and nicking extension amplification reaction (NEAR) use a nicking endonuclease to create nicks in the double stranded DNA structure, and a strand displacing polymerase that can initiate polymerization at these nicks [5]. Recombinase polymerase amplification (RPA) relies on recombinase enzymes to facilitate primer binding to a double stranded DNA, a single stranded DNA binding protein (SSB) to prevent reassociation with the displaced strand, and a strand displacing polymerase to create the DNA product [22]. While these techniques allow amplification to proceed without the high temperature denaturation step required during PCR, they can introduce non-specific amplification during which the reaction produces products in the absence of the intended target.

Isothermal nucleic acid amplification reaction products correlate with the signal of a sensor, such that nonspecific amplification products can increase limits of detection: nonspecific amplification creates noise in which low response signals are lost. Uncontrolled, unpredictable non-specific amplification can also impact specificity if a nonspecific signal is mistaken for a signal produced by the target molecule. This non-specific amplification is also often exacerbated at the low reaction temperatures used during isothermal amplification, where formation of secondary structures, primer dimers, and nonspecific binding is

magnified. Additionally, newly developed isothermal amplification reactions frequently require optimization to improve speed, robustness, and stability of the reaction reagents.

In this review, we have compiled a collection of reaction additives and enhancement strategies to improve speed, sensitivity, and specificity of isothermal nucleic acid amplification reactions used during sensing applications. Many of these reaction additives will also delay or prevent nonspecific reaction products that plague sensing applications. When known, the mechanism of each additive is included to make the overview of results broadly applicable to sensing applications that use nucleic acid amplification reactions. Table 1 provides a summary of reaction enhancement strategies that are discussed in this review. Although most additives are commonly applied for one specific effect, they often have multiple effects that should be considered during the selection process.

2. Enhancement strategies

2.1. Crowding agents

In nature, enzymes function inside crowded environments with high concentrations of macromolecules [79,80]; for example, an *E. coli* cell

Table 1

A summary of enhancement strategies for isothermal nucleic acid amplification reactions that are used during sensing applications.

Enhancement strategies	Purpose	Additive/modification	Reactions tested
Crowding agents	Increasing enzyme activity and accelerating reactions, resulting in a faster and potentially more specific assay.	Polyethylene glycol (PEG) [23–25] Ficoll [25–27] Dextran [25]	Padlock exponential RCA (P-ERCA) [23], Bead-based padlock RCA [24], HDA [25] HDA [25,26], SDA [27] HDA [25]
Reducing secondary structure and destabilizing dsDNA	Eliminating the denaturation step, decreasing nonspecific interactions, decreasing sequence dependence of DNA melting temperature. Can lead to increased sensitivity, specificity, and potentially increase speed and yield of an associated assay.	Helicase [19,28–32] Recombinases [22,33,34] Endonucleases [35–37] Ionic liquids [38] Betaine [39–41], Proline Trehalose [40] Proline [16,42] TMAC [40]	HDA [19,28–32] RPA [22] RCA [35], SDA [36,37] LAMP, Isothermal multiple-self-matching-initiated amplification (IMSA) [38] RPA [39], EXPAR [40], LAMP [41] EXPAR [40] LAMP [16,42] EXPAR [40]
Duplex strengtheners	Increasing specificity and decreasing sequence dependence of DNA melting temperature, which can improve multiplexing capabilities.		
Enzyme stabilizers	Increasing enzyme activity, enzyme stabilization, and enzyme protection, which can improve assay robustness and reproducibility. Use during lyophilization extends reagent storage times.	BSA [43–46] Trehalose [40,47–51] Pullulan [52,53]	LAMP [43], SDA [44], RCA [45] EXPAR [40], LAMP [47,48], SDA [49], real-time quantitative nicking endonuclease-mediated isothermal amplification with small molecular beacons (SMB-NEMA) [50] Cross-priming isothermal amplification (CPA) [52], RCA [53]
Template blocking	Decreasing nonspecific interactions between nucleic acids and polymerase or between nucleic acids, protecting nucleic acids from degradation, decreasing background fluorescent signals. Can improve sensitivity and specificity.	SSB [19,22,27,30,31,37,40,54–59], Graphene oxide (GO) [55,60,61], CoOOH nanoflakes [57] LNA [62–67]	HDA [19,30,31], RPA [22], SDA [27,37], EXPAR [40,55,57,58], Exonuclease chain reaction [54], RCA [56,59] EXPAR [55], Isothermal strand-displacement polymerase reaction (ISDPR) [60], RCA [61] EXPAR [57]
Oligonucleotide modifications and analogs	Increasing specificity of nucleic acid association, manipulating nucleic acid melting temperatures, manipulation of nucleic acid structure, prevention of undesired nuclease activity. Used to improve sensitivity and specificity, improve multiplexing capabilities, and tune the reaction temperature.	PTO [3,68,69] PNA [37,58,70–72]	Linear DNA amplification [62], EXPAR [63], Toehold-mediated strand displacement reaction (TMSDR) [64], LAMP [65], Plasmon-enhanced hybridization chain reaction (PE-HCR) [67] EXPAR [3,69], LAMP [68] SDA [37], EXPAR [58,72], LAMP [70], RCA [71]
Degradation of unwanted signal amplification	Decreasing background signal; increasing sensitivity, specificity, and robustness.	Carryover DNA degradation [73–75] Amplification sinks [3,76,77], Netropsin [3,69,78] dATP removal [69]	LAMP [73–75] EXPAR [3], RCA [76], LAMP [77] EXPAR [3,69,78] EXPAR [69]
<i>Ab initio</i> synthesis delay/prevention	Increasing robustness and increasing the allowable observation time.		

contains 300–400 g/L of protein and RNA [81]. Crowding agents are popular reaction enhancers for isothermal nucleic acid amplification reactions, as they can increase the activity of enzymes such as polymerases [82–85], ligases [86,87] and nicking endonucleases [88]. This enhancement occurs through mechanisms such as the excluded volume effect and enhanced protein stability by favoring folded states over unfolded states [84,85,89]. Crowding agents can therefore increase enzyme stability and amplification reaction rates if properly optimized. However, due to the complexities of crowding effects on reaction kinetics, the effect of crowding agent concentration on reaction kinetics is non-monotonic and the choice of crowding agent for each reaction is not always straightforward. This is due to effects such as potential non-specific associative reactions between the crowding agent and the reaction reagents [90] and reduced diffusion rates in a crowded environment [80,89].

Polyethylene glycol (PEG), Ficoll, and dextran are commonly used crowding agents, and have been shown to enhance RCA [23,24], HDA [25,26], SDA [27], and LAMP [91]. Addition of 5% Ficoll 400 was shown to increase the amplification speed for HDA [26], while PEG increased reaction yield and ligation efficiency for bead-based padlock RCA (Fig. 1.1) [24]. Cui et al. analyzed the effects of crowding agents on specificity of their single nucleotide variation (SNV) detection method by allele specific extension [90]. They investigated the effect of crowding agents on the extension kinetics of a target DNA strand bound to an ssDNA template by the strand displacing polymerase Klenow Fragment (exo-). PEG, Ficoll, dextran, and BSA were all found to decrease the extension kinetics of targets with SNV while retaining the extension kinetics of the perfectly matched targets (Fig. 1.2). The differential effect of crowding agents on matched and mismatched targets increased their discrimination factor and therefore increased the specificity of their detection method. The decrease in mismatched extension kinetics was attributed to a decrease in reagent diffusivity in the crowded environment. This kinetic decrease was hypothesized to be offset by the depletion effect that occurs when a polymerase binds to a fully matched target and forms a compact molecular complex, which was not as pronounced for a mismatched target.

2.2. Manipulation of DNA structure and stability

Eliminating the high temperature denaturation step of PCR during isothermal DNA amplification reactions creates the need for other strategies to dissociate double-stranded nucleic acid targets and reaction products. Lower reaction temperatures can additionally require temperature independent methods to disrupt secondary structures of long single stranded nucleic acids and tune the binding affinity of nucleic acids. Below are strategies to manipulate the DNA structure and stability during isothermal nucleic acid amplification reactions.

2.2.1. Enzymatic manipulation of DNA structure

Enzyme applications inspired by natural systems have been used both as reaction enhancement methods and to develop novel isothermal nucleic acid amplification strategies. This enzymatic approach is especially useful to eliminate an initial heat denaturation step but can also be employed to improve reaction sensitivity and specificity. Enzymatic enhancement of isothermal nucleic acid amplification reactions requires multiple enzymes to operate in the same reaction mix, however, can complicate optimization of temperature and buffer conditions as each enzyme can require different conditions for optimal function. Helicases, recombinases, and endonucleases are common enzymatic tools used to enhance isothermal nucleic acid amplification reactions.

