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**CRITICAL REVIEW**

# Miniaturized isothermal nucleic acid amplification, a review

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Micro-Total Analysis Systems (μTAS) for use in on-site rapid detection of DNA or RNA are increasingly being developed. Here, amplification of the target sequence is key to increasing sensitivity, enabling single-cell and few-copy nucleic acid detection. The several advantages to miniaturizing amplification reactions and coupling them with sample preparation and detection on the same chip are well known and include fewer manual steps, preventing contamination, and significantly reducing the volume of expensive reagents. To-date, the majority of miniaturized systems for nucleic acid analysis have used the polymerase chain reaction (PCR) for amplification and those systems are covered in previous reviews. This review provides a thorough overview of miniaturized analysis systems using alternatives to PCR, specifically isothermal amplification reactions. With no need for thermal cycling, isothermal microsystems can be designed to be simple and low-energy consuming and therefore may outperform PCR in portable, battery-operated detection systems in the future. The main isothermal methods as miniaturized systems reviewed here include nucleic acid sequence-based amplification (NASBA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), rolling circle amplification (RCA), and strand displacement amplification (SDA). Also, important design criteria for the miniaturized devices are discussed. Finally, the potential of miniaturization of some new isothermal methods such as the exponential amplification reaction (EXPAR), isothermal and chimeric primer-initiated amplification of nucleic acids (ICANs), signal-mediated amplification of RNA technology (SMART) and others is presented.

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## Introduction

Nucleic acid-based detection offers several advantages over traditional microbiological detection as a specific and sensitive

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method for rapid on-site detection of environmental, food-borne, and water-borne pathogens as well as for point-of-care clinical diagnostics.[1–7] A key method in nucleic acid detection is nucleic acid amplification because it generates a large number of target copies, greatly increasing assay sensitivity. While the requirement of a clean environment has limited the ability of amplification reactions to be used outside the laboratory, one solution has been the use of micro- and nanofabrication to reduce amplification reactions to fully enclosed microsystems, which reduce the risk of contamination (Fig. 1). Miniaturization also reduces the volume of expensive amplification reagents, which is important in amplification reactions in which costs are prohibitive to widespread use. In addition to preventing contamination, reducing cost, and simplifying the use of amplification reactions by eliminating the need for laboratory equipment, miniaturization also provides a compact format for running several reactions in parallel. Miniaturized amplification can be integrated with sample preparation and detection in what are known as micro-total analysis systems (μTAS) or labs-on-a-chip (LOC) (Fig. 2), which are designed to perform all of the bioanalytical steps that would normally be carried out in a laboratory on one hand-held chip.[8–13]

The most well known and widely used amplification technique is the polymerase chain reaction (PCR), which relies on thermal cycling and polymerase activity for primer-directed target amplification.[14] PCR is a simple reaction that is a cost-effective choice for laboratories because there are several automated instruments available along with a range of options for real-time detection chemistries.[15] A large number of microsystems for nucleic acid amplification using PCR have been developed since the first by Northrup *et al.* in 1993[16] including several recent examples,[17–24] some at various stages of integration with sample preparation and detection on the same chip. Impressive limits of detection have been achieved and rapid amplification times down to a few minutes have been realized by using microfluidics to optimize thermal cycling. Thermal cycling for on-chip PCR can be provided by rapidly heating and cooling static reactions in microchambers or by re-circulating the reaction in micro-channels across distinct temperature regions. In addition to PCR, reverse transcription polymerase chain reaction (RT-PCR) microfluidic devices for RNA amplification have also been developed.[25,26] Microfluidic PCR has been well reviewed in the literature, including several articles[27–32] and an excellent book chapter.[33] The reader is referred to those sources for information on PCR microsystems.

Limitations of the PCR reaction have led to the development of several alternative isothermal amplification methods.[34] One drawback of PCR is that it requires precision thermal cycling between three temperatures during the reaction. In contrast, isothermal amplification methods do not require thermal cycling and instead use proteins that use *in vivo* mechanisms of DNA/RNA synthesis.[35] Isothermal amplifications are different from PCR in that the rates of enzyme activity are limiting factors rather than the rate of thermal cycling; it is therefore unlikely that microfluidic isothermal amplification reactions can be significantly accelerated as has been achieved by optimizing heat transport in microfluidic PCR.[33] As a result, the systems developed to miniaturize isothermal amplification have used

