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To cite this article: Jörn Glökler, Theam Soon Lim, Jeunice Ida & Marcus Frohme (2021): Isothermal amplifications – a comprehensive review on current methods, Critical Reviews in Biochemistry and Molecular Biology, DOI: [10.1080/10409238.2021.1937927](https://doi.org/10.1080/10409238.2021.1937927)

To link to this article: <https://doi.org/10.1080/10409238.2021.1937927>



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Published online: 15 Jul 2021.



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Isothermal amplifications – a comprehensive review on current methods

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ABSTRACT

The introduction of nucleic acid amplification techniques has revolutionized the field of medical diagnostics in the last decade. The advent of PCR catalyzed the increasing application of DNA, not just for molecular cloning but also for molecular based diagnostics. Since the introduction of PCR, a deeper understanding of molecular mechanisms and enzymes involved in DNA/RNA replication has spurred the development of novel methods devoid of temperature cycling. Isothermal amplification methods have since been introduced utilizing different mechanisms, enzymes, and conditions. The ease with which isothermal amplification methods have allowed nucleic acid amplification to be carried out has had a profound impact on the way molecular diagnostics are being designed after the turn of the millennium. With all the advantages isothermal amplification brings, the issues or complications surrounding each method are heterogeneous making it difficult to identify the best approach for an end-user. This review pays special attention to the various isothermal amplification methods by classifying them based on the mechanistic characteristics which include reaction formats, amplification information, promoter, strand break, and refolding mechanisms. We would also compare the efficiencies and usefulness of each method while highlighting the potential applications and detection methods involved. This review will serve as an overall outlook on the journey and development of isothermal amplification methods as a whole.

ARTICLE HISTORY

Received 5 March 2021
Revised 12 May 2021
Accepted 31 May 2021

KEYWORDS



Isothermal amplification; exponential DNA amplification methods; origin of amplification; nucleic acid-based diagnostics; point-of-care diagnostics

Introduction

Nucleic acid amplification methods have transformed the way we perform research and have penetrated our life in areas such as recombinant DNA technology, molecular diagnostics, and forensics. The development of the polymerase chain reaction (PCR) by Cary Mullis (Saiki et al. 1985) rewarded him together with Michael Smith a Nobel Prize in Chemistry in 1993 and kick-started these transformations in molecular diagnostics and forensics. This was down to the highly efficient exponential amplification of templates that can be used to detect even a single molecule. The main disadvantage of PCR is the demand for a programmable thermocycler that can quickly change between the three different reaction temperatures at the expense of energy use and portability. Thus, despite the usefulness of PCR, there is an ongoing need for employing efficient nucleic acid amplification reactions that run at a constant temperature and require only simple energy-conserving incubators.

Today it is less well known that isothermal amplification preceded the invention of PCR. More specifically, it was the purified RNA-dependent-RNA-polymerase from the bacteriophage Qbeta (Q β), termed Q β replicase isolated from *Escherichia coli* that was shown by Sol Spiegelman to exponentially amplify the phage genome *in vitro* (Spiegelman et al. 1965). His group had later discovered that this *in vitro* replication accumulated many errors and deletions in the progeny and that minimal variants of only 218 nucleotides (nt) could be generated by serial transfer, thus demonstrating for the first time the feasibility of extracellular evolution (Kacian et al. 1972).

The first modern post-PCR era isothermal amplification methods that gained attention was the self-sustained sequence replication (3SR) published in 1990 (Guatelli et al. 1990) shortly followed by the very similar nucleic acid sequence based amplification (NASBA) in 1991 (Compton 1991) and the transcription-mediated amplification (TMA) (Kacian and Fultz 1995). NASBA

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became commercially successful for bioMérieux in the form of rapid diagnostic tests for several pathogenic viruses with single-stranded RNA genomes, whereas TMA was commercialized by Gen-Probe Inc. for the detection of sexually transmitted infections. The next important method to enter the field of isothermal amplification is the strand displacement amplification (SDA) published in 1992 which depends on performing enzymatic site-specific nicks for initiation in combination with a strand-displacing polymerase (Walker et al. 1992). The SDA has been commercialized by Becton Dickinson for the diagnosis of infectious diseases. The loop-mediated isothermal amplification (LAMP) was published in 2000 by Notomi et al. (2000) and became one of the most popular amplification methods due to the availability of components comprising one strand-displacing polymerase and unmodified primers, but mainly due to its high amplification efficiency. Yet despite its popularity mostly in academic applications, little commercialization beyond some reaction kits marketed by Eiken Chemical Co., Ltd. has been realized so far. The next popular and more commercially applied isothermal amplification method requires an auxiliary enzyme to the polymerase. More specifically, the helicase dependent amplification (HDA) was published in 2004 (Vincent et al. 2004) and marketed by BioHelix Corp. HDA is still widely applied in diagnostic assays for the detection of infectious diseases (Teo et al. 2015; Gaydos et al. 2017).

The most effective and promising isothermal amplification method so far is the recombinase primed amplification (RPA) first published in 2006 (Piepenburg et al. 2006) and commercialized by TwistDx. The RPA has already found applications beyond mere diagnostics such as cluster generation in next-generation sequencing (Shen et al. 2013). The critical differentiating factors between these methods are the enzymes involved in the process and the mechanism by which DNA strand displacement is being carried out.

The chronological development of all amplification methods discussed in this review is shown in Figure 1

and the most relevant details are listed in Table 1. Important features with respect to temperature, RNA or DNA target, product, typical polymerase(s) and auxiliary factors, theoretical specificity as primer/probe binding sites are listed, including seminal publication and patent applications (preferably a PCT application if found). Although very interesting for the experimenter, exact figures for sensitivity and amplicon size were excluded from the table as these features may not be directly comparable to the heterogeneous data available in the original publications. Considering the plethora of amplification strategies available, classification of these methods can be efficiently done by looking at their ability to generate either a linear or exponential amplification.

Amplification method categories

Amplification can occur under very different conditions. The conventional PCR requires repetitive temperature changes, whereas by definition isothermal amplifications must occur at a more or less constant temperature. However, some different categories of isothermal amplification methods can be distinguished. The most important distinction can be made between linear and exponential amplification. In linear amplifications such as rolling circle amplification (RCA), one target sequence is amplified only from its template and not from its progeny (Figure 2(a)). Respectively, in most exponential amplifications the copies generated in the process themselves can again become templates. However, unlike PCR, many of the exponential isothermal amplification methods do not strictly generate two copies per polymerase initiation and elongation step. This is especially the case for isothermal amplification cascades in which one amplified product triggers the generation of a different kind of product in a subsequent stage within the cascade. Such cascade amplification reactions may also comprise more than two stages and are not limited to polymerases to rapidly generate amplification products.

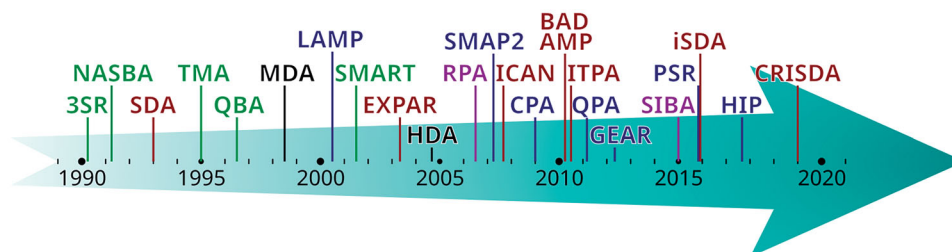


Figure 1. Timeline of isothermal nucleic acid amplification methods. Methods are ordered according to their first publication dates. Acronyms of individual methods are colored with respect to their underlying amplification mechanism: promoter-based in green, nicking-based in blue, refolding-based in red, strand-invasion-based in purple and unique mechanisms in black (see color version of this figure at www.tandfonline.com/ibmg).

Table 1. Isothermal and exponential amplification methods.

Name	Acronym	Temp. (°C)	Duration (min)	Initial denaturation	Mechanism	Polymerase	Auxiliary factors	Template	Information	Oligos	Patent application & priority date	Company/institution	Reference
Self-sustained sequence replication reaction	3SR	37–42	<60	No	Promoter	T7 RNA pol + AMV RT	RNase H	NA	Yes	2 primers	WO 90/06995 A1 1988-12-16	Siska Diagnostics	(Guatelli et al. 1990)
Nucleic acid sequence based amplification	NASBA	41	<120	Yes	Promoter	T7 RNA pol + AMV RT	RNase H	RNA, DNA	Yes	2 primers	WO 91/02814 A1 1988-02-24	BioMérieux	(Compton 1991)
Strand displacement amplification	SDA	37	<120	Yes	Nicking	Klenow exo-	Restriction enzyme	DNA	Yes	2 primers	US 5270184 A 1991-11-19	Becton Dickinson	(Walker et al. 1992)
Transcription-mediated amplification	TMA	41	<140	Yes	Promoter	T7 RNA pol + reverse transcriptase	N/A	RNA, DNA	Yes	2 primers	WO 91/01384 A1 1989-07-11	Gen-Probe	(Kacian and Fultz 1995)
Qbeta amplification	QBA	37	<30	Yes	Promoter	Q beta replicase	Ligase	RNA	Yes	2 primers	WO 94/16108 A1 1993-01-15	NA	(Tyagi et al. 1996)
Loop-mediated Amplification	LAMP	60–68	<60	(Yes)	Refolding	Bst DNA pol	N/A	DNA (RNA)	Yes	4–6 primers	WO 00/28082 A1 1998-11-09	Eiken	(Notomi et al. 2000)
Signal mediated amplification of RNA technology	SMART	41	60	Yes	Promoter	T7 RNA pol + Bst DNA pol	N/A	RNA, DNA	No	2 primers	WO 99/37806 A2 1998-01-27	Cytocell	(Wharam et al. 2001)
Ramification amplification	RAM/MDA	30, 63	60	Yes	Multiple priming	Phi29, Bst DNA pol	Coupled with ligase	DNA	Yes	2–6 primers /random hexamers	WO 98/04746 A1 1996-07-31	Thome Diagnostics	(Zhang et al. 2001)
Exponential amplification reaction	EXPAR	60	10	No	Nicking	Vent Exo- pol	Nicking enzyme	DNA	No	1 primer	WO 2004/06726 A2 2003-01-29	Ionian Technologies	(Van Ness et al. 2003)
Helicase-dependent amplification	HDA	65	<90	No	Strand unwinding	Bst DNA pol	UvrD or T7 gp4 helicase + SSB	DNA (RNA)	Yes	2 primers	WO 2004/027025 A2 2002-09-20	NEB, BioHelix	(Vincent et al. 2004)
Recombinase polymerase amplification	RPA	30–42	15–60	No	Strand invasion	Bsu DNA pol	RecA + SSB	DNA	Yes	2 primers	WO 03/072805 A2 2002-02-21	TwistDx	(Piepenburg et al. 2006)
Smart amplification process version 2	SMAP2	60	<60	Yes	Refolding	Aac DNA pol	Taq MutS	DNA	Yes	5 primers	WO 2005/063977 A1 2003-12-25	Riken licenses to DNAtform	(Mitani et al. 2007)
Isothermal and chimeric primer-initiated amplification of nucleic acids	ICAN	60–65	<80	Yes	Nicking/digest	Bst DNA pol	RNase H	DNA (RNA)	Yes	2 primers	WO 03/016569 A1 2001-08-20	TakARA	(Mukai et al. 2007)
Cross-priming amplification	CPA	63	60	No	Refolding	Bst DNA pol	N/A	DNA (RNA)	No	4 primers	WO 2010/080691 A1 2009-01-06	Ustar Biotechnologies	(Fang et al. 2009)
Beacon-assisted detection	BAD AMP	40	40	(Yes)	Nicking	Bst DNA pol	Nicking enzyme	DNA (RNA)	No	1 beacon	NA	NA	(Connolly and Trau 2010)
Nicking enzyme amplification reaction	NEAR	55	5–10	No	Nicking	Bst DNA pol	Nicking enzyme	DNA (RNA)	No	1 primer	WO 2009/012246 A2 2007-07-14	Ionian Technologies	NA
Isothermal chain amplification	iTPA	60	40	(Yes)	Nicking/digest	Bst DNA pol	RNase H, T4 gene 32 protein	DNA (RNA)	Yes	4 primers	WO 2009/072705 A1 2007-12-03	RapleGene	(Jung et al. 2010)
Quadruplex priming amplification	QPA	70	<120	No	Refolding	Taq DNA pol	N/A	DNA	No	2 primers	WO 2011/103407 A1 2010-02-19	NA	(Kankia 2011)
Genome exponential amplification reaction	GEAR	60	15–30	(Yes)	Refolding	Bst DNA pol	N/A	DNA (RNA)	No	2/4 primers	WO 2014/025337 A1 2012-08-06	NA	(Prithiviraj et al. 2012)
Strand invasion based amplification	SIBA	30–42	<30	No	Strand invasion	Bsu DNA pol	RecA + SSB	DNA	No	2 primers, 1 oligo	WO 2009/150467 A1 2008-06-11	Orion Diagnostica Oy	(Hoser et al. 2014)
Polymerase spiral reaction	PSR	60–65	<50	Yes	Refolding	Bst DNA pol	N/A	DNA (RNA)	Yes	2 primers	WO 2016/054870 A1 2014-09-24	NA	(Liu et al. 2015)
Isothermal SDA	iSDA	49	20–30	No	Nicking	Bst DNA pol	Nicking enzyme	dsDNA	Yes	2 primers	WO 2014/164479 A1 2013-03-11	Elietechgroup B V	(Toley et al. 2015)
Hinge-initiated primer-dependent amplification of nucleic acids	HIP	60–65	60	(Yes)	Refolding	Bst DNA pol	N/A	DNA (RNA)	Yes	2 primers	NA	NA	(Fischbach, Frohme, et al. 2017)
CRISPR-Cas9-triggered nicking strand displacement amplification mediated strand displacement amplification	CRISDA	37	90	No	Nicking	Klenow exo-	Nicking Cas9-sgRNA	DNA	Yes	2 primers	NA	NA	(Zhou et al. 2018)

Duration times for positive reactions were inferred from the publications. Template NA refers to the DNA or RNA templates used in absence of a dedicated RT and is put in parentheses when Bst DNA polymerase can be applied that has limited RT activity. Information denotes the amplification of sequence information not encoded in added oligonucleotides. Patent application refers to earliest PCT application numbers when present or national priority application numbers and first priority dates are provided. Reference refers to the seminal publication of the given method. All methods are ranked chronologically according to their publication date.

Many isothermal amplification methods, and all cascade amplifications, do not amplify any sequences beyond what is encoded by oligonucleotides added to the reaction. Thus, no additional information other than the presence or absence of a target sequence can be obtained in such a reaction. While some of these methods are very rapid, such as exponential amplification reaction (EXPAR) (Van Ness et al. 2003), they are refractory to false positives which cannot be discriminated against the presence of the real template (Figure 2(b)).

Special cases of isothermal amplification methods do not even require enzymes such as the original hybridization chain reaction (HCR) (Figure 2(c)) (Dirks and Pierce 2004) and the more efficient and sensitive catalyzed hairpin assembly (CHA) (Yin et al. 2008; Li et al. 2011). Depending on the design of these reactions, even an exponential amplification is feasible. However, it is also obvious that these enzyme-free isothermal amplification methods are not able to amplify any

sequence information other than the mere presence or absence of the target.

Another special case is the so-called bridge amplification which is employed for the generation of clonal sequence clusters in most of the Illumina sequencing machines. Although the amplification occurs at roughly the same temperature, the process is discontinuous and non-homogeneous due to cyclic denaturation steps achieved by alternating between buffers, one comprising a denaturing agent such as formamide (Quail et al. 2009). Such cyclic isothermal amplification is only possible due to amplification on the solid phase by using two template-specific immobilized primers (Figure 2(d)).

In a similar manner, many other isothermal amplification methods require, or are at least strongly enhanced by, a first denaturation step in order to anneal primers introducing additional sequences to the template. Once the additional sequences can constitute an active initiation site, the reaction can continue in a

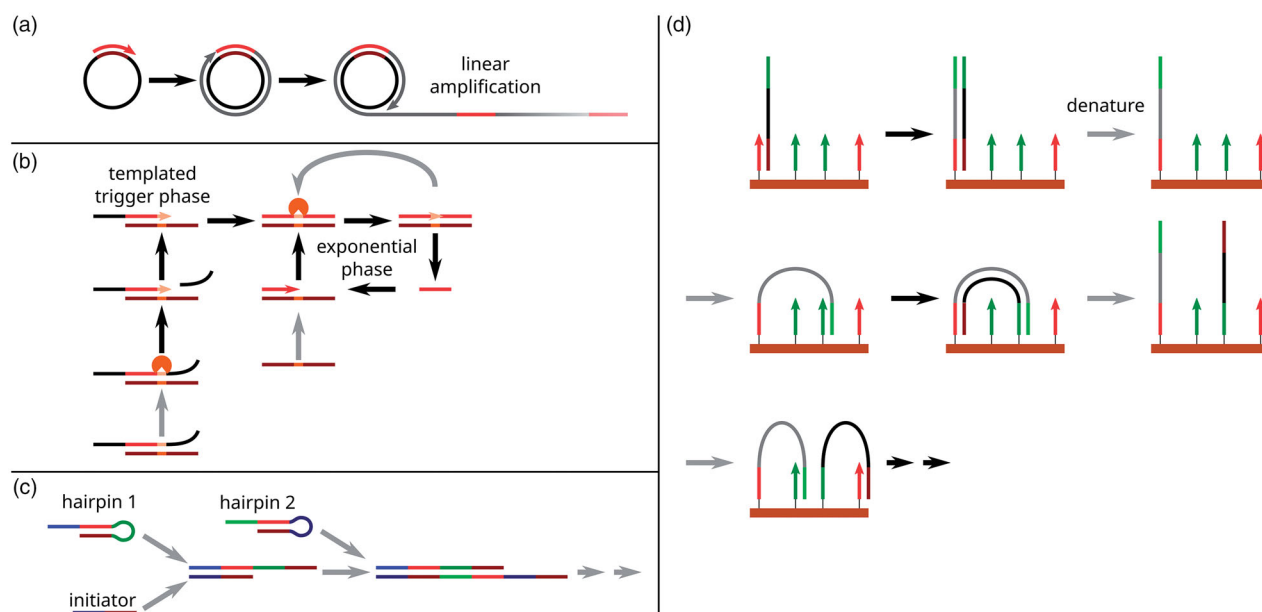


Figure 2. Examples for different isothermal amplification categories. Black arrows designate enzymatic steps as part of the amplification process and gray arrows indicate mostly reversible refolding or recycling steps. (a) Linear amplification by RCA. A circular single stranded DNA serves as a template after annealing of a primer leading to a concatemeric single stranded product. (b) Exponential amplification without template-derived information by EXPAR. A single-stranded template comprising a trigger sequence (bright red region) and a nicking enzyme recognition site (short beige region) is hybridized with a complementary oligonucleotide, comprising a repeat of the trigger sequence, for strand cleavage to enter the templated trigger phase. A polymerase elongates the template to copy the trigger sequence which is continuously cleaved off and displaced by the polymerase, binding to yet another oligonucleotide thus entering the exponential phase. (c) Enzyme-free amplification by HCR. Hairpin 1 changes conformation by annealing to an initiator sequence (dark blue and red), exposing a single-stranded region (dark green and red) for hairpin 2 to anneal and expose a region identical to the initiator that is again recognized by hairpin 1. Without the aid of an enzyme a linear double-stranded concatemer is assembled from the hairpins. (d) Discontinuous isothermal bridge amplification. A single stranded template is hybridized to an immobilized primer 1 (bright red) in a first priming and elongation step. The template is removed by chemical denaturation leaving the immobilized copied strand behind. Another immobilized primer 2 (dark green) hybridizes to the complementary region of the copied strand for a second priming and elongation step. The product is now forming a bridge-like structure in which both strands are immobilized by the 5' ends. Further denaturation and copy steps yield clonal amplification products that are clustered around the initial template copy (see color version of this figure at www.tandfonline.com/ibmg).

homogeneous manner without additional temperature changes. A final denaturation step can be introduced to stop the reaction for subsequent analysis.