Helicase proteins are important elements of cellular processes such as replication, repair and recombination with their ability to separate DNA duplexes in an ATP-dependent manner [19,92]. Vincent et al. utilized helicase to develop the helicase-dependent isothermal DNA amplification reaction (HDA) where *E. coli* UvrD helicase unwinds the target DNA, allowing the primers to anneal to the templates to amplify the target region at a single temperature [19]. HDA is now a commonly used isothermal amplification reaction that has been reported to detect bacterial DNA, RNA and miRNA [28–31]. Helicase can also be used as a reaction enhancer for LAMP that can significantly decrease nonspecific amplification, which has been optimized by New England Biolabs [32]. While the original no-target control amplified nonspecifically in 20 minutes, the addition of helicase allowed the reaction to run for over an

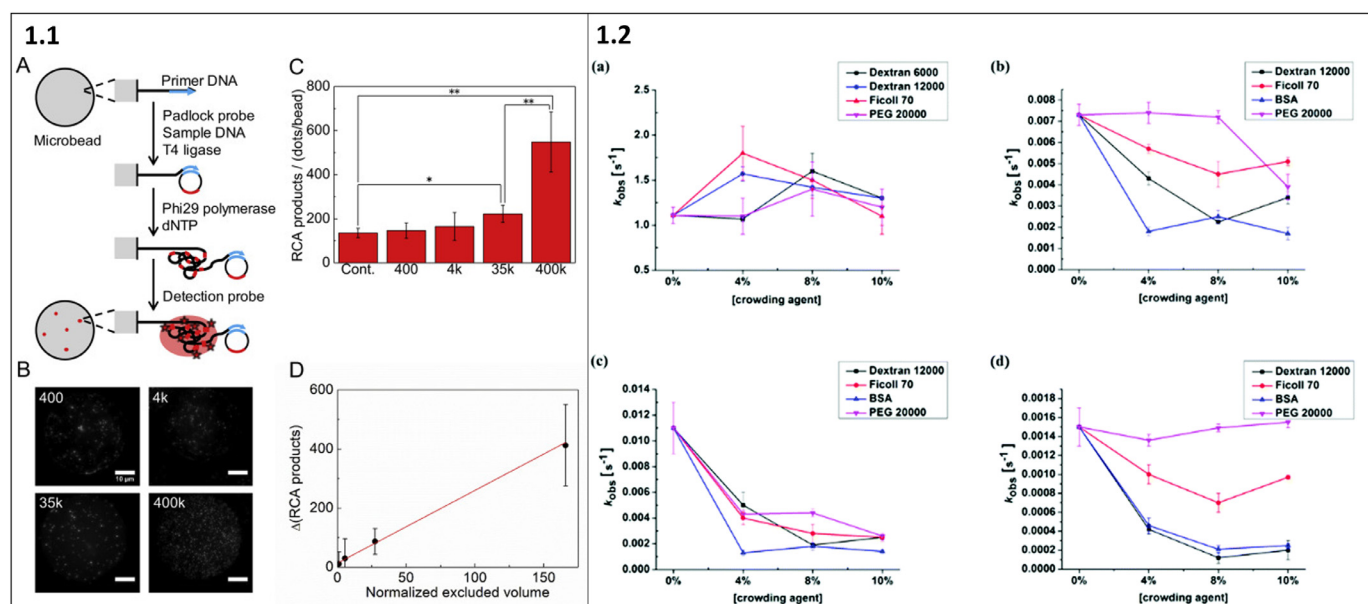


Fig. 1. 1) Effect of molecular weight of PEG on reaction characteristics of bead-based padlock RCA (A–D). **A**) Bead-based padlock RCA reaction scheme. **B**) Fluorescence images of beads following RCA. **C**) Effect of PEG molecular weight on RCA product number. [PEG] = 2% (except control experiments). N = 15. Error bars: ±1SD. (*: P < 0.05. **: P < 0.01). **(B) & (C)** suggests that product number increased with increasing PEG molecular weight. **D**) Normalized excluded volume-dependence of ΔRCA products. Higher product numbers were observed as excluded volume increased[24]. **1.2**) Effects of Dextran 6000 & 12000, Ficoll 70, BSA and PEG 20000 on Klenow fragment (-exo) DNA polymerase kinetic profiles with **a**) match sequence, **b**) A–C mismatch, **c**) T–C mismatch and **d**) C–C mismatch [90].

hour without nonspecific amplification. The reaction that included target had a decreased yield when compared to a target-triggered reaction with no helicase but maintained the time to positive of approximately 8 minutes. A published account also found that helicase improved speed of a LAMP-based assay [93].

Recombinase proteins promote strand exchange and can therefore also aid primer binding to dsDNA target sequences without a denaturation step. In nature, recombinase enzymes catalyze strand exchange during homologous recombination, which is critical during repair of double-stranded DNA breaks [94]. Biotechnology can also harness this function of recombinases: Recombinase polymerase amplification (RPA) is an isothermal amplification method that relies on the bacteriophage recombinase T4 uvsX and the recombinase loading factor T4 uvsY to promote strand exchange between a primer and a double stranded DNA, with the single stranded binding protein T4 gp32 binding the displaced strand to stabilize the replication complex [22]. Chen et al. found the nucleotide cofactors and optimal conditions to perform a similar function with RecA, which is an extensively studied and commercially available recombinase that is isolated from *E. coli* [33]. RecA improved ligation efficiency of two ssDNA strands using a dsDNA template and allowed extension of a ssDNA primer bound to a dsDNA template when using a polymerase with strand displacement activity. While RecA was not used in an isothermal amplification reaction to detect a specific molecule, the results implied that RecA and a commercially available single-stranded binding protein can be broadly used to open dsDNA during isothermal nucleic acid amplification reactions. RecA was also used to increase the kinetics of signal formation by the enzyme-free DNA circuit catalytic hairpin assembly (CHA), which can also be used for sensing applications [34]. It is worth noting that RecA exhibits sequence bias [94], which may be a consideration when designing an assay using RecA.

Endonucleases are another group of enzymes that are used to initiate specific amplification in the presence of a target molecule during isothermal reactions. Tong et al. used restriction enzymes to enhance amplification during HDA, as cleaving the DNA near the target site was hypothesized to enhance recruitment of helicase, allowing amplification by a polymerase with strand displacement activity [25]. Nicking endonucleases are generally used to open dsDNA for isothermal amplification reactions, as occurs during isothermal reactions such as SDA [20] and EXPAR [21] where nicking endonucleases nick one strand at a recognition sequence to initiate amplification. CRISPR/Cas systems can also perform this function using a synthetic single guide RNA (sgRNA) and a CRISPR associated (Cas) enzyme.

CRISPR is the adaptive immune system of many prokaryotes, which uses small RNAs to guide an endonuclease to a specific target sequence within a DNA or RNA. CRISPR-Cas systems are now widely commercially available, with Cas enzymes that can cleave or nick DNA, as well as enzymes that target RNA. As guide RNAs are readily synthesized to target a specific DNA sequence, CRISPR endonucleases can target virtually any arbitrary sequence. This provides an advantage over traditional restriction enzymes that are each confined to a specific recognition sequence. The target region must be next to a short protospacer adjacent motif (PAM), but with the expanding number of Cas enzymes, the number of potential DNA and RNA targets are also expanding [95]. The target sequences are also longer than a typical endonuclease recognition sequence, which provides additional selectivity to an assay. Chang et al. cleaved a *Phytophthora infestans* gene fragment near an Nb.BsrDI nicking endonuclease site, which created a short ssDNA that allowed padlock probe ligation and priming for RCA [35]. Nicks in a dsDNA created by Cas9 nickase were used to initiate isothermal SDA-RCA for detection of *E. coli* O157:H7 [36]. CRISPR-Cas9 nickase was also used to trigger SDA in a novel CRISPR-based method to detect DNA (Fig. 2) [37]. A pair of sgRNAs guided Cas9 nickase to a target dsDNA and nicked both strands around a sequence of interest. SDA primers with a nicking enzyme recognition site on their 5' ends bound the newly exposed ssDNA adjacent to the Cas9 nicking sites; the primers also bound inside the dsDNA next

the sgRNA binding site with the help of single stranded DNA binding proteins. This created two ssDNAs for a downstream exponential SDA reaction. SsDNA can also be freed from dsDNA for downstream isothermal DNA amplification using two adjacent Cas9 nickase cleavage sites on the same strand and a polymerase with strand displacement activity [96]; this strategy could be generally applied to initiate a variety of isothermal DNA amplification reactions. CRISPR-Cas systems can also be incorporated in to other steps of an isothermal amplification scheme, as discussed in this comprehensive review of CRISPR-based molecular diagnostics [97].

2.2.2. Chemical manipulation of DNA structure

Chemical manipulation of DNA structure can be an effective strategy to improve the specificity, sensitivity, and speed of isothermal nucleic acid amplification reactions without the need for expensive enzyme additives. These additives typically require optimization to determine concentrations that provide the maximum benefit without inhibiting the reaction. These optimization steps are usually less extensive than those required when adding additional enzymes to the reaction mix, however.

2.2.2.1. Destabilizing nucleic acid structure.

Destabilizing nucleic acid structure through chemical additives is a strategy to reduce undesirable secondary structures that can hinder primer binding and polymerase function. These additives also decrease nonspecific interactions between non-target sequences and reaction templates or primers, which can improve specificity. Destabilizing agents can additionally reduce primer-dimer formation, which can increase reaction efficiency and prevent the formation of nonspecific signals. An optimized concentration of a destabilization agent can therefore improve sensitivity and specificity while maintaining or accelerating reaction kinetics. The effect of destabilizers such as ionic liquids (ILs), betaine, trehalose and proline on isothermal amplification reactions are discussed in further detail below.

Ionic liquids (ILs) are non-molecular solvents composed of ions that are fluid around or below 100°C [98]. Example IL cations are imidazolium, pyridinium, pyrrolidinium, and phosphonium and anions are halides, tetrafluoroborate (BF_4^-), hexafluorophosphate (PF_6^-), and bis [(trifluoromethyl) sulfonyl]imide (NTf_2^-). ILs have gained popularity in nucleic acid extraction techniques [99], but were also shown to be beneficial to enzyme-based DNA amplification reactions. For example, ILs have been shown to improve PCR amplification of DNA by Shi et al., who demonstrated that poor amplification of high GC content DNA targets and unwanted nonspecific amplification could be countered by addition of ILs [100]. It is hypothesized that the addition of ILs destabilize DNA duplexes and reduce unwanted secondary structures, which could explain the effects of ILs on amplification reactions.