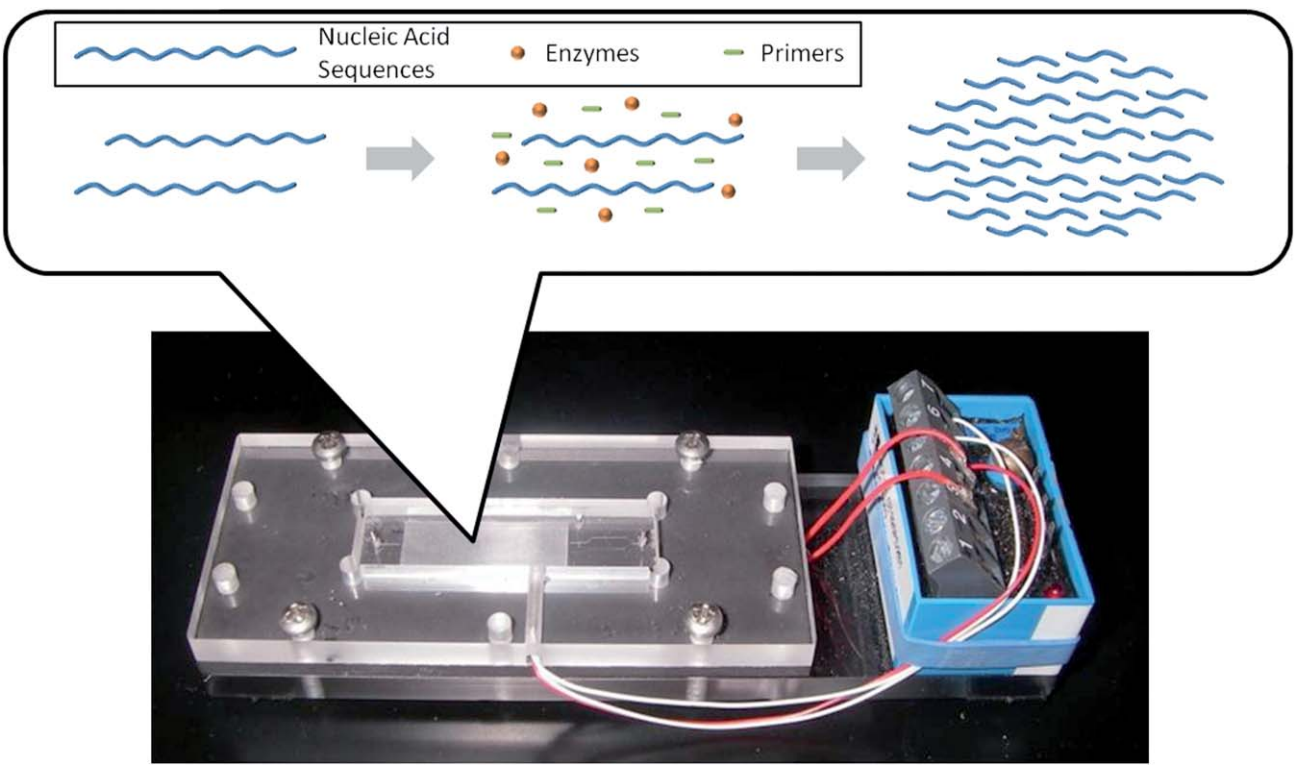


**Fig. 1** The principle of isothermal nucleic acid amplification is illustrated here in a polymer microchip mounted on a hand-held simple heater assembly that is used by the Baeumner group for NASBA reactions. While the mechanisms vary, all isothermal amplification reactions use specific primers and enzymes to synthesize several million to several billion copies of target RNA or DNA sequences at one constant temperature.

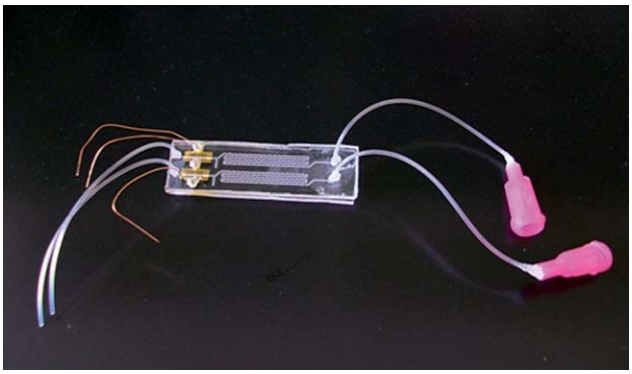

**Fig. 2** A polymer microchip with dimensions 4.5 cm × 1.0 cm is shown here that was developed by the Baeumner group to perform purification, NASBA, and electrochemical detection of pathogenic mRNA.



microchambers with no fluid motion during the reaction. This gives isothermal amplification systems the advantage of a simple design requiring less energy to operate than those that require rapid heating and cooling or re-circulation during amplification, which makes isothermal amplification attractive for use in battery-operated portable detection systems.

This review will first discuss the isothermal amplification microsystem design and will then discuss the miniaturization of isothermal amplification methods including nucleic acid sequence-based amplification (NASBA), loop-mediated amplification (LAMP), helicase-dependent amplification (HDA), rolling circle amplification (RCA), and strand displacement amplification (SDA). Since the focus here is on miniaturization, only the basic biological mechanism of each reaction will be discussed along with references to direct readers to more detailed information on the amplification reactions themselves. Attention is focused on device design and dimensions as well as integration of isothermal amplification with sample preparation and detection toward μTAS.

## Miniaturized isothermal amplification systems

### Design

As mentioned previously, there is no need in isothermal amplification microsystems for re-circulation across temperature zones or rapid heating and cooling mechanisms as in miniaturized PCR systems. This simplifies the design of miniaturized isothermal amplification systems, in which the reaction takes place in a microchamber or microchannel. Constant temperature can be provided by a small heating apparatus such as one using a thin film heater (*e.g.* Kapton® Insulated Flexible Heater) controlled by a thermocouple onto which the amplification microchip is mounted (Fig. 1).

The microsystem can be designed to achieve both parallel and multiplex amplification in a simple and compact format. For parallel analyses, the amplification reaction is split into several separate microchannels and in multiplex analyses, each of those channels can contain different primers for several targets of interest. The world-to-chip interface, which is an important design consideration for ease-of-use and preventing contamination, usually takes the form of a pipette loading port. A self-sealing PDMS septum for the inlets and outlets of microdevices has also been developed.[36] Reservoirs can be included to store and inject the enzymes, primers, and reagents necessary for the reaction and mix them with the sample. The procedure can also be automated to reduce the number of user-directed steps. The ultimate design for an amplification microchip is one in which several parallel reactions are run and primers, enzymes, and reagents are all pre-stored on the chip.

A complete μTAS for nucleic acid detection in biological samples first includes sample preparation steps prior to amplification such as cell pre-concentration and cell lysis to extract nucleic acids from pathogenic cells followed by purification to remove cellular debris and inhibit RNA or DNA degrading enzymes. This often takes place in chambers and channels upstream from the amplification chamber. There have been several advancements in microfluidic sample preparation methods that have been the subject of recent reviews[37–39] to which the reader is directed because a detailed discussion of that topic is beyond the scope of this paper. Following purification, nucleic acid targets are amplified for subsequent detection by one of several microchip methods that include electrochemical[40] and optical[41,42] systems as well as electrophoresis.[43] Detection can take place either directly in the amplification chamber as in real-time methods or in separate channels and chambers down-stream from the amplification chamber.

