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Isothermal Amplification and Quantification of Nucleic Acids and its Use in Microsystems

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Abstract

Nucleic acid amplification technologies (NAATs) offer the most sensitive tests in the clinical laboratory. These techniques are used as a powerful tool for screening and diagnosis of infectious diseases. Isothermal methods, as an alternative to polymerase chain reaction (PCR), require no thermocycling machine and can mostly be performed with reduced time, high throughput, and accurate and reliable results.

However, current molecular diagnostic approaches generally need manual analysis by qualified and experienced personal which is a highly complex, time-consuming and labor-intensive task. Thus, the demand for simpler, miniaturized systems and assays for pathogen detection is steadily increasing. Microfluidic platforms and lab-on-a-chip devices have many advantages such as small sample volume, portability and rapid detection time and enable point-of-care diagnosis.

In this article, we review several isothermal amplification methods and their implementation in microsystems in relation to quantification of nucleic acids.

Introduction

The idea of miniaturization of available technologies was created by Richard P. Feynman in his famous lecture in 1959, who started the ball rolling [1]. In 1982, Petersen described the entire potential of microsystem technology, and in the following years and decades a wealth of concepts for microsystems, possible applications and new miniaturized systems were developed by today [2]. This technology can combine electronic and non-electronic functions and achieve new mechanical, optical, fluidic, electronic functionalities or miniaturize already established applications. When integrating sensors and actuators a direct analysis can bring decisive advantage [3]. The microsystems in medicine and biology often belong to one of the four important areas of application: diagnostics, drug delivery, minimally invasive surgery, or neural prosthetics and tissue engineering [4]. Concerning the diagnostic area there are three categories, most of the test systems are based on: paper-based analytical devices, lab-on-chip, and micro total analysis system (µTAS) [5].

Miniaturized systems for the analysis of genetic sequences are a major cornerstone for the development and marketability of point-of-care (POC) devices [6]. These devices can be characterized by a high integration of several functionalities onto a single platform, e.g. cumbersome lab processes like sample preparation, nucleic acid amplification and detection. An integrated microfluidic device, for example, incorporates many of the required components of a typical room-sized laboratory on a small chip [7]. They offer the possibility to leave a clean lab environment, because one major target is the incorporation of as many necessary reagents as possible on the device. Therefore, contaminations are greatly reduced.

Microsystems lead to portable, easy-to-use devices, which might also be applied by lay users. These systems have shown that the analysis of samples can be moved from a specialized laboratory closer to the patient, aiming at POC, or even to in-field testing [8,9].

However, the key areas of application for integrated and miniaturized systems can be diverse. The majority of publications are focussed on clinical diagnostics including foodborne organisms for environmental monitoring [10-12]. In case of an infectious disease, cultivation and phenotypic characterization are still the standard methods of microbiologists which need days up to weeks to obtain a diagnostic result. Hence, scientists started to make use of nucleic acid amplification methods to accelerate the analysis. The most widespread and well known technique for this purpose is the polymerase chain reaction (PCR) [10,13], which exploits the activity of the DNA polymerase. The method is based on a thermal cycling process consisting of repeated cycles of heating and cooling steps allowing for DNA melting, sequence specific primer binding and enzymatic amplification of the target nucleic acid. The quantitative assessment of target copies is possible by using a real-time PCR approach, where a concentration dependent signal (e.g. fluorescence) increases over time [14]. The latter enables the user to easily assess the pathogen load of patient samples [15-18]. Since the first example of a PCR on a miniaturized system in 1994 [19,20], numerous devices were presented, which integrate not just the amplification, but also sample preparation and detection steps [9,21-28]. Just a few of those microsystems have reached the market so far, but some have shown a fascinating potential to replace the classical laboratory-oriented state-of-the art [29-33].

Miniaturization of biochemical reactions leads to major advantages, where a lower amount of valuable clinical sample and reagents is just one: approximately 1/1000 of the volume used in conventional lab

setups has been shown to obtain a similar result [34]. A smaller heat capacity allows for rapid changes in temperature beneficial for the PCR time as well as a higher parallelism of multiple genetic samples [34].

However, a major drawback of the integration of PCR to microsystems is the necessity of sophisticated instrumentation. The reaction requires a thermal cycling instrumentation, space and considerable expertise [35]. Therefore, isothermal reactions for the target specific amplification are alternative and valuable methods for the simplification of diagnostic devices.

This review will discuss different isothermal amplification methods for nucleic acids and their integration into miniaturized systems. Some already published reviews gave a broad overview of that field [36-39], however, we will have a special view on the most recent strategies to amplify and quantify target molecules in samples. Since the focus of this article is the integration of these technologies into miniaturized systems, the molecular background of the isothermal methods is just briefly described. Comprehensive references will direct the reader to the more detailed descriptions of the biology behind the amplification reactions.

Strategies to Miniaturize Isothermal Amplification Reactions

LAMP: Loop-mediated isothermal amplification

The loop-mediated isothermal amplification LAMP was firstly described in 2000 and developed by the Japanese Eiken Chemical Co, Ltd. (Tokyo) [40]. Various publications on LAMP describe it as a very promising tool for the use in microsystems. In principle, the reaction employs a DNA polymerase with strand displacement activity and 4 or 8 specially designed primers that recognize 6 or 8 distinct sequences on the target DNA under isothermal conditions (60-65°C). On average, the reaction runs for about 60 minutes [40-42], showing an extremely high specificity [43]. Also, LAMP method has a high amplification efficiency that allows for the synthesis of large amounts of DNA in a short time. Its detection limit is a few copies per reaction and therefore is comparable to that of PCR [40]. For the assay performance, only a heating block at a constant temperature or a water bath is necessary. Furthermore, LAMP can be adapted to RNA templates in combination with a reverse transcriptase under the same working temperature and without additional time for transcription [44].

LAMP amplification products can be detected either by gel electrophoresis, real-time monitoring of turbidity with a turbidimeter [43,45] or simply with the naked eye. During the reaction, a large amount of DNA is synthesized, yielding a large pyrophosphate ion by-product. It was observed that pyrophosphate forms an insoluble, observable white precipitate with divalent metallic ions [43]. Another visual detection method based on the formation of pyrophosphate can be accomplished by using the fluorescent metal indicator calcein, which binds free calcium ions. Calcein has been used for the real-time detection of DNA formation during LAMP [46]. Further methods apply intercalating DNA dyes such as SYBR Green I [47], FDR [48], or oligonucleotide probes labeled with different fluorescent markers as well as low molecular weight cationic polymers such as polyethylenimine [49].

To perform the reaction, a set of two specially designed inner and outer primer pairs and a DNA polymerase with strand displacement activity are required for the DNA synthesis. The initial reaction steps are illustrated in Figure 1. DNA regions F3 and R3 are complementary to F3c and R3c on the template, respectively. The F2 region in the

forward inner primer FIP is complementary to the F2c region followed by the F1c complementary to F1 of the target DNA. The same principle is used to design the backward primer. As a result, these four primers recognize six distinct sequences which ensure high specificity for target amplification. Moreover these primers enable generation of a stem-loop DNA for subsequent complex LAMP cycling including self-priming reactions. A mixture of DNA, differing in the number of loops and lengths of the stem loop, are produced as final products.

Due to the easy operation, high sensitivity and specificity, LAMP is an excellent choice for point-of-care applications. Thus, many microsystems that combined LAMP with different detection methods, newly designed lab-on-chips, microfluidic cartridges and automated techniques were reported. In many studies, the LAMP amplification signals were already detected after 15-40 minutes after the thermal reaction started [50-54].

The preferred and the most sensitive method for detection is based on fluorescence (with SYBR green, SYTO green or EvaGreen as fluorescent dyes) that can be detected as early as 15 minutes after the thermal reaction starts. For calcein, a limit of detection (LOD) of about 270 copies/μL was described [55]. Instead of fluorescence, Mori et al. developed a device capable of measuring the turbidity of multiple samples simultaneously. While maintaining a constant temperature, the LAMP reaction was followed in real-time by measuring changes in turbidity of solution [43].

Hsieh et al. presented a microfluidic LAMP device with an integrated electrochemical sensor [56]. Methylene blue, an electrochemically active DNA-binding compound, was added to the reaction mixture. A drop of current was measured due to the intercalation of methylene blue into the newly formed DNA, giving a LOD of 16 copies for *C. enterica* [56]. Ahmed et al used ruthenium hexaamine molecules as the redox active compounds for electrochemical sensing. The sensitivity referred to less than 24 CFU/mL for bacteria and 8.6 fg/μL for DNA [57]. Chuang et al. demonstrated a simple, low-cost surface plasmon resonance sensing cartridge based on the LAMP method for the one-site detection of hepatitis B virus [58-66]. The HBV template could be detected by an SPR-LAMP system in 17 minutes even at the detection limit of 2 fg/mL.

Several LAMP systems of on-chip detection modules were also combined with heating modules [52-54]. For the purpose of complete automatic control of the assay, Wang et al. integrated sample pre-treatment into the microfluidic LAMP device by using magnetic beads and a specially designed isolation membrane [53,54]. The entire diagnostic protocol including sample pre-treatment, LAMP reaction and optical detection was automatically completed in 60 minutes, which was twice as fast as with conventional devices [53].

Table 1 lists various strategies for the application of LAMP in microsystems which were developed in the last three years.

RPA: Recombinase Polymerase Amplification

RPA combines a low reaction temperature (about 37°C) with short reaction duration and thus makes it ideal for POC tests. The method couples isothermal recombinase-driven primer targeting of the template material with the strand-displacement DNA synthesis. It was patented by Piepenburg et al. including a novel probe-based detection approach [67].