Magnetic ionic liquids (MILs) are ILs with high-spin d^5 iron (III) ions that exhibit strong response to magnetic fields and have been reported to enhance isothermal amplification reactions. MILs are additionally advantageous due to their ability to capture target nucleic acids from biological samples. Hydrophobic MIL microdroplets dispersed in a water solution captured nucleic acids, which were then collected by a magnet into MIL droplets [101–105]. Ding et al. captured DNA targets from a cellular lysate using MIL on a magnetic rod, and added the MILs containing the DNA directly to an isothermal DNA amplification reaction to detect target DNA. MIL containing reactions with optimal buffers decreased nonspecific signal production of both LAMP and multiple-self-matching-initiated amplification (IMSA) while increasing the specific reaction kinetics (Fig. 3) [38]. Without buffer optimization, MILs inhibited the reaction, but in an optimized buffer it was hypothesized that the MILs destabilized dsDNA and thus decreased nonspecific amplification from primer-dimers. This demonstrated the ability of MILs to streamline the DNA purification step, increase the reaction speed, and decrease nonspecific amplification for both LAMP and IMSA reactions. ILs would likely be similarly beneficial in other isothermal amplifications schemes.

Betaine is a zwitterionic amino acid analogue that carries both a positive and a negative charge at pH values close to neutrality. Rees et al.

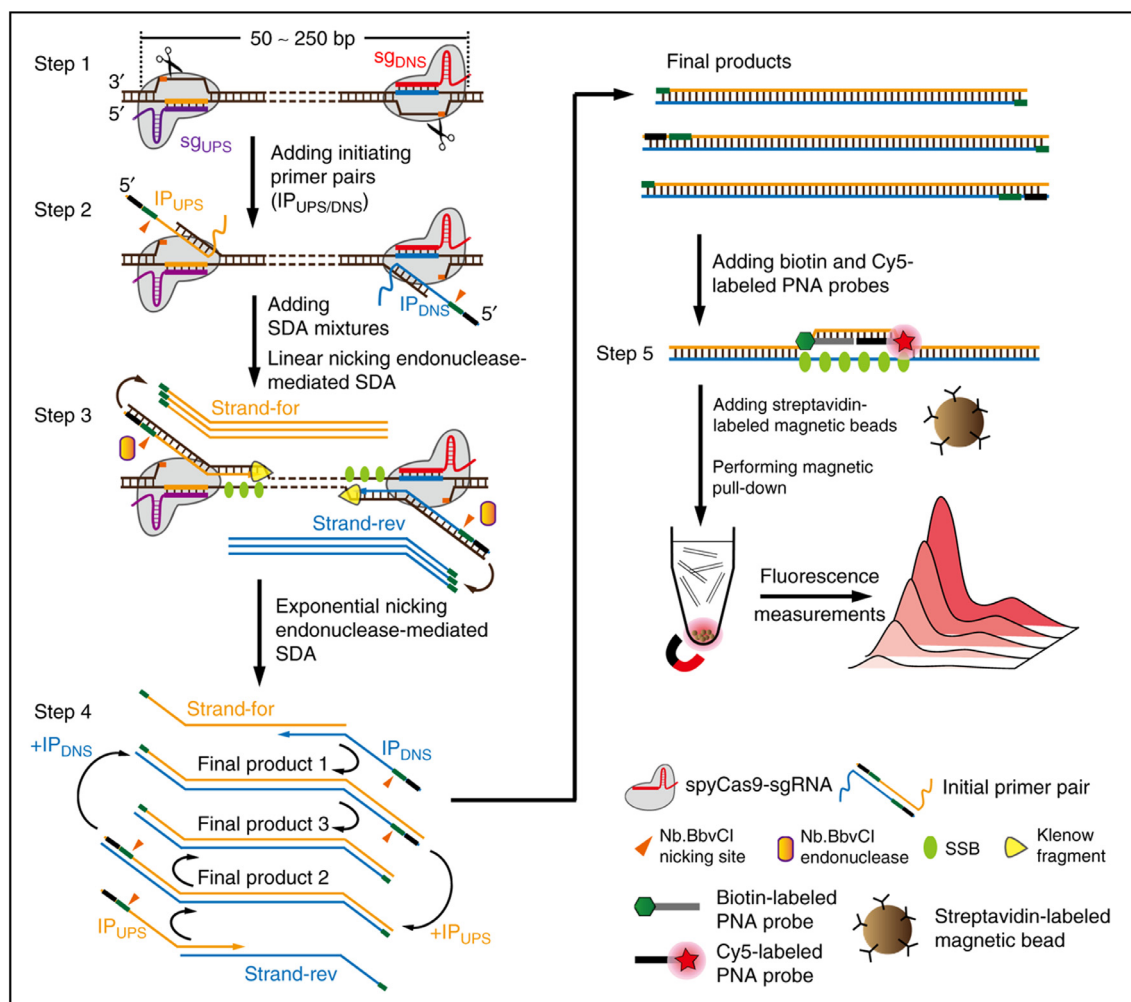


Fig. 2. CRISPR-Cas9-triggered SDA for DNA detection [37].

demonstrated that betaine is an isostabilizing agent for DNA that eliminates composition dependence of DNA melting temperature, with a minimal effect on DNA-protein electrostatic interactions [106]. Betaine sharpened and shifted DNA melting transition to a lower temperature, thus destabilizing the DNA duplex [106,107]. It has been used to improve PCR amplification of GC-rich sequences by reducing secondary structure of nucleic acids in the reaction [108], with a similar effect as DMSO albeit with a different mechanism [109]. Betaine has since been used to enhance isothermal amplification reactions such as RPA [39], EXPAR [40], LAMP [41], and RCA-LAMP [46], typically in the 0-1M concentration range. Excess betaine decreased reaction yield such that optimization was necessary to find the optimal concentration for an assay [40,41], and LAMP reactions to detect a target that is not GC rich may not require betaine enhancement [110]. Nonetheless, betaine reduced nonspecific amplification of EXPAR and increased efficiency and speed of LAMP by decreasing sequence bias and reducing secondary structure of the nucleic acids in the reactions.

Trehalose and proline have also been reported as additives in isothermal amplification reactions for their duplex destabilization effects along with their ability to improve thermal stability for enzymes [16,40,42]; these effects are discussed in further detail below. PNA probes and single stranded DNA binding proteins also provide multiple functions including DNA destabilization, and they are also discussed in more detail below. Additional substances are known to destabilize DNA duplexes, such as adenovirus DNA-Binding protein [111], calf thymus helix-destabilizing

protein [112], and ribonuclease [113], although they have not yet been reported as additives in isothermal DNA amplification reactions.

2.2.2.2. Stabilizing nucleic acid structure. While destabilizing nucleic acid bonds is more commonly required for reaction enhancement, duplex stabilization may also be advantageous for select applications. Duplex stabilization increases melting temperature of associated nucleic acids and can be used to improve isothermal nucleic acid amplification reaction kinetics, especially for weak primer:template complexes. These additives could also allow the operating temperature of a reaction to be increased without changing primer or template sequences, if necessary.

TMAC (Tetramethylammonium chloride) is a hygroscopic ammonium salt that increases DNA melting temperature by increasing AT base pair stability closer to that of a GC base pair. TMAC preferentially binds AT base pairs due to the attraction of the nonpolar arms of alkylammonium ions to the more hydrated AT base pairs [114,115]. TMAC addition decreased sequence bias for the detection of four different influenza A subtypes, which simplified multiplexing for subtyping influenza virus using a PCR-based assay [116]. Similar effects could aid in multiplexed assays using isothermal amplification techniques. Besides increasing specificity of probe hybridization [116–118]; TMAC increased specificity of PCR [119–121] and EXPAR [40] assays. Mok et al. showed that TMAC addition increased the time between reactions that contained the target molecule and reactions that did not contain the target [40].

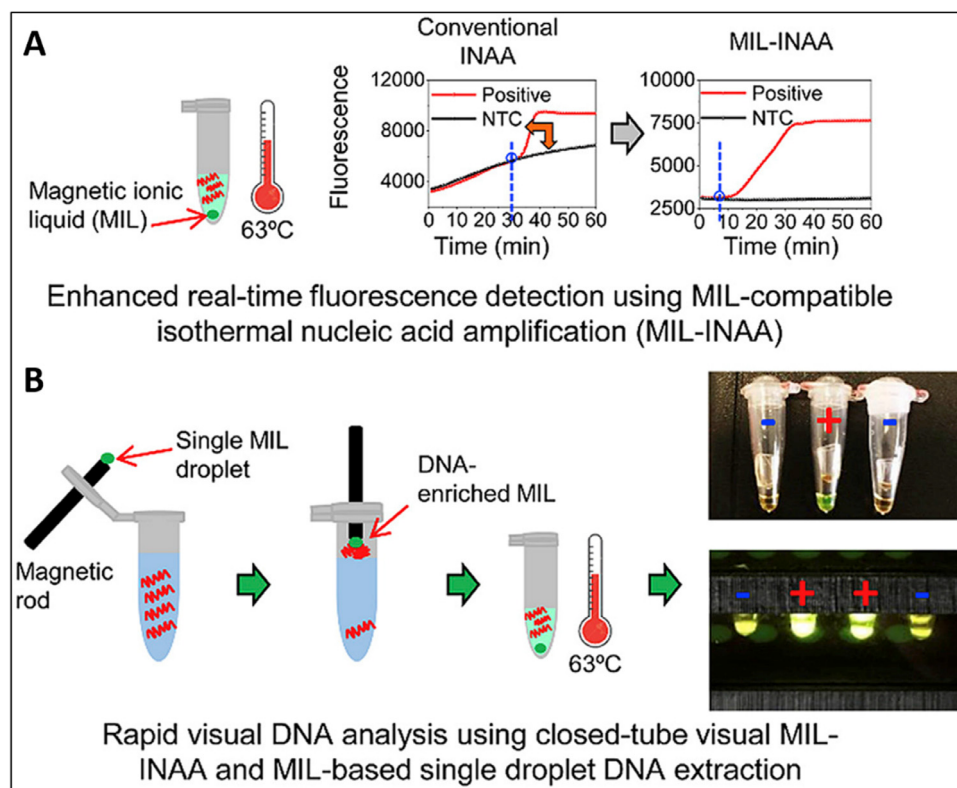


Fig. 3. Effect of MILs on isothermal nucleic acid amplification reaction. **A)** MIL addition lowered background fluorescence and decreased detection time. **B)** MIL-based DNA isolation scheme. MIL captured DNA was isolated with a magnetic rod, subsequently detected by isothermal amplification reaction [38].