### Amplification reaction

The choice of isothermal amplification reaction is primarily driven by the target of interest. For this reason, amplification microsystems usually adapt already established laboratory methods or commercial kits where primers have already been designed for target DNA or RNA sequences and the reaction has been optimized. Factors such as amplification time, reaction temperature, tolerance to substances in biological samples, length of the target, whether an initial heat denaturation step is required or not, and complexity (*i.e.* the number of enzymes and primers required) may also be taken into consideration when choosing an amplification reaction.

### Surface effects

While there are several advantages to miniaturized nucleic acid amplification that were indicated previously, several challenges need to be addressed in adapting amplification reactions to the microscale. The foremost challenge is the interaction of biomolecules with microfluidic channel walls. The extremely high surface area-to-volume ratio of microchannels can lead to a high incidence of non-specific adsorption and surface effects,[44–46] which can limit or inhibit reactions such as amplification reactions where maintaining primer and enzyme concentrations is vital. In microfluidic PCR devices, it was found that the adsorption of Taq polymerase on untreated microchannel surfaces inhibited the reaction.[47]

The large increase in surface area-to-volume ratio as systems are reduced to microscale dimensions is demonstrated in Table 1. The example shows that in a typical 1.5 mL

 

**Table 1** The large increase in surface area-to-volume ratio is demonstrated as a 20 μL (0.02 $cm^3$) reaction volume in a microcentrifuge tube is reduced to microscale vessels with the same volume

| Reaction vessel | Dimensions | Surface area | Surface/volume ratio |
|---|---|---|---|
| 1.5 mL microcentrifuge tube | Half-sphere[a] (4.2 mm diameter) | 0.3 $cm^2$ | 14 $cm^{-1}$ |
| Microchamber | 1.4 cm × 1.4 cm × 100 μm | 4 $cm^2$ | 200 $cm^{-1}$ |
| Microchannel | 2 m × 100 μm × 100 μm | 8 $cm^2$ | 400 $cm^{-1}$ |

[a] The bottom of a 1.5 mL microcentrifuge tube occupied by the reaction volume was approximated as a half-sphere with a volume of 20 μL.



microcentrifuge tube, a 20 μL volume (typical for NASBA reactions) is in contact with approximately 0.3 $cm^2$ of the tube surface while in a microfluidic channel 100 μm × 100 μm, the channel surface in contact with the same volume increases almost 30-fold to 8 $cm^2$.

In order to make microfluidic devices biocompatible, several strategies have been employed to treat microchannel surfaces to prevent biomolecule adsorption[48,49] either by permanent surface modification or by using molecules to block the surface of channel walls. Permanent modification includes polymer coatings such as polyethylene glycol (PEG)[50] and linear polyacrylamides (LPAs),[51] while a commonly used blocking molecule is bovine serum albumin (BSA).[52] The selection of an appropriate surface treatment, if necessary, is an important design consideration in amplification microsystems. The necessity of surface treatment for NASBA microsystems will be discussed in subsequent sections, as it may be that the impact of non-specific adsorption is greater on amplification reactions that rely on the co-operation of several enzymes.

### Fabrication

High fabrication costs were a challenge early on in making microsystems for biological analysis economically feasible. The first microfluidic systems were fabricated in silicon, quartz, and glass using microelectronic fabrication methods, but the need to prevent contamination by using disposable devices made from durable materials at low cost has led to the use of polymers and a variety of polymer microfabrication methods.[53,54] Thermoplastic microfluidic devices such as those in poly(methyl-methacrylate) (PMMA), polycarbonate (PC), polystyrene (PS), and cyclic olefin copolymer (COC) have been fabricated using methods including hot embossing,[55] laser micromachining,[56] and micro-injection molding[57] and are sealed by one of several bonding techniques.[58] Microdevices in polydimethylsiloxane (PDMS), an elastomer, can be fabricated by soft-lithography.[59–61] Another cost-effective method for microdevice fabrication uses thin, flexible films.[62]

It is important that walls of microchannels are made as smooth as possible during the fabrication process. While the increase in surface area created by surface irregularities during fabrication may be negligible on the macroscale, it becomes increasingly important as a system is scaled down and increases the surface effects discussed above. Sharp corners and junctions should also be avoided as they are regions where biomolecules could aggregate. The material and bonding method should be designed to be able to withstand the reaction temperature.

## Miniaturized isothermal amplification methods

### NASBA

Nucleic acid sequence-based amplification (NASBA)[63] is an isothermal amplification reaction mimicking retroviral RNA replication.[64] NASBA is specific for target RNA sequences and has been gaining popularity because it has been shown to have a wide range of applications for pathogen detection in clinical, environmental, and food samples.[65] It is also convenient to use with commercially available kits.[66–68] As an alternative to RT-PCR for RNA amplification, NASBA has a larger amplification factor and the advantage of not requiring thermal cycling.[69] Held at 41 °C, the reaction can produce about one billion RNA that are complimentary to the target RNA in 1.5 hours using two primers specific to the target RNA and three enzymes: reverse transcriptase, RNase H, and T7 DNA-dependent RNA polymerase (Fig. 3). During the reaction, the forward primer hybridizes to any target RNA present in a sample. The enzymes reverse transcriptase (RT) and RNase H along with the reverse primer then produce a dsDNA with the target sequence and a T7 promoter. T7 DNA-dependent RNA polymerase (DdRp) uses this dsDNA to produce many RNA strands complementary to the original target RNA. After this initial phase of NASBA, each newly synthesized RNA can be copied in a cyclical phase, resulting in an exponential amplification of RNA complimentary to the target.[70] The single-stranded RNA product can be detected directly with hybridization-based methods such as lateral flow assay,[71] electrochemiluminescence,[72–75] and microfluidic electrochemical detection.[76] Real-time NASBA using molecular beacons[77] is also a method that is widely used. The isothermal nature, the specificity for RNA *versus* DNA, and the immense amplification capability of NASBA make it an important method in RNA research and RNA diagnostics.