The RPA method amplifies DNA sequences by using a recombinase, DNA polymerase and single-stranded DNA-binding proteins (SSBs).
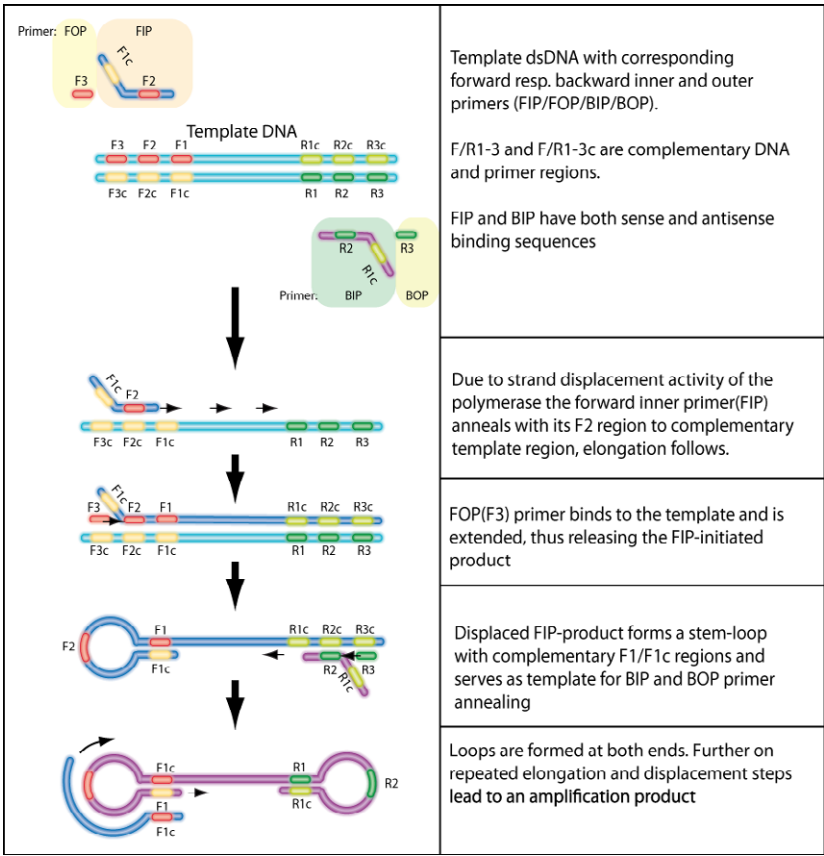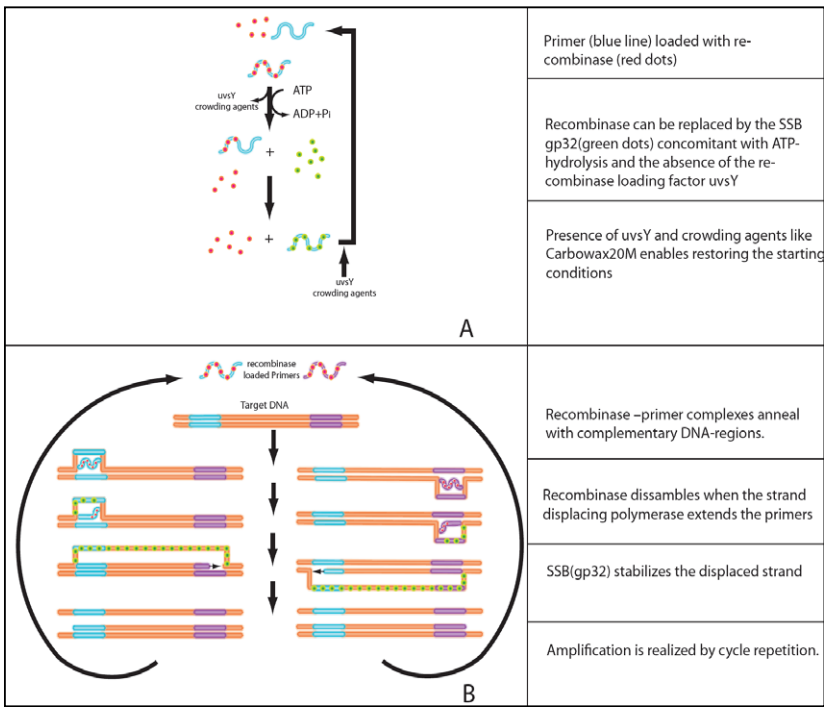
Figure 1: Initial steps of the LAMP reaction.

Figure 2: A) Combination of the primer-recombinase complex and replacement with SSBs; B) RPA cycle.

System design	Detection strategy	LOD	Target	Ref.
Microfluidic single chip consists of seven PDMS microchambers with on-chip RNA purification, RT-LAMP and optical detection	RT-LAMP and optical detection of turbidity change	35 pg	RNA extracted from CymMV-infected *Phalaenopsis orchids*	[59]
LAMP combined with lateral-flow dipstick	Lateral flow dipstick	<5 pg	IS6110 gene of *M. tuberculosis* 178 bp fragment	[60]
PDMS based microfluidic channels and membrane for three microchambers with on-chip RNA extraction and RT-LAMP	Real-time fluorescence	10-100 fg	cDNA of NNV RNA1	[53]
"μ-LAMP"; PDMS-glass hybrid microfluidic chip with eight 5 μl microchannels and on-chip real-time absorbance detection device by integrated optical fibers	Naked eye and real-time optical detection of turbidity change	10 fg	PRV genomic DNA ~ 108 bp fragment (Hinc II restriction enzyme digestion assay)	[50]
Magnetic bead-based microfluidic chip; five PDMS microchambers with on-chip cell lysis and DNA hybridization	Spectrophotometric analysis (OD_{260})	10 fg	DNA of MRSA	[54]
Ten microchamber mμLAMP system in a PDMS-glass format	Direct naked-eye determination and SYBR green I fluorescence	<10 copies	Conserved DNA fragments of three human influenza A substrains and eight important swine viruses	[50]
CCD-based fluorescence imaging system in disposable COP-microchips; for ds-DNA standard dilution series: 16 circular wells with 1 mm diameter and 2 μL volume per well; for real-time LAMP: Seven V-shaped reaction wells with a volume of 2 μL per well; fabricated with 100 μm thick ZeonorFilm®	Real-time detection; fluorescence imagining	Single copy	Genomic DNA from 12 virulence genes of major waterborne pathogens	[61]
Novel SPR-LAMP microfluidic cartridge integrated with a polycarbonate-based prism coated with a 50 nm Au film	Surface plasmon resonance (SPR)	2 fg	HBV fragment	[58]
Oxidized silicon well array (4 arrays of 6x6 wells) with dehydrated primers were covered with mineral oil; droplets (30 nL) are arrayed with an automated microinjection system	Real-time fluorescence of EvaGreen®	Not specified	*stx2* for *E. coli O157*, *hlyA* for *Listeria monocytogenes*, and *invA* for *Salmonella*	[62]
6x8 semisolid polyacrylamide gel post array; each post: 670 nL volume; Peltier element for heating, a diode laser as an excitation source, and a CCD camera for detecting fluorescence in real-time	Real-time fluorescence of the dye LCGreen Plus+, polymerized into the gel	63 fg	Six *M. tuberculosis* DNA samples with variable concentrations and target numbers of IS6110	[63]
46 mm x 36 mm x 3.4 mm cassette consists of three solvent-bonded layers of PC; valve was formed with a composite of PDMS and highly expandable microspheres; on-chip real-time monitoring	Real-time fluorescence with a portable, compact detector	10 copies	*E. coli* DNA/RNA-fragments	[64]
PMMA cartridge; exothermic reaction between Mg–Fe alloy and water as the heat source; reaction rate is controlled by using a filter paper; the amplification chambers' temperatures are regulated with a phase change material	Visual fluorescent detection with SYTO® 9 Green by naked eye and/or recorded with a portable digital camera	10 copies	Fragment of *E. coli*	[52]
FTA (Flinders Technology Associates (Whatman FTA®) membrane) disc was installed in the amplification reactor and operated in a flow-through (filtration) mode; DNA (captured on the FTA disc) were directly used as templates for LAMP without a need for elution and transfer of nucleic acids	Blue LED excitation light for end-point detection with a cell phone camera	Not specified	Genomic DNA from mosquito tissue (*Anopheles gambiae* and *An. grabiensis*)	[65]
Single reaction chamber with an integrated, flow-through FTO membrane for isolation, concentration and purification of DNA/RNA; thermal control by an external film heater	Portable optical detection system	<10 HIV particles	HIV particles suspended in raw saliva	[66]

Table 1: Comparison between different LAMP devices.

A RPA cycle is initiated by the binding of a recombinase to the primers in the presence of ATP, as shown in Figure 2A. After the recombinase-primer complex attaches sequence specifically to the double-stranded DNA, the 3'-ends of the primer are accessible to DNA polymerase. Its displacement activity creates a single strand, which is stabilized by SSBs. Both strands can be targeted, leading to an exponential amplification (Figure 2B).

Commercial Kits for RPA are available from TwistDX (Cambridge, UK). The Kits are conFigured for gel electrophoresis, lateral flow and fluorescence detection (www.twistdx.co.uk).

During the last years, RPA was described in some publications focusing on isothermal reactions for microsystems. Lutz et al. designed an integrated microfluidic assay based on RPA where all required dry and liquid reagents were pre-stored in the test carrier [68]. The fluidic cartridge included cavities for processing of up to 30 samples in parallel in separate 10 μL microchambers. This technology allowed for the detection of the antibiotic resistance gene *mecA* of *Staphylococcus aureus* from ≤ 20 starting copies in <15 minutes at a temperature of 37°C.

A phaseguided passive batch microfluidic mixing chamber

utilizing RPA was demonstrated by Hakenberg et al. The device was fabricated with a combination of dry film resist technology and direct wafer bonding. Fluorescent signals were measured directly on the chip afte one minute mixing [69].

Rohrmann and Richards-Kortum developed a paper- and plastic-based device that stored enzymes, mixed reagents, and supported RPA of HIV DNA [70]. This application was created to be compatible with DNA extraction from dried blood spots and detection using lateral flow strips. It indicated that isothermal, enzymatic amplification of DNA is practicable in a matrix-based format, serving as a new device for paper-based microfluidic techniques.

