frontiers in Sensors

REVIEW
published: 28 September 2021
doi: 10.3389/fsens.2021.752600

Isothermal Amplification of Nucleic Acids: The Race for the Next "Gold Standard"

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Nucleic acid amplification technologies (NAATs) have become fundamental tools in molecular diagnostics, due to their ability to detect small amounts of target molecules. Since its development, Polymerase Chain Reaction (PCR) has been the most exploited method, being stablished as the "gold standard" technique for DNA amplification. However, the requirement for different working temperatures leads to the need of a thermocycler machine or complex thermal apparatus, which have been preventing its application in novel integrated devices for single workflow and high throughput analysis. Conversely, isothermal amplification methods have been gaining attention, especially for point-of-care diagnosis and applications. These non-PCR based methods have been developed by mimicking the *in vivo* amplification mechanisms, while performing the amplification with high sensitivity, selectivity and allowing for high-throughput analysis. These favorable capabilities have pushed forward the implementation and commercialization of several platforms that exploit isothermal amplification methods, mostly against virus, bacteria and other pathogens in water, food, environmental and clinical samples. Nevertheless, the future of isothermal amplification methods is still dependent on achieving technical maturity and broader commercialization of enzymes and reagents.

Keywords: isothermal amplification, non-pcr amplification, point—of—care, nucleic acids amplification, NAAT

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Specialty section:
This article was submitted to Biosensors, a section of the journal Frontiers in Sensors

Received: *03 August 2021*
Accepted: *13 September 2021*
Published: *28 September 2021*

Citation:
Oliveira BB, Veigas B and Baptista PV (2021) Isothermal Amplification of Nucleic Acids: The Race for the Next "Gold Standard". Front. Sens. 2:752600. doi: 10.3389/fsens.2021.752600

INTRODUCTION

Innovations in biotechnology and molecular biology have push forward novel nucleic acid amplification technologies (NAATs) to meet the demands for more efficient, sensitive, specific protocols capable to be integrated into portable systems and for high-throughput analyses. Polymerase Chain Reaction (PCR), introduced by Mullis in 1985 (Mullis et al., 1986) has become the leading method for DNA amplification with application in all types of molecular detection strategies, such as recognition and identification of infectious pathogens, characterization of genetic disorders, identification of disease biomarkers, gene expression studies, sample preparation for downstream applications and techniques (e.g., sequencing, labelling, etc.), among many others (Klein 2002). In fact, PCR is considered the "gold standard" in molecular analysis of nucleic acids, owing to its capability to amplify from as few as 1 to 10 molecules of target, ultimately leading to an increase to the sensitivity of molecular assays. As a result of the hype regarding PCR, many variants have been developed to address the ever-growing need for additional features, such as

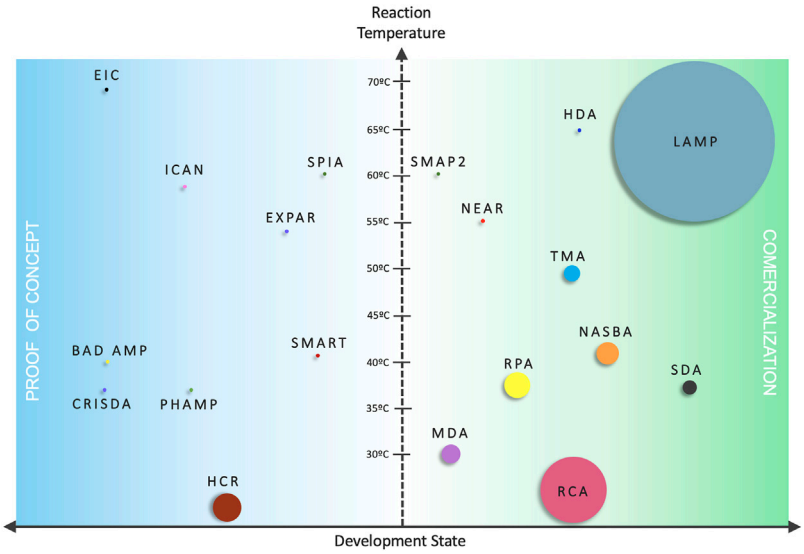

FIGURE 1 | Comparison between isothermal amplification mechanisms. Vertical axis represents the reaction temperature and the horizontal axis the development status—(Blue) IA methods not developed much beyond proof of concept/academic research only; (Green) IA methods commercialized under kit formats/exploited by biotech companies. The size of the circles is proportional to the number of scientific items in literature.

multiplex (Edwards and Gibbs 1994), nested (Porter-Jordan et al., 1990), quantitative (Porter-Jordan et al., 1990), reverse (Porter-Jordan et al., 1990) and digital forms (Kopp et al., 1998), which will be further addressed. This is indeed the technique against all others compare.

Still, inherent features to PCR require dedicated instrumentation capable of thermal cycling with appropriate temperature control, which is usually found in laboratory settings. This small but critical bottleneck has spurred the development of non-PCR based techniques, preferably suitable to be performed at a constant temperature—isothermal amplification (IA) methods, which aim at simplifying protocols for point-of-care (PoC) use, allowing to move the molecular diagnosis from centralized labs.

This review shall focus on the most representative and applied isothermal amplification methods, such as Nucleic Acid Sequence-based Amplification (NASBA), Loop-mediated Isothermal Amplification (LAMP), Strand Displacement Amplification (SDA), Recombinase Polymerase Amplification (RPA) and Rolling Circle Amplification (RCA), as well as their characteristics, applications and prospects to become the next gold standard amplification technique (**Figure 1**).

Despite the plethora of different IA concepts, they converge in the need to remove temperature cycling requirements for specific amplification (Li and Macdonald, 2015). While PCR requires the lowering and rising of two or three working temperatures, to allow annealing and denaturation of DNA strands and primers, and to facilitate strand displacement, IA methods proceed at a single working temperature, which removes the need for a thermocycler. Some of these isothermal amplification mechanisms also allow direct amplification from non-DNA targets, such as RNA (without the need for an additional retro transcription step) or protein-nucleic acids conjugates. Another interesting feature of these IA reactions is that they show increased tolerance to (bio-)chemical inhibitors often present in clinical samples (Bachmann et al., 2009; Craw and Balachandran 2012; Kaneko et al., 2007; Mori and Notomi 2009; Vincent et al., 2004). A summary of the main IA mechanisms proposed in the literature are presented in **Table 1**, together with key advantages and disadvantages.

Despite the exciting advantages of the IA schemes, these approaches also face some challenges, such as complex primer design, unspecific amplification, high background signal, confuse reaction mechanisms and the requirement for more enzymes and denaturing agents (Karami et al., 2011), These methods differ from each other in terms of primers, enzymes, reaction conditions, attainable sensitivity, specificity and ideal target molecules (miRNA, long DNA fragments, circular DNA and RNA targets) (Chang et al., 2012). Overall, isothermal amplification processes produce longer amplicons, have greater amplification efficiency, produce higher amplification yields and require less sample preparation steps than PCR (Klein 2002). In fact, it has been reported that non-PCR based clinical tests often outmatch the PCR results in clinical diagnosis (Aryan et al., 2010; Bachmann et al., 2009). From these, a risen trend concerning IA systems has been observable, over the last decades (**Figure 2**).

In fact, scientific literature has been reporting the application of isothermal amplification systems in a wide number of fields, among them: pathogen detection in clinical, environmental and food samples (Fang et al., 2010; Chow et al., 2008; Zhao et al., 2012; Mahmoudian et al., 2008a; Krõlov et al., 2014), diagnosis of various infectious diseases (Schopf et al., 2011; Hellyer and Nadeau 2004; B.

TABLE 1 | Summary of the characteristics of PCR and the main isothermal amplification systems, highlighting key advantages and disadvantages.

Property	PCR	NASBA	LAMP	SDA	RCA	RPA
Nr of required enzymes	1	3	1	2	2	2
Primer design (Nr of primers)	Simple 2	Simple 2	Complex 4 or 6	Complex 2 or 4	Simple 2	Simple 2
Temperature	Thermal Cycling (95, 50−65, 72) oC	Isothermal ~41°C	Isothermal 60−65°C	Isothermal 37°C	Isothermal 30°C	Isothermal 37−42°C
Reaction time	2–3 h	1.5−2 h	<1 h	2 h	1.5 h	20–40 min
Target	DNA (RNA)	ssRNA (DNA)	dsDNA (RNA)	ssDNA (RNA)	Circular DNA (RNA)	dsDNA
Amplicon	dsDNA	RNA, DNA	Concatenated DNA	dsDNA	Circular DNA	Long dsDNA
Sensitivity	1–10 copies	Single copy	Single copy	10 copies	10 copies	Single copy
Detection	Gel electrophoresis, Real-time and ELISA	Gel electrophoresis, Real-time and ELISA, ECL	Gel electrophoresis, Real-time and turbidity	Gel electrophoresis, Real-time	Gel electrophoresis, Real-time	Gel electrophoresis, Real-time and ELISA
Tolerance to cotaminants	Low	Medium	High	Low	Low	High
Initial template denaturation	Yes	No	No	Yes	No	No
Template processivity	Heat	Rnase H	Strand-displacement property of *Bst* polymerase	Restriction enzymes Heat	Strand-displacement property of ϕ29 DNA polymerase	Recombinase
Advantages	Accurate and robust quantification	Design to detect RNA targets	10^9-fold amplification in less than 1 h	Suitable for miRNA profiling	Low amplification temperature (~37°C) ssDNA products are compatible with other isothermal techniques	Low amplification temperature (~37°C)
	Simplicity of the procedure	10^7-fold amplification in 2 h	Tolerance to inhibitory substances	10^5-fold amplification in 2 h	Produce long DNA fragments	Selective
	Well-stablished method	Kits commercially available	Highly specific	Commercially available platform	Specific for circular targets	Fastest amplification system (20–40 min)
	Availability of wide number commercial kits	Power saving (41°C)	Allows naked-eye detection	Power saving (37–50°C)	—	Tolerance to high volume reduction
	—	—	Great variety of commercial kits	—	—	Tolerance to inhibitory substances
Disadvantages	High equipment cost	Not ideal for DNA targets	Not suitable for small targets	Initial denaturation required	Only works with circular templates	Stringent reaction condition
	Intolerant to many substances	Less efficient for long RNA targets	False positive phenomenon often occurs	Requires sample preparation	RNA amplification is complex and often problematic	Amplification products cannot be readily detected by electrophoresis
	Error prone polymerase	Prone to false positives	Complex primer design	Limitation to small targets	Linear targets require ligation reactions	Commercial kits are expensive
	Less efficient than most of the isothermal methods	Need to optimize 3 different enzymes	—	Prone to unspecific amplification	Linear amplification profile	—
	Long amplification time	—	—	—	—	—