2.3. Enzyme stabilization

Target analytes are often found in complex biological and environmental samples. However, many compounds found in blood, urine, feces, soil and milk exhibit inhibitory effects on polymerase activity [122–129]. Strategies to overcome these adverse effects has been well studied for PCR, and many of these techniques are also applicable to isothermal amplification reactions that require a polymerase. Furthermore, loss of enzymes due to adsorption to reaction containers or deactivation in sub-optimal reaction conditions can be problematic for sensing applications that require enzyme activity. Enzyme stabilization is particularly important for sensing applications outside of the laboratory, such as at the point of care. Sacrificial proteins and enzyme thermostabilizers are popular additives to counteract potential enzyme loss or deactivation during a sensing application.

2.3.1. Sacrificial proteins

One method to overcome polymerase inhibition is the addition of bovine serum albumin (BSA). BSA is a frequently used as an additive to PCR and isothermal DNA amplification reactions to alleviate potential inhibition from residual organics and thermal stress [130]. The inhibitory effects of hemoglobin, lactoferrin, iron chloride, hemin in blood, humic, tannic and fulvic acids in soil, and proteinase in milk were successfully suppressed with the addition of BSA for PCR and PCR-based TaqMan assays [123–125,127,128,131–133]. BSA serves as a sacrificial protein; it reduces interaction of the enzymes with inhibitory molecules as well as prevents enzyme loss from adsorption to solid surfaces. Walker et al. acknowledged BSA in SDA reactions as an enzyme stabilizer and a sacrificial protein. Likewise, Lee et al. used BSA in their electrochemical sequence-specific DNA detection platform, based on primer generation-rolling circle amplification (PG-RCA) reaction. The electrochemical detection chip was pretreated with BSA and BSA was included in the

PG-RCA reaction mix [44,45]. Most isothermal amplification reactions use BSA as a reagent, but few include concentration optimization studies. Lin et al. investigated two different concentrations of BSA for their digital LAMP on a commercial membrane and found that 1 mg/mL of BSA was optimal while 2 mg/mL BSA decreased reaction yield, likely due to decreased reactant diffusion rates caused by molecular crowding as discussed above [43].

2.3.2. Enzyme thermostabilization and thermoactivation

Enzyme stabilization is an effective method to increase reaction yield and speed by preventing loss of enzyme activity, especially during sub-optimal reaction or storage conditions. In addition, they aid in point-of-care applications by allowing reactants to be stored long term or at elevated temperatures. Trehalose and pullulan are two popular enzyme thermostabilizers used during sensing applications.

Trehalose is a non-reducing disaccharide, which is produced by the yeast *Saccharomyces cerevisiae* when exposed to heat shock. De Virgilio et al. showed that heat-triggered synthesis of trehalose by *S. cerevisiae* contributes to thermotolerance induction [134]. Carninci et al. showed thermostabilization of several enzymes in the presence of trehalose, with some enzymes showing increased activity at high trehalose concentrations (thermoactivation) [135]. Hedoux et al. investigated the mechanism behind thermostabilization effect of trehalose on BSA. They hypothesized that trehalose reduces the exposure of protein hydrophobic groups to water molecules and strengthens intermolecular O-H interactions in the hydrogen-bond network of water, which leads to the stabilization of the tertiary structure of the protein [136]. It should be noted that several studies have shown trehalose can also destabilize DNA duplexes [137,138], which will also affect nucleic acid amplification.

Addition of trehalose to isothermal amplification reactions usually results in increased reaction yield and faster reaction kinetics until trehalose concentration reaches a threshold level, at which point it can

inhibit the reaction. This inhibition is hypothesized to be either due to its DNA duplex destabilization effects or possible formation of trehalose clumps that limit diffusion rates [40,139]. Trehalose addition to EXPAR [40], NEMA (Nicking endonuclease-mediated isothermal amplification) [50], SDA [49], and LAMP [47] reactions were investigated and optimized for an increased reaction yield and speed. Furthermore, trehalose is widely used as an enzyme stabilizer for lyophilized LAMP reaction mix, in efforts to use LAMP for point of care applications [47,48,51]. Curtis et al. tested the stability of their lyophilized HIV-1 RT-LAMP reaction reagents containing 5% trehalose and found that the reagents remained stable at 30°C for up to a month of storage if the quencher probe was not included [51]. Wan et al. achieved a 3-day storage time at 30°C for their lyophilized LAMP reagents, hypothesizing that inclusion of a fluorescent reagent in the lyophilized reagents shortens the shelf life [47]. Effects of varying concentrations of glycine, trehalose and freeze-drying time on reaction kinetics can be found in Figure 4.1.

Pullulan is a non-ionic polysaccharide composed of maltotriose units, which is produced by the fungus *Aureobasidium pullulans* [140]. Aqueous solutions of pullulan are viscous, which can complicate reaction handling. However, pullulan confers thermal stability to biomolecules [140,141], and can stabilize enzymes and other assay reagents for long-term storage when dried [140]. Enhanced stability of reagents in pullulan suggests that pullulan can be used as a molecular enhancer for isothermal DNA amplification reactions operating at high temperatures. Liu et al. introduced a paper device for DNA or miRNA detection with

RCA, where a pullulan film was used to encapsulate reaction components. Pullulan film helped adapt RCA to point of care applications by preserving reaction reagents for 15 days when stored at 4°C [53]. Wang et al. used pullulan in a cross-priming isothermal amplification (CPA) reaction, which is similar to LAMP in that it creates looped structures in DNA reaction products and uses a polymerase with strand displacement activity. They found that pullulan increased the reaction yield and detection speed of genetically modified rice without increasing nonspecific amplification (Fig. 4.2-A) [52]. Melting curve analysis revealed that pullulan did not affect DNA melting temperature, which suggests that the improvements seen to the CPA reaction were due to enhanced enzyme stability and activity (Fig. 4.2-B). They noted that this differs from trehalose, which can both stabilize enzymes and lower DNA melting temperature. Another study showed that pullulan reduced nonspecific amplification of a LAMP-based assay in a concentration-dependent manner [142]. They have attributed this finding to pullulan decreasing primer-dimer formation, although pullulan is not known to affect the melting temperature of DNA. It is also possible that pullulan affected polymerase association with nucleic acids in the reaction.

Proline [42,143], glycine in combination with trehalose [47], betaine [143,144], sucrose, maltose, ectoine, hydroxyectoine, sorbitol, glycine betaine and homodeanol betaine have been shown to stabilize enzymes and they have been used as additives for isothermal amplification reactions [144,145]. However, their effects on reaction kinetics have not been extensively studied.