Due to the versatility of exponential reactions that also amplify sequences not encoded in added oligonucleotides, this review focuses mainly on this type of homogeneous isothermal amplification method. We will highlight the different mechanisms involved in homogeneous isothermal amplification methods as well as paying special attention to the various enzymes involved in the different processes. Each of these methods has their unique advantages and disadvantages which would be discussed together with examples of various applications afforded by these methods.

Reaction initiation mechanisms

The concept of PCR is very artificial because neither temperature change for denaturation and readily formed DNA primers usually exist in natural replication mechanisms. Most natural replication systems require the unwinding of double-stranded nucleic acids and the generation of short primer fragments on single-stranded regions by specialized enzymes termed primases. Thus, isothermal amplification methods must rely on different means to compensate especially for the denaturation step in order to generate accessible primer binding sites.

In the earliest publication of the 3SR amplification method (of which only the abstract is accessible), the inventors lay down their rationale behind the design of the initiation mechanism. Taking nature as an example, they wanted to modify a transcription amplification system in order to mimic the retroviral strategy of replication, resulting in the 3SR which operates under isothermal conditions (37 °C) (Gingeras et al. 1990). Indeed, most of the isothermal amplification methods exploit initiation mechanisms already known in natural replication systems. As polymerase initiation sites in these amplification methods bear a resemblance to typical origins of replication, we propose the term origin of amplification (or *oriA* for short) to signify this specific property and discriminate against natural replication systems.

Several mechanisms have been developed to generate one or more of such *oriA* to achieve a site-specific initiation of polymerases for the amplification of generic templates. Most amplification methods can be understood as a variation of a theme based on a panel of *oriA* mechanisms. The mechanisms can be either applied symmetrically to both ends of the amplicon or combined in an asymmetric fashion. Depending on the

type of mechanism, an initial denaturation of the target sequence is necessary to start the amplification. In addition, most mechanisms require the regeneration of terminal sequences to reconstitute an active *oriA*.

A list of the most distinctive initiation mechanisms has been compiled and is discussed in context with prominent examples in further detail below.

Promoter mechanism

The Q β and the 3SR/NASBA/TMA amplifications rely on a promoter-like mechanism for initiation of the polymerase at the *oriA*. In the case of the Q β amplification, it is the formation of a highly efficient three-dimensional structure of RNA arising from the termini that act as a promoter for the Q β replicase. Although the replicase is not limited to one type of structure and sequence, it strongly prefers certain structures as a promoter (Ugarov and Chetverin 2008). A notable attempt to employ Q β replicase in a selective and sensitive isothermal amplification was published in 1996 using a template-dependent ligation of binary probes comprising terminal promoter sequences (Tyagi et al. 1996). However, despite these encouraging results, no amplification system based on Q β replicase was ever commercialized.

Due to the plasticity of the Q β replicase, more specific and less structurally dependent promoters have been introduced. The most valuable promoter is based on the popular T7 RNA transcription system derived from the uneven *E. coli* bacteriophage T7. The optimal promoter sequence of the T7 RNA polymerase is known for a long time and the enzyme is commercially available from multiple sources to be easily integrated into an amplification composition. As the T7 promoter is usually absent in a given template and the T7 RNA polymerase initiates with guanine at the end of the promoter sequence, the promoter sequences have to be added to the template. This is achieved by designing primers having the promoter sequence 5' fused to the usual template-specific region. Such an approach has been first explored in the transcription-based amplification system (TAS) which performed alternating cycles of *in vitro* transcription and reverse transcription in a non-homogeneous manner which also involves heating steps for denaturation and primer annealing (Kwoh et al. 1989).

The original 3SR concept took advantage of the discontinuous TAS and turned it into an isothermal and homogeneous amplification method by regenerating a single-stranded DNA (ssDNA) for primer binding from the otherwise inaccessible RNA/cDNA-heteroduplex

using RNase H. Initially, a symmetric concept with terminal T7 promoter sequences acting as oriA was used in combination with T7 RNA polymerase, two unmodified DNA primer oligonucleotides (for the introduction of T7 promoter sequence), AMV reverse transcriptase and *E. coli* RNase H. The isothermal reactions were conducted at 37 °C in early versions of 3SR (Guatelli et al. 1990) (see Figure 3(a)). More optimized versions of 3SR later employed an asymmetric concept with one oriA with T7 promoter and relied exclusively on the activity of RNase H to constitute the other oriA as a simple primer binding site, and the reaction occurred at a more stringent temperature of 42 °C (Fahy et al. 1991) (see Figure 3(b)). Interestingly, the mechanism of other patented methods such as NASBA and TMA is more or less indistinguishable from this later format of 3SR.

The T7 promoter has also been introduced as an oriA in linear isothermal amplification methods. For instance, the linear amplification method single primer isothermal amplification (SPIA) was used for unbiased amplification of complex samples such as the whole

transcriptome due to the ability to preserve the stoichiometry of the input material (Kurn et al. 2005). However, signal-mediated amplification of RNA technology (SMART) (Wharam et al. 2001) is based on the template-dependent formation of a three-way junction (3WJ) structure comprising a T7 promoter sequence which serves as an oriA. The T7 RNA polymerase amplifies a signal RNA to be detected in a hybridization-based assay. The unbiased linear amplification of distinct signal RNAs allows multiplex detection. By using the second set of template oligonucleotide, exponential amplification can be achieved (see Figure 4). However, no information from the original template is amplified by SMART which makes the discrimination against artifacts difficult.

The main advantage of promoter-based oriA is the direct application for RNA template amplification without an initial denaturation step. In addition, the highly processive T7 RNA polymerase can be harnessed to efficiently generate thousands of transcripts from a single template.

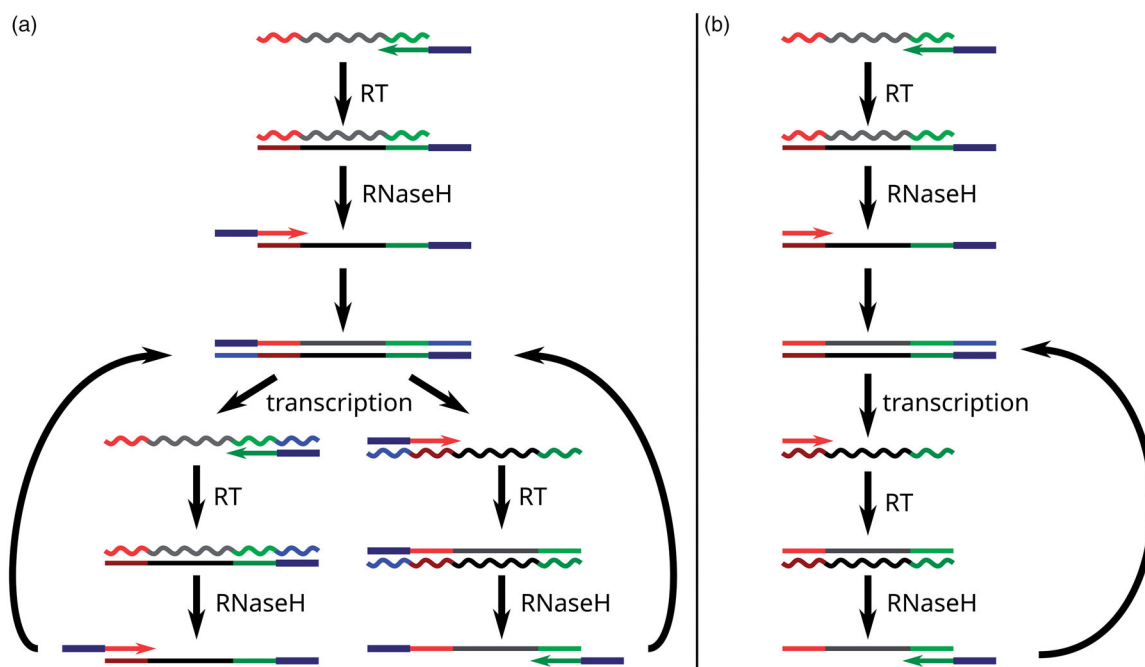


Figure 3. Self-sustained sequence replication (3SR). (a) Symmetrical 3SR with two T7 RNA polymerase promoter sequences. The single-stranded RNA template (shown as a wavy line) is first contacted with a first specific primer that initiates reverse transcription that also adds a T7 RNA promoter sequence (bold dark blue region). The RNA of the resulting heteroduplex is digested by RNaseH, yielding a single-stranded cDNA which is again contacted with second specific primer that initiates reverse transcription adding another T7 RNA promoter sequence. The symmetric dsDNA product is then transcribed by T7 RNA polymerase yielding two RNA strands. Both RNA strands become template for another round of reverse transcription, digest and copying steps, resulting in the exponential accumulation of the central symmetric dsDNA product. (b) Asymmetric 3SR with one T7 RNA polymerase promoter sequence. The single-stranded RNA template (shown as a wavy line) is first contacted with a first specific primer that initiates reverse transcription that also adds a T7 RNA promoter sequence (bold dark blue region). The RNA of the resulting heteroduplex is digested by RNaseH, yielding a single-stranded cDNA which is again contacted with second specific primer that initiates reverse transcription. The asymmetric dsDNA product is then transcribed by T7 RNA polymerase yielding one RNA strand which becomes template for another round of reverse transcription, digest and copying steps, resulting in the exponential accumulation of the asymmetric dsDNA product (see color version of this figure at www.tandfonline.com/ibmg).

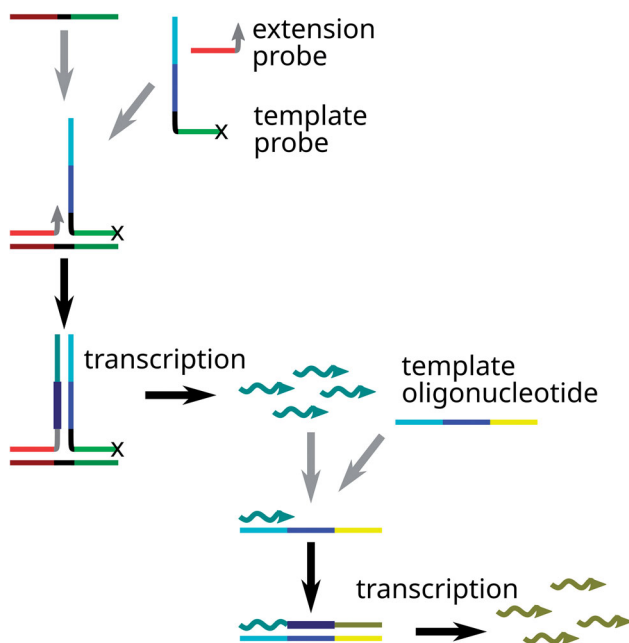


Figure 4. Signal Mediated Amplification of RNA Technology (SMART). The single-stranded template hybridizes to the extension probe and template comprising a T7 promoter sequence (bright blue line), a signal sequence (azure line) and a blocking modification (designated as x). The assembled complex constitutes a 3-way junction structure presenting a free 3'-end (gray arrow) that can prime elongation on the template probe. The Bst DNA polymerase elongates the extension probe and thus generates a T7 promoter (comprising the thick blue strand) which is recognized by T7 RNA polymerase. The RNA polymerase can initiate multiple rounds of transcription by which many copies of a first signal RNA (wavy blue-green arrows) is generated. The first signal RNA can then hybridize to another template oligo also comprising a T7 promoter sequence to act as a primer for elongation by Bst DNA polymerase. This newly generated template is again transcribed by T7 RNA polymerase, yielding multiple copies of a second signal RNA (wavy olive-colored arrows) for detection (see color version of this figure at www.tandfonline.com/ibmg).

Strand break mechanism

Many natural replication systems employ the use of nicking enzymes breaking one strand to initiate the replication process. For instance, conjugative plasmids often encode a site-specific nickase that cleaves at the origin of transfer. The resulting free 3' end can be used by polymerases to initiate a rolling circle replication. The first example of an artificial implementation of this mechanism as an *oriA* in an isothermal amplification method was the SDA (Walker et al. 1992). At the time of invention, no nicking enzymes were commercially available. Thus, a typical restriction enzyme was turned into a nicking enzyme by using a modified nucleotide that renders the newly synthesized strand resistant to cleavage. An exonuclease-deficient form of the large fragment of *Escherichia coli* DNA polymerase I (exo- Klenow

polymerase) with strand-displacing activity extends the 3' ends of the duplexes using dGTP, dCTP, TTP, and Rp- α -phosphorothioate-deoxyadenosine, which produces hemiphosphorothioate recognition sites at the cleavage sites. The restriction enzyme Hinc II nicks the unprotected primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands (see Figure 5(a)). Many restriction enzymes efficiently nick a non-palindromic hemiphosphorothioate recognition site but apparently dissociate too slowly from the nicked site for efficient operation in SDA. Still, the SDA required 4 h at 37°C to amplify a template derived from *Mycobacterium tuberculosis* approximately 10⁶-fold. Primers are used to introduce the recognition sites for the nicking and require an initial denaturation step for target hybridization. As the amplification product of SDA is linear, the recognition site has to be regenerated at the 5'-end with the primer also serving as a template after each copying step. Background amplification increases with increasing amounts of non-target DNA, which means amplification factors decrease with increasing amounts of non-target DNA. An important feature of this method that the amplification of target sequences is efficient up to a size of about 100 bp (Walker 1993). In order to efficiently generate a template that can be amplified by SDA, initial conversion steps are required to introduce nicking enzyme recognition sites. An important feature in this conversion is outer or bumper primers that can bind upstream to the SDA primers that speed up the generation of single-stranded intermediates without the need for additional denaturation steps. More recently, an improved isothermal SDA (iSDA) has been published that entirely skips the requirement for an initial denaturation step (Toley et al. 2015). It employs the use of one primer that initiates at a site where DNA base pairs spontaneously open or transiently convert into Hoogsteen pairs, that is, "breathe". Two additional bumper primers again help to accelerate the addition of nicking recognition sites to the template by the two main extension primers (see Figure 5(b)). A combination of DNA polymerase, Bst 2.0 WarmStart with the dedicated nicking enzyme Nt.BbvCI has allowed the detection of 10 copies of pathogenic MRSA genomes in about 20 min by lateral flow (Toley et al. 2015).

After the inception of the original SDA method, many variations of the theme have been developed. The first published follow-up method employing a nicking mechanism was EXPAR (Van Ness et al. 2003) which was commercialized by Ionian Technologies. Compared to SDA, EXPAR is a faster and simplified isothermal amplification method. It employs a real nicking enzyme (N BstNB), a strand-displacing DNA polymerase (Vent

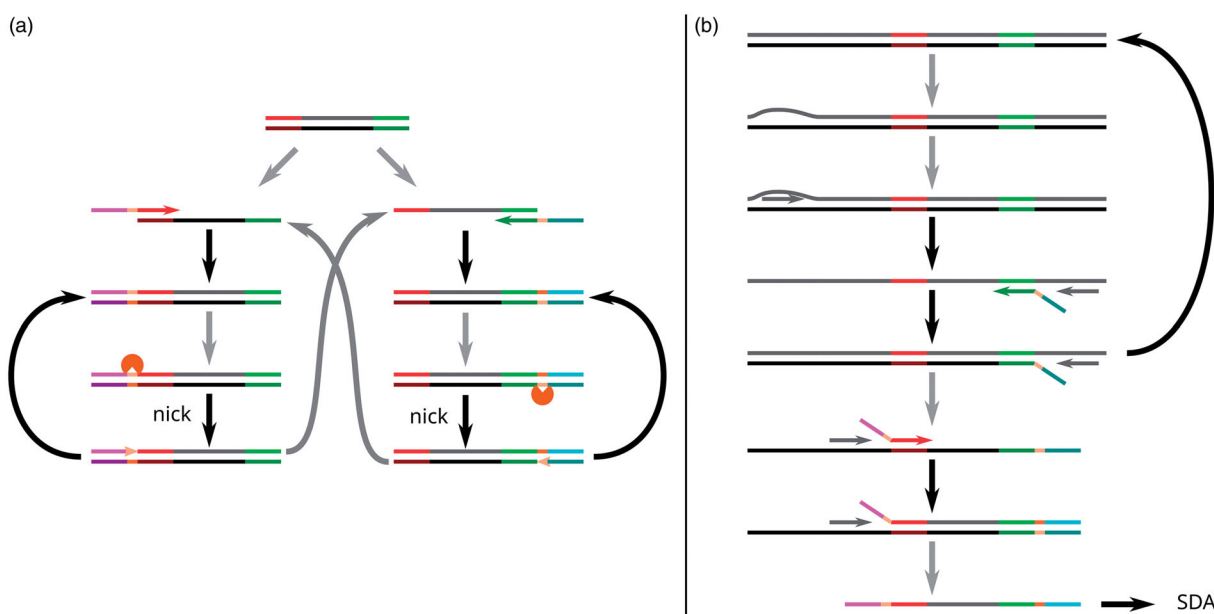


Figure 5. Strand displacement amplification (SDA). (a) Simplified mechanism of SDA. The template is initially denatured to make target sequences available for hybridization with SDA primers comprising a nicking enzyme recognition sequence (short beige-colored lines) and a 3' target-binding region. The mechanism for both strands is shown in a symmetric fashion. The template is extended by the nicking sequence copied from the target-bound SDA primer. The newly introduced nicking sequence is recognized and cleaved by a nicking enzyme, generating a nick with an extendable 3' end. The strand-displacing polymerase extends from the nick, thereby displacing the single-stranded template sequence lacking a portion of the SDA primer. The single-stranded template is again recycled by hybridization with SDA primers, while the newly generated double strand is repeatedly recognized by the nicking enzyme, thus leading to an exponential amplification of the template sequence. (b) Initial template conversion steps by iSDA. The template has to be converted by initial steps to introduce a nicking enzyme recognition sequence. In the case of iSDA, a specific “breathing” site is selected for a primer that can start the generation of a single strand for conversion by SDA primers based on bumper primers. The bumper primers accelerate the displacement of the newly synthesized strand with the nicking enzyme recognition sequence which are then further copied by the complementary SDA primers. The product can then directly enter the exponential SDA reaction (see color version of this figure at www.tandfonline.com/ibmg).

exo⁻), and only two rather short unmodified oligonucleotides. One oligonucleotide comprising a nicking enzyme recognition site acts as a probe that specifically hybridizes to a target sequence under stringent conditions. The resulting duplex is nicked by the enzyme and a runoff product of 12 bases is produced by displacement. This first product can either accumulate in a linear amplification or hybridize to another oligonucleotide and act as a trigger by causing another extension and nicking reaction to occur, thus resulting in an exponential amplification (see Figure 2(b)). The exponential reaction is carried out at 60 °C and is very fast, usually generating a detectable signal in under 10 min of incubation time. EXPAR has been slightly improved using Bst DNA polymerase and an initial template-dependent extension step as nicking enzyme amplification reaction (NEAR) (Maples et al. 2017). The main limitation of both EXPAR and NEAR is the lack of information amplified from the original template and the comparably short trigger oligonucleotides that could give rise to false-positive amplification reactions.