Abbreviations: PCR—Polymerase Chain Reaction; NASBA—Nucleic Acid Sequence-based Amplification; LAMP—Loop-mediated Isothermal Amplification; SDA—Strand Displacement Amplification; RPA—Recombinase Polymerase Amplification; RCA—Rolling Circle Amplification.

A. Rohrman and Richards-Kortum 2012; Torres-Chavolla and Alocilja 2011; S. Y. Lee et al., 2007) and Single-Nucleotide Polymorphisms (SNP) detection (Li et al., 2010). Furthermore, some clinical trials based on IA systems for diagnosis and detection of infectious diseases have been performed: LAMP—(ClinicalTrials.gov Identifier:NCT02371395 2018a; ClinicalTrials.gov Identifier: NCT04579549, 2021; ClinicalTrials.gov Identifier:NCT03829735, 2020a; NASBA—ClinicalTrials.gov Identifier: NCT01838902 2018a; HDA—ClinicalTrials.gov Identifier: NCT01838902 2018b; RPA—ClinicalTrials.gov Identifier: NCT04500873 2020b). Among the publications concerning these amplification techniques, most are focused on the analysis of water and food, with LAMP covering 67 and 59% of the publications, respectively. Then RPA covers for circa 20%, and the remainders, RPA, RCA, HDA and NASBA add up to 5%.

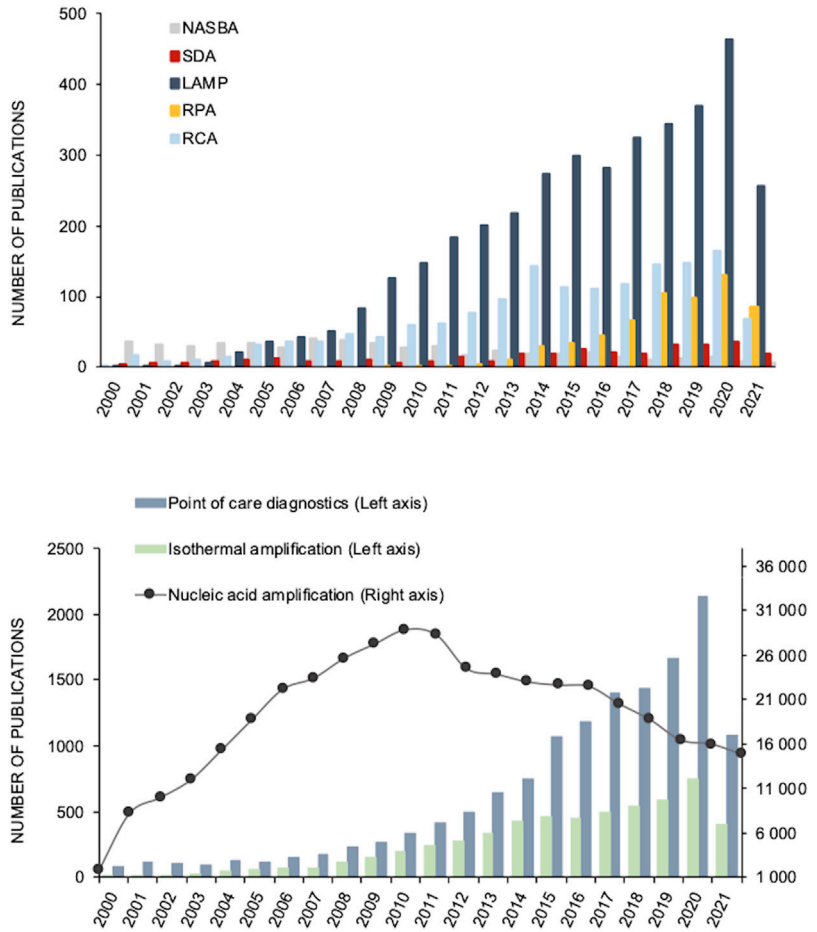

FIGURE 2 | Number of scientific publications per year concerning amplification systems from 2000 until 2021. **(A)** Number of publications with the key words "Nucleic acid sequence-based amplification (NASBA)", "Strand displacement amplification (SDA)", "Loop mediated isothermal amplification (LAMP)", "Rolling circle amplification (RCA)" and "Recombinase polymerase amplification (RPA)". **(B)** Number of with the key words "DNA amplification", "Point of care diagnosis" and "Isothermal amplification". All the data was obtained from PubMed (accessed May 21, 2021).

Despite the continuous efforts to make IA strategies more robust, there are still some hurdles before these approaches might eventually counteract current PCR limitations. Some constraints relate to complex primer design, different kinetics for template denaturation, the high proficiency of the amplification might indivertibly lead to unspecific amplification, and the need for multiple enzymes that are still not in the mainstream production (Karami et al., 2011). Other bottlenecks are shared between PCR and IA, such as requirements for apparatus towards test portability. Still, some IA schemes have been endorsed for particular applications, some already being approved by Regulatory Agencies, such as the Food and Drug Administration (FDA), European Medicines Agency (EMA), and even the World Health Organization (WHO) through the Foundation for Innovative New Diagnostics (FIND), for molecular diagnostics of critical pathogens (Guichón et al., 2004; Eiken 2021; Lucigen 2021; New England BioLabs, 2021), which has attracted further attention by diagnostics-oriented biotech companies (Karami et al., 2011). Some of these advances are depicted in **Table 2**.

MATURE ISOTHERMAL AMPLIFICATION SYSTEMS—HOW CLOSE TO BECOME THE NEXT "GOLD STANDARD"

Nucleic Acid Sequence-Based Amplification

Nucleic acid sequence-based amplification (NASBA) is an isothermal transcription-based technique, that mimics the retroviral RNA replication (Compton 1991). NASBA mechanism entails two phases: 1) non-cycling, where the target RNA is converted to dsDNA by reverse transcription; and 2) cycling, where the dsDNA molecules are actively transcribed into RNA products, leading to a yield of 10–100 copies of RNA from each template molecule (Hønsvall and Robertson 2017) (**Figure 3A**).

Even though this mechanism requires three enzymes - Reverse transcriptase, RNase H and T7 RNA polymerase, there are some advantages when compared to Reverse-transcription-PCR (RT-PCR), such as isothermal working temperature (41°C), which allows amplification on a simple heating block, shorter reaction

TABLE 2 | Summary of commercial test kits, respective targets and companies.

	Kit	Diagnostic use	Cost	FDA approval	CE market	Available tests	Biotech companies
NASBA	Nucli SENS EasyQ	No	$ 50,000	No	No	HIV	Bio Merieux, France
	OligoC-TesT	Yes	$26/test	No	Yes	*Leishmania* parasites	CorisBioConcept, Belgium
LAMP	Genie II	No	$13 000 Instrument	No	No	Compatible with most fluorescence-based assays	OptiGene, United Kingdom
	Loopamp™	Yes	€352.50/96 reactions	Yes	Yes	*Mycobacterium tuberculosis*, Plasmodium vivax, Plasmodium falciparum* and *Plasmodium pan* species, SARS-CoV-2*	Eiken Chemical Co., Japan
	Alethia (Illumigene)	Yes	—	Yes	Yes	*C. difficile*, *Herpes simplex virus (HSV) type 1 and 2*, Cytomegalovirus*, Streptococcus* agalactiae, *Mycoplasma* pneumonia, *Bordetella* pertussis, *Streptococcus* pyogenes, Plasmodium *spp*	Meridian Bioscience, 2021, United States
SDA	ProbeTec Becton	Yes	—	Yes	Yes	*Chlamydia trachomatis* Neisseria gonorrhoeae* HSV 1and2**	Dickinson and Co., United States
RCA	illustra TempliPhi 2000	No	$ 3,804/200 reactions	No	No	User design	GE Healthcare, United States
RPA	Twista	No	$160/1 00 reactions	No	No	User design	TwistDX, United Kingdom
MDA	REPLI-g Mini Kit	No	$807/100 reactions	No	No	User design	Qiagen, United States
NEAR	DNAble®	No	—	No	No	GMO testing	EnviroLogix, Brazil
SMAP 2	AccuLift Ultra-Sensitive RNA Amplification Kit	No	—	No	No	User design	Fluidigm, United States
HDA	BESt Cassette Type II	Yes	$10/test	Yes	No	*Neisseria gonorrhoeae Chlamydia trachomatis,* HSV 1and2*	BioHelix United States
	AmpliVue and Solana	Yes	—	Yes	No	*Bordetella pertussis, Trichomonas vaginalis* Clostridium difficile**, Herpes simplex virus type 1 and 2* Varicella-zoster virus, Group B *Streptococcus* Bordetella parapertussis, Streptococcus pyogenes** Influenza A, Influenza B respiratory syncytial virus Human metapneumovirus, *Streptococcus dysgalactia**	Quidel, United States
TMA	Aptima® assays	Yes	—	Yes	Yes	*Chlamydia trachomatis*, Neisseria gonorrhoeae* Mycoplasma genitalium, Trichomonas vaginalis* HSV 1 and 2*, Zika Virus, HIV-1*, HBV*, HCV, SARS-CoV-2*	Hologic® Inc., United States