Digital amplification is an attractive option for quantitative analysis of nucleic acids. However, the nucleic acid template must be compartmentalized prior to adding initial reagents realizing digital RPA without false-positive results. Shen et al. developed a microfluidic digital RPA SlipChip for simultaneous initiation of over 1000 nL-scale RPA reactions by adding a chemical initiator to each reaction compartment with a simple slipping step after instrument-free pipette loading [71]. Fluctuations of the incubation temperature ranking from 37°C to 42°C had no influence to the digital RPA. In order to monitor

the generation of amplified target material, end-point fluorescence readout was realized by applying a fluorophore/quencher bearing probe which was cut enzymatically in response to sequence-specific binding to amplified DNA. This proceeding led to an increase in observable fluorescence as a result of separation of the fluorophore and quencher groups.

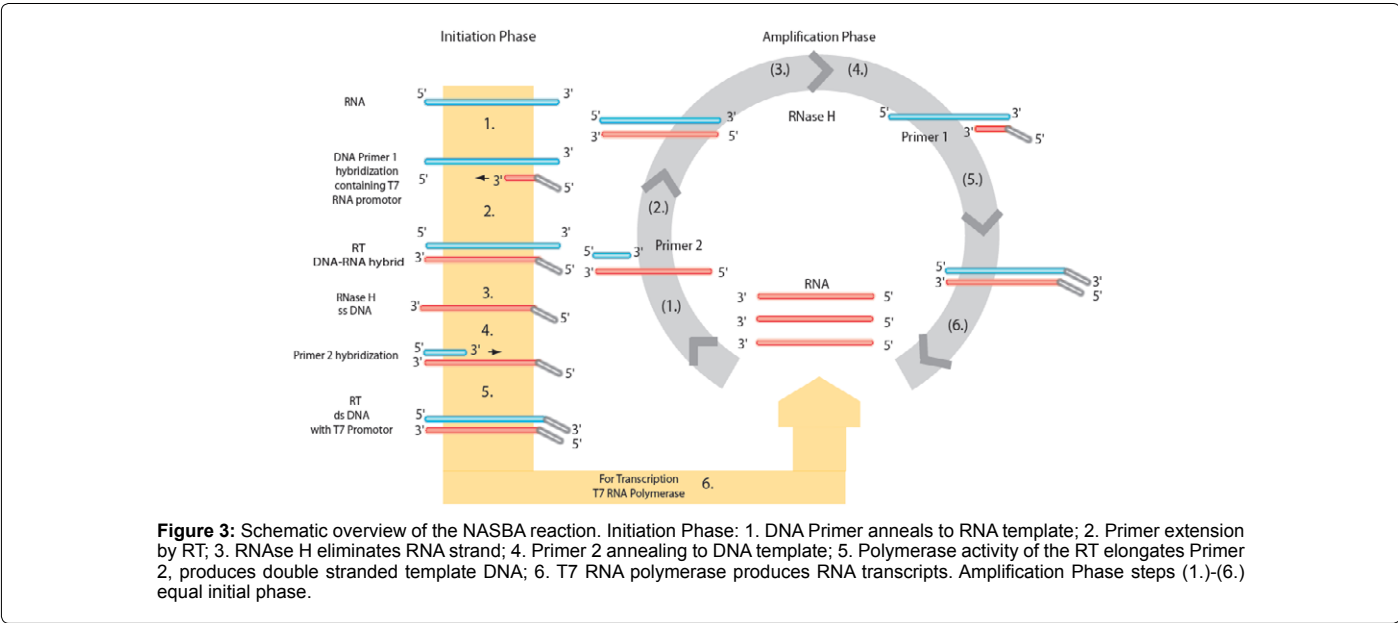Table 2 presents diverse devices for RPA in microsystems which were developed in the last four years.

NASBA: Nucleic acid sequence-based amplification

Nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR) and transcription mediated amplification (TMA) are very similar, based on same principle and derived from transcription based amplification system (TAS) described by Kwoh et al. [72]. NASBA was first described in 1991 [73], 3SR from Guatelli et al. [74] and TMA from Pfyffer et al. [75].

These types of isothermal amplification reactions combine three different enzymes to specifically amplify RNA or single-stranded DNA: an AMV (avian myeloblastosis virus) reverse transcriptase, RNase H, and a T7 RNA polymerase simultaneously conduct a billion-fold

System design	Detection strategy	LOD	Target	Ref.
Thermoformed foil-based centrifugal microfluidic lab-on-chip cartridge including prestored liquid and dry reagents, and a commercial available analyzer for incubation at 37°C; separate 10 µL microchambers for amplifying up to 30 samples in parallel	Real-time fluorescence	<10 copies	*mecA* of *Staphylococcus aureus*	[68]
Phase-guided microfluidic mixing chip; passive laminar flow mixing of two 6.5 µL batches in a microfluidic chamber	Inverted fluorescence microscope	Not specified	Not specified	[69]
Paper and plastic device assembled by stacking components made of acetate, double-sided adhesive, glass fiber matrix, and cellulose	Lateral flow strips; visual detection	10 copies	HIV *gag* DNA	[70]
Microfluidic digital RPA SlipChip; 1550 reaction compartments of 9 nL each, with 2 sets of wells for controls (50 wells each)	Yes or no digital readout of end-point fluorescence	300 copies/mL	*mecA* of *Staphylococcus aureus*	[71]

Table 2: Comparison between different RPA devices.

Figure 3: Schematic overview of the NASBA reaction. Initiation Phase: 1. DNA Primer anneals to RNA template; 2. Primer extension by RT; 3. RNAse H eliminates RNA strand; 4. Primer 2 annealing to DNA template; 5. Polymerase activity of the RT elongates Primer 2, produces double stranded template DNA; 6. T7 RNA polymerase produces RNA transcripts. Amplification Phase steps (1.)-(6.) equal initial phase.
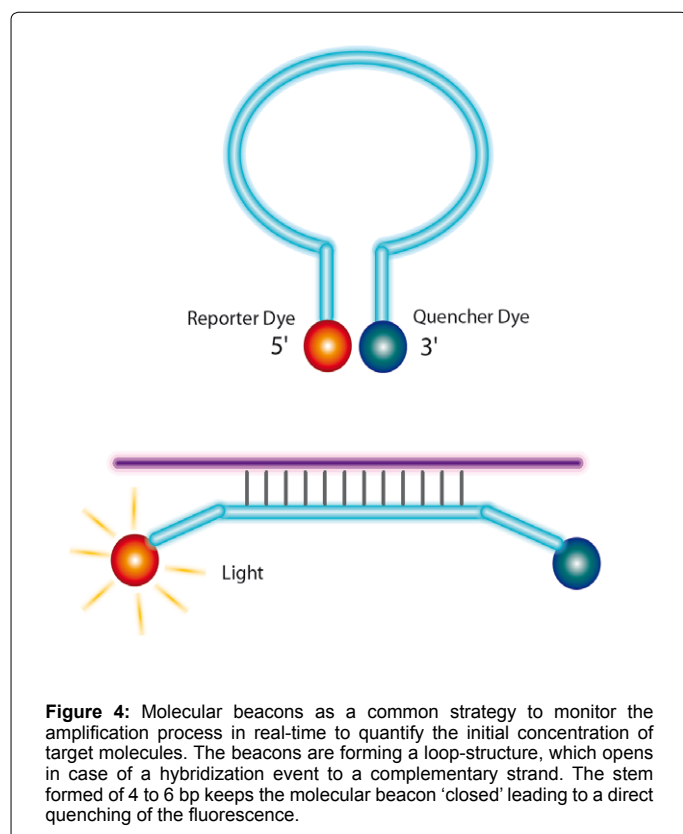
amplification of a single strand within a time range of up to 90 min (Figure 3).

The reaction requires an initial heating step of 95°C (for DNA as template) resp. 65°C (for RNA) to prepare accessible single strands, before the amplification takes place at a constant temperature of 41°C. During the initial phase, reverse DNA primers containing a T7 promoter region, bind to any available target sequence in the sample. The primers are extended by the reverse transcriptase (RT). The resulting RNA-cDNA hybrids are degraded by the activity of RNase H, leading to cDNA single strands. A forward DNA primer hybridizes to these targets forming a new template which can be elongated by the reverse transcriptase. This step integrates the T7 promoter region into the produced DNA, allowing a T7 RNA polymerase to bind, generating complementary copies of RNA. During the cyclic process, each synthesized RNA will initiate a new round of duplication, leading to an exponential amplification which is comparable to the RT-PCR performance [76-78].

Shortly after the development of NASBA it was already applied for the fast detection and quantification of HIV-1 in patient blood samples [79]. Due to the direct use of RNA as well, this isothermal amplification method is optimal for RNA virus detection, viability tests of cells and transcriptional characterization.

For the quantification of RNA molecules, molecular beacons probes have shown potential to monitor the reaction in real-time [80-82]. During the reaction the fluorescence increases due to the sequence-specific annealing of the probes to their target strands. This process separates the fluorescence label from its neighbouring quencher (Figure 4).

The first integration of a NASBA reaction into a microfluidic

Figure 4: Molecular beacons as a common strategy to monitor the amplification process in real-time to quantify the initial concentration of target molecules. The beacons are forming a loop-structure, which opens in case of a hybridization event to a complementary strand. The stem formed of 4 to 6 bp keeps the molecular beacon 'closed' leading to a direct quenching of the fluorescence.

silicon chip was shown in 2005 by Gulliksen et al. [83]. They have used the molecular beacon strategy to show sensitivities comparable to laboratory based NASBA reactions.

Zhao et al. [84] have reported a quantitative amplification microfluidic platform composed of a membrane-based sampling module, a sample preparation cassette, and a 24-channel Q-NASBA chip for environmental investigations on aquatic microorganisms. The multifunctional microfluidic system could detect a few microorganisms quantitatively and simultaneously. Also, they designed different aptamer- and immuno-NASBA assays, which can be used to monitor molecular profiling in serum samples [85] or detect waterborne pathogens [38].