Another example for a nicking enzyme-based method is primer generation-rolling circle amplification (PG-RCA) (Murakami et al. 2009). A special feature of this method is the combination of a strand break by nicking enzymes with the initiation of a linear rolling circle amplification that results in an exponential cascade reaction. The template is recognized by hybridization to a circular probe under stringent conditions which comprises a nicking enzyme recognition site in the double-stranded hybrid region. Stringency is controlled by hybridization to a single-stranded circular probe comprising a 19 base complementary sequence at an annealing temperature of 60 °C using the Vent exo⁻ DNA polymerase and a nicking enzyme with a 7 base recognition site. The nicking enzyme cleaves the template strand, thereby initiating a first linear rolling circle amplification step. This rolling circle amplification generates a concatemeric linear single-stranded product which now comprises multiple fragments containing the recognition site that can also anneal to multiple circular probes. By nicking of these strand-probe

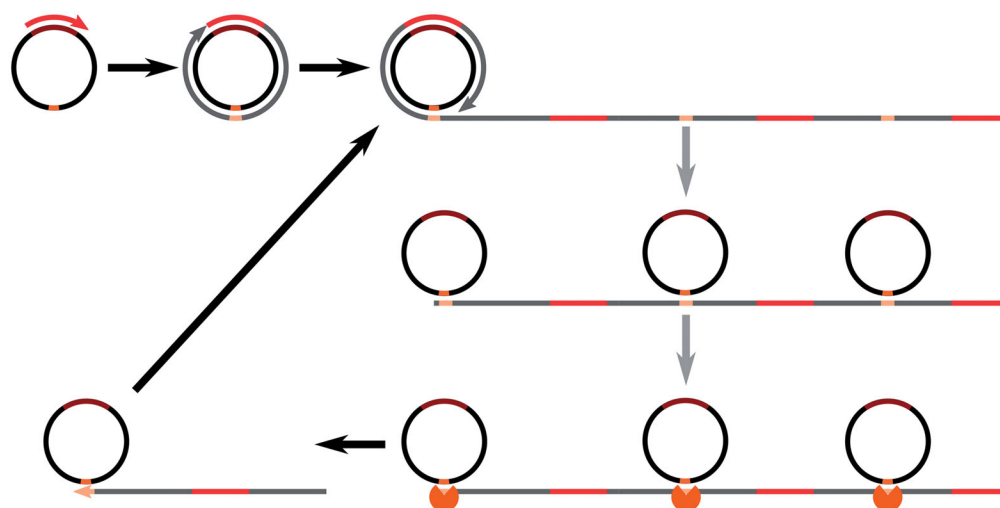


Figure 6. Primer generation-rolling circle amplification (PG-RCA). An initial template DNA (bright red) hybridizes to a single-stranded circular probe comprising a complementary sequence (dark red) and a nicking enzyme recognition site (beige-colored line), thereby priming a classical linear RCA. The concatemeric product can then multiply anneal to the same circular probe or other circular probes that share the same complementary sequence. By cleavage of the concatemer at the nicking sites multiple copies of a circular probe with annealed primer ends are generated. These copies themselves again generate concatemeric products, thus resulting in an exponential amplification (see color version of this figure at www.tandfonline.com/ibmg).

complexes, the second stage of amplification is triggered that yields additional concatemeric products independently. The efficiency of this reaction could be improved by using circular probes comprising several copies of the complementary target sequence (see Figure 6). This type of rolling circle cascade can therefore rapidly amplify a product for highly sensitive detection of even minute target amounts. However, as the product does not comprise any original template sequence other than that encoded by the circular probe, no information is amplified. Therefore, a false positive amplification product cannot be distinguished from the presence of the real target in a given sample.

The beacon-assisted detection amplification (BADAMP) method is also based on a nicking enzyme but is combined with a typical molecular-beacon-like signal detection system (Connolly and Trau 2010). The detection of the template is achieved by using a molecular beacon that switches its conformation in response to hybridization to the trigger sequence. The conformational switch also allows hybridization of an engaging primer which initiates DNA polymerization on the beacon. The polymerase will also displace the DNA trigger during the synthesis of the duplex DNA. As the beacon sequence comprises a nicking enzyme recognition site (e.g. NbBvC1 endonuclease), a strand-displacing polymerase (Bst large fragment DNA polymerase) can initiate from the nick on the double-stranded beacon and generate new DNA trigger molecules, thus effectively resulting in an exponential feed-back amplification (see Figure 7). Since the molecular beacon can be designed

to generate a strong fluorescent signal upon switching, the reaction can be well monitored in real-time. Despite the advantageous coupling with a sensitive detection system, the method does not amplify sequence information beyond the one encoded by the molecular beacon.

More related to original SDA is the isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) (Mukai et al. 2007) developed by Takara Bio Inc., Japan. This method allows the amplification of target DNA under isothermal conditions at around 55 °C with only a pair of 5'-DNA-RNA-3' chimeric primers, RNaseHIII (falsely attributed as RNaseH in the original publication) derived from a hyperthermophilic archaeon, and a polymerase with strand-displacing activity. RNaseHIII selectively recognizes single RNA bases in a dsDNA context, thus generating a nick on the 5' side of the ribonucleotide to serve as a strand break for polymerase elongation. The nicking efficiency can be increased by introducing up to 3 or 4 consecutive ribonucleotides in the primer (Haruki et al. 2002). Notably, ICAN amplifies target sequences of about 100 bp carrying sequence information with a very high sensitivity of several hundred template molecules. The product is monomeric, yet 3 bands are observed in a gel due to digested isoforms/intermediates. The mechanism is assumed to include template switching by the polymerase in order to regenerate the full-length product (Uemori et al. 2007) (see Figure 8).

The isothermal target and signaling probe amplification (iTPA) method developed by RapleGene, Korea is

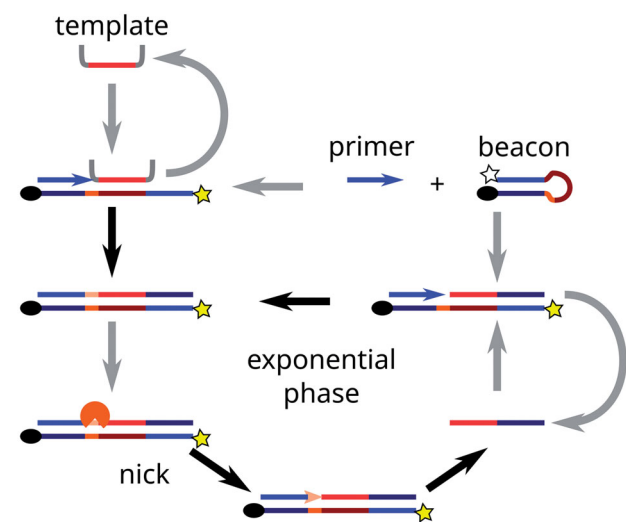


Figure 7. Beacon-assisted detection amplification (BAD AMP). The central component of the method is the molecular beacon comprising a template-specific sequence (dark red), a nicking enzyme recognition sequence folded in a loop structure, a stem-region comprising a primer binding sequence (dark blue) and a pair of dyes at the termini. The dyes in the folded molecular beacon are in close proximity at the end of the stem structure which causes the fluorophore (white star) to be quenched by the quencher (black ellipse). The molecular beacon is triggered by hybridization with the template strand comprising the recognition sequence (bright red), allowing the primer to anneal (bright blue) and unquenching the fluorophore (yellow star). A strand-displacing polymerase copies the molecular beacon while displacing the original template that can repeat the binding step with another beacon molecule. The product comprising a functional nicking enzyme recognition site (light and dark beige-colored lines) represents the replication intermediate that enters the exponential amplification phase. A nicking endonuclease cleaves the newly copied strand of the replication intermediate to initiate another copying step by which a new trigger comprising the original template sequence is released. This trigger can again bind to another beacon and primer molecules to prime the copying step, thereby closing the loop of the exponential amplification phase. The beacon becomes progressively unquenched during this amplification, allowing an online product detection by fluorescence measurement (see color version of this figure at www.tandfonline.com/ibmg).

similar to ICAN. Of several stand-displacing DNA polymerases tested, Bst DNA polymerase displayed the best amplification efficiency (Jung et al. 2010). The main difference to ICAN is the use of a set of DNA-RNA-DNA hybrid primers, RNase H, T4 gene 32 protein as a single-strand binding protein (SSB), and an additional set of unmodified outer primers. The latter accelerates the generation of a replication intermediate which is amplified with the chimeric primers. These doubly chimeric primers are internally digested by RNase in the next steps, apparently improving the specificity of the reaction over the original ICAN method (see Figure 9). In contrast to RNase HII, the RNase H (or RNase HI)

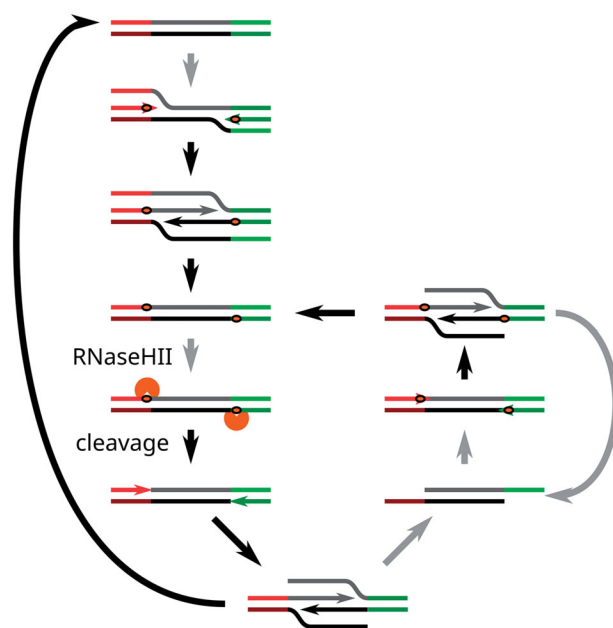


Figure 8. Proposed mechanism for isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN). The template is first invaded by specific hybridization of primers comprising a terminal short RNA sequence (beige ellipse) in a symmetric fashion. During elongation, the strand-displacing polymerase switches template in order to generate a product comprising RNA regions that represents the exponential phase intermediate. By RNaseHII cleavage, another round of copying with template switching is initiated. Both displaced strands can be contacted by the primers by simple hybridization to the single stranded regions. The elongation of the primers generates the exponential phase intermediate (see color version of this figure at www.tandfonline.com/ibmg).

requires at least 4 consecutive ribonucleotides for efficient cleavage followed by full degradation of hybridized RNA. The iTPA method requires that the 3' DNA section of the chimeric primer is complementary to the template, whereas the ribonucleotides and the 5' DNA section must not anneal to the original template sequence. Only by the synthesis of the second strand initiated by the chimeric inner primers, the full double-stranded replicative form with a cleavable RNA/DNA heteroduplex is generated. The combination with a fluorescence resonance energy transfer cycling probe technology (FRET CPT) further improves sensitivity, apparently comparable to PCR. Furthermore, a study of the iTPA method for the *Mycobacterium tuberculosis* detection in patient samples revealed a high concordance rate with real-time PCR (Kim et al. 2016).

Although primers comprising RNA bases appear to work well in some detection applications, they may prove difficult in assays using unpurified and partially purified samples due to the presence of ubiquitous RNases which can rapidly degrade these primers. In

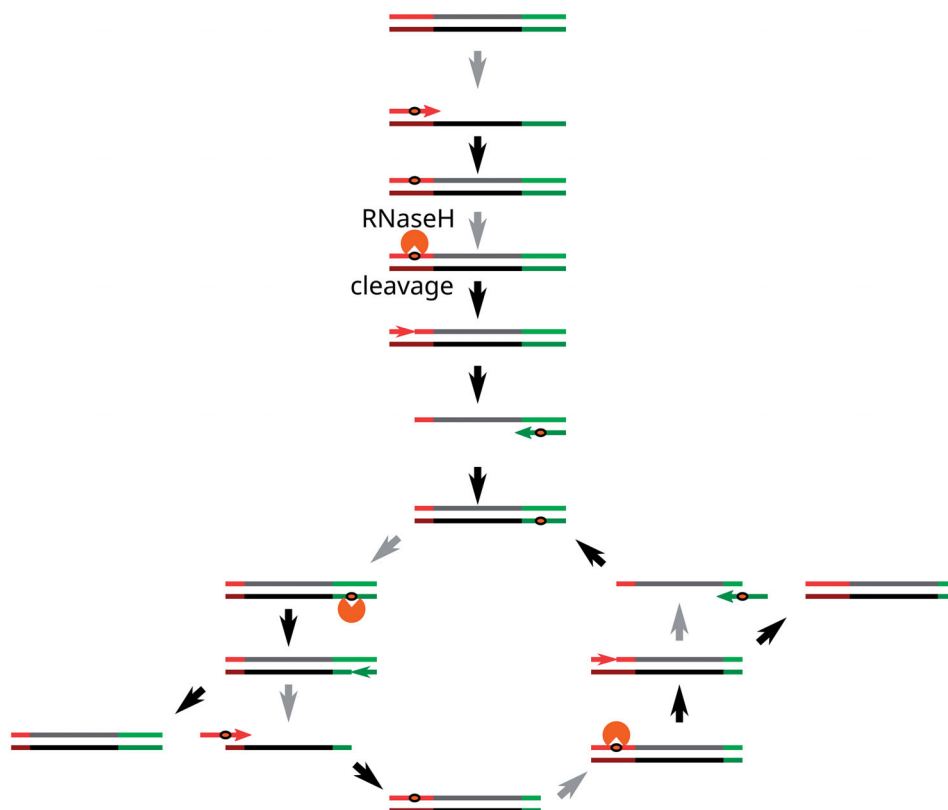


Figure 9. Isothermal target and signaling probe amplification (iTPA). The template is first denatured to generate a single strand that can be hybridized by a chimeric primer comprising an internal short RNA sequence (beige ellipse). By RNaseH digest, a short DNA primer is generated that initiates a first copying step by strand-displacing polymerase. The displaced single stranded copy is contacted with another chimeric primer that generates the central exponential phase intermediate. Repeated steps of RNaseH digest, priming and copying again generate the exponential phase intermediate while accumulating shorter sterile dsDNA end products (see color version of this figure at www.tandfonline.com/ibmg).

addition, RNA is chemically less stable under alkaline conditions and sensitive to some metal ions.

A rather recent strand cleavage-based amplification method uses the currently highly popular CRISPR-Cas technology to generate site-specific nicks in the target sequence. The CRISPR-Cas9-triggered nicking endonuclease-mediated strand displacement amplification (CRISDA) takes advantage of the high sensitivity/specificity and unique conformational rearrangements of CRISPR effectors in recognizing the target DNA (Zhou et al. 2018). The main difference to the other strand-breaking mechanisms is that the CRISPR-Cas9 nicking complexes stay bound to their recognition site and would therefore restrict efficient amplification of the template by themselves. Therefore, the nick is generated in the displaced strand at a position that leads out of the template sequence. This effectively allows the annealing of a pair of primers to initiate the SDA reactions (Initiating Primer pair, IP_{UPS/DNS}) to the exposed non-target strands for elongation. The IP introduces recognition sequences for the nicking enzyme Nb.BbvCI, which leads to a product that can be

amplified according to the well-known SDA method (see Figure 10). Thus, the advantage of the CRISDA over SDA is the skipping of the initial denaturation step otherwise required to efficiently initiate the first primer binding for the introduction of specific nicking enzyme recognition sites. Notably, the CRISDA allows the reaction to occur at only 37 °C due to the highly specific initiation step. The final detection step is performed by hybridization with a target-specific PNA probe.