(*) Tests with FDA approval; Abbreviations: MDA—Multiple Displacement Amplification; NEAR—Nicking and Extension Amplification Reaction; HDA—Helicase Dependent Amplification; SMAP2—Smart Amplification Process Version 2.

time and less prone to inhibitory components in clinical and environmental samples (Dyer et al., 1996; Rutjes et al., 2006). In addition, NASBA uses RNA as the main target molecule, removing the need to previously prepare cDNA, thus saving time, labor and reducing the risk of contamination (Hønsvall and Robertson 2017). Also, contamination with DNA does not interfere with the reaction, because the temperature is kept bellow the DNA melting temperature, so strand displacement does not occur (Simpkins et al., 2000) (**Table 3**).

Nevertheless, there are limitations concerning NASBA implementation, such as the challenge to optimize three different enzymes that leads to higher costs, and the susceptibility to false positives that require additional probe-specific detection methods, which are generally more expensive than non-specific dyes (Cordray and Richards-Kortum 2012). Once amplification has been achieved, there are several ways to detect and monitor products, such as: molecular beacons, which is the most commonly used (Leone et al., 1998), fluorescence markers

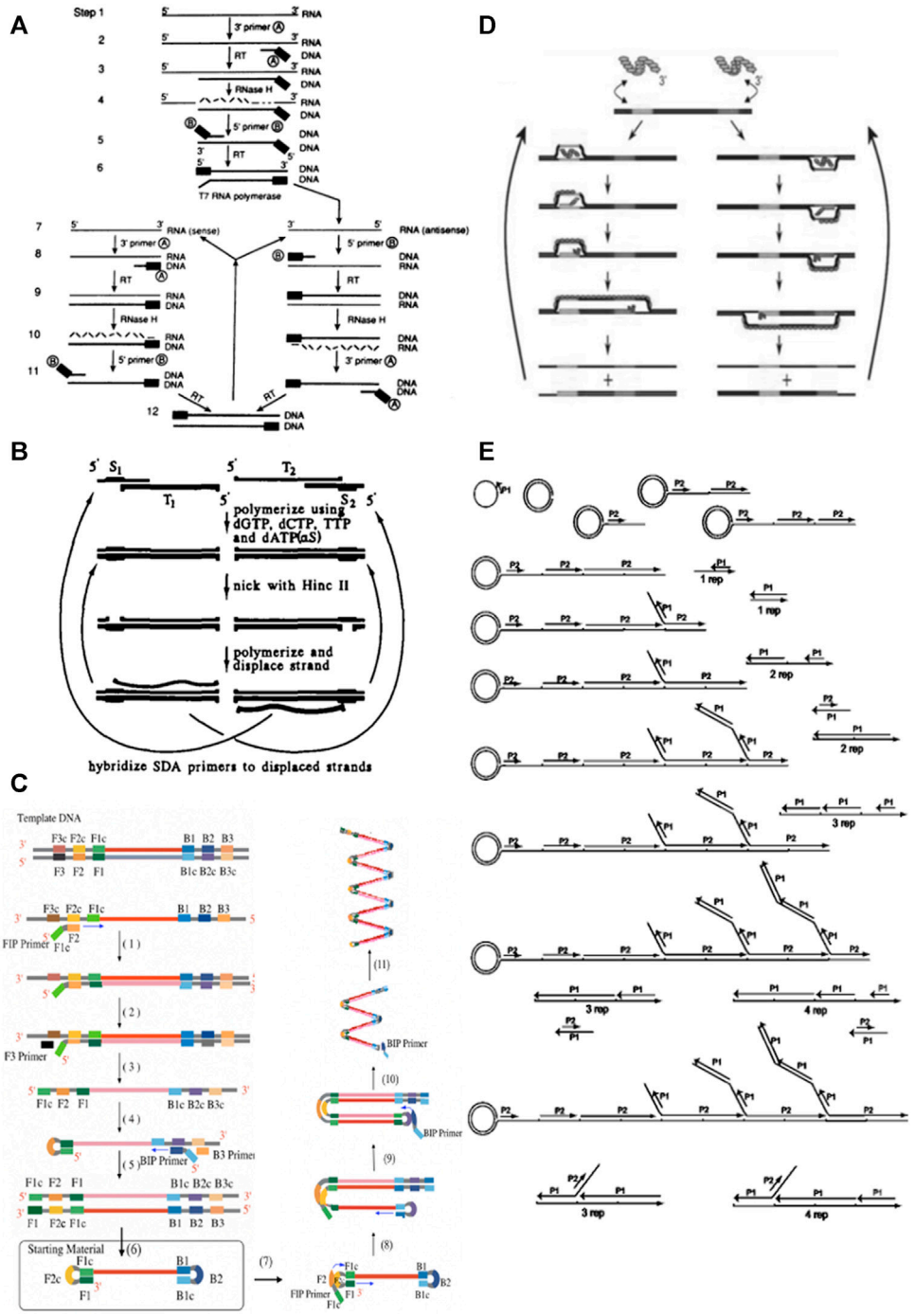

FIGURE 3 | Schematic representations of the main isothermal amplification techniques. **(A)** Schematic representation of NASBA. Initially, the forward primer hybridizes to the target RNA molecule, leading to the formation of complementary DNA (cDNA) intermediate by reverse transcriptase and RNase H activity. Secondly, the reverse primer hybridizes in the cDNA intermediate forming a double stranded (ds) cDNA intermediate with a promoter region, which is recognized by T7 DNA-dependent RNA polymerase to produce more initial RNA template targets by transcription of the cDNA intermediate. The newly formed antisense RNA targets and cDNA serve as templates for the continuous cycling of reverse transcription (rt) and transcription reactions, resulting on the exponential accumulation of antisense RNA molecules complementary to the initial RNA target. **(B)** Schematic representation of SDA. The SDA mechanism starts with heat denaturation in the presence of two primers. After this step, primers hybridize forming two primer-template duplexes with 5' overhangs, each containing one restriction site for the endonuclease (NEase) to cleave. Following, DNA polymerase extends the 3' ends of the duplexes to produce dsDNA with complete recognition sites, that will be cleaved after by NEase. These nicks create new 3' ends that promote new extension reaction with the displacement of the downstream fragment by DNA polymerase. The cycles of these sequenced events of cleavage and polymerization/displacement continuously produce a ssDNA molecule complementary to each of the primer-template duplexes, resulting in exponential accumulation of target sequences. **(C)** Schematic representation of LAMP. Steps (1) to (6) correspond to the first stage of LAMP—starting material producing stage. Steps (7) to (10) correspond to the second stage—Cycling amplification. Step (11) represent the third and final step of

(*Continued*)

FIGURE 3 | LAMP—Elongation and rolling cycle step. Adapted from reference (Notomi, 2000). **(D)** Schematic representation of RPA. The recombinase-primer filaments scan the dsDNA target molecule for the homologous site. Recombinase catalyzes the primer hybridization with the homologous template sequence. Strand exchange is promoted by recombinase in the cognate sites. The resulting structures are stabilized by ssDNA-binding proteins to prevent primers displacement by branch migration. The DNA polymerase recognize the 3' end of the primer and starts primer extension reaction. The binding/extension events of two opposing primers generate one complete copy amplicon together with the original template. **(E)** Schematic representation of RCA of a circularized probe using two primers. The first primer (P1) initiates an RCA reaction, and the reverse primer (P2) binds to each tandem repeat generated by the rolling circle. Multiple priming events are initiated by P2 as the original RCA strand elongates. As these priming events elongate and generate displaced DNA strands, new priming sites for the first primer (P1) are generated. To follow the sequence of strand displacement events, note that as the reverse primer P2 binds to the fifth repeat, the primer at the third repeat begins to displace a branch; subsequently, as P2 binds to the seventh repeat, the elongating strand at the fifth repeat begins to displace a branch, and so forth. By the time a reverse primer binds to the tenth repeat, the DNA product already contains three growing branches. New primer extension events initiated in released DNA molecules also generate branches, as shown at the bottom of figure. As the displaced DNA becomes completely double-stranded, it accumulates in fragments of unit length containing one, two, three or four repeats (shown as: 1 rep, 2 rep, 3 rep, 4 rep). Thus, in the presence of a circular template, the two primers generate a self-propagating, ever-increasing pattern of alternating strand-displacement, branching and DNA fragment release events, which we call hyperbranching.