McCalla et al. [86] tried to overcome amplification problems derived from the secondary structure of RNA by using a specific hybridization probe, which later serves as an easy accessible target. Different publications reported about the "sample-in, answer-out" diagnostic platforms, which integrate sample preparation, amplification using NASBA, and final detection [87]. To quantify and characterize human papilloma virus (HPV) successfully, they applied artificial and cervical smear samples and were able to measure up to 16 different targets simultaneously [87].

Instead of an optical detector, Nugen et al. showed the use of an integrated NASBA reaction to detect hsp70 mRNA from *Cryptosporidium parvum* by combining a low cost photolithographically produced electrochemical detector on a modified PMMA polymer substrate. The isothermal amplification enabled them to detect a single oocyst in a reaction volume of 4 μL [88].

Esch et al. developed a microfluidic PDMS chip using NASBA for the detection of viable *Cryptosporidium parvum* [89]. Sandwich hybridization of the generated amplicons between capture probes and reporter probes, tagged with carboxyfluorescein-filled liposomes, enabled the detection. The limit of detection of this system was 5 fmol/L for a sample size of 12.5 μL.

By using the molecular beacon fluorescent probe technology, Dimov et al. presented an on-chip purification and quantitative amplification cDNA from *E.coli* cells [90]. They were able to detect 1000 cfu/mL in less than 3 min by using tmRNA as target sequence. tmRNA contains organism-specific sequences, has a higher stability compared to mRNA and has a high copy number per bacterial cell. The same group has shown sample processing of cells, their cultivation and different bioassays including gene expression analysis in the nanoliter range using NASBA [91].

Finally, Smith et al. were focussed on the development of a compact hand-held heated fluorometric device for performing a real-time NASBA. This battery driven system can support nucleic acid analysis in the field [92,93].

Table 3 lists several platforms for NASBA in microsystems which were developed in the last eight years.

Multiplex NASBA was firstly shown in 1999 [94], but the lower efficacy in comparison to a singleplex reaction might be a reason for lacking publications of multiplex NASBA used in microsystems.

A general disadvantage of the NASBA reaction is the initial heating step to denature or to reduce secondary structure. A precise temperature control together with higher power consumption makes NASBA unfavourable, even though the actual reaction temperature (41°C) is relatively low. However, a major advantage of the method

System design	Detection strategy	LOD	Target	Ref.
COC microchip (50 x 40 mm); 10 parallel reaction channels; signals can simultaneously detected in 80 nL volumes; custom-made optical detection unit	Auto-fluorescence	10^{-6} µM 20 cells/µL	HPV 16 sequences SiHa cell line	[83]
Q-NASBA microfluidic platform combines a membrane-based sampling module, a sample preparation cassette, and a 24 channel Q-NASBA chip	Quantitatively by a common microplate reader	<10 copies	*Saccharomyces cerevisiae*; *E. coli*; *Staphylococcus aureus*	[84]
Aptamer-NASBA chip modeled on a 384-well microplate; fabricated by using MEMS technologies; bottom and cover layer: silicon wafer	Quantitatively by a common microplate reader	2×10^{-16} mol/L	Gonadotropin (GnRH)	[85]
Reactor design contained 11 parallel channels with 2 separate chambers (silicon-glass) for each heating step, separated by hydrophobic burst valves; second chamber included dehydrated enzymes; full reaction runs not on-chip; PDMS reactor for microfluidic separation	Molecular beacon fluorescence	Not specified	Influenza A/H5 vRNA	[86]
Automated LOC system for sample preparation, nucleic acid extraction, amplification and real-time fluorescence detection: sample preparation chip, NASBA chip (75 mm x 44 mm x 1,5 mm) consists of a disposable microfluidic cartridge composed of injection moulded COC; NASBA instrument	Real-time fluorescence	Not specified	HPV E6/E7 mRNA	[87]
Electrochemical biosensor based on a polymer substrate; channels fabricated in PMMA using hot embossing with a copper master; interdigitated ultramicroelectrode array (IDUA) realized directly on the PMMA surface; 2 detection channels	Electrochemical	Not specified	*hsp70* mRNA from *Cryptosporidium parvum*	[88]
Microfluidic chip (PDMS)	Real-time fluorescence by using carboxy-fluorescein-filled liposomes	5 fmol of ampolicon in 12.5 µL	Viable *Cryptosporidium parvum*	[89]
Integrated RNA purification chamber (0.25 µL) and real-time NASBA device (2 µL); channels and chambers: 80 µm high	Molecular beacon fluorescent probe technology	1000 CFU/mL	*E. coli*	[90]
Cellular and molecular analysis platform realized by an integrated microfluidic array plate (iMAP) consists of 64 processing modules, that can perfom 64 independent simultaneous integrated assays	End-point fluorescence by using a thermally controlled inverted fluorescence microscope	100 CFU/mL	*E. coli*	[93]
Hand-held heated fluorometric instrument (150 mm x 48 mm x 40 mm) combines a Printed Circuit Board/Micro Electro Mechanical System (PCB/MEMS) reaction detection/reaction chamber containing an integrated resistive heater with attached miniature LED light source and photo-detector and a disposable glass waveguide capillary to enable a mini-fluorometer	Real-time fluorescence	Not specified	Not specified	[92]

Table 3: Comparison between different NASBA devices.

is the production of single-stranded RNA molecules which can easily hybridize to fluorescently labelled probes without any necessary denaturation step. Though, the labile stability of RNA has to be considered, if the sampling-to-assay time is considerable. For point-of-care tests, where the diagnosis is close to the patient anyhow, the stability might be negligible.

Nowadays, some commercially available diagnostic products are based on real-time NASBA. BioMerieux (Marcy l'Etoile, France) is selling the NucleiSENSEasyQ system, which features the first automated system combining NASBA and real-time molecular beacon detection [95]. Up to 48 samples can be run in parallel with a detection of targets in less than 2 hours [96].

Coris BioConcept (Gembloux, Belgium) has also integrated NASBA in an oligochromatographic (OligoC) detection kit for *Trypanosoma cruzi* [97] and *Leishmania spp.* [98] in lateral flow format [99].

Furthermore TMA-based Kits are commercial available from Gen-Probe (San Diego, USA). *Neiserria gonorrhoea, Chlamydia trachomatis*, HPV, HIV, *Trichomonas vaginalis* and *M. tuberculosis* detection is available with this system [100,101].

HDA: Helicase-dependent amplification

The helicase-dependent amplification, firstly described in 2004 [102], makes use of the naturally occurring process of DNA replication. A helicase unwinds the target DNA strand at a temperature of 37°C to circumvent the heat-induced denaturation step of PCR. The MutL protein stimulates the helicase unwinding, whilst the single-stranded binding (SSB) protein prevents re-hybridization of the separated ssDNA targets [103] (Figure 5). The primers can hybridize to the free ssDNA and a DNA polymerase subsequently extends them. This exponential reaction can produce million-fold copies of target DNA in 60 to 120 min [104]. The development of a heat-stable helicase from *Thermoanaerobacter tengcongensis* allowed driving the reaction at a temperature of 45°C to 65°C [103,105] without the addition of MutL and SSB. Furthermore the performance of the HDA was improved [104], leading to longer amplification fragments from initially up to 400 bp to >2000 bp [104]. A sensitivity of as few as 10 copies of bacterial genomic DNA has been presented [103]. This thermophilic HDA system was further developed for diagnostic applications by Goldmeyer et al. [106,107]. They reported that the HDA is suitable for the rapid

Figure 5: Schematic overview of the Helicase-dependent amplification.
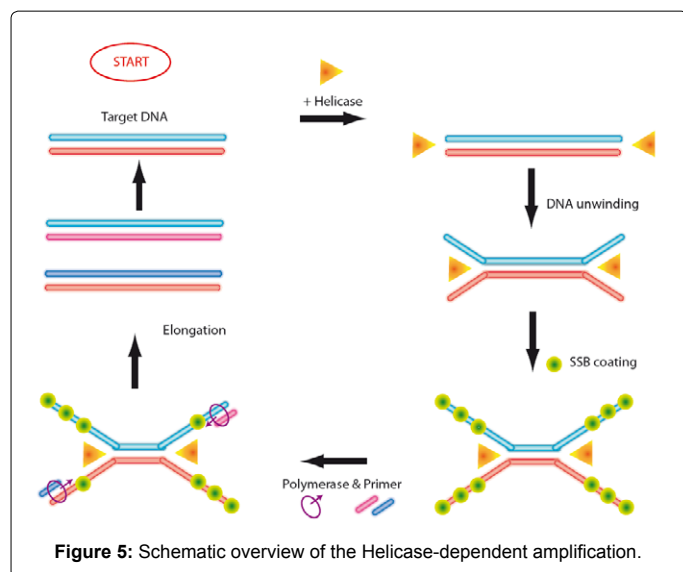
characterization of *Staphylococcus aureus* and the determination of methicillin resistance by the detection of *mecA* gene.

The simple reaction scheme is one of the major advantages of HDA over other methods that often include more than one set of primers and just a singular enzyme [39]. There is no need for an initial denaturation step for DNA and the reaction has the ability to detect RNA [108-111]. HDA has been shown to be effective for various samples, from urine [112] and vaginal swabs [112] to blood [110,111] and stool [108,109]. Therefore, the HDA has a great potential for using it in portable point-of-care devices [113].

A study of Andresen et al. has shown a solid phase helicase-dependent amplification using microarrays. One primer was immobilized to the surface of an epoxysilanized glass slide. The fluorescently-labelled second primer and the sample containing HDA enzymes were incubated for two hours to allow for bacterial pathogen monitoring [114]. The detection of *Staphylococcus aureus* from nasal swabs was presented in a study from Frech et al*.,* were an asymmetric HDA was used to amplify specific sequences, which hybridized to capture probes on a DNA array. The readout was visible to the naked eye by an enzymatic process employing the dye Tetramethylbenzidine (TMB) [115].