Strand refolding mechanism

A self-priming mechanism involving the formation of hairpin loops by folding back of newly generated inverted repeat termini has been long implicated in the replication of Poxviruses (Baroudy et al. 1982; Moss 2013). Furthermore, the folding back of 3' termini of cDNAs for self-priming has also been used first for the generation of double-stranded cDNA libraries in the absence of known sequences (Efstratiadis et al. 1976). Yet, the concept of using primers that can fold back on themselves while introducing additional primer binding

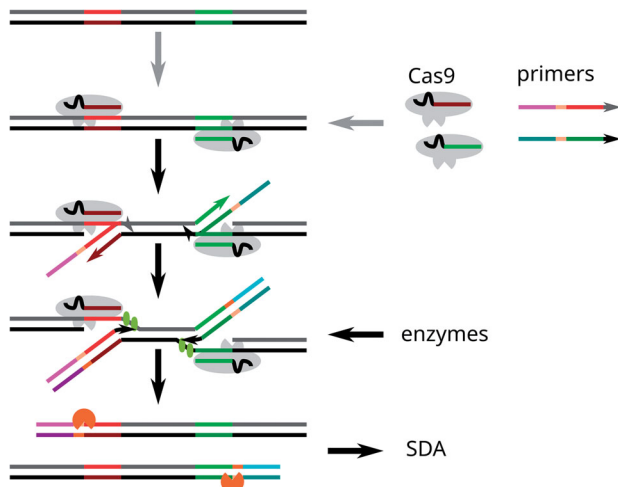


Figure 10. CRISPR-Cas9-triggered nicking endonuclease-mediated strand displacement amplification (CRISDA). The template is first contacted with Cas9 complexes (gray shapes) comprising target specific gRNAs (with dark red and bright green regions, respectively) and primers comprising a target-specific region and a nicking enzyme recognition sequence (beige). The Cas9 complexes bind tightly to the target sequences and after nicking displace the cleaved unbound strand. The cleaved strand then hybridizes to the primers. Upon addition of polymerase, SSB (green ellipses) and nicking enzyme (beige shapes), the annealed primers are copied by the nicked strands. The 3'-ends of the primers are aligned by SSB binding to the complementary regions of the template, allowing the template to be copied by the strand-displacing polymerase. This copying step generates the replicative intermediates that enter the exponential reaction according to the conventional SDA mechanism (see color version of this figure at www.tandfonline.com/ibmg).

sites to act as an *oriA* in isothermal amplification is rather novel. The publication of the LAMP method by Notomi et al. in 2000 (Notomi et al. 2000) kick-started the development of a whole plethora of similar isothermal amplification methods. The main driver for this is the requirement for simple unmodified primer oligonucleotides and a commercially available strand-displacing Bst DNA polymerase.

The concept of LAMP appears at first to be rather complicated, especially when looking at the main figure of the original publication with the schematic representation of the mechanism including proposed replication intermediates generated in the reaction of LAMP. Many different types of concatemeric molecules are generated from which both internal and terminal priming can occur. However, the most crucial step responsible for the actual success of the LAMP replication is the generation of a symmetrical single-stranded dumbbell structure as a replicative form comprising two-terminal loops (see Figure 11). In order to generate this symmetric replicative form, one set of inner primers (often designated BIP and FIP) is required to artificially introduce

complementary sequences that can hybridize as a stem with upstream target sequences, thereby forming stable loop structures comprising further priming binding sites. An additional set of outer primers (referred to as B and F) is required to accelerate the reaction and generate a first and second single-stranded intermediate. The second single-stranded intermediate can already form the symmetrical dumbbell structure. The reaction can be optionally further accelerated by the use of an additional loop primer pair (BL and FL) that targets a region in the loop between the stem hybridization region and the inner primer binding sites (Nagamine et al. 2002). It is also possible to accelerate the reaction using stem primers instead of loop primers that bind in the inner template region (Gandelman et al. 2011).

The generated amplicon size is limited to less than 300 bp (including outer primers) and the template region between the inner primer hybridization sites is mostly kept short (below 20 bp). This strongly reduces the amount of sequence information originating solely from the template that is amplified by LAMP. However, the sensitivity of LAMP can be very high, down to about 10 template molecules in a reaction. The target specificity is deemed to be very high as well, because of the many different sites (6 or optionally 8 target binding sites vs. 2 in PCR) that are recognized during the amplification process. Although a dedicated software is available for LAMP primer design based on a given sequence (<http://primerexplorer.jp>), it is recommended to evaluate 2–4 complete sets of LAMP primers for optimal sensitivity and specificity before choosing a final set. Already the seminal publication demonstrated the feasibility of RT-LAMP for the detection of RNA templates by introducing an initial reverse transcription step. More recently, a mutant of the Bst DNA polymerase has been made commercially available to efficiently perform the reverse transcription step and LAMP with the same enzyme (Lee et al. 2016). Due to its high popularity, availability of the Bst DNA polymerase, and potentially also driven by patent limitations, many other isothermal amplification methods based on the strand-refolding mechanism have been developed.

The smart amplification process version 2 (SMAP 2) is similar to LAMP as it retains one half of the dumbbell with a stem-loop structure while the other half is replaced with a more simple hairpin structure resulting in a more asymmetric structure (Mitani et al. 2007). Other notable differences are the use of a mismatch-binding protein such as Taq MutS and the *Alicyclobacillus acidocaldarius* DNA polymerase (Aac pol) instead of the default Bst DNA polymerase as a strand-displacing polymerase. The MutS protein is designed to

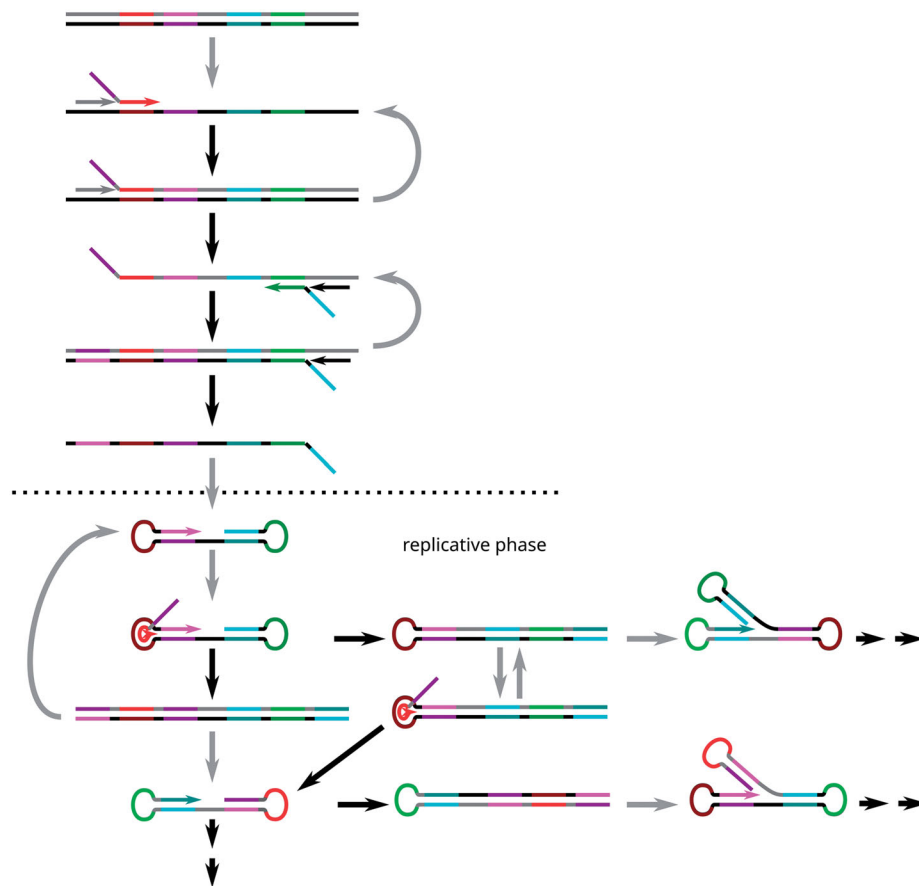


Figure 11. Loop-mediated isothermal amplification (LAMP). The first 6 steps show the generation of the replicative intermediate using bumper or outer primers (indicated by black and gray arrows) depending on strand specificity. The inner primers (comprising red and green recognition sequences) bind to the template and initiate synthesis of a first strand which remains bound to the double-stranded template. Outer primers binding upstream of the inner primers are used to displace the products comprising the newly added loop sequences. The replicative intermediate forms a dumbbell-like structure comprising one loop with a 3'-end that can be elongated to a first loop-hairpin structure, or bound by another inner primer that simply copies the dumbbell sequence to a double-stranded product. The dsDNA can refold to form two different dumbbells (with respective anti-sense sequences), whereas the extended loop-hairpin structure can refold to form an extended dumbbell repeat or be copied by an inner primer to regenerate a dumbbell. The reaction continues to produce more concatemeric sequences which are also partially resolved as the small dumbbell replicative intermediate by internal primer binding (see color version of this figure at www.tandfonline.com/ibmg).

tightly bind at primer-template mismatches and thereby inhibiting the amplification of single-nucleotide mutations. The hairpin is introduced into the asymmetric replication template by a folding primer (FP), while the stem-loop structure is added by a turn-back primer (TP) analogous to an inner primer in LAMP (see Figure 12). In the same manner, first and second single-stranded intermediates are generated by extension from the outer primers, yielding the asymmetric replication template. A single boost primer (BP) binding in the loop region is employed to further accelerate the amplification reaction analogous to the loop primers in LAMP. Although the general performance of SMAP 2 appears to be comparable to LAMP and adds an interesting feature with regard to SNP detection, it has not gained a similar degree of popularity. This may of

course be due to the later introduction of the method, but also because of the dependence on Aac pol and Taq MutS enzymes that are less readily available than Bst DNA polymerase. Yet, the method is marketed as SmartAmp[®] and its respective reagents are distributed by Kabushiki Kaisha DNAFORM.

The cross-priming amplification (CPA) technology is an invention from an isothermal DNA amplification system by Ustar Biotechnologies Co., Ltd., that relies on only one loop structure to function as a replicative form (see Figure 13). The first published CPA method employs two outer primers termed Displacement Primers (DP1s for sense strand and DP2a for anti-sense strand) and two inner Cross Primers (PFs for forward sense and PRa for reverse anti-sense strand). The outer primers are used for acceleration and generation of the

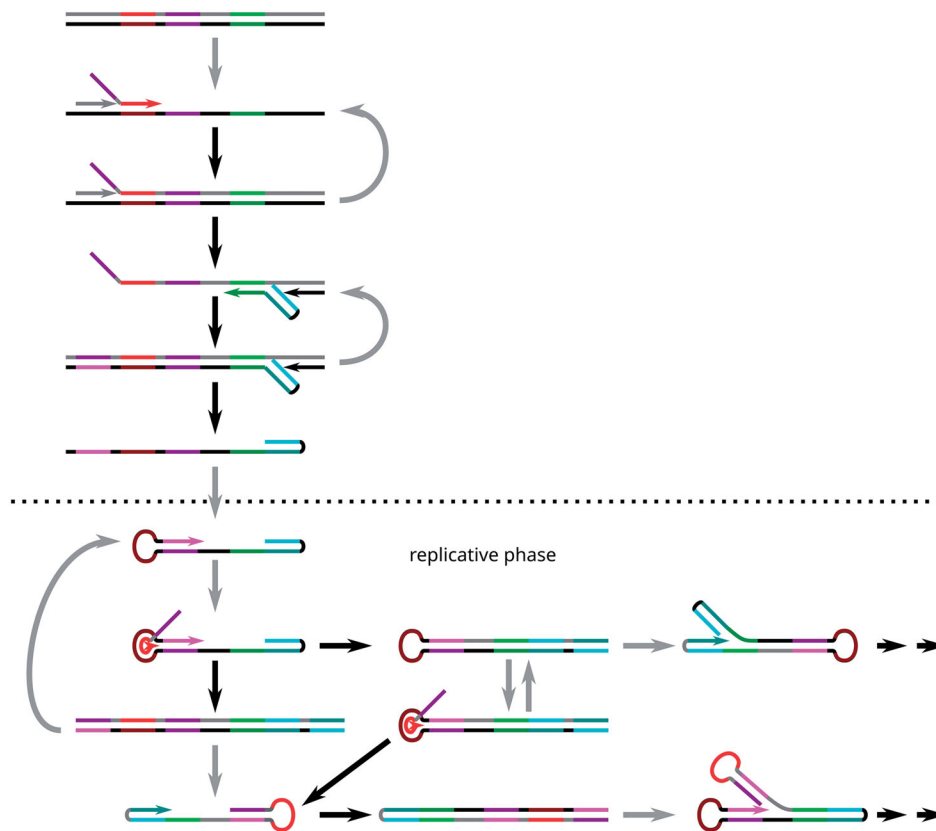


Figure 12. Smart amplification process version 2 (SMAP 2). The first 6 steps show the generation of the replicative intermediate using bumper or outer primers (indicated by black and gray arrows) depending on strand specificity. Inner primers bind to the template and initiate synthesis of a first strand which remains bound to the double-stranded template. One inner primer (with a red binding sequence) comprises a loop forming sequence, whereas the other inner primer is a turn-back primer that comprises a simple hairpin sequence. Outer primers binding upstream of the inner primers are used to displace the products comprising the newly added sequences. The replicative intermediate forms an asymmetric structure comprising a loop-hairpin and a folded-back hairpin structure with a 3'-end that can be elongated to a first loop hairpin structure, or bound by another inner primer that simply copies the sequence to a double-stranded product. The dsDNA can refold to form two different replicative intermediates (with respective antisense sequences), whereas the first extended loop structure can refold to form an extended repeat comprising two ends with refolded hairpins or be copied by an inner primer to regenerate another replicative intermediate. This can be elongated from the refolded hairpin to yield an extended dsDNA, which can refold to a form comprising two ends with loop-hairpin structures. The reaction continues to produce more concatemeric sequences which are also partially resolved as the small replicative intermediates by internal primer binding (see color version of this figure at www.tandfonline.com/ibmg).

first single-stranded intermediate forms. The cross primers introduce a complementary section that allows hairpin formation. As the complementary sections introduced by the primers do not form a direct repeat, two equally stable hairpin structures with a free primer binding site represent the exponentially amplifiable replicative form. The exposed primer binding sites can be efficiently primed by the matching cross primers which leads to a growing concatemeric sequence of priming sites that frame a central template-derived sequence for specific detection that is not present in any of the primers. This type of CPA has later been termed double-crossing CPA and another, more reduced single crossing CPA has been developed as well (Xu et al. 2012). The single CPA employs only one

cross primer and two additional short replicative primers that bind to complementary sections of the replicative form where two symmetric hairpin structures (derived from each strand separately) without any single strand extension is used for priming. However, the loop structure comprises a primer binding site that drives the exponential reaction. Single crossing CPA results in apparently concatemeric products which cannot be easily explained by the amplification scheme. As a tweak for breaking down this concatemer for correct product size analysis, a unique restriction site can be introduced in the cross-primer. As most of the methods are based on the strand refolding mechanism, both types of CPA rely on the large fragment of the Bst DNA polymerase as strand-displacing polymerase, working in

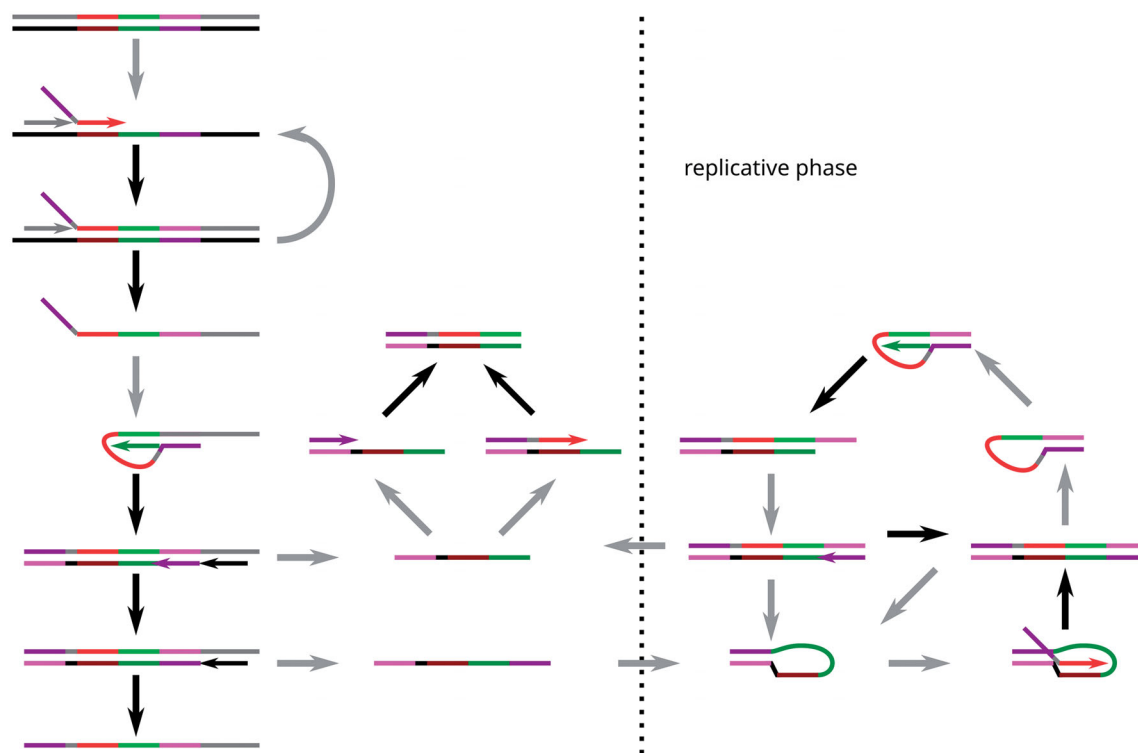


Figure 13. Cross-priming amplification (CPA). The first 6 steps show the generation of the single-stranded intermediate using bumper or outer primers (indicated by black and gray arrows) depending on strand specificity. Inner primers bind to the template and initiate synthesis of a first strand which remains bound to the double-stranded template. One inner primer (with a red binding sequence) comprises a loop forming sequence, whereas the other two inner primers comprise only one simple binding sequence (green for the first and purple for the second arrow). Outer primers binding upstream of the inner primers are used to displace the products comprising the newly added sequences. The single-stranded intermediate displaced after step 6 rapidly folds into the first replicative intermediate, entering the replicative phase. The replicative intermediate forms a loop-hairpin structure which is bound by the inner primer comprising the loop-forming sequence that yields a double-stranded product. The dsDNA can refold to form two different replicative intermediates (with respective antisense sequences), whereas one can refold to regenerate the first replicative intermediate and second replicative intermediate. This can be copied by the first simple inner primer to yield a partially double-stranded product with a free primer binding site for the second inner primer. This primer allows regeneration of the full-length dsDNA product, while displacing a sterile intermediate. The sterile intermediate is finally copied to a sterile dsDNA end product (see color version of this figure at www.tandfonline.com/ibmg).

the range between 60–65 °C. The authors state that the double-crossing CPA is faster, but that the probe binding sites can become hidden in the secondary structures produced (Xu et al. 2012). However, in contrast to the double-crossing CPA, the single crossing CPA does not comprise sequence information derived from the template that is not encoded by primers. Therefore, a probe cannot be used to distinguish template-derived products from artifacts.