TABLE 3 | Advantages and disadvantages of NASBA in comparison with PCR.

Advantages	Disadvantages
Sensitivity (detection limits <1 cell per reaction)	Reaction kits are expensive
Isothermal (does not require thermocycler)	False-positives more common than false-negatives
Shorter reaction time	Harder to calculate concentrations with qNASBA
Less labor-intensive	Often requires probe-specific detection
Does not require cDNA preparation	Requires the optimization of three different enzymes
Less susceptible to reaction inhibitors	—
Decreased risk of contamination	—

cDNA—Complementary DNA; qNASBA—Quantitative Nucleic Acid Sequence-based Amplification.

TABLE 4 | Top. Examples of LOC devices using NASBA for amplification and detection of targets with environmental interest. **Middle.** Examples of LOC devices using LAMP for the amplification and detection of targets relevant in infectious diseases and food quality control. **Bottom.** Examples of LOC devices using RPA for the amplification and detection of targets relevant in infectious diseases and antibiotic resistance.

	Target	Amplification	Detection	Device material	Ref
NASBA	*Escherichia coli*	NASBA (NucliSENS kit)	Molecular beacon	PDMS	Dimov et al. (2008)
	Norovirus	NASBA (NucliSENS Kit)	SYBRs Green II	PMMA and PDMS	Chung et al. (2015)
	hsp70 of *Cryptosporidium parvum*	NASBA (NucliSENS Kit)	Lateral flow assay	PMMA	Reinholt et al. (2014)
	rbcL of *Karenia brevis*	NASBA (gelled/modified reagents from Basic EasyQTM kit)	Molecular beacon (read by portable LabCardReader)	Labcard made with COC	Tsaloglou et al. (2013)
LAMP	*Salmonella spp.*	LAMP	Eva Green, SYBR Green I, SYTO-26, SYTO-62 and SYTO-82	COC	Y. Sun et al. (2015)
	Nervous necrosis virus	RT-LAMP	slab-electrophoresis	PDMS	C. H. Wang et al. (2011a)
	Salmonella enterica Typhimurium	Loopamp™ DNA Amplification Kit	SYBR Green I	PMMA and PSA	Uddin et al. (2015)
	Hepatitis B	LAMP	Electrochemical (square wave voltammetry)	Custom made portable device	Jayanath et al. (2018)
	Salmonella spp.	LAMP	Naked eye observation with AuNPs	PDMS and PMMA	Garrido-Maestu et al. (2017)
RPA	methicillin-resistant	RPA	RPA exo kit (TwistDx)	PDMS	Yeh et al. (2017)
	Staphylococcus aureus DNA	Digital RPA	Fluorophore/quencher system	SlipChip	Shen et al. (2011)
	$bla_{CTX\text{-}M\text{-}15}$ and $bla_{NDM\text{-}1}$ genes	RPA	ion-sensitive field effect transistor	TFT sensor, Nanoribbon transistors, PDMS and PMMA	Hu et al. (2017)
	F. tularensis holarctica	Solid phase RPA	Label-free detection	PMMA, PDMS, SOI wafer	Sabaté del Río et al. (2015)
	mecA gene	Multiplex solid-phase RPA	Fluorescence	poxy-silanated glass	Kersting et al. (2014a)
	Ebola virus	RT-RPA	Fluorescence	Paper microfluidics device	Magro et al. (2017)

NASBA—Nucleic Acid Sequence-based Amplification; PDMS—Polydimethylsiloxane; PMMA—Poly(methyl methacrylate); COC—Cyclic olefin co-polymer; LAMP—Loop-mediated Isothermal Amplification; RT-LAMP—Reverse-transcriptase LAMP; PDMS—Polydimethylsiloxane; PMMA—Poly(methyl methacrylate); COC—Cyclic olefin co-polymer; PSA—Pressure-sensitive adhesive; RPA—Recombinase Polymerase Amplification; RT-RPA—Reverse-transcriptase RPA; PDMS—Polydimethylsiloxane; TFT—Thin Film Technology; PMMA—Poly(methyl methacrylate); SOI—Silicon on Insulator.

that bind and/or intercalate into the produced amplicons (Hønsvall and Robertson 2017) and gel electrophoresis for end-point detection (Morabito et al., 2013). Additionally, other colorimetric methods have been proposed that rely on nanoparticles, e.g., gold nanorods (Niazi et al., 2013) and spherical gold nanoparticles functionalized with specific probes (Mollasalehi and Yazdanparast, 2012).

Despite its promising performance, NASBA has yet to be widely acknowledge by users in mainstream labs and thus it remains in the "shadow" of other amplification techniques. NASBA has already shown it usefulness for the detection of common waterborne pathogens, such as *Escherichia coli* (Heijnen and Medema, 2009), *Pseudo-nitschia multiseries* (Delaney et al., 2011), norovirus (Rutjes et al., 2006) and notoriously it has been adopted by international space agencies for monitorization of recycled water in the International Space Station (Bechy-Loizeau et al., 2015). Another example suitable for the standard user is the NucliSENS EasyQ, an automatized commercially available NASBA system directed at the real-time amplification detection by fluorescence (Yao et al., 2005), (Biomerieux, 2021).

Depending on the detection method used for monitorization of the isothermal amplification profile, there is the possibility of NASBA integration into Lab-on-chip (LOC) devices, suitable for point-of-need, low-cost and portable platforms. In fact, there are some examples of LOC devices using NASBA for the assessment of environmental pathogens (**Table 4**), among which the first integrated chip for RNA isolation, NASBA amplification and real-time detection proposed (Dimov et al., 2008).

In summary, NASBA has been reported as more sensitive and less prone to inhibitory substances than PCR, which in combination with the isothermal profile makes it a useful point-of-need (PoN) tool. Still, the high cost of the reaction kits, the need to optimize multiple enzymes and the lack of specificity are crucial factors that prevent its widespread uptake by the general community.

Strand Displacement Amplification

In 1992, Walker *et al.* proposed to use the strand displacement capability of primers and enzymes for amplification of a given target (Walker et al., 1992). The strand displacement amplification (SDA) combines the action of an endonuclease, an exonuclease deficient DNA polymerase and two sets of primers, one with single-stranded restriction site overhangs, and a second set called "bumper primers" to support the displacement of the amplification product from the first set of primers. Briefly, dsDNA is initially heat denatured, allowing for the hybridization of the first set of primers with 5′ overhangs with specific recognition site for HincII restriction enzyme. Because of thiol-modified dATP, only the original primer is cleaved by HincII but not the newly synthetized strand, resulting in 3' overhangs, which are extended by the exonuclease deficient klenow (exo-klenow) polymerase and further displace the downstream DNA strand, at temperatures ranging from 30—50°C. The exponential amplification is achieved though the coupling of sense and antisense reactions, in which the displaced sense strand serve as template for the antisense reaction and vice versa—(Deng and Gao 2015) for deeper insights into the molecular mechanism (**Figure 3B**).

Despite the advantages of isothermal reaction, the low temperature (between 30 and 55°C) makes it prone to unspecific primer-binding that might cause unspecific amplifications (Karami et al., 2011), similarly to what happens with NASBA. This is a critical aspect that prevent expansive implementation for diagnostics, since DNA/RNA retrieved from clinical samples usually contains several potential similar target sequences (e.g., gene families, homologous *loci*, etc.) that might miss-prime in relation to the desired target (de Pazet al., 2014). Generally, SDA presents high amplification efficiency for short target sequences that require initial template denaturation.

The high amplification efficiency of SDA associated to the capability to amplify small fragments (ranging from 50 to 120 bp) has highlighted its potential for miRNA expression profiling (Ye et al., 2019; Deng and Gao 2015). Still, SDA takes a long-time to detection (2.5 h), which makes SDA inadequate for on-site amplifications and detection. As such, SDA has been mainly applied to clinical diagnosis of *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and herpes simplex virus from urogenital samples, on a high throughput platform commercialized since 1999 (Little et al., 1999; Akduman et al., 2002). This semiautomated system offers several advantages over the traditional methods for STDs detection, such as the exploit of an alternative NAAT while maintaining high sensibility and specificity, offers high-throughput and removes the need for separate work areas or unidirectional workflow. Additionally, offers medium-free transport for swabs and room temperature storage and the total time of the assays ranges from 3 to 4 h (Van Der Pol et al., 2001). Another example refers SDA inclusion into an automated device that combines sample preparation from whole cells and detection (J. M. Yang et al., 2002).

Loop-Mediated Isothermal Amplification

Loop-Mediated Isothermal Amplification (LAMP) (Notomi et al., 2000) has been the most widely used IA method, referenced in approximately 3,700 scientific publications, 8 clinical trials (ClinicalTrials.gov, 2021) and approved by WHO as an alternative molecular diagnostics method for pulmonary tuberculosis (World Health Organization 2016) and SARS-CoV-2 (Kashir and Ahmed, 2020).