NASBA on a chip was demonstrated in 2004 by Gulliksen *et al.*[78] with real-time detection of 1.0 μM human papillomavirus (HPV) 16 at reaction volumes as low as 10 nL in 41 °C heated silicon–glass microchambers. While the NASBA reaction mixture was prepared by hand off-chip prior to being added to the device for incubation and detection, these results, which compared well with conventional NASBA methods with only 1/2000 the reaction volume, indicated for the first time the feasibility and potential use of NASBA in a miniaturized detection system. In order to prevent adsorption of molecules to chamber walls, it was necessary to use Sigmacote® and yeast tRNA. The devices were also used only once to prevent contamination.

This first NASBA device was followed up by a device fabricated in COC for the real-time NASBA detection of mRNA from SiHa cells with 1–2 copies HPV 16 DNA per cell with a detection

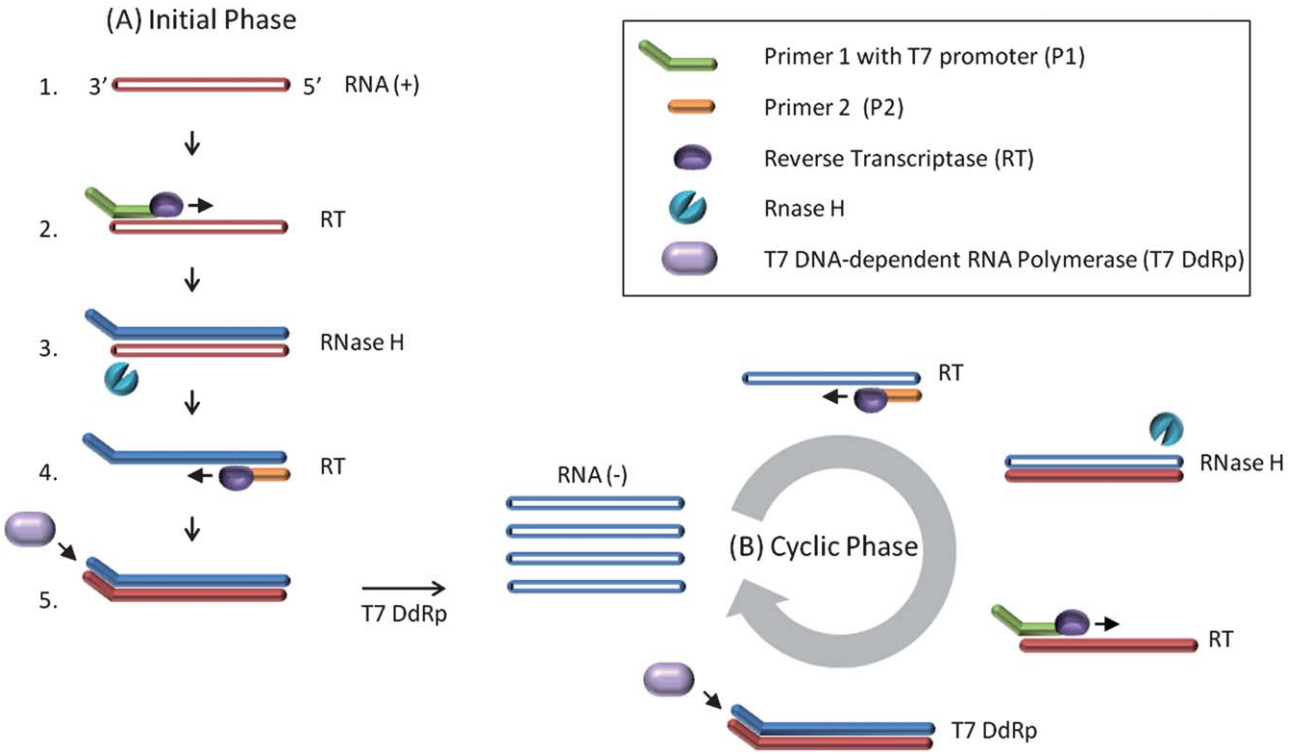


**Fig. 3** NASBA is shown. (A) The initial phase: (1) a target RNA; (2) reverse transcriptase extension of P1 that contains a T7 promoter (angled region) creates a cDNA–RNA hybrid; (3) RNase H degrades the RNA; (4) reverse transcriptase extends P2 to copy the newly synthesized cDNA; (5) dsDNA with T7 promoter is a template for T7 DNA-dependent RNA polymerase to generate complimentary RNA. (B) In the cyclic phase, each newly synthesized RNA can be copied, leading to exponential amplification.

limit of 20 cells per μL.[79] In an effort toward a multiplex NASBA device, the chip featured ten parallel reaction channels that filled simultaneously when the NASBA mixture was added to the device and remained segregated throughout the reaction. A PEG coating was required to prevent adsorption on channel walls. Furthering the development toward a complete, disposable NASBA microchip detection system, it was shown that NASBA reagents, primers, and enzymes can be dried and stored in COC microchannels and re-hydrated by addition of sample to begin amplification and analysis.[80]

The first integrated system combining RNA isolation, NASBA amplification, and real-time detection on a single chip was developed in 2008 by Dimov *et al.*[81] On this PDMS chip with 80 μm tall chambers and connecting channels, solid phase extraction of RNA from cell lysate in a 0.25 μL chamber containing silica beads was followed by NASBA amplification of tmRNA in a 2 μL 41 °C heated reaction chamber. To prevent inhibition of NASBA, the chamber walls were blocked with BSA prior to the reaction. The response from the NASBA reaction, as observed by the real-time monitoring of molecular beacons, was very rapid at less than three minutes. The time for the entire analysis of 100 *E. coli*, from input of sample to result, was 30 minutes.