A less obvious type of isothermal amplification method based on strand refolding is quadruplex priming amplification (QPA) (Kankia 2011). The method is based on the principle that sequences rich in consecutive guanines can form alternative structures termed G-quadruplex (G-4) that are more stable than the conventional duplex in the presence of cations such as potassium. Thus, sequences comprising such G-rich patterns form G-quadruplexes at elevated

temperatures under favorable buffer conditions. QPA requires a template comprising sequences that can form stable G-4 structures (see Figure 14). These can also be introduced by template-specific primers in an initial step, for instance by conventional PCR-like cycles (Kankia 2011). A QPA primer comprises a G-rich sequence lacking at least two guanines of the full G-4 motif at the 3' end. Thus, it is not able to form a unimolecular G-4 structure by itself in the absence of a target. Upon binding to the target and elongation, the missing guanines are added which drives the G-4 structure formation. By folding the complete G-4 in the primer strand, the primer binding site is liberated as a single-strand, ready for the next binding and extension cycle of the QPA primer. The folding and priming kinetics can be optimized by choosing a highly stable G-4 forming sequence, cation concentration, and temperature. The special feature of QPA with respect to most

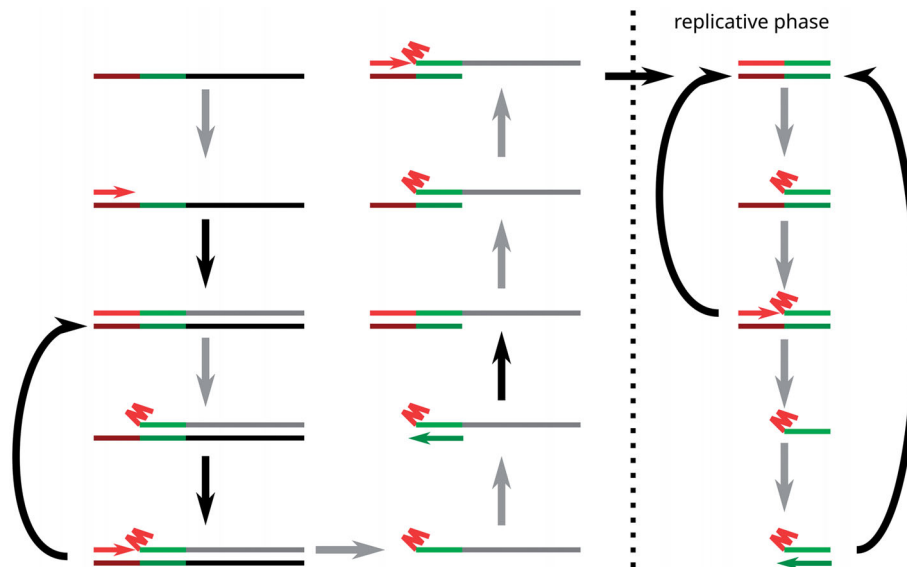


Figure 14. Quadruplex priming amplification (QPA). The initial ssDNA QPA probe comprises a quadruplex antisense sequence (red), a primer binding region (green) and a target-specific region. The QPA primer with an incomplete quadruplex sequence (bright red arrow) binds to the template to initiate the first strand synthesis, thereby completing the quadruplex sequence (bright red). The quadruplex sequence folds up as a compact quadruplex structure (bright red zigzag line), freeing the quadruplex antisense sequence for another binding step of the quadruplex primer. The QPA primer is elongated by a strand-displacing polymerase to displace the antisense strand of the QPA probe. The 2nd primer (bright green arrow) can bind to this strand and copies the full quadruplex antisense sequence. The quadruplex folding, QPA primer annealing step can occur again. Primer extension liberates the short replicative intermediate comprised of the quadruplex and primer binding sequence alone. This replicative intermediate can undergo recurrent steps of folding, QPA primer binding, strand displacement and regeneration by the 2nd primer (see color version of this figure at www.tandfonline.com/ibmg).

other isothermal amplification methods is the use of conventional polymerases such as Taq that do not possess a strand-displacing activity, potentially due to the relatively high reaction temperature (>70 °C) at which especially a short DNA duplex template is already more destabilized. The QPA can be designed either as a linear or exponential amplification method by introducing one or two G-4 sequences for QPA primer binding. The linear amplification was shown to be useful for longer DNA segments (Lomidze et al. 2018). So far no commercialization of the method was made and no kits for QPA are readily available.

A more recent isothermal amplification method is the genome exponential amplification reaction (GEAR) (Prithviraj et al. 2012). The method is very similar to LAMP as it relies on the generation of a dumbbell-like exponential replication form and loop-forming primers (see Figure 15). The main difference is the reduction of the two inner loop end hybridization sites in LAMP in the template to a single site in GEAR. Thus, both loop ends compete with each other in the formation of a dumbbell-like structure. The loops are formed with two loop-forming Tab primers (FT and BT) comprising two different template-specific priming sites and one region complementary to a common “Tab” binding site termed (T and Tc). GEAR does not require outer primers, but

relies primarily on the use of two inner primers (IF and IR) that accelerate the reaction by helping to displace the template strands. However, auxiliary primers like outer primers in LAMP have been shown to speed up the GEAR reaction as well (Jothikumar et al. 2014). Although GEAR allows amplification of template-derived information (between Tab and inner primers), no specific probes have been employed for detection so far. GEAR is mostly compatible with Bst DNA polymerase but has been shown to work best as a combination of AMV reverse transcriptase with *Geobacillus* species DNA polymerase in an optimized one-tube RT-GEAR mixture (Guan et al. 2016). Generally, the sensitivity and speed of the GEAR method appears to be high, even in the absence of outer primers and using RNA as a template with an intermediate reverse transcription step (Guan et al. 2016). Despite interesting publications, no commercial product using GEAR is yet available.

A novel, but partially controversial amplification method is the polymerase spiral reaction (PSR). The original publication suggests that the mechanism is based on a spiral arrangement of the template generated by primers that introduce direct and not reverse complementary sequences. The forward and reverse Tab primer sequences are reverse to each other at their 5' end (Nr and N), whereas their 3' end sequences are

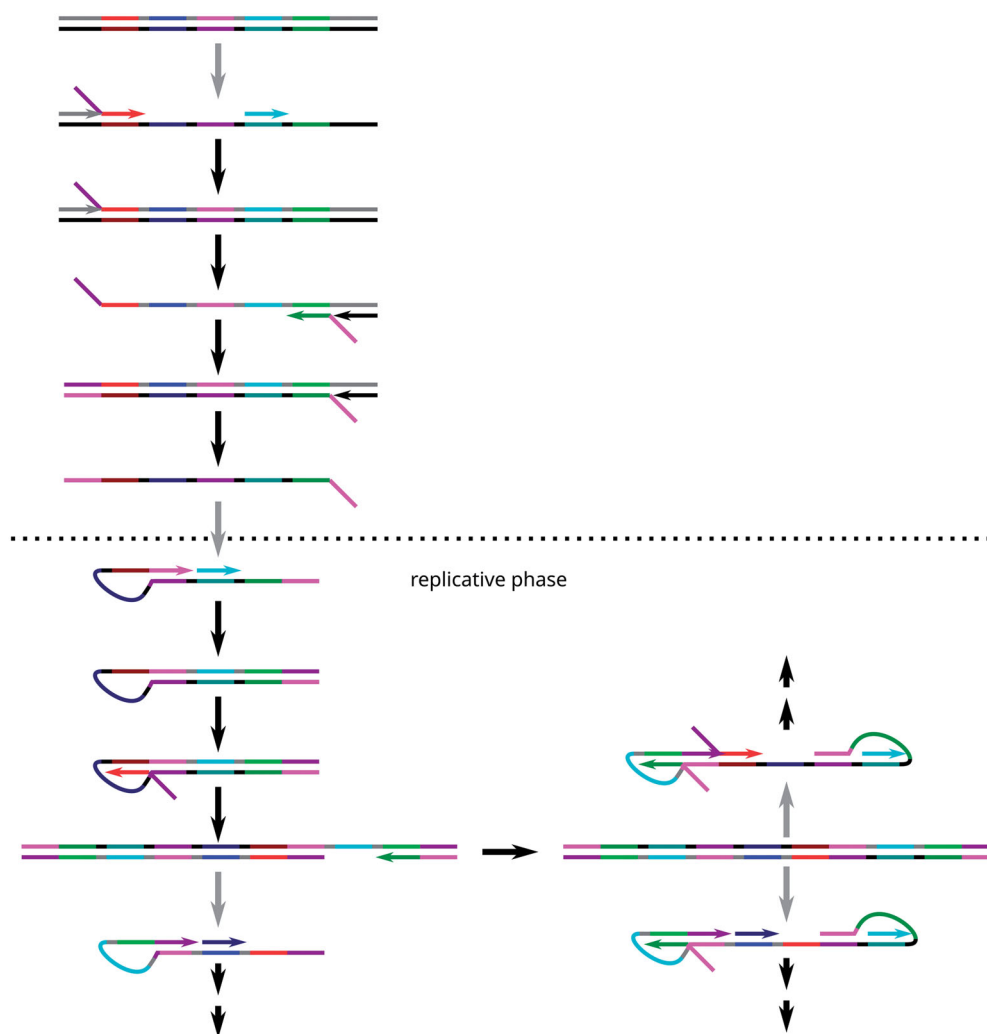


Figure 15. Genome exponential amplification reaction (GEAR). The first 6 steps show the generation of the asymmetric replicative intermediate using bumper or displacement primers (indicated by black and gray arrows) and inner primers (azure and blue) mainly to invade the target double strand and to displace newly synthesized strands. Tab primers (comprising red and green recognition sequences) bind to the template and initiate synthesis of a first strand which remains bound to the double-stranded template and is then displaced by synthesis from an outer primer. The replicative intermediate forms an asymmetric club-like structure comprising one loop with a 3'-end that can be elongated to a first loop-hairpin structure, or bound by another Tab primer that simply copies the intermediate to a mainly double-stranded product. This mainly double-stranded product can bind a Tab-primer at one end for another elongation step to displace one strand that can form a complementary club-like intermediate and generate a completely double-stranded product. The complementary club-like intermediate can follow the same replication steps as described before, while the completely double-stranded product can refold to yield elongated symmetric structures with loops that can either be directly elongated or primed after annealing of Tab-primers. This leads to generation of concatemeric sequences that are partially resolved into the smaller by internal Tab and inner primer binding (see color version of this figure at www.tandfonline.com/ibmg).

complementary to their respective target nucleic acid sequences (Liu et al. 2015). The mechanism is deemed to resemble a rolling circle amplification initiated from a spiral replicative form, resulting in direct concatemeric repeats (see Figure 16). However, during the adaption of the original over-simplified figure to this manuscript, we realized that the proposed mechanism is not feasible without violating so-far unchallenged rules in biochemistry. A more detailed discussion can be found at PubPeer (Liu et al. 2015). If the tab sequences Nr and N

indeed form a double-stranded helix, the complementary strands must be arranged in a parallel direction and not in the conventional anti-parallel fashion. Parallel-stranded double-helices are generally feasible, but usually at lower melting temperatures and under acidic buffer conditions (Szabat and Kierzek 2017). If the assumption of a spiral replication intermediate that is amplified by a rolling circle mechanism were true, the reaction primed by the 3' end would be required to run "upstream" in the so far unobserved direction on the

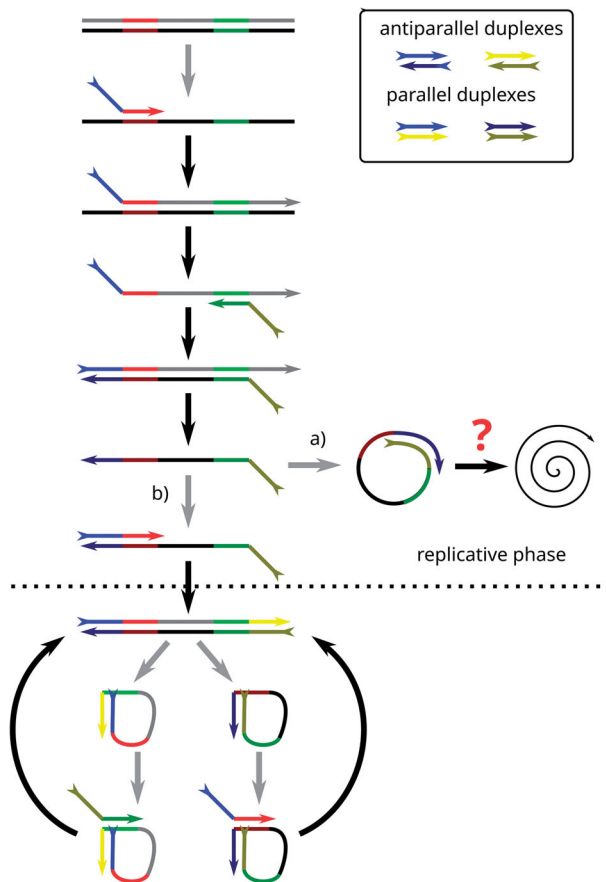


Figure 16. Polymerase spiral reaction (PSR). The special annealing mechanism of the direct complementary sequences require a more detailed annotation of the strand orientation by adding both arrow and tail symbols to all depicted nucleic acids. Matching sequences that can anneal in a parallel-stranded duplex are highlighted by complementary colors (blue with yellow and dark blue with olive). The respective parallel and anti-parallel duplexes are depicted in the box in the upper right. The PSR starts with the conversion of the template using forward and reverse primers comprising an additional 5' region with a defined sense and a reverse sequence, respectively. The publications suggest that the replicative form is a spiral that can amplify in a rolling circle-like mechanism as shown in a), which means that the polymerase must copy the template in the reverse direction (indicated by the red question mark). Alternatively shown in b), the double-stranded intermediate may refold into separate loops that simply allow primers to anneal for exponential amplification without violating any biochemical rules that are deemed to apply to polymerases (see color version of this figure at www.tandfonline.com/ibmg).

template (see pathway a in Figure 16). Nevertheless, as at least one independent publication also states that the amplification product comprises the correct template sequence, it can be assumed that a different mechanism may be at play (Gupta et al. 2017). It is indeed very likely that the termini form a parallel stranded duplex that results in the claimed spiral

structure. However, rather than amplifying the template by rolling circle, the primer binding sites are simply free for repeated hybridization and priming cycles that drive the exponential reaction (see pathway b in Figure 16).

A different approach of using the refolding mechanism for an isothermal amplification method was demonstrated by the hinge-initiated primer-dependent (HIP) amplification (Fischbach, Frohme, et al. 2017). Other than most of the other refolding-based methods, the alternative structure required for the reaction is much less complicated to design. So-called scorpion primers are used in PCR to specifically detect amplified sequences employing a fold-back mechanism of primer regions, thereby un-quenching a molecular beacon (Thelwell et al. 2000). The scorpion primer folding mechanism was adapted to generate a single-stranded region for repeated primer binding and elongation cycles under isothermal conditions (see Figure 17). In order to facilitate the refolding, the primer extension complementary to an upstream sequence remains single-stranded due to a modification that cannot be read-through by a given strand-displacing polymerase. It was established that this approach is generally feasible, albeit in a less efficient manner than other methods in the current format. However, it has also come to our attention that this method was difficult to reproduce. It appears that the main cause is the ability of the Bst DNA polymerase to bypass the blocking modification, thus leading to artifacts in the absence of a template. In addition, it may be helpful to modify the refolding region in the primer so that the polymerase cannot use it as a template in order to avoid primer dimers.

Strand unwinding mechanism

In nature, most processive DNA copying processes like genome replication, require helicase to support strand unwinding, thus allowing the polymerase to proceed through long stretches of the double-stranded template. Helicases are very diverse in function and some of which, such as the bacterial DnaB, are even directly involved in unwinding the duplex at natural replication origins so that priming can occur (Seitz et al. 2000). The natural mechanism has been first adapted to an isothermal amplification method termed HDA by using UvrD helicase that unwinds blunt-ended DNA fragments starting from the 3' ends using ATP as fuel (Vincent et al. 2004). The ends would then open up and become accessible for primer binding, thus acting as an oriA for isothermal amplification (see Figure 18). In order to enhance the reaction, additional proteins are required including a single-strand DNA binding protein (SSB),

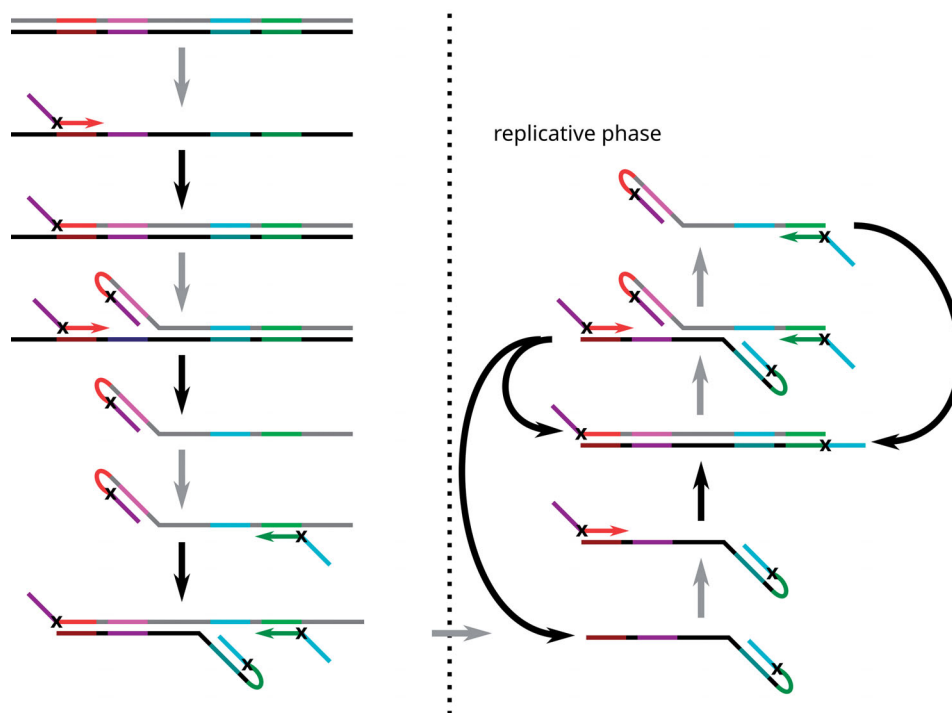


Figure 17. Hinge-initiated primer-dependent (HIP). The hinge primers comprise one conventional priming region (red or green arrows), a hinge modification (x) and an upstream hybridizing region (purple or azure lines). Upon elongation of a first hinge primer (with red arrow), the newly synthesized strand comprises a region which is complementary to the upstream hybridizing region of the primer. The primer is able to refold by forming a hairpin structure of these complementary regions which frees the primer binding site in the target strand for another hybridization of the same hinge primer. Said primer can serve as a bumper primer to displace the refolded strand, allowing a second hinge primer to bind (with green arrow). The second hinge primer is elongated, but terminated by the hinge modification of the first primer while the second hinge primer can refold and another primer can hybridize to the green primer binding region. Another priming step displaces the first replicative intermediate to enter the exponential replicative phase. Further hinge primer binding and elongation steps yield complementary strands which are amplified in the same manner, thus exponentially accumulating replicative intermediates of both complementary strands comprising terminal hairpin structures (see color version of this figure at www.tandfonline.com/ibmg).

such as T4 gene 32 protein, and MutL protein that stimulates UvrD helicase activity more than tenfold by loading it onto the DNA substrate.