LAMP relies on the strand-displacement activity of a DNA polymerase combined with a set of four unique primers, that may be extended to six for additional acceleration of amplification (Notomi et al., 2000; Mori and Notomi 2009). Compared to other NAATs, LAMP is 10 to 100-fold more sensitive than PCR (X. Wang X et al., 2014), with a detection limit as low as 1 copy per μL of template (H. Zhang et al., 2019), showing a higher specificity (Fujino et al., 2005) with an amplification time usually less than 1 h at 60–65°C, yielding 10^9 copies (Notomi et al., 2000). Additional advantages include not requiring an initial template denaturation step at 95°C and being less prone to inhibitory substances often present in biological samples (Enomoto et al., 2005; Kaneko et al., 2007). Perhaps one of the most interesting

TABLE 5 | Examples of commercial DNA and RNA amplification kits based on LAMP method.

Target	Name of the kit	Company	Ref
Tuberculosis	Loopamp™ MTBC Detection kit	Eiken Chemical Co.	Nguyen et al. (2018)
Malaria	Loopamp™ MALARIA Pan	Eiken Chemical Co.	Cuadros et al. (2017)
Leishmania	Loopamp™ Leishmania Detection Kit	Eiken Chemical Co.	Ibarra-Meneses et al. (2018)
koi herpesvirus	Loopamp™ Koi Herpesvirus Detection Kit	Eiken Chemical Co.	Yoshino et al. (2009)
West Nile Virus	Loopamp™ RNA Amplification Kit	Eiken Chemical Co.	J. S. Kumar et al. (2018b)
Trypanosoma cruzi	Loopamp™ *Trypanosoma cruzi* kit	Eiken Chemical Co.	Besuschio et al. (2017)
Clostridium difficile	Illumigene *C. difficile* DNA assay	Meridian Bioscience	Pancholi et al. (2012)
Mycoplasma pneumoniae	Illumigene *Mycoplasma* DNA assay	Meridian Bioscience	Ratliff, Duffy, and Waites (2014)
bla carbapenemase genes	eazyplex® SuperBug CRE system	Amplex Biosystems	García-Fernández et al. (2014)

benefits is that LAMP output may be visualized by the naked eye (e.g., turbidimetry), making it ideal for poor resource settings and PoC applications (Besuschio et al., 2017).

In brief, LAMP takes advantage of a polymerase with strand displacement activity and four pairs of primers, that recognize a total of six distinct sequences on the target DNA. The forward inner primer (FIP) can hybridize on the dsDNA target an initiate LAMP. The DNA synthesis is initiated by the DNA polymerase upon the annealing of the outer primer (F3) to its complementary region and its subsequent extension causes strand displacement, releasing a ssDNA molecule. This molecule serves as template for the annealing on the other end of the second inner (BIP) and outer (B3) primers, producing a stem-loop DNA structure. After this step, LAMP enters in the cycling phase in which one inner primer hybridizes to the stem-loop and initiates displacement DNA synthesis, yielding the original stem–loop molecule and a new stem–loop DNA with a stem twice as long. The cycling reaction continues leading to an accumulation of around 10^9 copies of target under the form of concatemers in less than 1 h (Notomi et al., 2000) (**Figure 3C**).

The main features of LAMP, and in particular its outstanding specificity and ability to amplify without highly sophisticated equipment, makes it a valuable alternative to PCR (H. Zhang et al., 2019). In fact, LAMP has been used for detection of pathogens, e.g., detection of RNA from severe acute respiratory syndrome virus (M. M. Parida et al., 2008), identification of Methicillin Resistant *Staphylococcus aureus* (MRSA) (C. H. Wang et al., 2011a), tuberculosis (M. F. Lee et al., 2009), influenza A (Jung et al., 2015), Zika (J. Song et al., 2016), nervous necrosis (C. H. Wang et al., 2011b), biomarkers of disease such as Adenoviral keratoconjunctivitis (Wakabayashi et al., 2004), Varicella-zoster virus (Okamoto et al., 2004) and SNP genotyping (Iwasaki et al., 2003) and cancer diagnostics like detecting metastasis of gastric cancer (Horibe et al., 2007), *KRas* gene mutation (Itonaga et al., 2016) and screening of other cancer-related mutations (Srividya et al., 2019). More recently, LAMP as also been applied to the molecular detection of SARS-CoV-2 (Srividya et al., 2019), due to the rapidness and specificity of its amplification mechanisms, being exploited by many biotech-oriented companies.

Additionally, LAMP has also played an important role in quality control of food and dietary products, such as rapid detection of *Salmonella* (Q. Yang et al., 2018), *Staphylococcus aureus* (H. Yang et al., 2011), *E. coli* O157 (Zhao et al., 2010), *Vibrio parahaemolyticus* (Oh et al., 2016) and food allergens (Yuan et al., 2018).

Continuous evolution of LAMP led to improvements to reaction conditions and accuracy, which spun it into a mature and reliable assay, suitable for stand-alone molecular diagnostics (Eiken 2021; Meridian Bioscience, 2021) (**Table 5).** Some of the available commercially and certified detections assays take between 15 and 30 min to result. Additionally, for real-time monitorization of LAMP reaction, low complexity apparatus are also available, such as Instrument Genie II from Optigene (Horsham, United Kingdom) for fluorescence-based measurements and Ilumipro-10 from Meridian Bioscience that detects amplification products based on turbidimetry (de Paz et al., 2014).

Due to its robust and fast isothermal amplification profile, LAMP has become a suitable solution for on field molecular assays (M. Parida et al., 2004), especially for integration in lab-on-chip platforms based on microfluidics. Since the development of the first microfluidic device for the detection of *hepatitis B* virus, that accomplished a sensitivity of about 50 copies per reaction within 60 min (S. Y. Lee et al., 2008), other PoC devices based on LAMP have been developed targeting relevant biomarkers in infectious diseases and food quality control (**Table 4**).

Over the years, several modifications have been made to LAMP, such as digital LAMP where reactions occur in individualized "reactors" (micelles) yielding a 0/1 output, ideal for applications requiring quantification of target (Rane et al., 2015; Gansen et al., 2012), reverse transcription LAMP for assessing gene expression (C. H. Wang et al., 2011a), multiplex LAMP for simultaneous detection of pathogens using dedicated platforms (Abe et al., 2011) and LAMP-on-a-chip (Xuzhi Zhang et al., 2014). These developments allow for high throughput, up to 1,200 samples simultaneously, while remaining extremely sensitive (less than 10 fg of target DNA) in small sample volumes (~10 pL) (Q. Zhu et al., 2012; C. H. Wang et al., 2011b; Rane et al., 2015). However, the complex primer design and the susceptibility to false positives still need to be addressed before LAMP is routinely applied globally (Tanner et al., 2012).

Perhaps one of the most relevant acknowledgements to LAMP has been its recognition by the WHO to fulfil all the criteria for an *ideal* NAAT for diagnostics (Wong et al., 2018). In fact, considering the continuous evolution of LAMP will allow expansion to other targets of relevance, which in turn will push costs of reagents down and make them more accessible,

TABLE 6 | Incorporation of RPA with other technologies.

Detection platform	Unconventional RPA platforms	References
Agar gel electrophoresis	RPA-Agar 3 gel	Piepenburg et al. (2006)
Lateral flow strip	RPA-Lateral flow	Kersting et al. (2014b)
Lateral flow dipstick	RPA-LFD	Tu et al. (2017)
Solid phase	RPA-ELISA	Santiago-Felipe et al. (2014)
	RPA-Electrochemical	Sabaté del Río et al. (2015)
	RPA-Paper	B. A. Rohrman and Richards-Kortum (2012)
Microfluidics	RPA on a chip	Lutz et al. (2010), Escadafal et al. (2014), T. H. Kim et al. (2014)
Fluorescence dye	Real time -RPA	Piepenburg et al. (2006),
Capillary electrophoresis	RPA-sequencing	Oyola et al. (2012)
Microarray	RPA-Microarray	Kersting et al. (2014a)

RPA—Recombinase Polymerase Amplification; LFD—Lateral flow dipstick; ELISA—Enzyme-Linked Immunosorbent Assay. Adapted from: Ameh James and Joanne Macdonald (2015): Recombinase polymerase amplification: Emergence as a critical molecular technology for rapid, low-resource diagnostics, Expert Review of Molecular Diagnostics, DOI: 10.1586/14737159.2015.1090877.

it is expected that LAMP will become a universal tool (H. Zhang et al., 2019).

Recombinase Polymerase Amplification

Recombinase Polymerase Amplification (RPA) is an isothermal amplification mechanism that operates at low temperature, usually between 37°C and 42°C, which exploits the activity of two different enzymes: a recombinase and a DNA polymerase and single stranded DNA-binding proteins. (SSBs)to ensure ssDNA stabilization (Piepenburg et al., 2006). RPA allows amplification to occur in 20—40 min, exhibiting a detection limit of 1 copy of target, being considered as one of the fastest NAATs available (de Paz et al., 2014). Briefly, RPA amplification begins with the binding of a recombinase protein (RecA from *E. coli* or as usually used uvsX from T4-like bacteriophages) to primers in the presence of ATP and a crowding agent (high molecular weight PEG or Carbowax20M), leading to the formation of a recombinase-primer complex. Then the complex scans the dsDNA target seeking for a homologous sequence, once homology is found the complex promotes strand displacement, forming a D-loop structure, which is stabilized by SSBs and further promotes primer-target hybridization. Finally, recombinase disassembly allows for a DNA polymerase with strand displacement activity (Bacillus subtilis Pol 1 or *Sau* Recombinase polymerase) to bind to the 3' end of the primer and elongate it in the presence of dNTPs. An exponential amplification is achieved by cyclic repetition of this process (Lobato et al., 2018) (**Figure 3D**).