## LAMP

Loop-mediated isothermal amplification (LAMP) uses two sets of primers and a DNA polymerase with strand displacement activity to amplify a target one billion times in less than an hour at a temperature around 60 °C.[82] The amplification is highly specific because four primers recognize six separate regions of the target sequence in order for the amplification to proceed. Rounds of displacement DNA synthesis initiated by the primer sets produce stem-loop DNAs with several inverted repeats of the target sequence (Fig. 4). The addition of a primer set that anneals to the LAMP amplicon loop structure increases efficiency and specificity of the reaction.[83] LAMP has been applied to detect a variety of pathogens including viruses and bacteria and can also amplify RNA with the addition of reverse transcriptase.[84] The byproduct pyrophosphate that is produced during the reaction and forms a white precipitate can be used to detect positive reactions both by visually observing the precipitate[85] and quantitatively by measuring the turbidity change for real-time analysis.[86] LAMP products have also been detected electrochemically in a microchip.[87]

Hataoka *et al.* combined LAMP with electrophoresis in a simple cross-channel PMMA microchip in 2004.[88] The 10 μL LAMP reaction was prepared by hand prior to loading into a heated reaction well on the chip. Following amplification at 65 °C, on-chip electrophoresis was performed in the 100 μm wide, 30 μm deep cross-channel and successful amplification of an initial concentration of 23 fg $\mu L^{-1}$ prostate specific antigen (PSA) cDNA in 15 min was shown.

Another PMMA micro-reactor for LAMP incorporated a different approach for detection, taking advantage of the turbidity change caused by the pyrophosphate byproduct of the

 

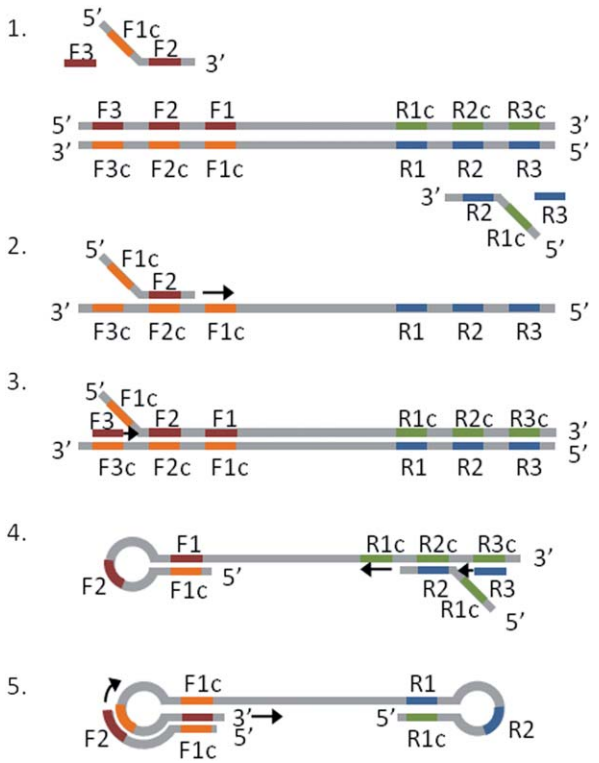


**Fig. 4** LAMP initial step is shown. For clarity, only the process initiated by the forward primer set is shown. (1) Primer design with inner (F1c–F2 and R1c–R2) and outer (F3 and R3) primer sets flanking the target DNA. (2) F2 region of inner primer hybridizes to F2c and is extended by polymerase. (3) Outer primer F3 hybridizes to F3c and its extension causes strand displacement. (4) Displaced strand forms loop at one end by F1c hybridizing to F1. At other end, inner primer R1c–R2 is extended and then displaced by extension of primer R3. (5) The resulting structure with loops on both ends enters the cycling step and a stem-loop structure with several inverted repeats of the target DNA is produced by repeated elongation and strand displacement.

reaction. This device by Lee *et al.* in 2007[89] consisted of a 25 μL micro-reactor with an external temperature controller to heat to 65 °C and a mini spectrometer for real-time monitoring and quantification. Hepatitis B virus (HBV) DNA was amplified and detected on the chip in 60 minutes. The detection of HBV DNA isolated from clinical serum samples has also been shown using this LAMP microchip.[90]

Fang *et al.* developed a microfluidic device for running several LAMP reactions in parallel on a single chip that can be used in multiple, high-throughput analysis.[91] The PDMS device featured eight parallel reaction chambers 1 mm × 0.8 mm × 0.6 mm, each holding a reaction volume of 5 μL. After sample was added to each chamber and LAMP reaction mixture was drawn in by capillary force, the chambers were sealed with uncured PDMS to prevent evaporation, contamination, and bubbles. Detection was by monitoring pyrophosphate production in each heated channel during the 63 °C LAMP amplification, which could be visualized by the naked eye in positive reactions or could be quantified by measuring absorbance. Amplification and detection of concentrations as low as 10 fg $\mu L^{-1}$ pseudorabies virus (PRV) DNA was shown in parallel with a negative control on the chip in less than 1 hour. LAMP also has much potential for further miniaturization as has been demonstrated by the successful LAMP amplification of single DNA templates encapsulated in polyacrylamide (PAA) microchambers as small as 1.5 μm tall × 17.5 μm diameter.[92]

## HDA

Helicase-Dependent Amplification (HDA) is an isothermal amplification reaction based on the natural mechanism of the DNA replication fork.[93] DNA helicase unwinds dsDNA to allow hybridization of target specific primers that are extended by DNA polymerase to produce two dsDNA target copies (Fig. 5). Each of those products can then be copied, which results in exponential amplification of the target. HDA has been used with lateral flow in assays on real samples to detect HIV-1 in human plasma[94] and *C. difficile* in fecal samples.[95] Since its introduction, advancements in helicase selection and the efficiency of helicase–polymerase fusion proteins have improved speed and processivity, allowing for the amplification of DNA fragments up to 2.3 kb compared to the original limit of 400 bp.[96] The simple reaction, when also combined with other advancements such as the ability to detect RNA with reverse transcription, gives HDA much potential for use in a portable nucleic acid detection system.[97]

Andresen *et al.* incorporated HDA on a microarray in an effort toward a LOC for rapid diagnostics.[98] One of the HDA primers was immobilized directly on the microarray surface while the labeled second primer was added with the HDA reaction mixture and sample. The labeled amplification product remained anchored, allowing for subsequent quantitative analysis. The detection of *N. gonorrhoeae* and *S. aureus* in single- and duplex-format on the HDA chip was shown in 2 hours.