The original format also utilized the exo- Klenow fragment of DNA polymerase I, a strand-displacing mesophilic polymerase (Vincent et al. 2004). To render the reaction more specific for a given template, more stringent primer hybridization conditions were introduced by performing HDA at higher temperatures. The thermophilic HDA (tHDA) requires an exchange of components with thermostable enzymes and proteins. The current format employs the *Thermoanaerobacter tengcongensis* uvrD and mutL homologs (Tte-UvrD and Tte-MutL) in combination with the Deep Vent DNA polymerase to work in an optimal temperature range of 60–65 °C (An et al. 2005). The newer tHDA format does not require the accessory protein MutL, but SSB can still enhance the reaction. Mostly, the tHDA is a robust reaction that generates a visible product in 30–90 min from very few templates. Additional improvements to tHDA were made by a fusion of UvrD helicase with Bst DNA

polymerase termed helimerase (Motré et al. 2008), but the enzyme is not commercially available. As the UvrD helicase can only enter dsDNA from blunt ends, the initial priming step is unaided in long intact dsDNA. It was found that the reaction can be accelerated by an enzyme that nicks in the proximity of the target sequence from which a first-strand synthesis can be initiated (Tong et al. 2011). As already mentioned above, the HDA has matured and is employed successfully in several commercial diagnostic assays (Barreda-García et al. 2018).

Strand invasion mechanism

A novel way to perform exponential isothermal amplification at low temperatures with high specificity employs the natural principle based on a recombinase that binds to single-stranded nucleic acids targeting double-stranded complementary sequences by a strand invasion mechanism. In nature, recombinases of the RecA/RAD51-family play a central role in homology

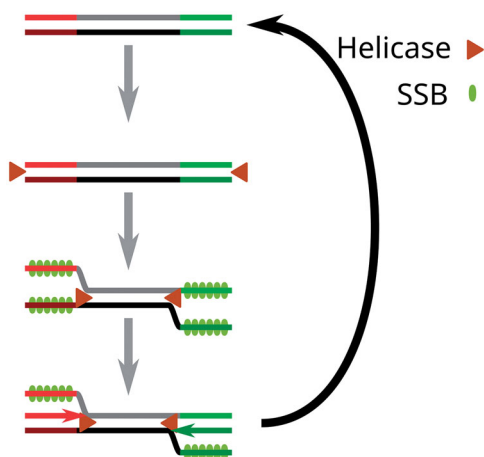


Figure 18. Helicase dependent amplification (HDA). The HDA is initiated by helicase (brown triangles) that can enter the double-stranded substrate by free 3' termini to unwind and displace one strand by translocation. Displaced single-stranded regions are stabilized by SSB (olive ellipses). The single-stranded primer binding regions in the target molecule can be hybridized by the respective complementary primers (red and green arrows). Elongation of the primers displaces one strand and thereby recycles the double-stranded substrate which can undergo another cycle of unwinding, priming and displacement by synthesis for exponential amplification (see color version of this figure at www.tandfonline.com/ibmg).

recombination. The mechanism of the recombinases is highly conserved and enzyme homologs can be found in all domains of life. In order to initiate recombination, RecA forms nucleoprotein filaments using single-stranded DNA as a scaffold. The RecA-ssDNA filament searches for sequence similarity along the dsDNA. The search process induces stretching of the DNA duplex, which enhances sequence complementarity recognition (De Vlaminck et al. 2012). The nucleoprotein filament assembles, disassembles, and promotes strand exchange with a predominant 5'-to-3' polarity. The observed polarity of RecA-mediated DNA strand exchange and filament disassembly from DNA relies on ATP hydrolysis to a large extent (Lin Y-H et al. 2019). RecA associates more tightly with DNA bound to ATP than with ADP. ATP hydrolysis by RecA occurs when DNA is bound to the filament, either as a single-strand, a double-strand or during strand exchange (Boyer et al. 2019).

The first method to employ RecA for an isothermal amplification was invented by the team of Niall Armes at ASM Scientific and published as recombinase polymerase amplification (RPA) in 2006 (Piepenburg et al. 2006). It relied on the components Bsu DNA polymerase, T4 uvsX recombinase, the single-stranded DNA binding protein T4 gp32, and ATP. Creatine kinase is also present in the reaction as it uses phosphocreatine to regenerate ATP from ADP and fuel the enzymes of the system. T4 uvsY, a

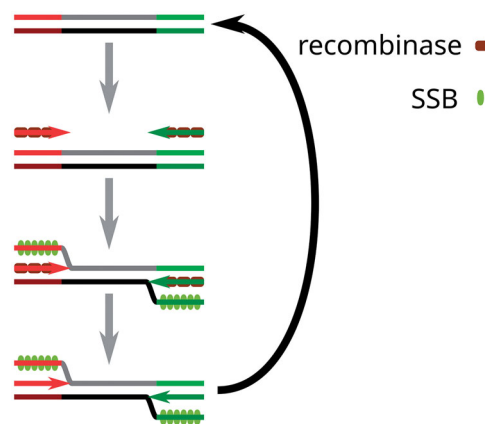


Figure 19. Recombinase primed amplification (RPA) The primers are coated by recombinase oligomers (brown rectangular shapes) that are able to invade the double-stranded template. Upon correct hybridization of primers (red and green arrows) to their complementary strand, the displaced template strand is coated by SSB (indicated by olive ellipses) and the recombinase dissociates. A strand-displacing polymerase can elongate from the hybridized primers to copy both of the annealed template strands. The generated copies can again enter further cycles of exponential amplification (see color version of this figure at www.tandfonline.com/ibmg).

recombinase loading factor, and a particular crowding agent (Carbowax20M) establish favorable reaction conditions that support RPA (see Figure 19). It was also established that primers had to be more than 30 bases long to be effective in the reaction. The current format as sold by TwistDx is supposed to be substantially enhanced, but no specific data as to the formulation is available. The main advantage of RPA over most other isothermal amplification methods is the comparably simple design of only two primers, rapid reaction, and high target specificity despite a low reaction temperature (around 37°C). The complex composition of enzymes and other additives makes it more difficult to establish the reaction without buying the commercial kit from TwistDx. The success of the method has already led to an ever-increasing list of publication and commercialized diagnostic kits (Li et al. 2018).

The current only other distinct method employing a strand invasion mechanism based on a recombinase is strand invasion based amplification (SIBA) (Hoser et al. 2014). Other than guiding the specific strand invasion of the primer as in RPA, a blocked oligonucleotide termed invasion oligonucleotide (IO) is used to displace the template strand in the target duplex for primer binding. Due to the size restriction of RecA, oligonucleotides shorter than 30 bases are not substrate to the recombinase and are thus available for efficient hybridization in the single-stranded target region for priming of a strand displacing polymerase (see Figure 20). The IO comprises 2'-O-methyl RNA that cannot be copied

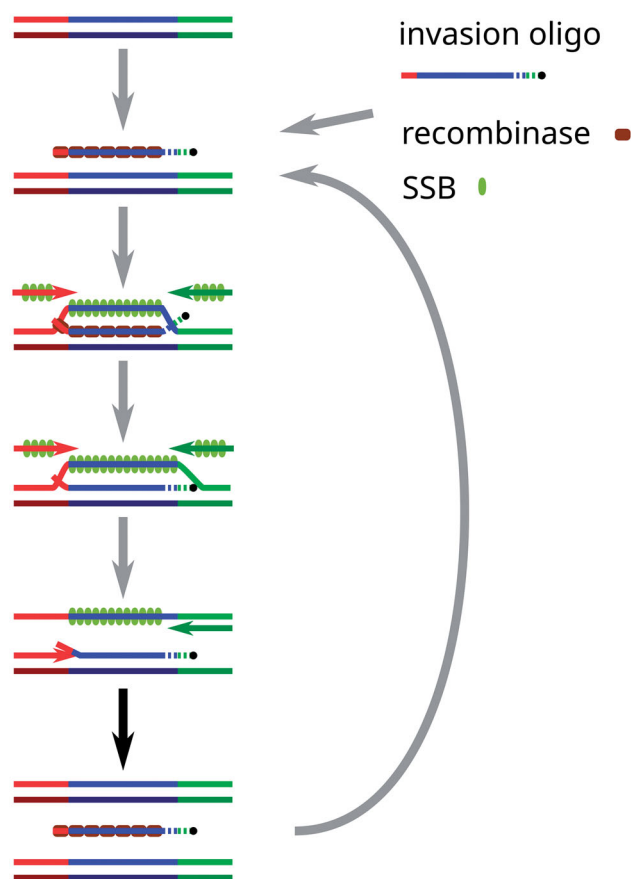


Figure 20. Strand invasion based amplification (SIBA) The double-stranded template is contacted with the invasion oligo (comprising a blocking 3' modification indicated by a black dot and a 2'-O-methyl RNA region indicated by a dotted line) coated by recombinase oligomers (brown rectangular shapes). The invasion oligo spans the entire target region including parts of the primer binding sites. The recombinase mediates invasion of the template, displacing one strand which is coated by the SSB (indicated by green olive ellipses) and annealing the the invasion oligo. Primers coated with SSB added already at the beginning of the reaction are finally able to hybridize to the complementary sequences on the template strands and initiate elongation by a strand-displacing polymerase. The displaced invasion oligo is thus recycled to enter further rounds of exponential amplification of the template and its copies (see color version of this figure at www.tandfonline.com/ibmg).

by the polymerase used in the reaction. Otherwise, the components such as Bsu DNA polymerase, T4 uvsX recombinase, T4 uvsY, the single-stranded DNA binding protein T4 gp32, creatine kinase, creatine-phosphate and ATP are the same as in RPA. Yet another important difference is that the amplicon in SIBA is limited mostly to the short region displaced by the IO and the two primers. SIBA has been commercialized by Orion Diagnostica Oy (now Aidian Oy) mainly for testing pathogens, for example, human *in vitro* diagnostics (IVD) for RSV (Alidjinou et al. 2019). The performance of

SIBA appears to be comparable to RPA with respect to speed, temperature, and specificity.

Enzyme features

The enzyme central to all isothermal amplification methods is the polymerase. Most isothermal amplifications employ a DNA polymerase and for some, an RNA polymerase. If RNA is used as a template, then a reverse transcriptase is required as well. Some methods also generate RNA copies from DNA templates and therefore also require an RNA transcriptase. Relevant features of polymerases, especially for isothermal amplification methods, in decreasing order of importance are strand-displacing activity, processivity, optimum temperature or stability and lastly, fidelity. In some isothermal amplification methods, additional enzymes are required to start and/or sustain the reaction. These are mainly nicking enzymes, helicases and recombinases. The main problem with multiple enzymes in a reaction is that it can be understood as a chain of events that work together, and as such, the weakest link in the chain determines the general efficiency of the reaction. Thus, a careful balance must be established with respect to optimal reaction temperature, buffer and respective activity in order to achieve a highly effective amplification reaction.

DNA polymerases

Almost all DNA polymerases used in isothermal amplifications are modified derivatives of naturally occurring enzymes to adapt their properties accordingly. A notable exception is the $\Phi 29$ DNA polymerase, a family B DNA polymerase, which naturally possesses a high strand-displacing activity, very high processivity and high fidelity (Ibarra et al. 2009). Due to its very low-temperature stability and optimum activity at 30 °C it is only applied to MDA and RCA and requires 3' end stabilized primers due to its high 3' exonuclease activity (Nelson et al. 2002).

In order to engineer strand-displacing activity in DNA polymerases, the natural enzymes are mutated to delete 3' exonuclease activity which prevents slippage (Viguera et al. 2001). The 3' exonuclease activity can be generally deleted by the exchange of at least two conserved aspartic acid residues (Bernad et al. 1989). Any potential 5' exonuclease activity, which would otherwise degrade the non-template strand instead of displacing it, is normally present in a structurally separate N-terminal domain which can be simply deleted resulting in the so-called large fragment of a polymerase

(Jacobsen et al. 1974; Joyce and Grindley 1983). A more recent engineering approach is to substitute Asp with Ala at position 422 to significantly increase the strand-displacing activity of prokaryotic A-family DNA polymerases (Piotrowski et al. 2019).

One of the first such engineered strand-displacing DNA polymerase is the Klenow exo-fragment derived from the *E. coli* DNA polymerase I, a DNA polymerase family A enzyme (Derbyshire et al. 1988; Walker et al. 1992). As the enzyme stems from a mesophilic organism, its activity and stability are limited at elevated temperature. This is why another large fragment family A enzyme was derived from a more thermophilic organism.

A very important enzyme in isothermal amplification is the Bst DNA polymerase, derived from thermophilic *Bacillus stearothermophilus* N3468. It is also a family A enzyme and naturally lacks a 3' exonuclease activity, which was originally postulated to enhance its thermostability (Aliotta et al. 1996). The large fragment of Bst DNA polymerase has been found early on to be an exceptionally efficient enzyme in terms of processivity and strand-displacing activity. The first isothermal amplification to use this polymerase was hyperbranched RCA (HRCA) (Lizardi et al. 1998), but its popularity truly took off with the introduction of LAMP in 2000 (Notomi et al. 2000). Since then, almost all isothermal amplifications typically working in the temperature range of 55–65 °C have adopted this polymerase (see Table 1). The enzyme has some quite unique properties such as the requirement of a high magnesium concentration from 4 to 8 mM, a high buffer pH of 9, and a tolerance for lower concentrations of manganese. It is also robust with respect to many inhibitors that are known to compromise PCR amplification (Francois et al. 2011). One of the more surprising features of Bst DNA polymerase is the innate reverse transcriptase activity (Shi et al. 2015). As some polymerases are known to display this activity in the presence of manganese at the expense of fidelity (Myers and Gelfand 1991; Grabko et al. 1996), the Bst DNA polymerase does not require any special buffer conditions and is, therefore, useful in amplification reactions with RNA templates. In addition, it has been found that the Bst DNA polymerase can accept a very wide range of unusual nucleic acids as templates (Chaput et al. 2003; Jackson et al. 2019) and incorporates modified nucleotides (Kumar et al. 2013; Röthlisberger et al. 2017). The great impact in isothermal amplification methods of the Bst DNA polymerase, and thus its commercial success, has led to the development of modified versions comprising useful features such as “warm

start”, improved thermal stability, and reverse transcriptase activity (Lee et al. 2016).

Yet despite those many advantageous properties, some other features also strongly limit its use. One problem is its inactivation at high temperatures normally required for an initial denaturation step in order to allow primers to anneal. Therefore, a denaturation step must be performed before the enzyme is added to the reaction. Much more problematic is the generation of amplification artifacts by Bst DNA polymerase that can even arise in the absence of a template. Potential mechanisms behind these artifacts are template switching (Lage et al. 2003), multimerization in the presence of short duplexes (Garafutdinov et al. 2020), and *ab initio* synthesis (Zyrina et al. 2014). Even in the absence of a clear mechanism, the property to generate a certain kind of artifact has prompted researchers to devise an unusual amplification method requiring only one primer (Wang et al. 2017).

All these properties together have led to the community working with the Bst DNA polymerase dubbing it unofficially “The Beast Polymerase” as a moniker.

A mesophilic enzyme related to the Bst DNA polymerase is the Bsu DNA polymerase first described as early as 1964 (Okazaki and Kornberg 1964). The large fragment also has similar properties, but even requires higher magnesium concentrations for optimal activity. The first isothermal amplification to employ the Bsu DNA polymerase was RPA, demonstrating its superior performance at 37 °C (Piepenburg et al. 2006). The other main isothermal amplification method so far to harness the enzyme is SIBA (Hoser et al. 2014).

A notable strand-displacing DNA polymerase that is active at elevated temperature and compatible with denaturation close to boiling temperature is the Vent exo⁻ DNA polymerase. It is derived from *Thermococcus litoralis* isolated from a submarine hydrothermal vent which is referred to as Vent DNA polymerase (Kong et al. 1993). As a typical family B DNA polymerase, it naturally lacks a 5' exonuclease domain. The exonuclease mutant is Vent exo⁻ DNA polymerase and displays strand-displacing activity. It has been applied in some earlier versions of isothermal amplification methods (see Table 1) but is less frequently used due to the mostly superior properties of the Bst DNA polymerase.

More recently, a novel method of engineering has been applied to the Taq DNA polymerase, a family A polymerase. Like the Bst DNA polymerase, the Taq DNA polymerase naturally lacks a proof-reading 3' exonuclease activity. However, the large fragment of the Taq DNA polymerase, also referred to as Stoffel fragment, does not have a strand-displacing activity. The solution

to achieve this goal was to generate a chimeric enzyme with parts of both Bst and Taq DNA polymerases (Ignatov et al. 2014). The resulting SD DNA polymerase showcases better thermostability, strand-displacing activity and a higher processivity. The SD DNA polymerase has been applied mainly to LAMP reactions so far (Ignatov et al. 2014; Shchit et al. 2017).

Reverse transcriptases

Any amplification method that uses RNA as initial templates require a reverse transcription step usually performed by a dedicated reverse transcriptase. Many different reverse transcriptases are commercially available. The choice of the optimal enzyme will depend mostly on buffer conditions required for other enzymes, temperature, strand-displacing activity, and RNaseH activity. For instance, many methods require only one transcription step to generate cDNA in order to continue with DNA amplification. For this purpose, strand-displacing activity is not required and RNaseH activity may speed up primer hybridization to the cDNA. The most popular reverse transcriptase for these methods is avian myeloblastosis virus reverse transcriptase (AMV-RT) (Gracias and Mckillip 2007; Clancy et al. 2016; Ma et al. 2017), which requires a separate RNaseH to digest the template RNA. As outlined above, in some methods like LAMP, the reverse transcription step can be performed by a DNA polymerase such as the Bst DNA polymerase.