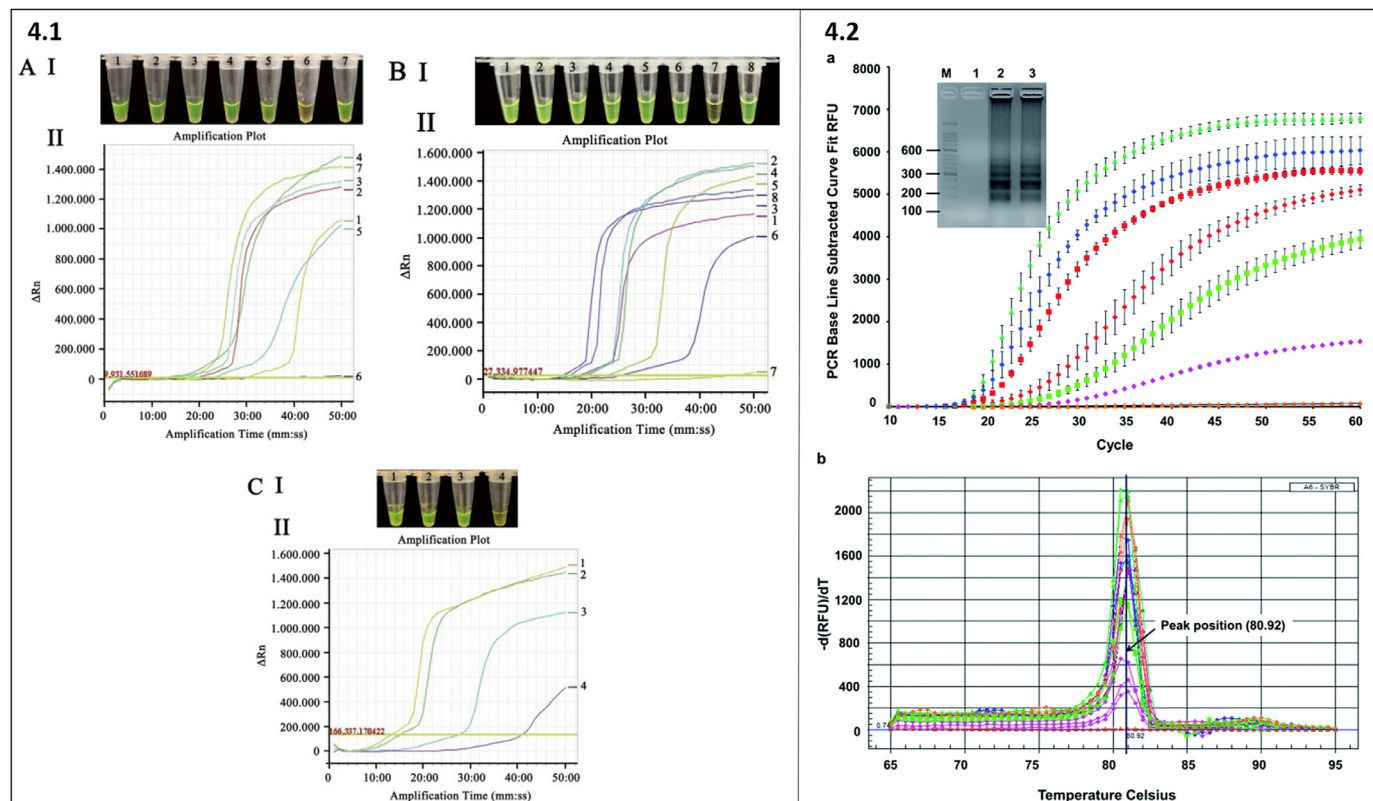


Fig. 4. 1) Change in LAMP reaction characteristics with different concentrations of glycine, trehalose and freeze-drying time on LAMP lyophilized reaction mixtures for *Salmonella* DNA detection. I: end-point sample images; II: fluorescence-time plots. A) 1–6: [glycine] = 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%; 7: the LAMP wet kit as a reference. B) 1–7: [trehalose] = 0%, 2.5%, 5.0%, 7.5%, 10.0%, 12.5% and 15.0%; 8: the LAMP wet kit as a reference. C) 1–4: freeze-drying time = 2.5 h, 3 h, 3.5 h and 4 h. [47] **4.2)** Effect of pullulan on real-time fluorescent cross-priming isothermal amplification reaction (CPA) kinetics. a) Amplification curves of *Agrobacterium tumefaciens* nopaline synthase terminator (T-Nos) gene in GM rice. [pullulan] = green triangle, 1% (w/v); blue diamond, 0.5% (w/v); red square, 2% (w/v); green square, 4% (w/v); pink diamond, 5% (w/v); red diamond, pullulan-free (0% (w/v)); no template control (0% (w/v)). Inset shows gel electrophoresis for CPA products after amplification for one hour. M is DNA marker (50 bp), lane 1 is no template control, lane 2 is [pullulan] = 1% (w/v) and lane 3 is pullulan-free condition. b) Melt curves with varying pullulan concentrations, showing that pullulan did not affect melting temperature [52]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Oligonucleotide modifications and analogs

Using modified oligonucleotide primers, probes, and templates provides another mechanism to control nucleic acid hybridization specificity and stability, and to prevent unwanted enzyme activity. These modifications are therefore commonly used to improve reaction specificity, particularly when detecting single nucleotide mismatches. Other notable enhancements include lowering limits of detection, modifying the optimal reaction temperatures, and decreasing nonspecific signal formation. Below are a summary of oligonucleotide modifications and analogs that have been incorporated into isothermal nucleic acid amplification reactions.

2.4.1. Locked nucleic acids (LNA)

Locked nucleic acids (LNA) are nucleic acid analogues that are modified by the addition of O2'- to C4' methylene-link on ribose ring, which locks them in an N-type conformation that facilitates duplex formation with DNA and RNA complements [146–149]. LNA modified oligonucleotides have higher duplex stability and mismatch discrimination [148,150]. However, You et al. reported an exception on the mismatch discrimination of LNA modified sequences and showed that discrimination of a G-T mismatch decreases with LNA modification [151].

Usage of chimeric probes [67], beacons [65,66] or reaction templates [62–64] that contain LNA modifications in strategic locations is a popular method to increase sensitivity and specificity of isothermal DNA amplification reactions. LNA modified oligos are especially advantageous for miRNA detection since their small size, sequence similarities, and low abundance results in challenges achieving high sensitivity and specificity during miRNA detection [63,152,153]. Yin et al. utilized LNA modified probes in their plasmon-enhanced hybridization chain reaction (PE-HCR) for quantification of miRNAs. Despite HCR being an enzyme-free method, they were able to achieve a detection limit of 0.043 fM (Fig. 5.1) [67]. Huang et al. established a miRNA detection system called miRNA-triggered real-time fluorescent isothermal reaction with

exponential amplification (ReFIRE) which uses LNA modified primers. *Taq* MutS, which strongly binds mismatched DNA, can suppress nonspecific amplification from primer dimers or mishybridization events. When paired with *Taq* MutS, ReFIRE was able to detect miRNAs down to 10 ymol in 20 μ L reactions with ability to differentiate between homologous miRNA in the let7 family [63].

2.4.2. Phosphorothioate (PTO) modification

Phosphorothioate nucleotide analogues (PTO) replaces a nonbridging oxygen atom of a phosphate group with a sulfur atom. The resulting nucleotides are resistant to nuclease activity [154–156]. Urtel et al. utilized PTO modification in the nicking enzyme recognition site of a synthetic ssDNA template to block undesired off-target restriction activity of Nb.BssSI during EXPAR [69]. PTO modification enabled Gines et al. to create an exonuclease-assisted method for isothermal digital detection of miRNAs using a molecular circuit [3]. Their circuit required the thermostable exonuclease *tttRecJ* to degrade DNA product, where all four synthetic ssDNA reaction templates were protected from exonuclease digestion by incorporating 5' PTO backbone modifications. PTO residues also destabilize DNA helices when bound to naturally occurring nucleotides. Incorporation of PTO modification on the 5' end of the inner and outer LAMP primers created more efficient foldback and loop formation of intermediate products during a LAMP reaction. When PTO primers were used jointly with SSB and urea, the operating temperature of a LAMP reaction could be decreased from 60–65°C to 40°C [68]. This may be advantageous if lower energy requirements are desired.

2.4.3. Peptide nucleic acids (PNA)

Peptide nucleic acids (PNAs) are created by replacing the sugar-phosphate backbone of DNA with an achiral polyamide backbone [157]. Since the peptide backbone of a PNA oligonucleotide is not negatively charged, they form highly stable duplexes with PNA, DNA, and RNA sequences due to lack of electro-repulsion [157,158]. While PNAs cannot be used as primers [58], their enhanced duplex stability makes PNA

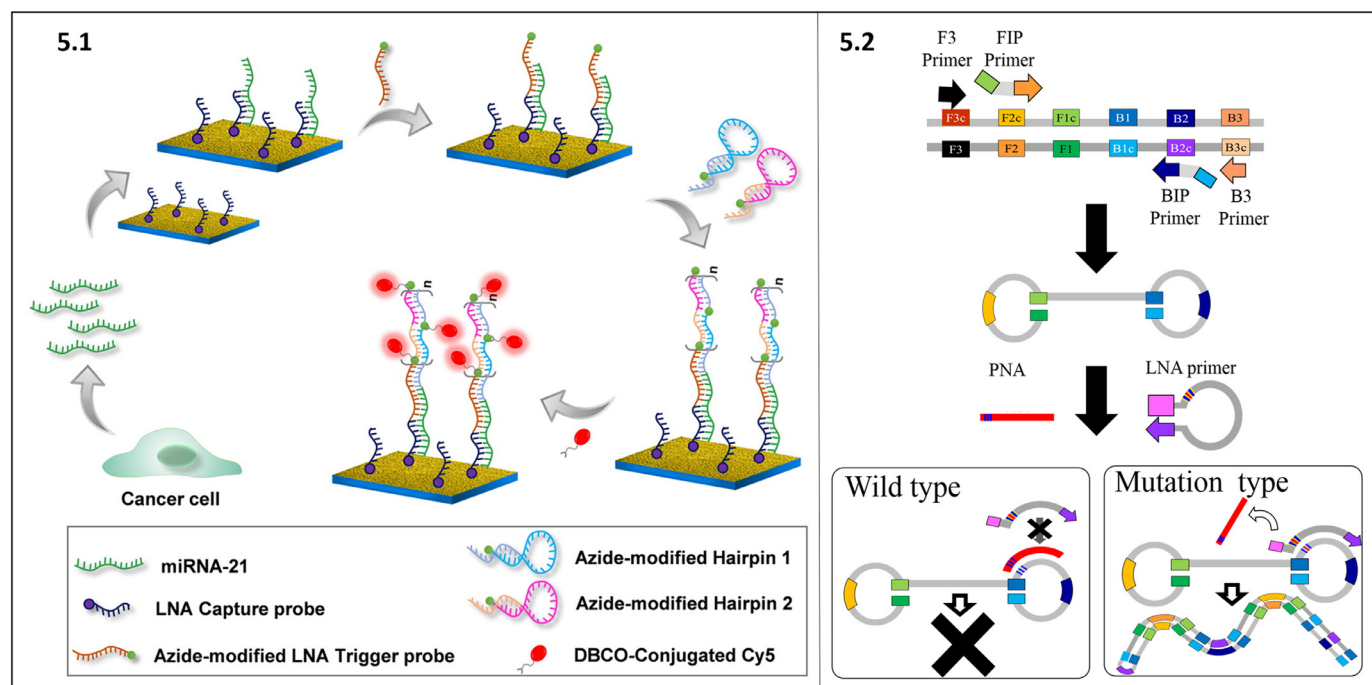


Fig. 5. 1) Reaction mechanism of PE-HCR miRNA detection assay. A plasmonic chip covered with LNA capture probes reacts with the target sequence, miRNA-21. Azide-modified LNA trigger probes and azide-modified Hairpin 1 and Hairpin 2 are added on the substrate and recognized by the immobilized miRNA-21 sequences, initiating the formation of HCR products, which are labeled by a Cy5 fluorophore via copper-free click chemistry [67]. **5.2)** PNA-LNA mediated LAMP scheme for KRAS mutation detection. Wild-type target results in a stable duplex of a PNA probe with the dumbbell structure, blocking the annealing and extension of the LNA primer. A mutated target allows binding of the LNA primer, such that the LAMP reaction can proceed [70].

sequences well suited for probes, blockers, or mechanisms to open dsDNA during isothermal DNA amplification reactions [37,58,70,72]. PNA clamps aided in the opening of a dsDNA target, which allowed binding of a circular oligonucleotide probe within a specific dsDNA sequence; these probes can be isothermally amplified using RCA to label specific regions of DNA [71]. While PNA probes are commonly used in nucleic acid amplification, PNA can also be used to block unwanted amplification. Reid et al. lowered nonspecific amplification of EXPAR by binding a complementary PNA strand on the 3' end of the synthetic DNA reaction template, which was hypothesized to block transient template hybridizations that can create nonspecific amplification [58]. Given that nonspecific amplification in EXPAR is largely due to nonspecific interactions between polymerase and the synthetic ssDNA template [159], it is possible that the PNA strand also prevented undesirable polymerase association with the ssDNA template. Itonaga et al. developed PNA-LNA mediated LAMP to detect a *KRAS* mutation, where they used a PNA sequence as an amplification blocker (Fig. 5.2) [70]. The reaction used LNA primers that bind in the loop region of the primary LAMP product, which cannot bind when blocked by a complementary PNA. The PNA clamp binds and blocks hybridization of the LNA primer during amplification of a wild type target gene, due to the perfect match between PNA clamp and target gene complementary sequence. In the case of a mutation, even a single mismatch between the PNA clamp and the target prevented the PNA from blocking LNA primer hybridization, which allowed amplification to proceed.