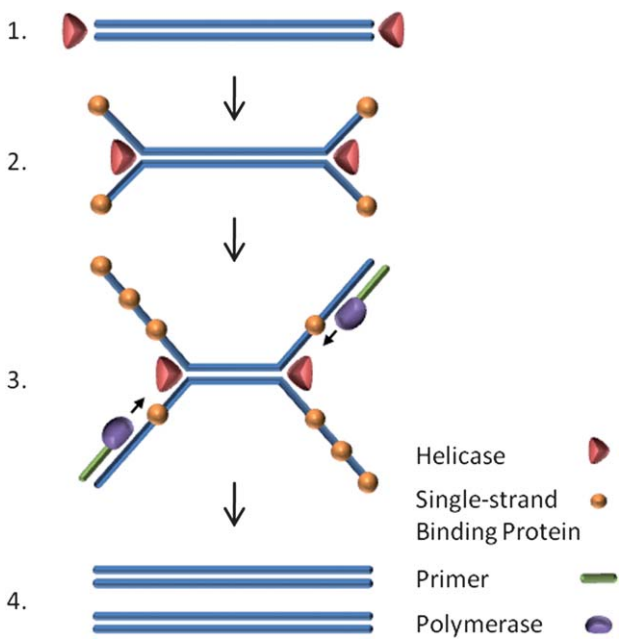


**Fig. 5** HDA is shown. (1) Helicase unwinds dsDNA. (2) As helicase unwinds DNA, the strands are stabilized by single-strand binding proteins. (3) Primers hybridize to the target DNA and are extended by polymerase. (4) A double-stranded copy of the target DNA is produced.

Microfluidic chips have also been developed for HDA. Ramalingam *et al.* designed a real-time HDA microfluidic device fabricated in PDMS and sealed with glass that had four parallel 5 μL microchambers fed by microchannels from a single pipette loading port.[99] No pumps or valves were required as all flow was by capillary action and an absorbent pad prevented cross-contamination between microchambers during amplification. An interesting feature of this design is that the HDA primers were dried onto the glass surface during fabrication so that each microchamber was pre-loaded with HDA primers. This approach allows for simple multiplex analysis of one sample if different primer pairs are pre-loaded into each microchamber. The microfluidic chip was demonstrated by successful HDA at 62 °C and a real-time quantification of 0.1 ng $\mu L^{-1}$ SARS cDNA and was also shown using smaller 192 nL microchambers.

A fully integrated microfluidic device that was the first to combine sample preparation and real-time HDA on the same chip starting from whole bacteria was developed by Mahalanabis *et al.*[100] The disposable cyclic olefin polymer (COP) device used flap valves to separate chambers and could run three simultaneous reactions as well as a control. DNA extracted from lysed bacteria in a micro-solid phase extraction (micro-SPE) chamber was amplified at 65 °C in 25 μL reaction chambers with fluorescent reporters. The detection of *E. coli* was demonstrated on the device in 50 min from the time of sample input.

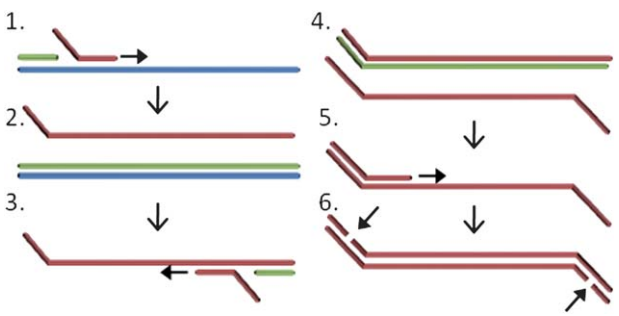


**Fig. 6** SDA initial steps are shown. For clarity, the process is shown only in the forward direction. (1) Forward primer (red) with restriction site sequence (angled region) and forward bumper primer (green) hybridize to ssDNA. (2) Extension of bumper primer releases the primer extension product (red). (3) Reverse primer and reverse bumper primer hybridize to primer extension product. (4) Primer extension releases the single-stranded product. (5) Forward primer hybridizes to this product and extension forms dsDNA with nickable restriction sites on each end. (6) Nicking and strand displacing polymerase extension from nick sites (arrows) release ssDNA that enters into exponential phase while regenerating the dsDNA for repeating rounds of nicking, extension, and displacement.