RNA polymerases

The earliest methods have employed the highly efficient Q β bacteriophage replicase, an RNA-dependent RNA polymerase, for RNA amplification. Many of its unusual features are still not fully understood (Chetverin et al. 2019). However, due to the intrinsic problems with contaminations and artifacts, this enzyme is not in use anymore. Instead, more specific DNA-dependent RNA polymerases that require a unique promoter are used. T7 RNA polymerase is an exceptional enzyme as it is highly processive and efficient (Borkotoky and Murali 2018). The main weakness as a mesophilic enzyme is its low thermostability. For this reason, special mutant versions have been engineered to allow slightly elevated temperatures to allow more specific primer annealing. Little literature is available on these, but some of the commercially available enzymes have been compared more recently with respect to the incorporation of unnatural base nucleotides (Kimoto

et al. 2017) and avoiding dsRNA artifacts during transcription (Wu et al. 2020).

Auxiliary enzymes and factors

Auxiliary enzymes are required in some isothermal amplification methods. These are mainly RNases, nicking enzymes, helicases, and recombinases as already discussed above. Sometimes, less obvious auxiliary enzymes are working in the background as they are required in either signal generation or energy recycling. For instance, recombinases require ATP which is constantly recycled from ADP and phosphocreatine by creatine kinase (Li et al. 2018). Non-enzymatic proteins such as single-strand binding proteins (SSBs) are also often central components that determine the efficiency and specificity of a given reaction. The selection of the optimal SSB may be tricky as they come in very different structures and binding mechanisms (Arif and Vijayan 2012).

Amplification detection methods

Provided that background levels are low for the respective amplification method, a general product detection can be sufficient for evaluation. In some cases, it is useful to distinguish similar templates from another which mostly requires an additional probe for verification. Especially, single nucleotide polymorphisms (SNPs) are difficult to detect in an isothermal amplification.

On-line (or real-time) detection also allows quantification, which should correlate with the rate of product formation. Yet, the means for detection must not compromise the efficiency and specificity of the reaction. Ideally, detection is performed as a homogeneous assay that does not require opening of the reaction vessels after the amplification in order to avoid any contamination of other assays conducted at the same place or with the same equipment.

Reaction product-based detection methods

Typical products of amplification reactions are DNA and/or RNA, inorganic pyrophosphate (PPi), and protons. As a result, the level of multivalent cations such as magnesium ions is reduced and the pH decreased which can be used to detect product formation (see Figure 21). The production and change in by-product levels during amplification have been well adapted to function as means to detect amplification. Most isothermal amplification methods using the Bst DNA

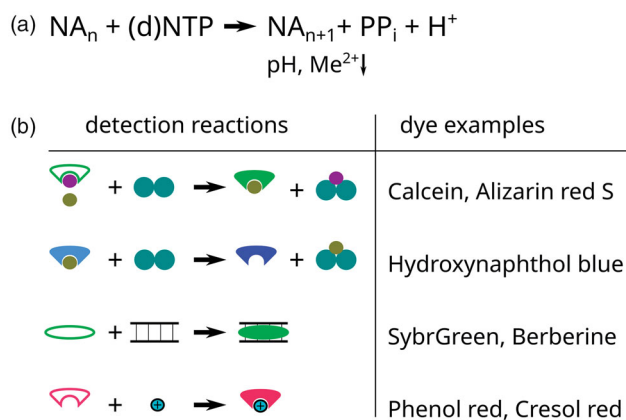


Figure 21. Polymerase reactions and respective detection methods. (a) Reaction equation of RNA and DNA polymerases with resulting conditions in the second line. (b) Symbolic detection reactions with pyrophosphate (blue-green double circles), manganese ions (purple circle), magnesium ions (olive circle), dsDNA (bold horizontal lines connected with parallel vertical lines), proton (azure circle with +) and ligand-responsive dyes (outlined shapes in turned off state and filled shapes in turned on state) (see color version of this figure at www.tandfonline.com/ibmg).

polymerase, such as LAMP, generate large amounts of products which makes simple detection methods possible.

The most simple and noninvasive optical means for detection is the increasing turbidity due to the precipitation of PPI (Mori et al. 2001). However, not all isothermal reactions yield such high concentrations of pyrophosphate and precipitation occurs comparably late. The majority of PPI detection has been reported for LAMP-based methods. However, the concept of PPI detection has evolved beyond turbidity detection. An indirect detection method resulting from PPI detection involves the conversion of PPI into ATP in the presence of adenosine 5'-phosphosulfate (APS) and ATP sulfurylase. Thereafter, the converted ATP was detected by constructing an electrochemical sandwich aptasensor (Xie et al. 2018).

General product formation can be simply monitored during the reaction by using dyes that respond to either product detection, or more indirectly, by educt depletion. A typical example for the detection by educt depletion is monitoring of free magnesium ions by a specific dye such as HNB (Goto et al. 2009), whereas product detection is more commonly employed. Especially DNA-responsive dyes that do not inhibit or affect the fidelity of the polymerase such as SybrGreen, EvaGreen, or even berberine are compatible with most amplification methods (Fischbach et al. 2014). Another popular product detection method is based on the formation of PPI. PPI can act as a selective chelator and binds avidly to certain polyvalent cations such as Mn^{2+} .

Certain dyes such as Calcein are quenched by free manganese ions which are sequestered by newly generated PPI. Thus, PPI formation in an isothermal amplification is indirectly detected by the depletion of free manganese ions (Tomita et al. 2008). Other dyes such as Alizarin Red S (ARS) are also useful to detect PPI in combination with manganese (Fischbach, Loh, et al. 2017). Accumulation of protons in the typical polymerase reaction can be detected by pH indicators such as Phenol red or Cresol red (Tanner et al. 2015). Alternatively, PPI-formation is detected by potentiometry using MnO_2 as a selective electrode (Glökler and Lisdat 2015). Acidification due to proton accumulation during the amplification reaction can also be detected by potentiometry using an ISFET (Toumazou et al. 2013).

A common feature of most methods based on the promoter and strand break mechanisms is the generation of a monomeric product. In some cases, a restriction enzyme can be used to convert a concatemer product into fragments of defined length (Notomi et al. 2000). Thus, correct product formation can be confirmed and even isolated based on fragment size other than using a probe. However, product size analysis is less practical than most other product verification methods and SNPs cannot be distinguished either.

Sequence-based detection method

In order to discriminate correctly amplified product from artifacts, sequence-specific probes can be used. Such probes are also a preferred means of detection to be used in reactions that do not yield high concentrations of end-products. A main problem of probes is their potential interference with the amplification reaction that can generate artifacts by themselves and give rise to false positives. Typical TaqManTM probes are not feasible as none of the strand-displacing polymerases used in isothermal amplifications comprise a 5'-exonuclease domain. Therefore, other formats must be used in a homogeneous assay (Kouguchi et al. 2010). Mostly, highly specific molecular beacons with dye and quencher pairs are compatible with many of the different methods (see Figure 22(a)) (Leone et al. 1998; Liu et al. 2017) and they may even be used for multiplex detection (Bakthavathsalam et al. 2018). In the case of the BAD-AMP, its molecular beacon is even directly integrated into the amplification mechanism itself (Connolly and Trau 2010) (see Figure 7). It is also possible to specifically cleave probes comprising dye/quencher pairs. For instance, endonuclease IV is used in RPA to cleave a tetrahydrofuran abasic site-mimick (THF) if hybridized to a complementary strand

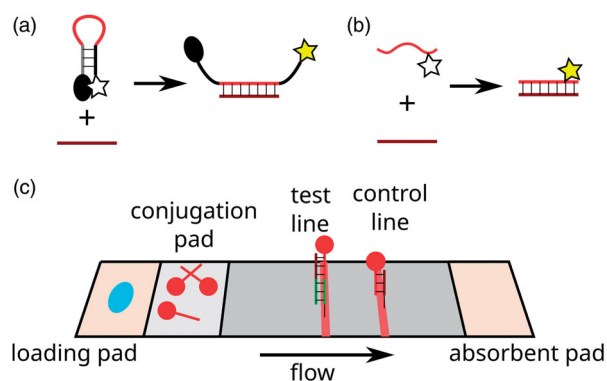


Figure 22. Probe-based detection methods. (a) Detection principle of molecular beacons. In absence of a matching target sequence, the molecular beacon forms a hairpin-like structure which brings a terminally attached quencher-dye pair (quencher as black ellipse, dye as outlined star shape) in direct proximity that is non-fluorescent. Upon specific target binding, the hairpin unfolds and the fluorescence of the dye is turned on (yellow star) by moving away the quencher moiety. (b) Self-quenching probe. In absence of a matching target sequence, the dye linked to a base within the self-quenching probe is non-fluorescent (outlined star shape). Upon specific target binding, the dye is presented outside the newly formed duplex and fluorescence is turned on (yellow star). (c) Detection principle of lateral flow devices. An aqueous sample is placed on the loading pad which starts to flow in the direction of the absorbent pad by capillary forces. Nanoparticles comprising one complementary probe situated on the conjugation pad come in contact with the sample and migrate along the flow direction. Target-hybridized nanoparticles are captured at test line by immobilized target-specific probes and a control line is used to capture free nanoparticles to serve as a negative control for the assay (see color version of this figure at www.tandfonline.com/ibmg).

(Piepenburg et al. 2006). More recently, a thermophilic recombinase has been employed to cleave the annealed probe at loop regions in LAMP reactions and convert it to a primer for additional extension (Chen et al. 2020; Ding et al. 2021). In many other methods, the detection probe forms an integral part of one or more primers based on luminescence upon extension technology (see Figure 22(b)) (Nazarenko et al. 2002; Taylor et al. 2013; Gadkar et al. 2018). In order to make sure that the probe does not interfere with the amplification and even cause artifacts on its own, detection can be performed after the reaction. A common approach utilizes colloidal gold nanoparticles as probes in lateral flow devices that capture amplification products at defined positions after specific probe hybridization (see Figure 22(c)) (Kiatpathomchai et al. 2008; Roskos et al. 2013; Kersting et al. 2014). More recently, an interesting approach combining RPA with a novel lateral flow-based format to perform multiplex assays has been shown (Li et al. 2019). However, more integrated devices have been developed for specialized

applications which are described in greater detail below.

Special applications and combinations

Considering the advantages of these isothermal methods and the urgent need for simple point-of-care diagnostics, a number of rapid detection assays targeting various target organisms applying isothermal amplification have been introduced. A major step toward making an assay or experimental study faster would be to simplify the analysis platform, and this creates a wide application for isothermal amplification. The potential application encompasses a wide field of studies, covering mostly clinical, food and feed, and environmental diagnostics. The involvement of isothermal amplification has brought a significant impact to various fields of research and development which include sensor development, quantification, molecular cloning, next-generation sequencing (NGS), whole genome amplification (WGA), immunoassays, Specific High Sensitivity Enzymatic Reporter Unlocking (SHERLOCK) and DNA nanotechnology.

Sensor development

Isothermal amplification has been widely applied for the development of molecular diagnostic assays with a focus on the point-of-care format (Zanoli and Spoto 2012; Tanner et al. 2015; Suea-Ngam et al. 2020). A frequent method applied is LAMP for the detection of different infectious diseases (Etchebarne et al. 2017; Kaarj et al. 2018). Recently, a simple, fast, and sensitive assay utilizing LAMP was developed for Zika virus assay. The assay was designed on paper microfluidic chips integrating a smartphone detection system. ZIKV RNA would flow and once it reached the detection area of the paper microfluidic chip, the paper was cut before adding RT-LAMP mixture and a pH indicator dye. This assay would then be conducted on a hotplate at 68 °C. The color change indicating successful amplification could be observed in 15 min and the quantification was done by smartphone imaging (Kaarj et al. 2018). A Tuberculosis diagnosis detection kit that uses LAMP has also been commercialized (Mitarai et al. 2011) as an alternative to other available kits such as Cobas Amplicor MTB (based on PCR technique) and TRC Rapid MTB (based on Transcription Reverse Transcription Concerted Reaction Technology) (Boehme et al. 2007). This kit would cater for primary testing for TB as it is easy to be adapted as a hand-held device with rapid results in just 1 h, and provide 100% specificity of

diagnosing TB (Mitarai et al. 2011). Several assays based on different isothermal amplification methods have also been developed for the detection of Salmonella (Hoser et al. 2014; Lim et al. 2015; Yang et al. 2016, 2018; Martzy et al. 2019). However, LAMP has well established itself in the field and several assays have been legitimately approved through multi-laboratory testing, especially for applications such as food pathogen detection (Yang et al. 2015). In a study, the performance of the LAMP-based commercial kit has been evaluated in comparison with the International Organization for Standardization (ISO) method for detecting uninjured and sub-lethally injured Salmonella cells artificially inoculated at different levels. The validation study showed that the commercial LAMP-based kit performed effectively with the ISO method as the reference (Lim et al. 2015). Hence, this LAMP-based diagnostic kit would be a reliable cost-effective approach for rapid detection of Salmonella.

Quantitative assay

A number of quantitative assays especially at nucleic acid level have adopted isothermal amplification in their designs (Shen et al. 2011; Mauk et al. 2015; Tröger et al. 2015). Generally, quantitative detection can be achieved by a more conventional real-time monitoring or the product accumulation or by digital end point analysis of compartmentalized parallel reactions. The former may suffer from a poorly timed initiation of the first reaction step, variations in the reaction kinetics and should ideally comprise an internal control. The latter also requires the reaction to start after compartmentalization in order to correctly count template molecules.

Real-time assays

Monitoring product formation in real-time to quantify nucleic acids has been developed for PCR originally as early as 1993 (Higuchi et al. 1993) and has since become the gold standard for quantification. The cycle of quantification/qualification (Cq) is an important parameter used in real-time polymerase chain reaction techniques, indicating the cycle number where a PCR amplification curve meets a predefined mathematical criterion. As isothermal amplification reactions represent a single cycle, another parameter has been introduced for quantification. The term “time to positivity” (T_p) was first introduced to relatively quantify the RNA of West Nile Virus (Parida et al. 2004): Positive real-time RT-LAMP assay results were determined by taking into account the time to positivity (T_p ; in minutes), at which

the turbidity increased above the threshold value fixed at 0.1.

Real-time quantitative analysis applying isothermal amplification has also been demonstrated for the detection of *Aspergillus burdinus* in lungs and bronchoalveolar lavage (BAL) fluids of rats with experimental invasive pulmonary aspergillosis (IPA) (Zhao and Perlin 2013). Rapid and quantitative detection is very crucial as it may facilitate early detection of invasive pulmonary aspergillosis (IPA). This study combined nucleic acid sequence-based amplification (NASBA) with molecular beacon (MB) technology. In just 30 min, this amplification method may yield amplicons of up to 10^{12} with a high degree of specificity (>95%). However, cross-reaction among the same species might occur with *Penicillium chrysogenum* and *Aspergillus spp.* with a 99% similarity in the amplifying region. Nevertheless, no cross-reactivity was detected when tested with other bacteria species and fungi (Zhao and Perlin 2013).

Utilizing NASBA, a detection approach has been developed to theoretically detect and quantify mutations using the common K103N as a model for HIV-1 reverse transcriptase drug-resistance mutation (Morabito et al. 2013). It was demonstrated that ssDNA hybridized efficiently with the probes at clinically relevant concentrations. This simple method of amplifying RNA target, termed SMART (McCalla et al. 2012), is an isothermal and exponential process that does not promote rapid degradation of enzymes and unwanted hybridization involving nucleic acids. This approach was able to take into consideration the need to lower the assay cost as well as the absence of proper laboratory infrastructure settings. This was accomplished by incorporating the advantages of microfluidics whilst avoiding the limitation of NASBA. The major challenge with NASBA is the need to use a 65 °C heating step to disrupt the secondary structure of DNA/RNA before enzymes are added. The incorporation of microfluidics has circumvented this issue. This assay was performed on a closed microfluidics chip device that used isothermal heating in a low temperature (41 °C) for 180 min without thermal cycling or preheating, thus driving the cost down. The sample-to-result time of this assay was comparable or compare favorably with most commercially available HIV genotyping assays to monitor drug resistance, such as ViroSeq (Abbott) and TRUGENE (Siemens, Washington, DC), which are based on PCR and DNA sequencing platforms respectively (Grant et al. 2003; Bilé et al. 2005; Church et al. 2006). This method is expected to be applied to clinical HIV-samples, other RNA targets, such as influenza virus and biothreats in the future (Morabito et al. 2013).

Digital assays

Digital quantification can surpass the accuracy of real-time assays as the exact number of template molecules is counted. For quantification, a sample is separated into a multitude of compartments in which the amplification is performed in parallel. At the endpoint of the amplification reaction, all compartments with positive signals are counted in order to determine the number of templates initially present in the sample. This has been first introduced for PCR and coined as digital PCR (dPCR) by Vogelstein and Kinzler (Vogelstein and Kinzler 1999). This principle can be applied to all kinds of different amplification reactions, provided that the method can reliably amplify a single molecule to a visible product in the given volume of a compartment. An important limitation is that the reaction must not start before the sample is dispensed into the departments. In order to achieve this, a trigger must ensure that the reaction starts after compartmentalization. The trigger used in most of the mesophilic isothermal reactions is an admixture of magnesium to activate the polymerase. The main advantage of digital assays over real-time assays for quantification in isothermal reactions is that competition between different amplicons such as artifacts and variation in amplification kinetics can be safely excluded.