Besides the established RPA reaction setup, several improvements have been made allowing RPA to evolve to different formats, such as RT-RPA (Magro et al., 2017), digital RPA (Yeh et al., 2017) and multiplex RPA, an example is the study conducted by Kersting *et al.* for multiplex detection of *Neisseria gonorrhoeae*, *Salmonella enterica* and *Staphylococcus aureus* (Kersting et al., 2014a). Additionally, several proof of concepts on RPA incorporation with varied detection technologies have been described for improved sensitivity, reduced manual handling, improved cost effectiveness and are suitable for rapid molecular diagnosis (James and MacDonald 2015) (**Table 6**).

These RPA-based assays attain comparable sensitivities to those obtained with PCR, but with a significant reduction in the reaction time—time to full reaction between 10 and 20 min (Kunze et al., 2016). Moreover, RPA assays show a detection limit around 10–20 copies of target, reduced sample and reagent volumes, and the capability to amplify under high concentrations of known PCR inhibitors (Polymerase and Rpa 2017; Kersting et al., 2014b; Crannell et al., 2014a). Also, RPA does not require an initial template denaturation step and complex thermal instrumentation (Deng and Gao 2015).

RPA has been mainly applied to the detection of pathogens (Shen et al., 2011; Crannell et al., 2014b; B. Rohrman and Richards-Kortum, 2015). For example, Rohrman and Richards-Kortum accomplished the detection of 10 copies of HIV DNA in under 15 min through RPA incorporated with a lateral flow assay allowing to perform HIV diagnostics compatible with resource poor settings (B. A. Rohrman and Richards-Kortum, 2012). Additionally, an RPA assay was also developed for the detection of Tuberculosis under 20 min with a limit of detection in the femtograms of template (Boyle et al., 2014). RPA has long been translated to the commercial and laboratory stetting - TwistDX (Cambridge, United Kingdom) for both end-point detection (TwistAmp^{TM} LF probe) and real-time monitorization (TwistAmp^{TM} exo probe) (TwistDx 2021).

Apart from the traditional detection methods usually applied for PCR, a unique FRET-based fluorescence probe and a lateral-flow strip system have also been employed in RPA detection of SARS-CoV-2 N gene, which decrease background noise and enable an instrumentation-free readout (Xia and Chen 2020). In fact, the RPA's low temperature reaction profile coupled to the high efficiency and specificity have been supporting the growing application of RPA in LOC devices for the amplification and detection of targets relevant in infectious diseases and antibiotic resistance (**Table 4**).

These examples demonstrate the advantages of RPA, especially as the quest for decentralized NAATs for diagnostics at PoC (Lobato et al., 2018; Daher et al., 2016). Additional reports show the possibility to use RPA for biomedical applications in surveillance of disease biomarkers (Euler et al., 2012), in the food industry (T. H. Kim et al., 2014) and in the agriculture sector

TABLE 7 | Examples of RCA applications.

Application	Target	Amplification	Detection	References
Detection of cancer cells	MCF-7 cells	RCA-directed enzyme-catalyzed polymerization	Electrochemical	Sheng et al. (2015)
	HeLa and Ramos cells	Cascade-RCA	Chemiluminescence	Li Y et al. (2012)
Detection pathogens	*Bacillus globigii* spores	RCA	PLA	Gómez De La Torre et al. (2012)
DNA amplification	M13 phage and pUC16	Multiply primed RCA	Gel electrophoresis	Dean et al. (2001)
DNA methylation	DNA methylation in CpG sites	HRCA	Fluorescent-labeled C-probes	Cao and Zhang (2012)
	DNA methytransferase	PG-RCA	Chemiluminescence	Zeng et al. (2013)
DNA microarray	4,308 mapped RPCI-11 BAC clones	Multiple-primed RCA	Fluorescent dyes	Smirnov et al. (2004)
Genome sequencing	Point-mutations sequencing in breast cancer tissues	RCA	Fluorescent dyes	Ke et al. (2013)
	V. cholerae	RCA and C2CA	Electrophoretic analysis	Mahmoudian et al. (2008b)
Immuno-RCA	Cytokine detection (e.g. MCP-1 and FGP-7)	RCA	Fluorescence sandwich immunoassay	Schweitzer et al. (2002)
In situ detection	*Tp53* gene	RCA	Fluorescent probes	Christian et al. (2001)
	Human centromeric alpha satellite DNA of chromosome 13 and 21		Fluorescent-labeled C-probes	Nilsson et al. (1997)
miRNA detection	miR-16	RCA	Slot-blot	Jonstrup et al. (2006)
	Let-7	BRCA	SybrGreen I dye	Cheng et al. (2009)
Real time monitorization	Synthetic target DNA	Exponentially BRCA	PNA beacon	Smolina et al. (2004)
		HRA	FRET-beacon	Yi et al. (2006)
Small molecules detection	Cocaine	RCA	Molecular beacons	Ma et al. (2011)
	Glucose	RCA-based one-pot method to prepare the micron-sized DNA flowers	Electrochemical biosensor	Yan et al. (2021)

RCA—Rolling Circle Amplification; ERCA—Exponential RCA; BRCA - Branched-RCA; PG-RCA—Primer generation RCA; C2CA—Circle-to-circle Amplification; HRCA—Hyperbranched RCA; PNA—Peptide Nucleic Acid; MCP—Macrophage Chemoattractant Protein 1; FGF-7—Fibroblast Growth Factor; PLA—Proximity Ligation Assay.

(Mekuria et al., 2014). Although, no RPA assays have been approved by FDA or CE market thus far, it is anticipated that its operational advantages will prompt for the uptake of this technology for the detection of human pathogens in low resource settings, which would have a tremendous impact in reducing the high burden of infectious diseases and associated morbidity.

Rolling Circle Amplification

Rolling Circle Amplification (RCA) is an isothermal amplification protocol that operates at low temperature (23°C—60°C), which has been adapted from the *in vivo* RCA mechanism (Fire and Xu, 1995). This system requires a DNA polymerase with strand displacement activity (such as Phi29 DNA polymerase), that in presence of a circular template (e.g., plasmid, bacteriophages, DNA/RNA from virus or bacteria, etc.) and a specific primer generates a long DNA molecule with tandem repeats (D. Liu et al., 1996) (**Figure 3E**). The RCA mechanism has circular DNA as the predominant type of template, but in presence of linear DNA, a surrogate circular intermediate template is formed, that is then used for the subsequent amplification step. The RCA architecture offers two main benefits: 1) the low temperature isothermal scheme, with incubation temperatures as low as 23°C; and 2) the simplicity of its mechanism requirements (single primer and a productive DNA polymerase) (Li and Macdonald 2015), which yields high amplification efficiencies (~10^3 copies in under 1 h), that could be enhanced though the addition of ssDNA-binding proteins (Y. Zhao et al., 2015).

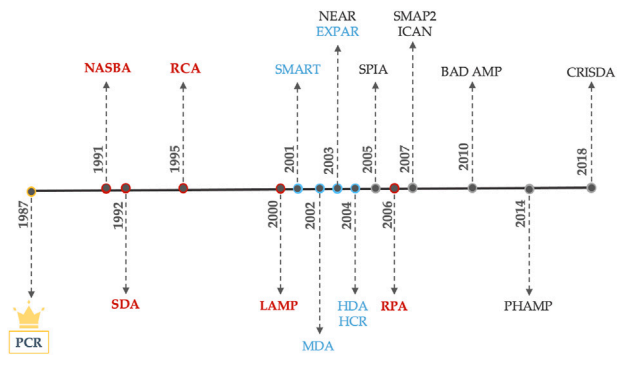

FIGURE 4 | Development year of each amplification scheme. (Red) Main nucleic acids amplification techniques. (Blue) Blooming NA amplification techniques. (Black) Poorly applied NA amplification schemes.

Interestingly, the early RCA mechanism results in a linear amplification profile over time. In order to overcome this hurdle, several techniques have been developed to allow for an exponential mechanism, such as ramification amplification (RAM) (D. Y. Zhang et al., 2001), hyper-branched RCA (HRCA) (Lizardi et al., 1998), cascade RCA (Thomas et al., 1999) and multiply primed RCA (Dean et al., 2001).

One key feature of RCA, i.e., the capability to produce long fragments has been proven of great value to produce material for whole genome amplification, especially in the analysis of

viral DNA genomes (Rector et al., 2004; Inoue-Nagata et al., 2004). RCA can also be performed on a solid support or complex biological matrixes (e.g., inside the cell or on cell surfaces) allowing for molecular level detection (Konry et al., 2011).

These advantageous characteristics have spurred the development of highly sensitive RCA-based detection methods (Yue et al., 2021) (**Table 7**).

The low temperature isothermal profile of RCA also makes it an attractive candidate for integration in point-of-care devices (Giuffrida and Giuseppe Spoto, 2017), for example for the detection of bacteria and virus (Sato et al., 2010; Schopf et al., 2008). Other schemes have evolved from the original RCA, such as C2CA, digital RCA, microbead-based RCA, particle-based RCA, performed in a great diversity of device formats, e.g., centrifugal valve-less (Heo et al., 2016) and droplet-based devices (Giuffrida and Giuseppe Spoto, 2017), DMF chips and paper-strips (Ali et al., 2009).