## SDA

Strand displacement amplification (SDA) uses the activity of a restriction endonuclease and a strand displacing DNA polymerase to generate copies of a target DNA sequence.[101,102] After an initial heat denaturation to separate dsDNA, the extension of SDA primers containing restriction sites and their displacement by extension of flanking bumper primers produce double-stranded target sequences flanked by nickable restriction sites (Fig. 6). These sites are nicked and the free 3′ end is extended, displacing a single-stranded copy and regenerating the dsDNA. Each ssDNA product can be copied for an exponential $10^{10}$-fold amplification of the target. SDA amplicon can be detected in real-time and the reaction has been used in assays to detect infectious diseases including a widely used kit for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.[103]

Burns *et al.* described a nanoliter glass–silicon device for DNA detection using SDA and electrophoresis with an amplification time of 17 minutes at 50 °C.[104] A droplet of SDA restriction enzymes and polymerase loaded from one microchannel was metered by hydrophobic patches and mixed with a droplet containing DNA template and primers from another channel to form a 240 nL total reaction volume. Yang *et al.* developed an automated stacked microlaboratory with several assay capabilities that combined sample preparation with SDA and detection for analysis starting with intact whole cells.[105] The device was demonstrated by detecting SLT1 gene from *E. coli* in 2.5 hours first by dielectrophoretic concentration and lysing of bacteria followed by a 60 μL SDA reaction at 60 °C in a PS microwell and detection by hybridization with fluorescent DNA probe.

In another design, anchored SDA on a microarray chip with immobilized amplification primers allowed for multiplex detection reactions on the chip with shared reagents[106,107] and the stability of anchored SDA product was improved with the addition of non-amplifiable primers[108] that cannot be nicked and improved detection signals 20-fold.

## RCA

Rolling circle amplification (RCA) uses a strand displacing DNA polymerase to continuously amplify a circular DNA template at a constant low temperature, producing a long DNA molecule with tandem repeats of the circular template.[109] This method can be used to amplify padlock probes, which are designed to circularize upon binding to a target sequence and seal by


**Fig. 7** RCA of a padlock probe in the presence of a DNA target. (1) Padlock probe anneals to ssDNA target, forming a circle with small gap that is sealed by ligation. (2) RCA is initiated by polymerase extension of primer. (3) Continuous strand-displacing synthesis forms a long DNA product with tandem repeats of the padlock probe that collapse into a sphere.

 

ligation.[110] RCA of padlock probes (Fig. 7) results in a long DNA product for each recognized target that collapses into a sphere that can be tagged and analyzed for single molecule detection. This has been done using a microfluidic channel to count individual RCA products.[111] RCA has also been used to amplify DNA viruses with circular genomes without using specific primers[112] and to prepare high quality templates for DNA sequencing.[113] The reaction has also been combined with magnetic beads and reporter DNA probes in a sandwich assay to detect viral DNA.[114]

Mahmoudian *et al.* introduced a PMMA chip with a 10 μL well for RCA connected to a 100 μm × 30 μm cross-channel for electrophoresis.[115] The detection of *V. cholerae* isolated from clinical samples was shown by RCA amplification of a padlock probe at 37 °C directly followed by electrophoresis in less than 65 min. This microchip electrophoresis has also been applied to detect product from circle-to-circle amplification (C2CA), an amplification method based on RCA.[116]

Reiß *et al.* performed a surface-tethered RCA from a DNA primer immobilized on a 20 nm fluorescent bead in a flow cell and



**Table 2** Summary of isothermal amplification microsystems

| Amplification | Microdevice design | Target | Ref. |
|---|---|---|---|
| NASBA | 10 nL and 50 nL silicon–glass microchambers, real-time molecular beacon detection | HPV 16 ssDNA, 118-bp artificial ssDNA | 78 |
| | Ten parallel 80 nL COC microchannels with shared inlet, real-time molecular beacon detection | HPV 16 mRNA | 79 |
| | Parallel 500 nL COC microchannels with dried NASBA enzymes, real-time molecular beacon detection | HPV 16 ssDNA | 80 |
| | 2 μL PDMS microchamber integrated with on-chip RNA isolation, real-time molecular beacon detection | 363-bp *E. coli* tmRNA (*SsrA*) fragment | 81 |
| LAMP | 10 μL well on PMMA microchip, SYBR Green and on-chip electrophoresis | 136-bp PSA cDNA fragment, 132-bp λ-DNA | 88 |
| | 25 μL disposable PMMA micro-reactor, real-time optical detection of turbidity change | HBV DNA fragment (polymerase gene) | 89 and 90 |
| | Eight separate parallel 5 μL PDMS microchannels, naked eye and real-time optical detection of turbidity change | 188-bp PRV DNA fragment (DNA-binding protein gene) | 91 |
| HDA | Multiplex microarray with immobilized primers, fluorophore labeled primers detected by laser scanner | *S. aureus* (*mec A*), *N. gonorrhoeae* ($piv_{Ng}$) DNA | 98 |
| | Four parallel 5 μL PDMS–glass microchannels with pre-loaded dried primers, real-time detection of SYBR Green/EvaGreen | 78-bp SARS cDNA (BNI-1) fragment | 99 |
| | Three 25 μL chambers on disposable COP microfluidic chip with on-chip cell lysis and RNA purification, UV illumination to visualize EvaGreen/ROX fluorescence | 70-bp *E. coli* DNA (*dxs*) fragment | 100 |
| SDA | 240 nL silicon–glass microchannel that mixes enzyme with sample, SYBR Green and on-chip electrophoresis | 106-bp *M. tuberculosis* DNA fragment | 104 |
| | 60 μL reaction in PS microwell incorporated into automated integrated microlaboratory with cell lysis, concentration, and purification; fluorescent reporter probe detection | 75-bp *E. coli* DNA (SLT1) fragment | 105 |
| RCA | 10 μL well on PMMA microchip, SYBR Gold and on-chip electrophoresis | *V. cholerae* 16S rRNA | 115 |
| | 0.5 μL glass microchannel with microbead-tethered amplification, fluorescent reporter probe detection | *Salmonella* 16S rRNA | 118 |
| RPA | 30 parallel 10 μL COC microchambers in automated device with pre-stored liquids and dry reagents, real-time fluorescent detection of reporter probe | 420-bp *S. aureus* DNA (*mec A*) fragment | 120 |

were able to stretch and visualize a 15 μm long anchored ssDNA product by passing an air bubble by it.[117] Surface-tethered RCA was used by Sato *et al.* to develop a fully integrated RCA microchip using padlock probes hybridized to 34 μm diameter beads to serve as capture oligonucleotides and surfaces for RCA.[118] A microchannel 210 μm wide, 40 μm deep holding a volume of 0.5 μL was fabricated in glass with a dam structure to hold the beads in place during several wash steps, injection of RCA reagents, and injection of fluorescent probe for detection. The reaction temperature was 30 °C and operation of the RCA microchip was demonstrated by detection of *Salmonella*.