2.4.4. Zip nucleic acids (ZNAs)

Zip nucleic acids (ZNAs) are conjugated with repeated cationic spermine units in efforts to decrease electrostatic repulsion between duplex sequences [160]. ZNA modification allows fine tuning of hybridization temperatures that is not sequence or location dependent by changing the number of implanted spermine units, which simplifies design when compared to LNA modifications [151,161]. ZNAs were added to PCR and reverse transcription primers since they can operate at higher annealing temperatures, lower primer concentrations, and lower magnesium concentrations [162]. Furthermore, ZNAs can also be used as capture probes [163,164] and real-time PCR probes [165–168] for detecting miRNA, viral RNA, and single nucleotide mutations. Despite easier design constraints with comparable specificity enhancements to more conventional oligo modifications, to our knowledge there are no reported applications of ZNAs to isothermal DNA amplification methods.

2.5. Template blockers

Template blocking is a multipurpose strategy for isothermal reaction enhancement: blocking a nucleic acid template can reduce nonspecific amplification, prevent unwanted annealing of nucleic acid strands, assist in breaking dsDNA structure, and reduce background fluorescent noise. Single-stranded binding proteins, graphene oxide sheets and CoOOH nanoflakes are a selection of template blocking additives discussed below.

2.5.1. Single-stranded binding proteins

Single-stranded DNA-binding proteins (SSBs) bind with strong affinity to ssDNA, remove secondary structures in ssDNA, and protect DNA from nuclease digestion. SSBs are therefore vital elements of DNA replication, recombination, and repair in bacteria, eukaryotes, and viruses [169–179]. While sequence-specific SSBs exist [172,173], they typically bind nonspecifically to ssDNA [170,174–176,178] and with much lower affinity to dsDNA and RNA [169]. SSBs are popular reaction additives for isothermal DNA amplification reactions either for facilitating strand displacement activities [19,22,27,30,31,37], expediting ssDNA desorption from a surface [55,57], or decreasing background signal caused by unwanted enzymatic activities [40,54,56,58,59].

Reactions that are dependent on strand displacement or primer invasion into dsDNA are also often dependent on the presence of SSBs. HDA using Klenow fragment (exo-) [19] and RPA using Bsu polymerase [22] produced no measurable amplification without SSB. Amplification efficiencies dropped dramatically without SSB for linear nicking endonuclease mediated SDA using Sequenase 2.0 [27] and reverse transcription thermophilic HDA using Bst polymerase [30]. Zhou et al. utilized the SSB protein TP32 for their CRISPR-initiated SDA assay, which is described in more detail above. Without SSBs, the 3' end of the primers could not efficiently bind inside the dsDNA target, which was critical to allow the polymerase to extend past the Cas9 complex and initiate product formation (Fig. 2) [37]. SSBs also aided the binding of PNA probes within the dsDNA products, which labeled reaction products with biotin and fluorescent Cy5 and enabled endpoint detection of target DNA. SSB was therefore instrumental for two steps of the DNA detection reaction: allowing the invasion of the 3' primer into the dsDNA immediately next to the tightly bound Cas9/sgrRNA complex and aiding in PNA probe invasion into dsDNA products.

SSBs are also shown to reduce background noise in isothermal DNA amplification reactions, especially for reactions that use ssDNA reactants or have ssDNA products [40,54,56,58,59]. Background noise is particularly problematic when adding synthetic DNA templates to a reaction due to unwanted polymerase activity; for example EXPAR uses synthetic ssDNA templates to amplify signal molecules, and interaction between the polymerase and the unprimed ssDNA template results in nonspecific product formation. This nonspecific product formation was the most significant source of early phase nonspecific amplification: one study found that significant product formation was not caused by contaminating nucleic acids in the reaction reagents or spurious hybridization between templates [159]. In fact, templates capped with a 3' amine group that could not be extended by a polymerase did not have significantly lower NSA when compared to templates with a free 3' end. Reid et al. demonstrated *E. coli* single-stranded binding protein decreases nonspecific amplification by 3 orders of magnitude for EXPAR [58], while Inoue et al. almost entirely eliminated nonspecific product formation for RCA with a *Tth*SSB mutant protein [56]. SSBs can also protect DNA from degradation; SSBs were used by Liu et al. to protect ssDNAs from residual ssDNA degradation activity of Exonuclease III for the exonuclease III-assisted multiple cycle amplification reaction, which resulted in zero background signal [54].

2.5.2. Graphene oxide

Graphene is a two-dimensional (2D) honeycomb lattice consisting of tightly packed carbon atoms [180]. While graphene is hydrophobic, graphene oxide (GO) can form colloidal suspensions in water, which makes it more suitable for use in aqueous molecular detection reactions [181]. GO binds ssDNA with π - π stacking and hydrogen bonding [181–184], and has a weaker interaction with dsDNA through partial deformation of the double-helix [185,186]. Adsorption of DNA on GO is also length-dependent; the affinity of a 20mer ssDNA to GO is much higher than a 10mer or 5mer ssDNA [183]. GO can also quench fluorescent molecules [182,187,188]. With the ability to preferentially sequester ssDNA and quench fluorescence, GO is a promising candidate to reduce background signal in many isothermal DNA amplification reactions [55,60,61,189–191].

Wang et al. suppressed nonspecific amplification of EXPAR with the addition of GO to the reaction, lowering the limit of detection to 5 aM [55], as ssDNA templates are adsorbed to GO until they are specifically bound by a target molecule. Single-stranded binding proteins aided desorption of target-bound template molecules from GO, which decreased the reaction time while maintaining the suppression of nonspecific amplification. Release from GO can also be detected through fluorescent quenching over a broad wavelength, which allows multiplexing by using multiple fluorophores. Multiple miRNA probes with different attached fluorophores were used to detect miRNA during isothermal strand-displacement polymerase reaction (ISDPR). The miRNA

stabilized adjacent primer binding on the probe, such that Klenow fragment exo- could extend the primer only in the presence of the target miRNA [60]. GO was then added to the reaction; double-stranded products were weakly absorbed by GO, preventing quenching and resulting in a significant increase of the fluorescence signal. The remaining single-stranded probes are captured by GO, thus preventing their fluorescence. A similar concept is applied to RCA-based detection of Ebola virus to reduce background fluorescence upon capturing unbound fluorescence probes by Wen et al.; this mechanism is depicted in Figure 6.2 [61].

2.5.3. CoOOH nanoflakes

Another nanomaterial that has fluorescent quenching ability and selective absorption of ssDNA is cobalt oxyhydroxide (CoOOH) [192]. Similar to GO, CoOOH nanoflakes can capture ssDNA while maintaining low dsDNA absorption. Trinh et al. demonstrated usage of CoOOH nanoflakes to isolate circulating miRNAs before detection with an EXPAR [57]. CoOOH nanoflakes were placed in a clarified serum sample and incubated for 10 minutes to allow capture of single-stranded nucleic acids such as miRNAs. After a PBS wash, the CoOOH nanoflakes were placed in an EXPAR master mix that

contained a specific template for the target miRNA. Once the target miRNAs were hybridized with the reaction template, they detach from the CoOOH nanoflakes with help of the SSB protein T4 gp32, allowing enzymatic activity and exponential amplification of the DNA product. CoOOH and GO were both tested for their ability to suppress nonspecific amplification of EXPAR triggered by miRNA in solution, and CoOOH was found to have a higher signal-to-noise ratio for this application.

2.6. Strategies to remove nonspecific amplification and unwanted reaction products

Most isothermal amplification reactions suffer from nonspecific amplification (NSA), where the reaction produces signal in the absence of the target molecule. This issue curtails sensitivity of isothermal amplification reactions and can also reduce their reliability. Nonspecific amplification can occur from spurious interaction between a synthetic DNA template and a polymerase, partial hybridization of a nonspecific target DNA to a primer or a synthetic template, or primer dimer formation. Below are strategies to degrade undesired amplification products,