A variety of different microfluidic devices was recently published, showing the attractiveness of an implemented HDA reaction. Ramalingam et al. have applied a PDMS-glass hybrid to integrate the amplification, but pre-purified the target DNA without integrating the process on the chip [116]. They built four parallel 5 µL containing reaction chambers with pre-dried primers. The DNA sample was injected using a pipette and a real-time quantification of severe acute respiratory syndrome (SARS) coronavirus DNA was possible after 30 min reaction time. A detection of 0.1 µg DNA/mL at a reaction temperature of 62°C for the HDA was demonstrated.

Mahalanabis et al. [117] describe the combination of a micro solid phase extraction (µSPE) for DNA isolation from whole bacteria and HDA in one disposable, flap-valve controlled thermoplastic cartridge (Figure 6). The µSPE column is used to lyse and extract DNA from whole bacteria in a liquid sample. The integrated process of detection of *E. coli* in broth medium was demonstrated in as little as 50 min at 65 °C

including all sample preparation steps.

Lately, the same group has demonstrated a low-cost device, which can be used for the detection of *Clostridium difficile* DNA in the stool of infected patients. A thermoplastic chip for the HDA was combined with commercially available toe warmers, which were able to keep the temperature stable at 65°C ± 2°C for at least 55 min. A similar limit of detection of 1.25×10^{-2} pg of *C. difficile* DNA was obtained compared to standard laboratory procedure [109].

Zhang et al. developed a droplet microfluidic system consisting of a PDMS chip including cell lysis, DNA extraction with superparamagnetic particles, and the HDA reaction visualized via fluorescence detection [118]. Here all reagents for analysis of pathogens or disease biomarkers are stored in small droplets, increasing the simplicity of this system.

A vertical-flow strip was also used by Tang et al. to amplify HIV-1 *gag* gene (IsoAmp® HIV-1 assay, BioHelix Corp., Veverly, MA) by a reverse transcription HDA [119]. The detection was done with a Type I BEST™ Cassette which is based on a sandwich immunoassay with capture probes (fluorescein isothiocyanate (FITC)-labelled) and detection probes (biotin-labelled). In 75% of these assays a positive result was shown for not more than 50 RNA copies.

An alternative to fluorescence detection was shown by Kivlehan et al. describing an electrochemical method for real-time detection with the possibility of quantification and melting curve analysis [120]. This method is based on a dsDNA intercalating redox probe that becomes less detectable upon binding. This decrease is measured by square-wave voltammetry (SWV) and compared to free counterparts.
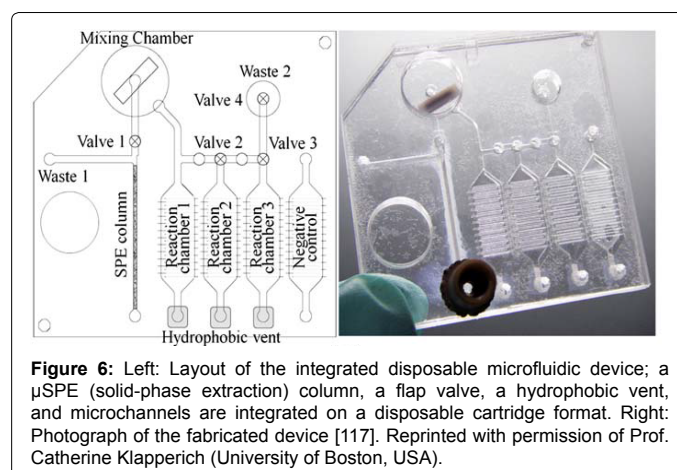
Also Torres-Chavolla and Alocilja described an electrochemical method of HDA-amplicons using gold nanoparticles [121].

Table 4 displays several designs for the application of HDA which were developed in the last five years.

Already commercially available are standardized reagents and research kits from BioHelix (Beverly, MA, USA), the inventors of HDA. They offer tests for e.g. *Staphylococcus aureus* and MRSA, *Clostridium difficile, HIV, Herpes Simplex, Chlamydia trachomatis* and *Neisseria gonorrhoeae* based on their BESt™ Cassette [108,122-124].

SDA: Strand Displacement Amplification

The reaction, which can be performed over a broad temperature range (37°C to 70°C), was already described in the early nineties applying multifunctional primers, both having target sequences for the

Figure 6: Left: Layout of the integrated disposable microfluidic device; a µSPE (solid-phase extraction) column, a flap valve, a hydrophobic vent, and microchannels are integrated on a disposable cartridge format. Right: Photograph of the fabricated device [117]. Reprinted with permission of Prof. Catherine Klapperich (University of Boston, USA).

System design	Detection strategy	LOD	Target	Ref.
OnChip-HDA amplification, or solid-phase amplification on a microarray	Real-time fluorescence	1 ng 250 pg	*Neisseria gonorrhea*; *Staphylococcus aureus*	[114]
T-structure poly-dimethylsiloxane (TSPS) coated wafer (aldehyde-functionalized chip surface); poly (lys-phe) coated wafer (succimidyl-4-formyl benzoate (SFB) functionalized)	Naked eye by an enzymatic process employing the dye Tetramethylbenzi-dine (TMB)	≤ 2 CFU per swab	*nuc* of *Staphylococcus aureus*	[115]
PDMS-glass hybrid; 4 parallel reaction chambers (5 µL volume)	Real-time fluorescence	0,1 µg DNA/mL	Severe acute respiratory syndrome (SARS) DNA	[116]
Disposable, flap-valve controlled thermoplastic cartridge; includes a µSPE column (COP; 0.55 x 0.55 x 20 mm), flap valves, hydrophobic vents, microchannels, 4 HDA reaction chambers (á 25 µL)	Real-time fluorescence	≤ 10 CFU	*E. coli*	[117]
Polymer-based microfluidic chip (COP) with multiple reaction chambers (à 25 µL); toe warmer as heat source in a Styrofoam cup	Real-time fluorescence	1.25×10^{-2} pg	*Clostridium difficile* DNA	[109]
Droplet microfluidic, sample-to-answer system (PDMS) including cell lysis, DNA extraction with superparama-gnetic particles	Real-time fluorescence	Not specified	Ovarian cancer biomarker Rsf-1; *E. coli*	[118]
Vertical-flow DNA detection strip	Visual detection	50 copies	HIV-1 *gag* gene	[119]
Screen printed carbon electrode (SPCE) chip; dextrin coated gold nanoparticles as electrochemical reporter	Electrochemical	0.01 ng/µL	*IS6110* gene of. *M. tuberculosis*	[121]

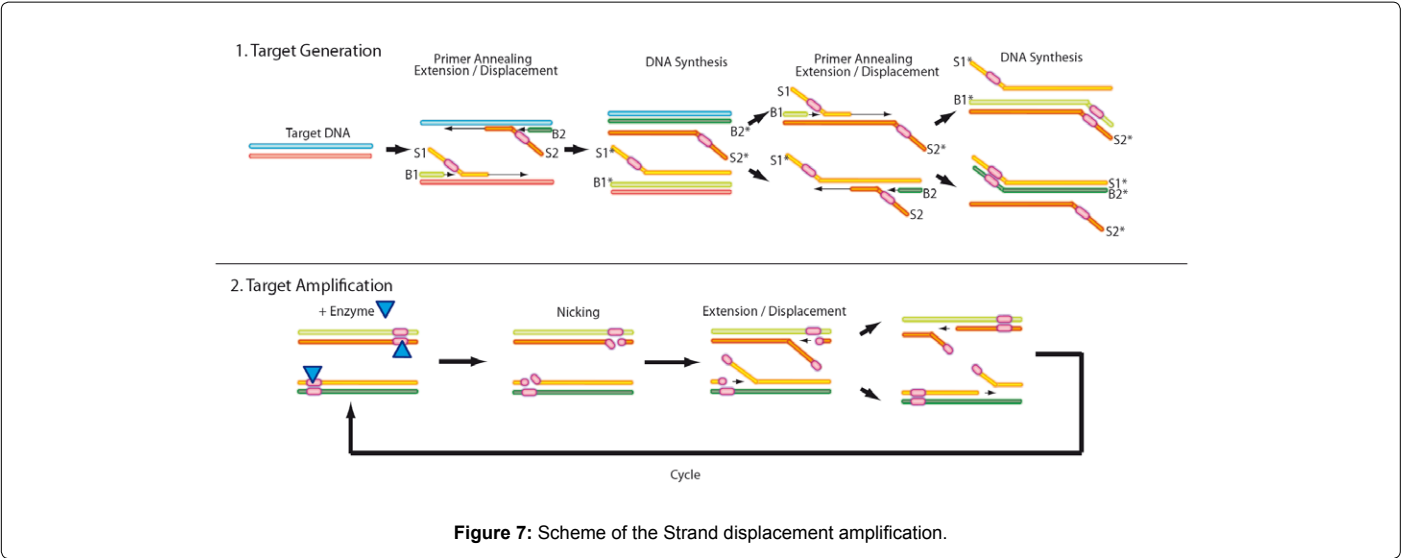**Table 4:** Comparison between different HDA devices.

Figure 7: Scheme of the Strand displacement amplification.

directed hybridization to the DNA strand of interest and a restriction site for endonucleases [125,126] (Figure 7).

After the heat induced strand separation of the dsDNA, the primers bind sequence-specifically introducing a restriction site into the product. Bumper primers, which bind adjacent to the first primer, will be elongated by a polymerase with strand displacement activity, releasing the first single-stranded amplicon. In a second step, the reverse primer – also with an included nickable restriction site, will be elongated (Figure 7). An endonuclease will cleave the restriction sites only at one strand, because thiol-modified nucleotides are incorporated to prevent cutting of the whole ds DNA strand. The free 3'-end is subsequently extended, displacing the new single stranded copy molecule. This process of nicking and displacing will lead to an exponential amplification of DNA.

However, SDA is not a common method for point-of-care or microfluidic devices [38]. The need of an initial denaturation step and a longer processing time of two hours may be a reason. Additionally, some reports have shown a co-amplified background signal due to unspecific primer binding [125,127]. Due to the excess of human DNA in clinical samples [128,129], the large background nucleic acid can hamper the efficiency of the SDA considerably.