### Additional isothermal amplification methods

Recombinase polymerase amplification (RPA) is a low temperature isothermal method to amplify specific DNA using recombinase, a DNA polymerase and DNA-binding proteins; all of which are necessary in the cell for DNA synthesis, recombination, and repair.[119] At 37 °C, recombinase–primer complexes promote primer binding at the target sequence of dsDNA and displacement of the non-template strand. The displaced strand is stabilized by single-strand DNA binding proteins and the primer is extended by strand displacing DNA polymerase. The two dsDNA products can each be copied, resulting in exponential amplification. RPA has been applied to a variety of targets in DNA down to less than 10 copies and amplified targets have been detected by using a nucleic acid dye for real-time analysis, a specific fluorophore/quencher probe, and a lateral flow assay.

Lutz *et al.* have developed a fully automated COC chip for RPA that has all liquids and dry reagents for the amplification pre-stored on the chip and can run up to 30 reactions simultaneously in separate 10 μL microchambers arranged in a circular pattern that allows for controlling fluid flow between chambers by spinning the chip at different frequencies.[120] DNA sample was added directly to each microchamber and the automatic sequence started with stored RPA buffer dissolving dried RPA reagents and primers. Operation of the chip was demonstrated with detection of the antibiotic resistant gene *mecA* of *S. aureus* in a total time of 20 minutes. A BSA wall coating helped to prevent non-specific adsorption.

Multiple displacement amplification (MDA) is an isothermal method used in whole genome amplification from few DNA copies by the strand displacement activity of $\phi$29 DNA polymerase and multiple random primers.[121] Marcy *et al.* showed that 60 nL microfluidic MDA was more specific than standard 50 μL reactions in single-cell genome amplification of isolated individual *E. coli*.[122] Microsystems based on MDA could be useful in forensics, clinical diagnosis, and sequencing single cells.

Along with the above mentioned techniques, there are numerous additional isothermal amplification methods that could be useful in future microsystems; a few will be mentioned here to demonstrate the wide range of available technologies. Isothermal exponential amplification reaction (EXPAR) can amplify a 10–20 bp trigger oligonucleotide generated from a genomic target more than $10^6$ times in less than 10 minutes at 55 °C by repeating cycles of polymerase and endonuclease activity[123,124] and has been coupled with DNA-functionalized gold nanospheres for the detection of herpes simplex virus.[125] Isothermal and chimeric primer-initiated amplification of nucleic acids (ICANs) amplify target DNA at 55 °C using a pair of 5′-DNA-RNA-3′ primers and the activity of RNase H and strand-displacing polymerase.[126] Signal-mediated amplification of RNA technology (SMART) produces many copies of an RNA signal at 41 °C in the presence of an RNA or DNA target by way of the three-way junction formed between the target and two probes, one of which contains the RNA signal sequence and a T7 promoter sequence for T7 RNA polymerase.[127] The single-stranded RNA product can be detected by hybridization-based methods and because the signal is independent of the target, SMART can be easily adapted for detection of different target sequences. Cyclic enzymatic amplification method (CEAM) detects nucleic acids in the picomolar range in less than 20 minutes at 37 °C using a displacing probe and Exonuclease III (Exo III) to generate amplification of fluorescent signal in the presence of a target.[128] Isothermal target and signaling probe amplification (iTPA) combines the principle of ICAN and the inner–outer probe concept of LAMP along with fluorescence resonance energy transfer cycling probe technology (FRET CPT) for simultaneous target and signal amplification in 90 minutes at 60 °C and has been shown to detect *Chlamydia trachomatis* at single copy level.[129]

## Conclusions

This overview of miniaturized isothermal amplification, summarized in Table 2, demonstrates the potential of these amplification methods in a μTAS for on-site detection. With no need for thermal cycling, isothermal microsystems can be designed to be simple and low-energy consuming and therefore may outperform PCR in portable, battery-operated detection systems. It should be noted that low temperature isothermal amplification (*i.e.* NASBA, RCA, and RPA) requires less energy to operate than higher temperature isothermal amplification (*i.e.* LAMP and HDA), an advantage to consider when designing a microsystem for use with limited resources. Miniaturized isothermal amplification gives researchers more options when designing microsystems for nucleic acid analysis because there are numerous existing methods and commercial kits currently available for pathogen detection that use isothermal amplification. The significant volume reduction achieved by miniaturization may make some of these methods more cost efficient and lead to their wider use.

While there is a great potential for systems incorporating miniaturized isothermal amplification, there is still much room for improvement in pre-concentration and on-chip sample preparation prior to the amplification reaction. Real samples that would need to be analyzed by a μTAS include dirty water and clinical specimens. Complex matrices are also particularly challenging when analyzing food samples.[130] In the examples reviewed here, most systems used clean spiked samples in proof of design, but for a μTAS to be practical it must have the capability of analyzing real samples and future systems will need to continue to address this area. While this task is complex, sample preparation methods developed for any nucleic acid amplification reaction will most likely be adaptable to all other methods.

Toward an easy-to-use disposable μTAS, of particular interest are nucleic acid amplification microchips in which reagents,

 

enzymes and primers are pre-stored on the chip. These devices, a few examples of which were discussed, reduce user error and potential sources of contamination. It also provides a method for multiplex detection if different primer pairs are loaded in different reaction chambers on the chip. One can envision pre-packaged disposable nucleic acid amplification chips that can be incorporated into an automated μTAS.

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