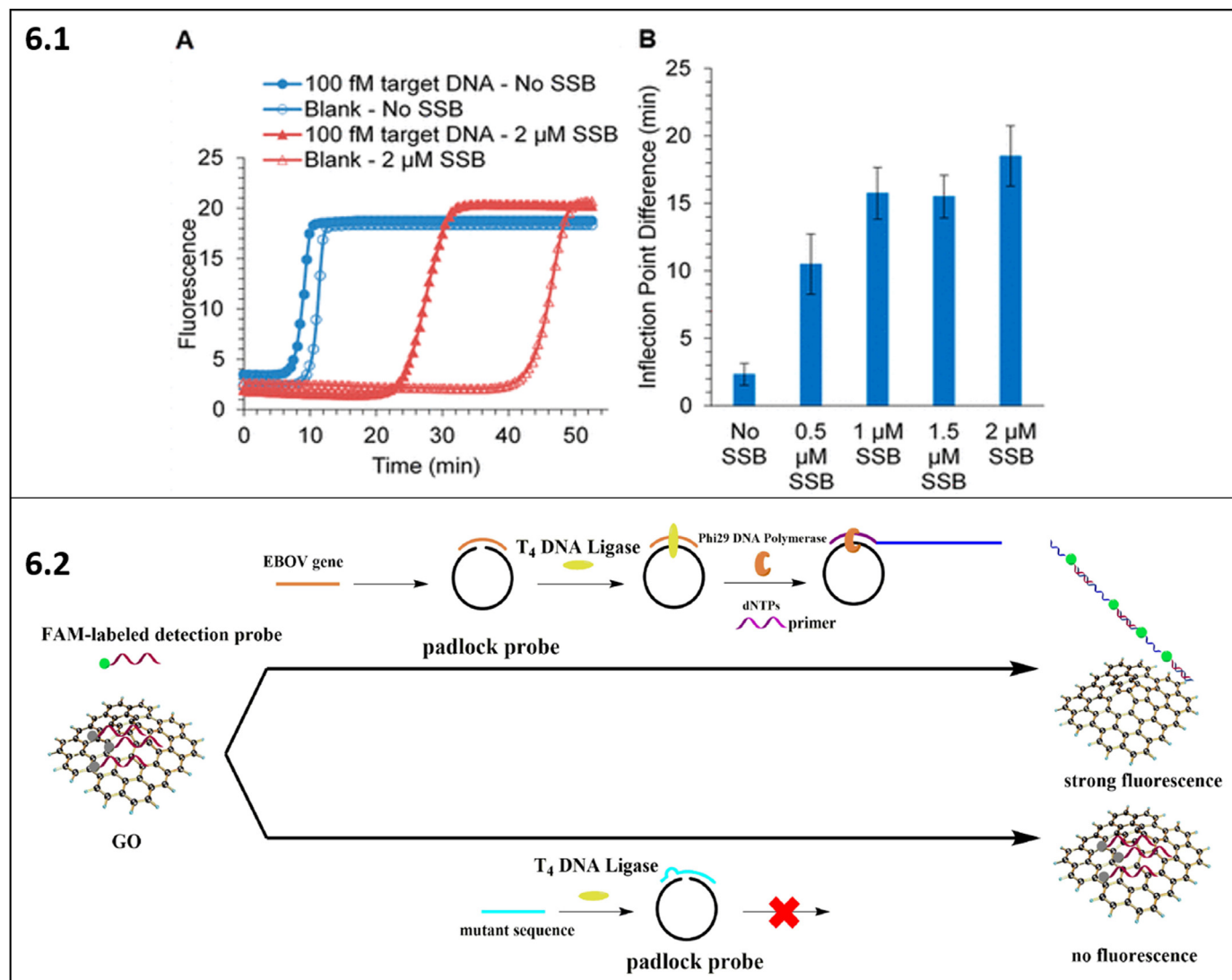


Fig. 6. 1) Effect of SSB on EXPAR reaction kinetics. **A)** Fluorescence-time graph where delayed amplification is observed for reactions with added SSB. **B)** Inflection point differences between the signal (100 fM target) and background (blank). Data represent the mean $\pm$ the standard deviation [58]. **6.2)** Mechanism of GO assisted RCA based biosensor for Ebola Virus (EBOV). Unincorporated fluorescent probes and mismatch target sequences were sequestered by GO sheets to decrease background noise [61].

which can lower limits of detection and create a more robust, specific assay.

2.6.1. Degradation of carryover contamination

Carryover contamination from previous amplification reactions can create undesired signal during nucleic acid amplification. The amplified reaction templates and their products can contaminate laboratory surfaces, reaction reagents, equipment, and operator skin and clothes; this results in increased background signal and false positives [193–195]. He et al. aimed to prevent this type of contamination for their multiplex loop-mediated isothermal amplification (mLAMP) by replacing dTTPs with dUTPs so that any product produced by mLAMP can be degraded with uracil-N-glycoside (UNG) treatment, similar to technologies used during PCR [74]. Pre-treating mLAMP reaction mixtures with UNG prevented amplification of carryover contaminants. Hsieh et al. further enhanced this method by incorporating UNG in the LAMP reaction mix, eliminating the pre-treatment step (Fig. 7) [75]. They showed that incubating the reaction mix with UNG at room temperature for 5 minutes significantly decreased the background signal.

Targeted removal of carryover contamination was also achieved using a CRISPR/Cas9 system. Bao et al. incorporated CRISPR/Cas9 recognition sites to the inner primers of LAMP, resulting in multiple cleavage sites for a Cas9/sgRNA complex within the LAMP products [73]. Before starting a fresh LAMP reaction, possible contaminant DNA carryover is removed by incubating the samples with the Cas9/sgRNA complex, where the carryover DNA is degraded and cannot contribute to signal. This was a very similar mechanism to dUTP/UNG degradation of reaction products, but it lacked the possible inhibitory effects of dUTPs on the reaction.

2.6.2. Amplification sinks

Another strategy to suppress nonspecific amplification is creating reaction “sinks” that remove unwanted amplification products [3,76,77]. Degrading products at the same rate as nonspecific amplification can increase specificity, increase robustness, and lower limit of detection. Gines et al. introduced a pseudotemplate to their EXPAR-based miRNA detection assay, which captures and deactivates reporter molecules [3]. The pseudotemplate consists of the reporter molecule complementary strand along with a random sequence tail; the binding of the reporter molecule primes extension of the pseudotemplate, leaving the reporter molecule with a random sequence tail and rendering it unable to prime extension of new EXPAR templates. All ssDNA products were degraded at a low rate using a thermostable exonuclease, such that the pseudotemplate continuously bound and suppressed low level

amplification events. Exponential production and linear degradation of reporter molecules created a bistable switch: in the absence of the target molecule, the low-level NSA signal was absorbed by the pseudotemplate (Fig. 8.1). With this method, they were able to suppress NSA for up to 1200 minutes, decreasing the limit of detection from 3.7 pM to 1 fM.

A CRISPR-Cas12a system was also used to nonspecifically degrade reaction products during an RCA reaction, creating an internal control that suppressed nonspecific amplification [76]. A detection loop specifically created ssDNA product using RCA in the presence of a synthetic Dengue DNA sequence, but nonspecific amplification also occurred, especially in the presence of contaminating non-target DNA. A reference DNA loop served as an internal control: it nonspecifically created DNA product at a similar rate as the detection loop, and this reference loop product could activate a Cas12a complex. When activated, Cas12a nonspecifically cleaves ssDNA in the sample, thus degrading the DNA products that produced signal. The use of an internal control is beneficial, as isothermal amplification reactions can exhibit variable kinetics day to day. While amplification sinks that continuously degrade signal can be tuned to remove products at the rate of nonspecific amplification, they will still be limited by nonspecific amplification that is rapid or variable. The amplification reaction may therefore need optimization before amplification sinks are employed.

Restriction endonucleases can also provide a targeted competitive sink for products during isothermal amplification. Sun et al. incorporated three different restriction enzymes into single-molecule RT-LAMP, which suppressed amplification of specific hepatitis C (HCV) genotypes if the recognition sequence was present in the target (Fig. 8.2). By observing which restriction enzyme significantly decreased the number of amplified HCV RNA molecules, the same base LAMP reaction was able to distinguish between four different HCV genotypes [77].

2.7. Delay or prevention of *ab initio* synthesis

Ab initio synthesis occurs when polymerase produces long stretches of poly d(A-T)s from nucleotides without primer or template DNAs [196,197]. It has been hypothesized that *ab initio* DNA synthesis was the driving mechanism to create primordial genetic information on the early earth [197,198]. *Ab initio* DNA synthesis was attributed to enzyme contamination with DNA until it was observed that purified enzymes showed the same behavior [199,200]. When a nicking endonuclease is introduced in addition to a polymerase, the *ab initio* synthesis rate accelerates with incorporation of nicking enzyme recognition sites to the poly d(A-T)s [201,202]. Zyrina et al. proposed that during *ab initio* DNA synthesis, some ssDNA *ab initio* products form hairpins that are self-priming and can create dsDNA. In the presence of a nicking endonuclease, numerous *ab initio* synthesis products are produced that can prime further amplification [197]. Kaboev et al. demonstrated that a helicase (DnaB) can enhance *ab initio* synthesis by a thermophilic polymerase [203].

Ab initio synthesis causes unwanted background noise for isothermal DNA amplification reactions such as EXPAR and SDA, where a thermophilic polymerase and a nicking endonuclease are utilized simultaneously [159,204–207]. Some strategies to overcome this issue include decreasing polymerase and nicking endonuclease concentrations, addition of netropsin, removing dATPs from reaction mix, and using LNA modified templates.

Netropsin is an oligopeptide antibiotic that has a high affinity for A/T-rich regions of helical DNA [208,209]. Netropsin has been shown to reduce *ab initio* synthesis during EXPAR [3,69,78]. Although netropsin can suppress *ab initio* synthesis, its biased affinity to A/T-rich regions could be problematic for reaction design, especially during a multiplex detection assay.

Urtel et al. investigated methods to overcome *ab initio* synthesis during EXPAR, starting with decreased enzyme concentrations and the addition of netropsin [69]. Although the *ab initio* synthesis onset time was successfully delayed, it was not fully suppressed with these methods. If

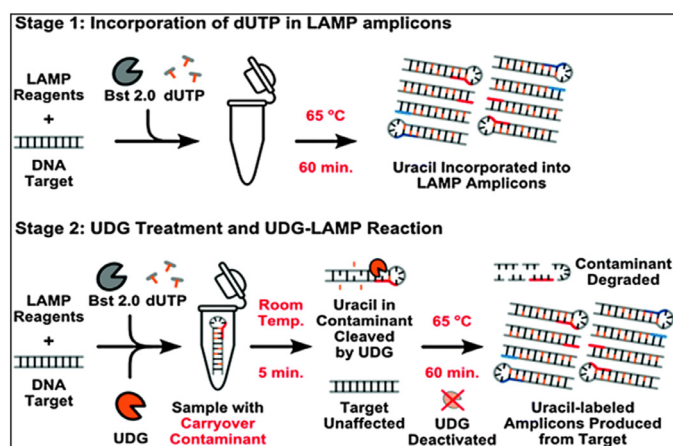


Fig. 7. Working principle of uracil-DNA-glycosylase-supplemented LAMP reaction. LAMP products amplified with dUTPs make DNA products prone to UDG degradation, enabling removal from the LAMP master mix with UDG treatment prior to a fresh LAMP reaction [75].