Another major disadvantage is the limited use of suitable restriction enzymes. A recognition sequence of about 5 bp occurs on average every 1024 nucleotides, thus templates longer than 1000 nts cannot be used. Furthermore thiol-modified nucleotides might influence downstream applications [130].

Nonetheless, the integration of the SDA on lateral flow strips was demonstrated by He et al. They combined the isothermal amplification of a human gene with a visual, gold-nanoparticle-based detection of a mutation within the keratin 10 gene [131]. A detection limit of 1 fM of the R156H-mutant DNA within 75 min without the use of any instrumentation was shown. Already in 1998, an integration of the reaction on a glass-silicon hybrid was demonstrated by Burns et al. A 106-bp DNA fragment was amplified in the nanoliter range and

elctrophorectically analyzed in only 17 min [132]. Precise metering of reaction mixtures was done by using hydrophobic patches and an air-pressure source. Volumes below 1 nL can be separated and moved within the channels. In 2002, Nanogen, Inc. presented the integration of a SDA module into an electric-field-driven DNA hybridization assay. The identification of the Shiga-like toxin gene from *E. coli* was accomplished within 2.5 h starting from a dielectrophoretic concentration of intact *E. coli* bacteria and finishing with an electric-field-driven DNA hybridization assay, detected by fluorescently labelled DNA reporter probes [133].

A multiplex SDA for up to ten targets was shown by Westin et al. in a microarray chip with anchored primers [134].

Table 5 lists different strategies for the application of SDA in microsystems.

The SDA is commercialized by Becton Dickinson since 1999. The BD ProbeTec™ ET System enables the user to characterize and quantify in real-time of up to 564 samples in eight hours [135]. Its usefulness has been shown in clinical setups especially for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* and other pathogens [136-141]. The technology is utilized to generate billions of copies of target molecules from a single DNA or RNA template in the one-hour assay time [142]. The high throughput platform is FDA cleared/CE marked and supports tests for HSV from urogenital samples as well. The quantitative detection can be done by using the molecular beacon technique [135].

RCA: Rolling circle amplification

RCA, first described 1998 [143,144], exploits the excellent strand displacement activity of a Phi29 bacteriophage polymerase on target molecules. The isothermal reaction allows for the amplification of a single-stranded DNA to generate a continuous catenated product of up to 0.5 Mbases. Different approaches to perform the reaction have been reported [145-147], whereas the here described padlock probes have been used very successfully for linear DNA. These probes are linear oligonucleotides containing two target specific sequences, which are designed to circularize after hybridization and subsequent ligation (Figure 8). The dual recognition in combination with a ligation reaction ensures specificity of detection. After that, the circular padlock probe serves as template for the polymerase, which continuously elongates the product and displaces the generated strand [35]. It is also possible to use further primers binding the generated product and thereby producing hyper branching structures. This reaction then is called ramification

amplification and was described 2001 by Zhang et al. [148].

Optimized RCA was able to detect 0.163 pg (~ 60 molecules) of genomic DNA from *Listeria monocytogenes* [149], or 143 zmol (8.6 x 10^4 molecules) of in vitro transcribed human CD4 mRNA [150].

Sato et al. described a solid phase RCA for the on-chip detection of *Salmonella enterica* sequences from samples within [34]. Figure 9 shows the capture and ligation of a specific target DNA at 55°C. A circular primer is pre-hybridized to the 34 μm-capture bead. After a ligation step, the linear strand is produced by RCA. To finally quantify the product, a fluorescent probe was hybridized to the amplicon.

Mahmoudian et al. reported about combining circle-to-circle amplification (C2CA), a variant of RCA, with microchips using an electrophoretic port as a RCA reaction chamber. 25 ng of bacterial genomic DNA was detected in less than 65 min (including RCA and PMMA-based microchip electrophoresis) [151,152].

MicroRNAs (miRNAs) are non-coding small RNAs which play a central role in cellular regulation [153]. Providing the necessary sensitivity for the detection of miRNA in single cells, Wu et al. introduced a flow cytometry-on-a-chip device to detect the presence and localize the miRNA. A combination of rolling circle amplification and locked-nucleic acid probes allowed for the characterization of relative miRNA changes at a single cell resolution. The approach makes use of a 10-chamber microfluidic chip platform and a small amount of only 170 nL of reagent per single experiment. Konry et al. have demonstrated a similar droplet-based microfluidic device which enabled the user to detect protein markers in a nanoliter reaction volume. After highly specific antigen-antibody recognition, a combined RCA allowed the visual fluorescence detection of less than ten EpCAM surface tumor markers per cell [154].

The integration of RCA for a completely different strategy was shown by Zhao et al. [155]. The group demonstrated the coating of a microfluidic surface by using rolling circle amplification. The resulting 3D DNA network extends over tens of micrometer into the solution comprising of repeating adhesive aptamer domains. These structures were able to bind the protein tyrosine kinase-7 (PTK7) which is overexpressed on many human cancer cells [155]. The work impressively demonstrated the capture of target cells from biological sample by mimicking the strategies of marine organisms.

A similar technique was reported by Barbee et al., who fabricated DNA polymer brush arrays on a solid glass support. Oligonucleotides were covalently immobilized, a spin-coated photoresist covered the

System design	Detection strategy	LOD	Target	Ref.
Lateral flow strip through dual immunoreactions	Lateral flow dipstick; visual detection	<1 fM	R156H-mutant gene of keratin 10 in Epiderm-olytic hyperkeratosis	[131]
Glass-silicon hybrid includes a nanoliter liquid injector, a sample mixing, a positioning system, a tempe-rature-controlled reaction chamber, an electrophoretic separation system, and a fluorescence detector	Electrophoretical	Not specified	Not specified	[132]
Integrated, stacked microlaboratory; fabricated by orderly laminating several different functional layers (all 76 x 76 mm²) ; SDA module (76 x 298.5 mm²) consists of a denaturation chamber, an amplification chamber, two pinch, two peristaltic pumps, and an electronic control circuit for fluidic and thermal control	Electric-field-driven immunoassay	Not specified	Shiga-like toxin gene (SLT1) from *E. coli*	[133]
Microchip array; electronic anchoring of sets of amplification primers in distinct areas	Fluorescence-based by an electronic hybridization assay	Not specified	Chlamydia; *E. coli parC*; *Pseudomonas*; *Salmonella*; *E. coli gyrA*	[134]

Table 5: Comparison between different SDA devices.
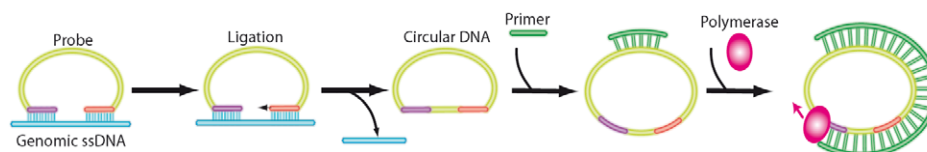
Figure 8: Rolling circle amplification. The padlock probe, one of various different strategies to use this isothermal amplification technique, hybridizes sequence specifically to the target strand.
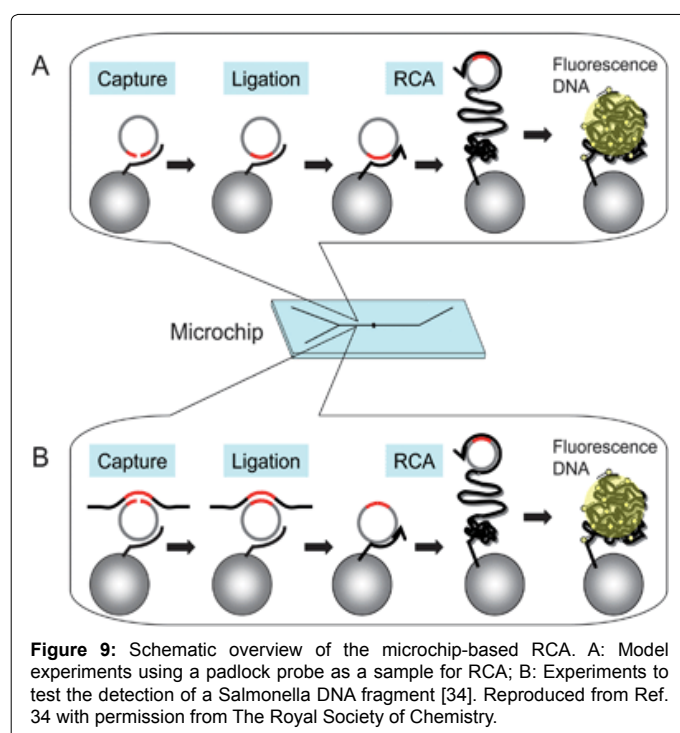
Figure 9: Schematic overview of the microchip-based RCA. A: Model experiments using a padlock probe as a sample for RCA; B: Experiments to test the detection of a Salmonella DNA fragment [34]. Reproduced from Ref. 34 with permission from The Royal Society of Chemistry.

DNA and oxygen-based plasma was used to destroy the exposed oligonucleotide primers. After assembly into a microfluidic chip, the DNA brushes were synthesized on the oligonucleotide array by rolling-circle DNA amplification. Barbee discussed various applications for these arrays [156].

Another unusual application for RCA on microfluidics was lately described by Juul et al. [157]. Microfluidics fabricated by conventional PDMS-based soft lithography techniques and channel heights at 25 µm was used to study enzymatic activities down to the single cell level. A picoliter droplet-based approach separated the sample solution, whereas the RCA was used to monitor the functionality of recombinase or topoisomerase-like proteins. The latter support the linkage of a circular probe, which can serve as a template for the RCA.