Despite RCA use has been evolving over 2 decades, no RCA-based diagnostic kits have yet reached the market (de Paz et al., 2014). So far, there are only RCA research-type kits being commercialized, such as the Illustra TempliPhi DNA amplification kit from GE HealthCare (Buckinghamshire, United Kingdom) (Reagin et al., 2003).

Presently, the large number of RCA developments have focused on biotechnology applications and molecular detection schemes, several innovative concepts have been merging RCA and nanotechnology that promise extension of RCA potential to new a totally different scale and scope.

BLOOMING CONTENDERS TO THE "GOLD STANDARD" TITLE

Despite the great number of isothermal amplification systems already showing technological maturity and consolidated applications, such as the reviewed before, the quest for improved systems and specific application still encourages the development of more isothermal amplification methods. The ones with most expression have been the Multiple Displacement Amplification (MDA) (Spits et al., 2006), Exponential Amplification Reaction (EXPAR) and Nicking and Extension Amplification Reaction (NEAR) (Van Ness et al., 2003), Helicase Dependent Amplification (HDA) (Vincent et al., 2004) and Hybridization Chain Reaction (HCR) (Evanko 2004; Dirks and Pierce 2004) (**Figure 4**). Next, these methods are briefly overviewed.

The ability to amplify really short fragments (~10 bp), perform whole genome amplification, use miRNA as targets, generate amplification products under linear ss-DNA forms, error prone reduction and the pursue for more efficient and specific reactions were the driving forces behind the development of these methods.

- Signal-Mediated Amplification of RNA Technology (**SMART**)—Used for detecting both DNA and RNA targets by a tree-way junction mechanism (Wharam et al., 2001).
- Multiple Displacement Amplification (**MDA**)—Ability to amplify very long fragments (around 50 kilobases) (Paul M. Lizardi 1997; Maragh et al., 2008; Y. Q. Sun et al., 2007).
- Nicking and Extension Amplification Reaction **(NEAR)**—Most efficient with short fragments (21–28 bp). Amplifies directly from RNA. Generates ssDNA products (Maples et al., 2007).
- Exponential Amplification Reaction (**EXPAR)**—Most applied to miRNA detection, DNA, proteins, enzyme activity and metal ions. Exponential version of NEAR (R. D. Li et al., 2016; Jia et al., 2010; Van Ness et al., 2003; Wang K et al., 2014; Zhang et al., 2015; J. Chen et al., 2016; H. Liu et al., 2017; Q. Xue et al., 2015; Jia et al., 2014).
- Helicase Dependent Amplification (**HDA**)—Diagnosis of dsDNA pathogens such as *Neisseria gonorrhoeae*, *Clostridium difficile*, *Staphylococcus aureus* and HIV-1 (Goldmeyer et al., 2008; Chow et al., 2008; Pandori and Branson 2010; Tang et al., 2010).
- Hybridization Chain Reaction **(HCR)**—Non-enzymatic mechanism based on successive hairpin hybridization. Applied to mRNA imaging, signal amplification and photoelectrochemical detection (Wu et al., 2015; Sha, Zhang, and Wang 2016; Chu et al., 2019).
- Single Chimeric Primer Isothermal Amplification **(SPIA)**—Specifically used to amplify linear mRNA. It has been applied to Whole genome amplification and Whole transcriptome amplification (Peng et al., 2018; Myrmel et al., 2017).
- Smart Amplification Process Version 2 (**SMAP 2)**—SPN identification and genotyping (Watanabe et al., 2007; Tatsumi et al., 2008; Araki et al., 2010).
- Isothermal and Chimeric primer-initiated Amplification of Nucleic acids **(ICAN)**—Specifically amplify short DNA sequences from complex DNA samples, (Mukai et al., 2007; Shimada et al., 2002).
- Beacon-assisted molecular detection **(BAD AMP)**—Emerging method to detect and amplify short DNA fragments (<22 bp) (Connolly and Matt, 2010; Connolly and Matt, 2011).
- Hairpin Florescence Probe Assisted Isothermal Amplification **(PHAMP)**—Detect and amplify microRNA (<22 bb). Mechanism similar to BADAMP (Ma, Liu, and Shi 2014)
- Exonuclease III-induced cascade two-stage isothermal amplification-mediated zinc (II) protoporphyrin IX/G-quadruplex supramolecular fluorescent nanotags **(EIC)**—Emerging method for early diagnosis of gene-related diseases (Q. Xue et al., 2014).
- CRISPR-Cas9-triggered nicking endonuclease-mediated strand displacement amplification mediated strand displacement amplification (**CRISDA**)—Emerging method with single nucleotide specificity and attomolar sensitivity (Zhou et al., 2018).

Multiple Displacement Amplification (2002)

MDA is an alternative DNA amplification based on random hexamer primers and the activity of a high-fidelity enzyme, such as Phi29 DNA polymerase (Spits et al., 2006). Compared with PCR and other amplification techniques, MDA do not require

sequence specific primers, once its mechanism exploits the random hybridization to generate large sized DNA fragments. Beside the isothermal profile (30°C), Phi 29 polymerase has proofreading activity, consequently MDA products contain less errors and higher sizes than the ones obtain *via* Taq amplification for the same time interval (2–3 h) (Blanco et al., 1989; Paez et al., 2004; Esteban, Salas, and Blanco 1993).

These beneficial features have spurred the application of MDA for Single Cell Genome Sequencing (Dean et al., 2002; Yoon et al., 2011; Rinke et al., 2013). Since it yields roughly 1–2 μg of DNA (enough for sequencing studies) with a genome coverage around 99% (Paez et al., 2004; Hellani et al., 2005), which allowed for the screening of genetic health condition in early-staged embryos before implantation (Hellani et al., 2005). MDA is also valuable for SPN allele detection (Ling et al., 2012) and for identifying the size of allelic polymorphic repeats (Ballantyne et al., 2006).

Exponential Amplification Reaction and Nicking and Extension Amplification Reaction (2003)

EXPAR is the exponential version of NEAR system, both simulate the SDA mechanism, where the difference in the melting temperature is exploited for target release and regeneration (Van Ness et al., 2003). A target template comprising a nicking site is designed to hybridize with the desire target. The polymerase replicates the template target, which is then nicked, producing a short fragment (8–16 bp) that is released by duplex instability, rather than by strand displacement activity (Van Ness et al., 2003). Conversely, the exponential version of NEAR uses a target template with two target complementary regions, instead of one, which after being nicked, releases a synthetized target, that can also pair up with the template target, creating a cycle of target regeneration and empowering NEAR to the desired exponential scale (Jia et al., 2010).

NEAR has a linear amplification profile, nevertheless it allows the amplification of any small targets by adding restriction sequences in the flanking regions (Maples et al., 2007). On the other hand, EXPAR is strongly limited by the requirement of targets natively flanked by restriction sites (Jia et al., 2010). Both NEAR and EXPAR (Tan et al., 2005) are particularly well suited for the detection of small fragments (8–16 bp) with high analytical sensitivity. Applications of NEAR include the detection of DNA (Zhou et al., 2014; Y. Song et al., 2014), proteins (A. X. Zheng et al., 2012; L. Xue, Zhou, and Xing 2010; Y. Huang et al., 2013; L. Xue, Zhou, and Xing 2012) and enzymes (Y. Zhao et al., 2013; X. Liu et al., 2014). Additionally, NEAR's ability to amplify at room temperature, without initial template denaturation and higher tolerance to reaction inhibitors are two beneficial criteria for POC integration. In this regard, Wang *et a*l., (L. Wang et al., 2017) reported on a highly specific detection assay for transgenic soya based on NEAR that is promising for PoC analysis. NEAR system is also being commercialized under kit formats. DNAble® by EnviroLogix (Portland, United States) test kit detects *Salmonella* and *Clavibacter michiganesis* (a plant pathogen) in 15 min with a sensitivity of 10^3 cfu per reaction (Envirologix, 2021). Alere-i POC allows the detection of influenza A and B virus in 15 min (Alere, Waltham, United States) (Nie et al., 2014; Alere™ now Abbott, 2021). Even though, not much is known about NEAR and EXPAR technology, their simplicity, specificity, and high adaptability are attractive features pushing for wider application.

Helicase Dependent Amplification (2004)

HDA is regarded as one of the simplest isothermal amplification schemes since it is based on the *in vivo* human DNA replication process (Vincent, Xu, and Kong 2004). HDA retains most of the PCR advantages, while improving the efficiency and selectivity, reducing the reaction and analysis complexity and requires less sample preparation steps, denoting the potential for integration in decentralized settings (An et al., 2005).

In the same fashion as PCR, HDA is compatible with different detection mechanisms such as gel electrophoresis for end-point measurement, fluorescent detection [with fluorescent DNA intercalator (Barbieri et al., 2014) and specific probes (Tong et al., 2008)] and electrochemical detection [with electroactive intercalator (Kivlehan et al., 2011)]], for real-time monitorization. However, HDA can also be monitored though Lateral Flow Devices (LFDs), which are much more suitable for low-cost and point-of-need scenario, than real-time assays (Du et al., 2017; Mahalanabis et al., 2010; Kolm et al., 2019; Tomlinson, Dickinson, and Boonham 2010).