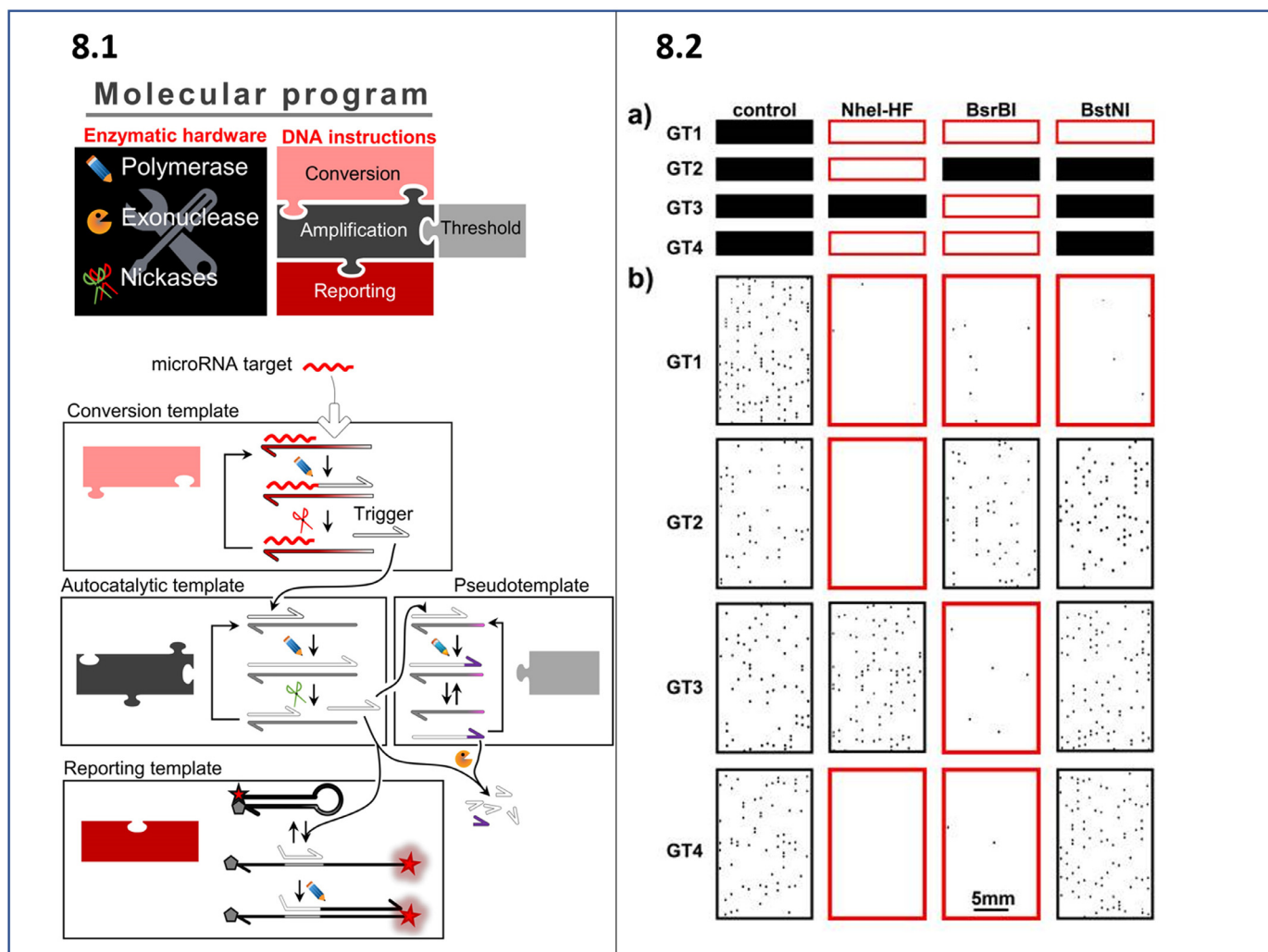


Fig. 8. 1 Background free-EXPAR based miRNA detection with an amplification sink. The system consists of three types of enzymes: a polymerase, an exonuclease, and an endonuclease. The conversion template is primed by the target microRNA to generate a reporter molecule for the autocatalytic template; starting exponential amplification. Signal from the target-independent amplification is suppressed by a pseudotemplate, which leaves the reporter molecules unable to amplify. Both the reporter and the extended reporter molecules are degraded by an exonuclease. Fluorescence signal is produced when the reporter molecule hybridizes to the reporting template and is extended by polymerase [3]. 8.2) Competition network-based RT-LAMP amplification for detection of Hepatitis C viral RNA molecules. Three different restriction enzymes were included in the reaction mix to inhibit amplification of different HCV genotypes by LAMP, which indicates the presence of specific HCV genotypes a) Predicted HCV genotyping pattern b) end-point digital LAMP images. Each row represents a genotype (GT) of HCV RNA and predicted signal inhibition is indicated by a red frame [77].

the template was designed to amplify without dATPs, then *ab initio* synthesis was effectively abolished: without dATPs, *ab initio* synthesis did not occur during the full 5000 minute observation period, while the

reaction that contained dATPs had an *ab initio* DNA synthesis onset time of approximately 300 minutes (Fig. 9). While promising, this method is not applicable to broad sensing applications that would require the use of dATPs.

3. Conclusions

Due to the speed and simplicity afforded by removing the thermal denaturation step, isothermal reactions have become a popular alternative to PCR for applications that require a rapid, low-cost technique. The methods discussed above aim to maintain or improve the speed and simplicity of an isothermal system while emulating the robust output of PCR. As PCR and isothermal amplification strategies share common mechanisms, enhancement strategies used for PCR may foreshadow isothermal enhancement strategies.

Targeted improvements of enzymes for PCR helped drive better PCR-based technologies, and similar tactics could be further employed for isothermal amplification. Hot start polymerases were used for PCR to prevent nonspecific production of DNA at low temperatures, where primer dimers or nonspecific priming were more likely to occur [210].

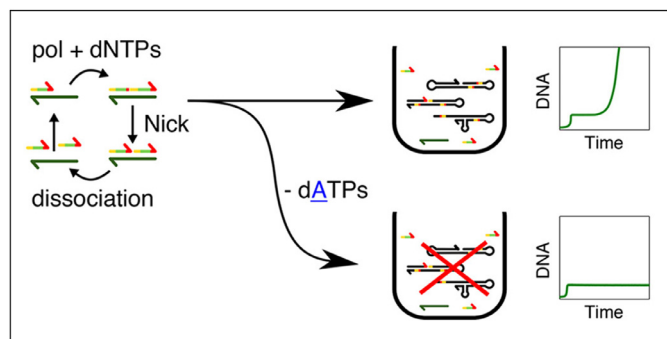


Fig. 9. *Ab initio* synthesis was prevented by removing dATPs from EXPAR reaction mix (rEXPAR). The large, second rise in signal that occurs with dATPs is caused by *ab initio* synthesis [69].

Analogous technologies for warm start polymerases and reverse transcriptases were developed for LAMP, and they are now available through New England BioLabs [211]. These enzymes contain aptamers that reversibly bind the active site, thus inhibiting enzyme activity at low temperatures. Another protein engineering method for PCR optimization was screening mutants of Taq DNA polymerase for resistance to reaction inhibitors [212]; further protein engineering could continue to optimize proteins for a variety of isothermal amplification reactions as well. For example, polymerases that are commonly used for isothermal amplification reactions could be further optimized to resist inhibition, function optimally at a variety of temperatures, or have decreased non-specific activity. Other protein engineering strategies have already been put into use for isothermal amplification enhancement: fusing a helicase and polymerase was shown to increase the product length of HDA reactions, albeit with lower enzyme activity [213]. These enhancement strategies could be improved upon in the future, especially for isothermal reactions that rely on the action of multiple enzymes.

PCR reactions often require optimization of reactant concentrations such as DMSO and magnesium. Likewise, isothermal nucleic acid amplifications also often benefit from individual optimization of each reactant. The many choices of reaction additives above may therefore seem daunting when choosing optimal reaction conditions, especially considering that many of the enhancement effects are dependent on the additive concentration. The choice of each enhancer will be determined by the desired effect and the mechanism of the enhancer, however. For example, detection of GC rich targets or countering excessive primer dimer formation would make it appropriate to choose an additive that reduces secondary structure of nucleic acids. For high temperature reactions, long assays, or assays that use lyophilized agents an enzyme stabilization agent would be appropriate. Reactions that require a synthetic ssDNA template, such as EXPAR, would benefit from template blocking strategies to prevent nonspecific amplification.

The reaction enhancement strategies detailed here can be selected or combined as discussed above to create an optimal detection system that is rapid, sensitive, and specific. Improved target isolation methods and single molecule confinement can also be combined with these optimized detection reactions to create faster, more sensitive sensing platforms. Methods to robustly suppress nonspecific amplification need further development, however, as nonspecific amplification remains a critical challenge for sensing applications that are based on isothermal nucleic acid amplification. This review provides a toolbox of additives that can enhance sensitivity, specificity, robustness, and speed of isothermal nucleic acid amplification reactions. The information provided can be used to guide optimization efforts for sensing applications that require isothermal nucleic acid amplification techniques.

Declaration of Competing Interest

The authors declare no competing interests.

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