Protein detection was shown by Yan et al., who capture target molecules, carcinoembryonic antigen (CEA), a clinically important tumour marker, from biological samples by using magnetic particles. A second antibody immobilized to a gold nanoparticle, which is covered with primers for RCA enabled the single amplification by integration of biotinylated dNTPs. Latter were bound by avidin-HRP conjugates resulting in an observable coloured product [158]. The use of RCA on protein microarrays to increase the efficiency of Raman detection was demonstrated by the same group. Less than 10 zeptomol of protein

molecules were detectable by using the isothermal amplification method for signal enhancement [159].

Various other readout systems for RCA are nicely described in Stougaard et al. [160].

Mazutis et al. described a droplet-based microfluidic system for single molecule amplification by RCA [161]. Quantification was done by analyzing an intercalating fluorochrome. The 2 pL sample droplets were fused with a 15 pL droplet consisting of an in vitro translation system and a fluorescein-di-β-D-galactopyranoside (FDG). During the formation of fluorescein β-galactosidase from FDG the enzyme activity can be measured by fluorescence detection.

In a sandwich capture assay streptavidin-coated magnetic beads are immobilized to biotinylated virus capture DNA. This capture DNA then binds reporter and viral target DNA through incubation. By adding the RCA buffer the target and the reporter DNA is released into a single well and RCA can be accomplished, whereby the reporter DNA is extended and detected by real-time fluorescence measurement [162]. Also Li et al. used streptavidin coated magnetic beads for DNA isolation by annealing of a biotin labelled capture probe to the target DNA [118]. Here a padlock probe is used as well as additional primers for ramification amplification (RAM).

A multiplex RCA assay is shown by Nallur et al. using primers anchored on surface, in a microarray [163]. Detection was done by fluorescence labelled probe and laser scanning. Also quantification was accomplished with the help of quantification software.

Table 6 presents diverse platforms for the application of RCA which were developed in the last five years.
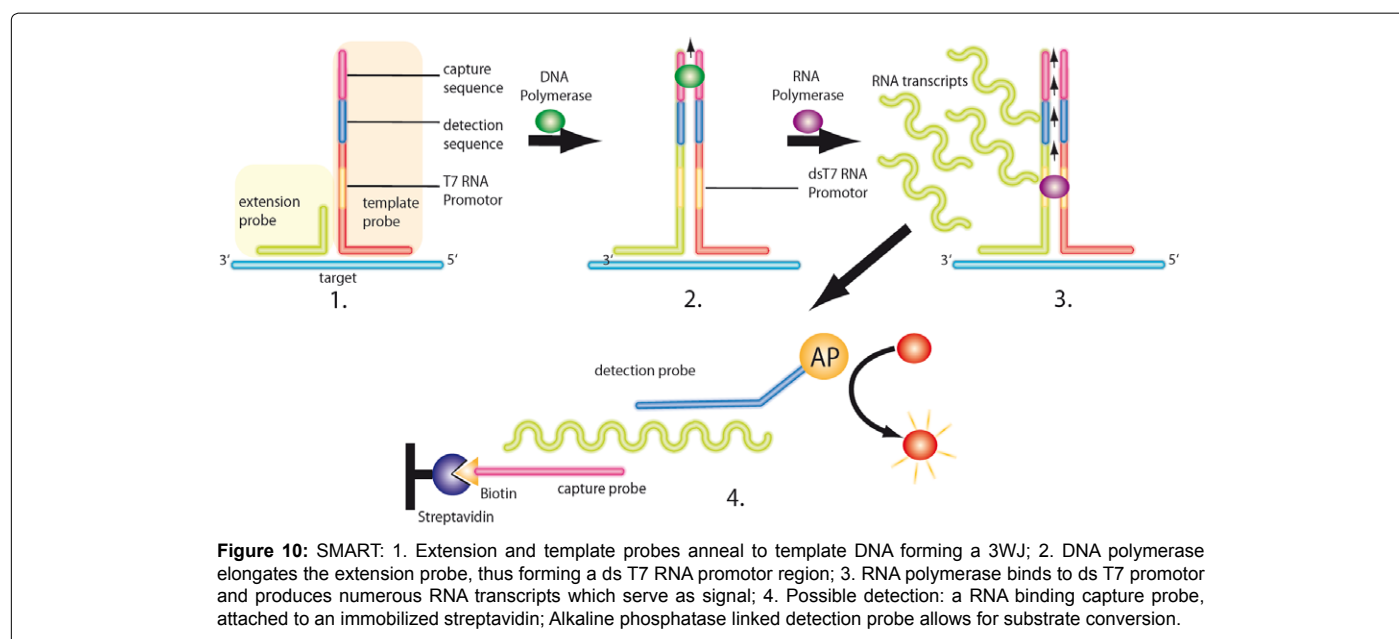
GE Healthcare & Life Science (Piscataway, USA) for example made rolling circle amplification commercially available and sell it under the brand name illustra TempliPhi 100 Amplification Kit.

SMART: Signal-mediated amplification of RNA technology

SMART is an isothermal amplification technology, developed for the detection of specific target sequences, either RNA (for expression) or DNA [164]. The SMART assay consists of two single stranded oligonucleotide probes ("extension" and "template"), which both can hybridize abreast to the target strand (Figure 10). One part of each primer can hybridize to each other to form a three-way junction (3WJ). After the three-way junction formation, a DNA polymerase elongates the shorter extension probe, meanwhile synthesising the complementary strand of the template probe, which includes a T7 RNA polymerase promotor sequence. Subsequently, the transcription template with the promotor sequence will be produced, allowing for a multiplicative production of transcription RNAs by a T7 RNA polymerase. It is also possible to increase the yield and make this reaction more sensitive by binding another probe with a T7 RNA polymerase promotor sequence

System design	Detection strategy	LOD	Target	Ref.
Glass microchip; Y-shapes channels with a dam structure for bead retention; Peltier temperature controller to control the temperature	Fluorescence and phase-contrast images	88 ng	*Salmonella enterica* sequences	[34]
PMMA microchip; on-chip detection by using a polymer separation matrix and fluorescence dye	Electrophoretic	25 ng	Bacterial genomic DNA	[151,152]
Ten-chamber microfluidic chip; fluidically-isolatable chambers (up to 2000 cells);	Fluorescence image; flow cytometry	Not specified	miR155 and CD69	[153]
Droplet-based microfluidic device (PDMS)	Visual fluorescence	<10 molecules/cell	EpCAM surface tumor markers	[154]
Microfluidic device to capture and isolate cells using a 3D DNA network comprising repeating adhesive aptamer domains that extend over 10 μm into the solution	Fluorescence image	Not specified	Minimal residual disease (MRD)	[155]
DNA polymer brush arrays; fabricated on glass coverslips using a destructive micropatterning technique and solid-phase RCA; additional microfluidic device with temperature control	Fluorescence image; gel electrophoresis; atomic force microscopy	Not specified	Not specified	[156]
Flow focusing droplet generator and a droptrap; channel height: 25 μm	Fluorescence image	Few cells	*TopI*, *Flp*, and *Cre*	[157]
Droplet-based microfluidic system (PDMS) for generation spheroids and their separation	Real-time fluorescence; cytotoxicity test	Not specified	HeLa; HEK293	[161]
Microarrays printed on streptavidin-coated glass microscope slides	Laser scanning and image analysis	≤ 150 molecules	Not specified	[163]

Table 6: Comparison between different RCA devices.

Figure 10: SMART: 1. Extension and template probes anneal to template DNA forming a 3WJ; 2. DNA polymerase elongates the extension probe, thus forming a ds T7 RNA promotor region; 3. RNA polymerase binds to ds T7 promotor and produces numerous RNA transcripts which serve as signal; 4. Possible detection: a RNA binding capture probe, attached to an immobilized streptavidin; Alkaline phosphatase linked detection probe allows for substrate conversion.

to the amplified RNAs [164].

The detection of RNA can be done efficiently in approx. 1 h at 41°C, whereas the detection of DNA still needs an initial denaturation step of >90°C [165]. To further increase the sensitivity, Hall et al. [165] described an enzyme-linked oligosorbent assay. Alternatively, the amplified RNA complex is captured on magnetic beads using a sequence-specific capture probe and is separated from unbound probe using microfluidics [86]. A rapid detection of 100 fmol/L from an artificial influenza A H5 vRNA sequence requiring only 5 μL of reaction volume has been demonstrated. Morabito et al. have presented the

detection of a HIV-1 reverse transcriptase drug-resistance mutation, which was partly done on a microfluidic platform in less than 180 min [81].

For the quantification of the reaction, molecular beacon approach has been applied successfully [81,86].

Table 7 lists several strategies for the application of SMART which were developed in the last three years.

Various other methods

Whole genome amplification by isothermal methods was reported lately by Li et al. [166]. The so called primase-based whole genome

System design	Detection strategy	LOD	Target	Ref.
Microfluidic reactor with 4 channels (PDMS)	Real time fluorescence	Not specified	Influenza A H5 vRNA	[86]
Microchip reservoirs; simple 4-channel device, in which each channel is tapered and designed for adequate separation of conjugated magnetic beads from unbound SMART probes using a magnetic bar	Gel electrophoresis; real-time fluorescence	Not specified	K103N (HIV-1 reverse transcriptase drug-resistance mutation)	[81]

Table 7: Comparison between different SMART devices.

amplification (pWGA) method from Harvard University has shown promising results in case when total DNA needs to be amplified (e.g. DNA archiving, single cell analysis, tracing of DNA contaminations, or forensics). The method employs the functionality of a DNA primase, which synthesizes primers in vitro. No need of added primers, no thermocycling nor prior heat-denaturation make that principle very promising for the transfer into microfluidic devices. The reaction has initially shown an over thousand-fold amplification after 1 h at 37°C [166]. By using circular DNA as template, a 10^8 fold amplification of as low as 100 initial target strands has been achieved. In addition to amplifying total genomic DNA, pWGA can also be used for detection and quantification of contaminant DNA in a sample when combined with a fluorescent reporter dye [166]. The commercialization is covered by BioHelix (Beverly, MA, USA).