Regarding HDA applications, the majority are devoted to the detection of infectious diseases. In fact, HDA has found a market niche in point-of-need testing (Craw et al., 2015), driven forward the development of commercial test kits (Gaydos et al., 2017) for pathogens such as, Herpes simplex [approved by FDA in 2011 (Lemieux et al., 2012); H. J. Kim et al., 2011)], *Bordetella pertussis, Clostridium difficile* Toxin A and *Streptococcus* from vaginal/rectal swabs (Faron et al., 2015). Quidel (proprietary for HDA technology) commercializes the AmpliVue and Solana test kits (Quidel 2021), based on HDA-Lateral Flow Devices, allowing the DNA amplification to occur in a CLIA-waiver, meaning without any sample preparation step (Weber et al., 2016). Additionally, BioHelix developed qualitative amplification detection kits (New England BioLabs, 2021) that recently receive FDA 501 (k) approval (Craw and Balachandran 2012).

Despite the usefulness of HDA in pathogen detection, it can be easily misinterpreted, since HDA presents issues often posed by the isothermal profile, such as the high risk of primer-dimer artifacts and off-target effects, ultimately leading to false positive phenomena, high background signal, inability to multiplex, low sensibility and selectivity (Barreda-García et al., 2018). Together, single-propriety commercialization, the false positive phenomena and the lack of validated assays have been hampered HDA widespread. However, if developers pushed forward the integration with LOC systems, HDA will likely show a significant increase in point-of-need pathogen detection,

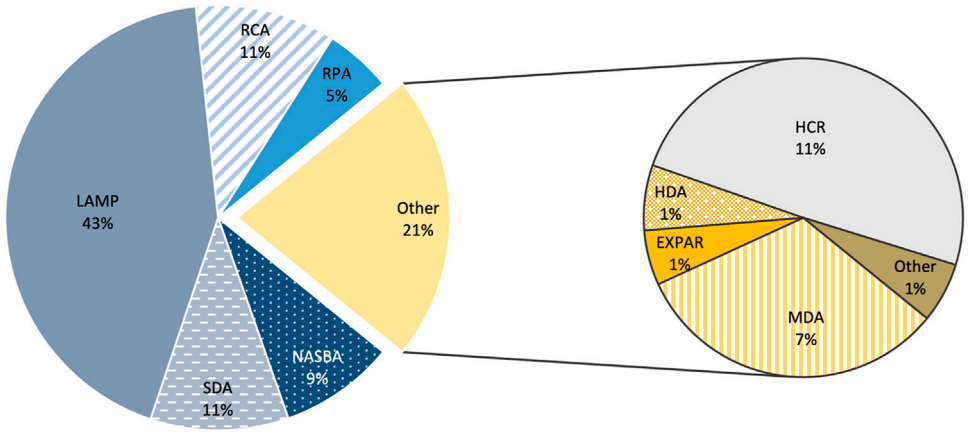

FIGURE 5 | Expression of each amplification scheme. Percentage of the Number of publications until 28 of October of 2020 in PUBMED. On the second plot, "Other" comprise to the following schemes: SMART, NEAR, SPIA, ICAN, BAD-AMP, SMAP 2, Exonuclease III-induced cascade two-stage isothermal amplification-mediated zinc (II) protoporphyrin IX/G-quadruplex supramolecular fluorescent nanotags, Hairpin Florescence Probe Assisted Isothermal Amplification and CRISDA.

ultimately fueling the development of more isothermal amplification schemes.

Hybridization Chain Reaction (HCR) (2004)

HCR Evanko (2004), Dirks and Pierce (2004) differs from all the previously reported methods, since isothermal amplification is achieved *via* signal amplification, without requiring the generation of DNA or RNA products. Comparing to the polymerase-based methods, the signal amplification schemes are not subject to product or enzyme inhibition. Depending on the strategy for signal amplification mechanism, there are three categories: nuclease-assisted, DNAzyme-assisted and enzyme-free reactions. Regarding the last type, HCR was the first being proposed in 2004 (Dirks and Pierce, 2004) being based in the differential hybridization of two partially complementary probes (hairpin form), that can only attain the energy required for conformational change (at room temperature) when in the presence of target ssDNA or RNA, generating a long-nicked copolymer. Trough the introduction of a fluorescent label on a specific hairpin moiety, real-time tracking of HCR can be achieved (Chemeris, Nikonorov, and Vakhitov 2008). This method pioneered the development of nanostructures from DNA self-assembly (Xuan and Hsing 2014; G. Zhu et al., 2013; Ding et al., 2015; J. Zheng et al., 2013; Xuan, Fan, and Hsing 2015) and introduced DNA as an amplifying transducer for biosensing (J. Huang et al., 2011; Y. Chen et al., 2012; L. Yang et al., 2012; B. Zhang et al., 2012; P. Liu et al., 2013) and bioimaging (Choi et al., 2010; Choi, Beck, and Pierce 2014; G. Zhu et al., 2013). HCR have shown applicability in *in situ* analysis (Choi et al., 2010; Choi, Beck, and Pierce 2014), miRNA (L. Zhou et al., 2019) and mRNA (Choi et al., 2010) detection. Additionally, the combination of HCR with other methods such as Catalyze Hairpin Assembly for detection single nucleotide polymorphisms (Li B et al., 2012), immuno-HCR (B. Zhang et al., 2012; J. Zhou et al., 2012) and AuNPs-supporting HCR (W. J. Wang et al., 2015) as electrochemical immunoassays and RCA incorporating HCR (Bi et al., 2013).

HCR's attractive features such as enzyme-free, isothermal profile and excellent efficiency, have contributed for its rapid gain of popularity (Deng and Gao 2015). Despite all the hype around non-enzymatic methods, they have been put aside due to leakage problems (Bi, Yue, and Zhang 2017).

Other Isothermal Amplification Schemes

Beside the up-mentioned methods, some other "niche" methods have also been developed, among them are SMART, SPIA, ICAN, SMAP 2 and BAD AMP (**Figure 5**).

These new isothermal amplification schemes cover broad applications, from nucleic acids detection (DNA, RNA, long RNA, SNPs, miRNA, DNA methylation) to the detection of proteins, enzymes, cancer cells, pathogens, small molecules and metal-ions (Y. Zhao et al., 2015; S. Kumar J. et al., 2018). Despite their range of applications, not all of these "blooming" amplification schemes have been applied much beyond their initial "proof-of-concept" purpose, since many of these methods still lack technical maturity (e.g., PHAMP, EIC, SPIA, ICAN and BAD AMP). Conversely, others have been endorsed over the years (e.g., MDA, SMART, HDA, EXPAR and HCR), nevertheless the strictness of conditions and reagents and the lack of accessible amplification kits and literature has been preventing wider use, forcing them to set as "niche" application schemes. As so, all schemes must undergo through improvements before they can emerge as competitors to the previously stablished methods (Li and Macdonald 2015).

CONCLUSIONS AND FUTURE PERSPECTIVES

PCR is the most widespread method and the current standard for nucleic acids amplification, its inherent features, such as the requirement for a thermocycler and the strict reaction conditions, increase the complexity of device integration and operating settings, overall rising the cost of PCR-based devices and assays. Conversely, isothermal schemes have constant and low temperature profiles, ability to use different types of molecules as starting material, low sensibility to inhibitors and

offer a wide range of detection methods, which are crucial features rendering for a significantly easier LOC integration, ultimately pushing forward their implementation and rising consideration. Furthermore, the integration under chip formats contributes to enhance the sensibility, reproducibility, rapidness, cost-effectiveness and achieve more accurate results, reducing the amplification bias. All together, these features are true "game changers", defying PCR technique for the "gold standard" title.

Generally, isothermal amplification methods offer a great potential for lower-cost, higher speed, smaller consumption of sample and reagents and the opportunity to automation of all processes from sample preparation to signal detection under lab-on-chip formats. In this regard, the isothermal amplification schemes were grouped in two main categories: the mature and consolidated ones, such as SDA, NASBA, LAMP, RPA and RCA; and the blooming schemes, that despite their promising features, still lack technical maturity and widespread application or commercialization under kit formats. This last group comprises SMART, MDA, EXPAR, HDA, HCR, SPIA and others.

Besides the nucleic acid detection purposes, isothermal amplification schemes have also been applied much further than this initial concept, such as: whole genome sequencing, single cell sequencing, electrochemical immunoassays, single-nucleotide polymorphism detection, virus and bacterial detection from biological swabs, environmental point-of-need niche applications, amplification of non-DNA templates and amplification of long, short and micro-RNA fragments. Nevertheless, the ultimate success of the isothermal schemes is still dependent on wider demonstration of specificity and cost-effectiveness. In fact, most of these methods show unspecific products formation and high background noise, due to the low amplification temperature, issues that need to be addressed before translation to the clinical scenario. Additionally, many of the schemes discussed are still protected by patents or licensed by a single proprietary company, which may be limiting the access to the necessary enzymes and reagents, ultimately leading to high market prices, thus preventing broader application. Once these rights have expired, IA-based methods should meet the requirements to become a reference to nucleic acid diagnosis and POC applications, with a profitable commercialization worldwide.

AUTHOR CONTRIBUTIONS

BO, BV, and PB planned and conceptualized the work, drafted the manuscript, and edited the final version. BO designed the schematics and figures.

FUNDING

This work is financed by national funds from FCT—Fundação para a Ciência e a Tecnologia, I.P., in the scope of the project UIDP/04378/2020 and UIDB/04378/2020 of the Research Unit on Applied Molecular Biosciences—UCIBIO and the project LA/P/0140/2020 of the Associate Laboratory Institute for Health and Bioeconomy—i4HB; and for 2020.07660. BD to BO.

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