The Japanese RIKEN Institute has developed the SmartAmp or Smart Amplification Process version 2 (SMAP2), which is based on self-priming, loop-forming motifs similar to the LAMP process. It allows a rapid detection in 15 to 30 min at with a complete suppression of disturbing background [167,168]. To further improve the specificity of the reaction and to suppress the background signal, the mutation binding protein MutS is employed [169]. MutS, which is a part of the naturally occurring mismatch repair system, identifies mismatched target strands or primers. The amplification is processed by the strand-displacing *Alicyclobacillus acidocaldarius* polymerase. If a MutS protein detects a mismatched duplex, it binds irreversibly to that position and blocks any further amplification. Non-specific sequences result therefore in an inhibition of amplification resp. product development. A variety of different SNP detection methods have lately been described [169-171]. Kawai et al. report about a first Influenza A (H1N1) assay, combining both a reverse transcriptase and a SmartAmp amplification in one-step setup. After a simple gel-filtration step of the swap sample, the reaction time was 40 min to result [172]. Monitoring of the amplification is easily possible by using dsDNA intercalating fluorescent dyes. The SmartAmp or SWAP2 has demonstrated an excellent specificity and sensitivity (3 copies), but is slower in comparison to LAMP [38,169]. However, due to its advantages including the reduction of background effects, the reaction might be very useful for integrated microsystems in future. The Japanese company DNAFORM will offer kits for SMAP2 soon, whereas the RIKEN institute supports the potential users with an online tool for primer design (http://smap.gsc.riken.jp/smap/PrimerDesign.do).

Jung et al. described another unique isothermal target and signaling probe amplification method, called iTPA, in combination with a facile gold nanoparticle (AuNP)-mediated colorimetric method for real-time detection of target DNA [173]. The iTPA simultaneously amplifies the target and signaling probe through two displacement events under isothermal conditions. Due to this method, as low as 10^2 copies of target *Chlamydia trachomatis* plasmid were successfully detected in a colorimetric manner. The strategy does not require a thermo-cycler or other devices and is remarkable simple and convenient. Therefore, this approach is a promising application in portable sensor systems such as microfluidic devices or POCT diagnostic.

A rapid on-site detection of *Acidovorax citrulli by* cross-priming amplification (CPA) was presented by Zang et al. [174]. The CPA is an isothermal DNA amplification method developed by Ustar Biotechnologies Co., Ltd., China. It belongs to a class of isothermal amplification reactions that is realized by a strand displacement DNA polymerase and does not require an initial denaturation step or the addition of a nicking enzyme [175]. The detection of resulting amplicons is visualized on a lateral flow strip housed in an enclosed, sealed plastic device in order to prevent the leaking of products. The sensitivity of CPA for pure culture was 3.7×10^3 CFU/mL. Consequently, it is a valuable alternative to immunoassays and PCR-based tests for diagnosis of *A. citrulli*.

Recently, aptamer-based analytical methods have been developed for protein detection. Ma et al. elaborated a cascade signal amplification strategy based on molecular switches and aptamers to improve protein detection [176]. This strategy consisted of two steps, including the recognition and the triggering of a polymerase reaction. The approach was constructed to simplify the analysis by detecting trace amounts of target isothermally, in real-time, and in a homogeneous solution. Ma and co-workers applied this method to measure thrombin in human serum samples and determined detection at a concentration as low as 0.17 nmol/L thrombin within 60 minutes.

An isothermal reaction for simultaneous amplification and detection of DNA is the beacon assisted detection amplification (BAD AMP). Connolly and Trau designed an integrated "biological circuit" composed of two molecular switches to detect, amplify and measure a specific DNA sequence in a cellular extract [177].

In 2003, Van et al. developed an exponential amplification reaction (EXPAR) for short oligonucleotides (called triggers) combining polymerase strand extension and single-strand nicking [178]. The reaction yields the 10^6 to 10^9-fold DNA amount under isothermal conditions within minutes. Jia and co-workers demonstrated that the EXPAR method is particularly suitable for the efficient amplification of small miRNAs [179]. By real-time measurement of fluorescence intensity, the presence of as low as 0.1 zmol of a miRNA (~ less than 100 copies of miRNA molecule in a volume of 10 mL) can be exactly defined. The assay displayed a great dynamic range over 10 orders of magnitude and high specificity to clearly discriminate a one-base difference in miRNA sequences. Furthermore, this method can be performed by using SYBR Green I as a fluorescent dye. Consequently, it is a simple, low-cost and highly sensitive method that should contribute significantly to future advances in research on the biological roles of miRNAs as applications in clinical diagnostics with miRNA as target. Nicking enzyme amplification reaction (NEAR) is a recent improvement of EXPAR, because it allows the any target amplification by inserting nicking enzyme recognition sites inside of the targeted gene sequence [178]. The NEAR technology is based on the very fast detection of small DNA or RNA fragments generated directly from the target nucleic acid. One main target of the proposed NEARs is

therefore small microRNAs [179], which can be amplified by >10^6-fold within minutes [178]. A combination of different isothermal amplification methods for the detection of microRNA has given a limit of detection of 2 aM [180]. Ménová et al. used that reaction to produce short diverse base-modified single-stranded oligonucleotides that are of potential interest as labelled primers or functionalized aptamers [181,182]. Although there are still not many publications on NEAR, two companies are very active in developing assays based on NEAR. Ionian Technologies Inc. (San Diego, USA) founded by the inventor of the technology, is currently focused on diagnostic products. They demonstrated the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in 5 min with a LOD of 10 copies [38]. EnvirLogix Inc. (Portland, USA) have shown the use of NEAR for the point-of-testing of plant pathogens [183]. The amplification products can be detected by a variety of standard methods, including LC-MS, real-time fluorescence, and capillary electrophoresis detection.

In addition to NEAR, the nicking enzyme mediated amplification (NEMA) is a similar reaction that was developed in 2006 from You et al. (Hagzhou Yousida Biotechnology Co. Ltd.) [184]. Both NEAR and NEMA use nicking enzymes instead of restriction enzymes, so there is no need for modified nucleotides like it is in the SDA reaction. The difference between both reactions is the application of only inner primer pairs in NEAR, while NEMA needs two primer pairs like in the SDA reaction [185].

The isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) was developed by Takara Bio Inc. and amplifies DNA targets by employing Rnase H and a strand displacing polymerase [186-188] at 55°C after an initial denaturation step. This method uses a single pair of 5'-DNA-RNA-3' chimeric primers and has given a signal at 100 fg resp. a few hundred copies of template DNA [186]. Practical work in the area of diagnostics was done on the amplification of 16S rDNA [189] or semi-automated bacterial spore detection systems for bio-warfare agents (e.g., Bacillus anthracis) including an aerosol sampler [190]. The detection of 10^4 spores was achieved within 2 h employing microfluidics. Quantification of targets was possible by measuring an increase of fluorescence. Commercial kits are available from Takara Bio (Otsu, Japan), e.g. gene polymorphism typing systems.

Isothermal chain amplification (ICA) was developed by RapleGene Inc. in 2010. A DNA-RNA-DNA chimeric primer set and a further outer primer set for strand displacement are used. RNase H cuts the RNA, anneals to the single strand and a strand-displacing polymerase generates the amplicon [191].

The single primer isothermal amplification (SPIA) was developed by NuGen™ in 2005, which provide various SPIA products [192]. This reaction uses a chimeric primer consisting of DNA at the 3′-end and RNA at the 5′-end. After the primer annealing and the elongation by a DNA polymerase the RNA at the 5′-end is cleaved by RNase H and the strand displacement activity of the polymerase displaces the synthesized stand by elongation of the new annealed primer. This reaction is done by 45-50°C for approximately 4 h. RNA as well as DNA can serve as amplification target [39]. Integration into a point-of-care system has not yet been shown.

Dirks and Pierce described in 2004 the hybridization chain reaction (HCR) for linear amplification of DNA without the need of enzymes. Two stable DNA hairpins are opened in the presence of the target DNA. These leads in a cascade of hybridization events to a growing DNA chain, that can be detected [193].

Conclusion

In this study, we highlighted the best known isothermal amplification methods for nucleic acids in combination with miniaturized systems. Several different solutions for microfluidic devices capable for particulary LAMP, RPA, NASBA, HDA, SDA, RCA and SMART were demonstrated.

The benefit of isothermal amplification methods compared to PCR is the constant temperature of amplification. This will result in a high potential for a simple integration in point-of-care devices and a reduction of complexity.

Microfluidic-based systems for isothermal amplification of nucleic acids have generally many advantages to the user, including lower costs, minimum consumption of samples, faster analysis and automation of all steps from sample preparation to signal detection. In comparison to PCR, there is no need for a sophisticated heating and cooling device, which controls the temperature cycles accurately. However, some isothermal reactions need a precise temperature control as well. In order to achieve an optimal and efficient heat transfer into the reaction chamber, the contact of the heating source to the microfluidic devices needs to be considered. Nevertheless, the thermal design of the microfluidic device is far more simplified in comparison to a PCR chip: the bonding methods as well as the choice of materials (e.g. for a valve) are by far broader due to the lower temperature used. Temperature sensitive reagents can be used and the power consumption of the device is reduced.

Chemical reactions or latent heat storages are suitable to generate heat for isothermal amplification techniques at constant temperatures. Several chemical and especially exothermal reactions, partly in combination with phase-change material (PCM) to maintain temperature have been reported to produce heat and to keep foods and coffee warm or for the development of hand warmers. The released heat can also be used for isothermal amplification strategies. Especially LAMP was demonstrated to work in combination with such heating methods. The chemical reactions include (amongst others): the hydration of calcium oxide, calcium chloride or of magnesium-iron coatings as well as the oxidation of iron and the crystallization of sodium acetate. These exothermal reactions have the potential to reduce the complexity and the costs of a point-of-care device, are working independently from electricity and present a special option for the development of single-use systems.

In the future, isothermal amplification methods for nucleic acids integrated in portable, easy-to-use microsystems offer great opportunities for home-care DNA diagnostic devices.

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