A first quantitative digital isothermal amplification method was developed using microfluidic digital recombinase polymerase amplification (RPA) SlipChip for the quantitative determination of nucleic acids first published in 2010 (Shen et al. 2010). The system is triggered by the parallel initiation of pre-compartmentalized reactions on the SlipChip, done by the addition of magnesium acetate as the chemical initiator. Validation of the performance of digital RPA in the SlipChip was performed by amplifying and counting single molecules of the target nucleic acid, methicillin-resistant *Staphylococcus aureus* (MRSA) genomic DNA. This isothermal SlipChip RPA approach can be performed at an incubation temperature range of 37–42 °C. The amplified target material or the end product generated can be monitored by a well-suited oligonucleotide-probe-based fluorescence detection system. It was also reported that the increase in temperature decreases the incubation time. This was critical as an incubation temperature under 42 °C was able to generate quantitative results in 30 min (Shen et al. 2011). A more recent water in oil emulsion-based method employs capillaries that control the initiation of RPA reactions by encapsulating the chemical initiator to each reaction compartment using shear force when passing through a cross connector (Li and Manz 2019). However, the generated

droplets were very heterogeneous in size and make an automated enumeration of positive reactions difficult. Another approach using LAMP and formation of droplets on a commercial track-etched polycarbonate (PCTE) membrane yielded highly uniform nanoreactors for accurate quantification without the use of microfluidic devices (see Figure 23) (Lin X et al. 2019). The so-called membrane digital LAMP (mdLAMP) system is inexpensive compared to other digital assays, yet it remains to be seen whether the manual removal step of excess reagent can be replaced with a different technique to allow full automation. Another newly published digital LAMP-based assay employs a nanoporous hydrogel in which amplification can occur directly without any sample pretreatment or DNA extraction (Yi et al. 2021). However, the robustness of this promising approach will yet have to be determined.

Whole genome and transcriptome amplification

The advanced amplification ability of multiple strand displacement (MDA) enables the amplification of minute quantities of nucleic acids in the genomic analysis even at whole-genome levels directly from single cells without the need of isolating and purifying nucleic acid (Deng and Gao 2015). The application of high processive and high fidelity DNA polymerases enables the development of an isothermal whole genome amplification (iWGA) (Ahrabi et al. 2010). Thomas and coworkers described a simple and low-budget method for single-cell iWGA by MDA upon random hexamer primer binding (see Figure 24). This approach used a Φ 29 DNA polymerase-based kit (Illustra GenomiPhi V2 DNA Amplification Kit) and yielded up to 5 μ g of iWGA product with 10 kb fragment lengths and longer (Kroneis and El-Heliebi 2015). This approach has been widely used and permits researchers to examine an entire genome in various downstream applications (Deng and Gao 2015) such as DNA profiling and sequencing, as this amplification method does not involve DNA fragmentation (Kroneis and El-Heliebi 2015). In addition, this method is well-suited for those lacking access to standard micromanipulation equipment (Kroneis and El-Heliebi 2015).

As RNA amplification can be biased by many different effects, it is advantageous to only perform a linear rather than exponential amplification for whole transcriptome sequencing. To this end, a single primer isothermal amplification of RNA (3'-Ribo-SPIA) has been developed for highly multiplexed applications (Kurn et al. 2005). The method comprises a cDNA generation step using a single chimeric primer, DNA polymerase

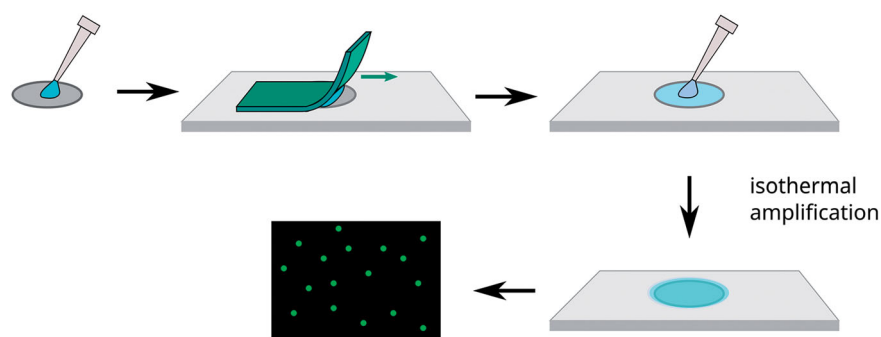


Figure 23. Membrane-based digital LAMP. A sample is first dispensed on an PCTE membrane (gray ellipse) which in the next step is put on a PDMS surface (light gray surface) and covered by a PDMS film (dark green) in one direction to effectively remove excess solution from the membrane. After peeling off the PDMS film, the membrane is overlaid with mineral oil to seal the separate compartments in the membrane. After the isothermal amplification step the PCTE membrane can be evaluated for digital amplification in compartments by fluorescence microscopy (see color version of this figure at www.tandfonline.com/ibmg).

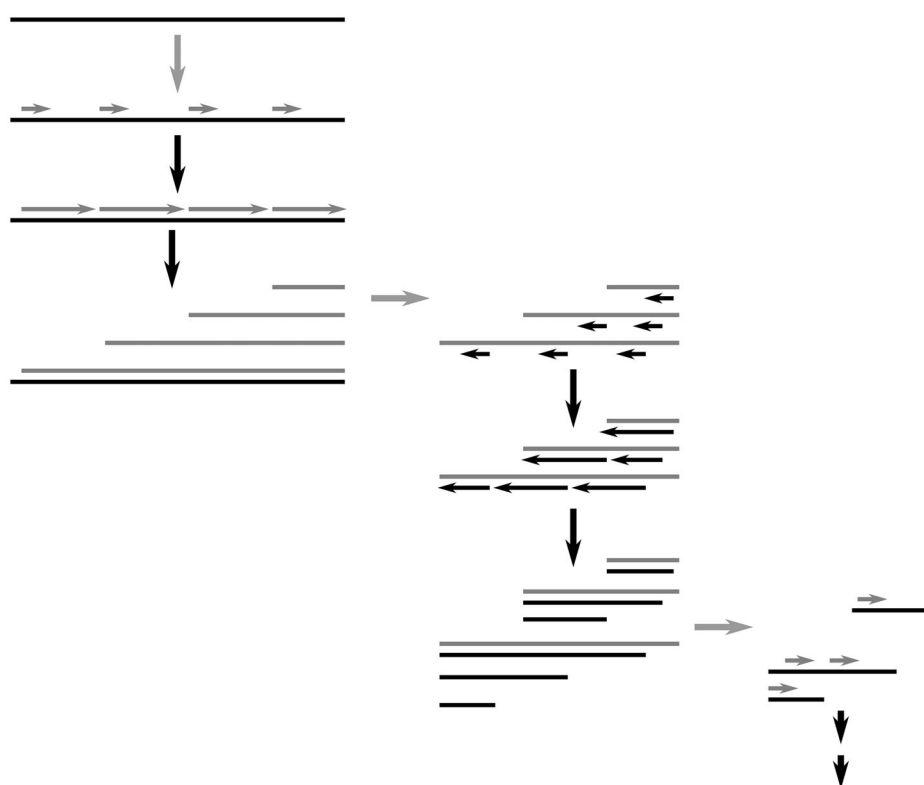


Figure 24. MDA-based whole genome amplification. A long target strand (black line) is bound by multiple short random primers (gray arrows) which are used for elongation by a strand-displacing polymerase. After the strand-displacing polymerase reaches a double-strand it continues elongation until the end of the template, thereby displacing previously elongated single strands (gray lines). These strands can again be bound by random primers (black arrows to indicate complementary orientation) to repeat the elongation and displacement steps, accumulating multiple copies of the initial template with decreasing lengths (see color version of this figure at www.tandfonline.com/ibmg).

with strand displacement activity, and RNase H (see [Figure 25](#)). The method has been commercialized by NuGen in combination with next-generation sequencing as the Ovation RNA-Seq System. It has been applied for complete bovine coronavirus genome coverage with higher sequence depth. This coupled assay was

evaluated by comparing the efficiency to the PCR-like sequence-independent single primer amplification (SISPA) and it was concluded that this method has high efficiency for amplification of viral RNA from high and low copy number samples, compared to the SISPA protocol (Myrmel et al. 2017).

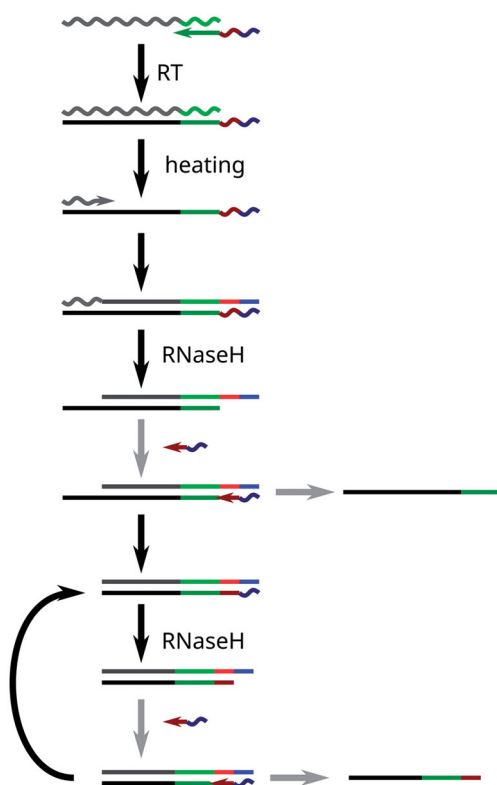


Figure 25. Ribo-SPIA. The mRNA template (gray wavy line with a polyA tail in green) is first contacted with a first chimeric primer comprising DNA (dark green arrow) and RNA regions (wavy red and blue line) for reverse transcription. The resulting cDNA (straight black line) represents a full-length copy of the template. By heating, parts of the RNA template is fragmented and removed from the cDNA, leaving a small portion that can act as a primer (gray wavy arrow) for second strand synthesis. By addition of RNaseH, the RNA region of the first chimeric is removed so that a complementary second hybrid primer comprising DNA (dark red arrow) and RNA region (wavy blue line) can anneal. Elongation by a strand-displacing polymerase displaces the first cDNA strand and regenerates the cDNA without the RNA primer region of the original template. The RNA primer region of the regenerated double-stranded cDNA is digested by RNaseH so that another second chimeric primer can anneal by partially displacing the strand that covers the DNA region of the primer (red line). Elongation of the primer recycles one copy of the regenerated cDNA and displaces one previously hybridized copy (black line with additional dark green and red regions) to accumulate in the linear amplification reaction (see color version of this figure at www.tandfonline.com/ibmg).

Next-generation sequencing

The massive-parallel sequencing or next-generation sequencing (NGS) methods have gained traction over the last decade. A central feature is molecular cloning and parallel sequence amplification which can be achieved by isothermal amplification. Among the first isothermal amplification in NGS, the use of the linear rolling circle amplification allowed the generation of

so-called DNA nanoballs comprising direct sequence repeats that can be arranged at high densities in a pre-patterned array for sequencing (Drmanac et al. 2010) (see Figure 26). The nanoballs were sequenced by hybridization (SBH) and were offered as a commercial sequencing service by Complete Genomics. The company was acquired in 2013 by BGI Group which combined the nanoball array with sequencing by synthesis (SBS) method, resulting in the BGISEQ-500 (Huang et al. 2017). In parallel, a platform based on RCA-generated colonies is termed “rolonies” in combination with SBS which was acquired by Qiagen AG in 2012. The resulting new commercial sequencing platform GeneReader is more specifically applied to targeted sequencing in a diagnostic environment (Darwanto et al. 2017).

A novel *in situ* isothermal amplification method was developed for NGS to produce billions of monoclonal colonies. Not only it is bead-free and cost-effective, it also favors less hands-on time, requiring no more than 30 min. Over one billion monoclonal colonies in a lane of 5500 flowchips could be generated through this solid-phase amplification approach, even no multiple cycles of denaturation and amplification are involved. The principle utilizes a DNA breathing mechanism manipulating a low-melting temperature homopolymer primer on the solid surface, for instance, poly-A (Figure 27). Besides, a low- T_m solution-phase primer is also used, for example, a 27-mer primer e (Pe), which contains a CAA repeat. The template switching strategy with inter-strand photo-cross linking further enables paired-end sequencing. This method can be used on NGS platforms to simplify the workflow for clinical genomic applications (Ma et al. 2013). The method was first established on the SOLiD sequencing platform termed “WildFire” and is currently available for the IonTorrent sequencing platform in the form of the Ion PGM™ Template IA 500 Kit.

Another group of studies has described the ability of amplification to occur at 37 °C in the presence of a high concentration of gp32 (a single-stranded binding protein), DNA polymerase, and two sequence-specific primers, to synthesize long and discrete dsDNA. This study has unveiled the unexpected roles of gp32 in triggering primer invasion on a dsDNA template, promoting specific binding and inhibiting nonspecific primer annealing. This strand invasion was suggested to occur by annealing gp32-bound oligos to the target by stabilizing ssDNA in the duplex or taking advantage of temporary DNA relaxation. This provided stable priming sites for the DNA polymerase to initiate DNA synthesis before the DNA synthesis was achieved by strand-displacing DNA polymerase. The method yields long

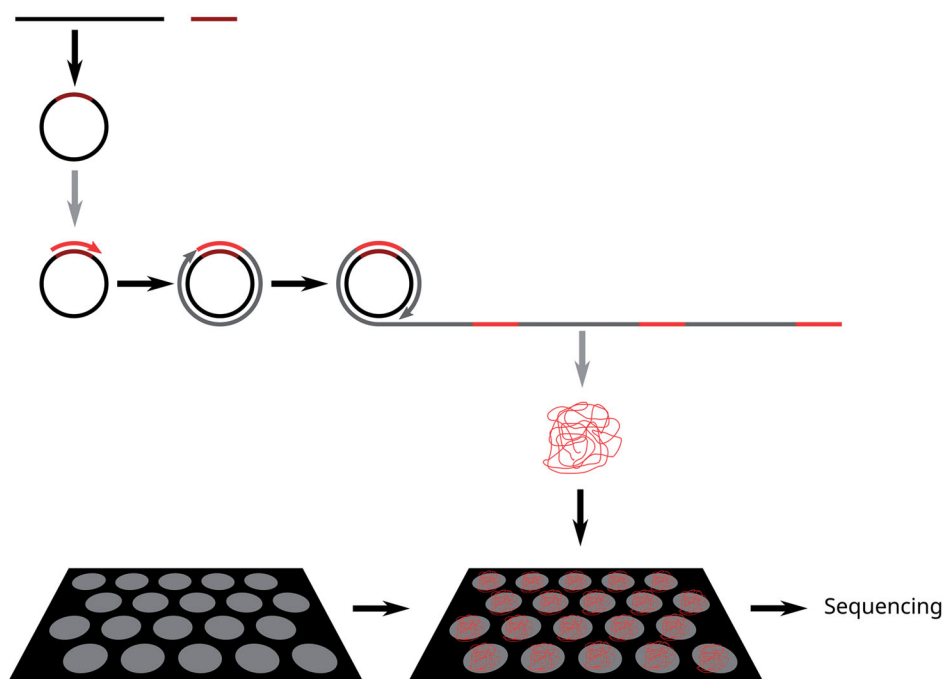


Figure 26. Nanoball-based sequencing. In a first step, the nucleic acid to be sequenced (black line) is circularized by incorporation a short constant region (dark red line). The RCA primer (red arrow) is annealed to the circular template and RCA is initiated by a strand-displacing polymerase. The long concatemeric ssDNA copy randomly compacts to form a nanoball (ball of red string). A solid surface (black field) comprising a pattern of preformed sticky features (gray circles) with diameters of about one nanoball is then charged with a batch of nanoballs. As each feature cannot occupy more than one nanoball, high density single-molecule sequencing of a large plurality of clones is possible (see color version of this figure at www.tandfonline.com/ibmg).

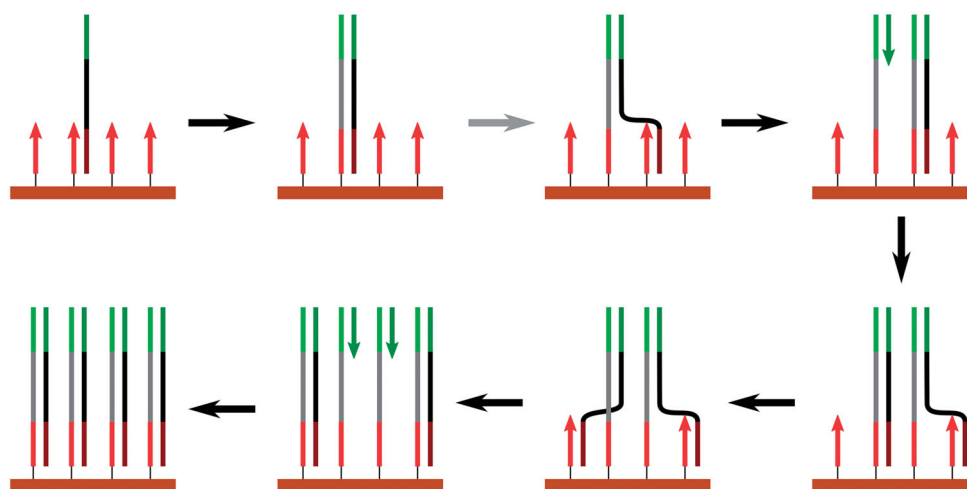


Figure 27. WildFire solid phase amplification. A solid surface (brown bar) comprising one type of immobilized primer molecules (red arrows) is contacted with a template molecule (black line with red and green end regions). In a first step, the template is copied to a double strand. The surface primer region can melt and one stand can hybridize to a neighboring primer. This primer can be elongated to copy the template strand while displacing the first immobilized copy. This immobilized single strand can be copied by a primer that hybridizes in solution (green arrow). The double-stranded immobilized copies are again able to melt and hybridize to yet another immobilized primer to allow further steps of isothermal "template walking". This isothermal reaction stops when no further surface immobilized primers are left for re-hybridization (see color version of this figure at www.tandfonline.com/ibmg).

single-stranded products in a mechanism similar to RCA. The ability of this DNA amplification method to synthesize long, discrete, and concatemeric dsDNA at low temperature expands the capability of it to be

utilized in clonal amplification for next-generation sequencing where long DNA is desired such as nanopore and Pacific Biosciences platforms (Zhang and Tanner 2017).

As already pointed out above, the bridge-amplification used in Illumina sequencers for cluster generation is isothermal but discontinuous due to intermediate incubation steps with denaturing agents. However, the new patterned flowcells are seeded by a mechanism called kinetic exclusion in order to achieve a very high density of clonal clusters with a defined regular distribution pattern (Shen et al. 2013). The method is automated by the cBot 2 system for cluster generation, in which the molecules of the library are applied to the pattern continuously at a low concentration. The isothermal RPA method is combined with bridge amplification by using two anchored primers confined to nano wells in a pattern. As soon as one template molecule binds to primers in the nanowell, the bridge RPA is initiated to rapidly seed the cluster before any other incoming template can interfere to generate a polyclonal cluster (see Figure 28). By slow seeding of templates, monoclonal clusters are generated by kinetic exclusion. There seems to be some disputed that this method may lead to an increase of so-called "index hopping" in comparison to the discontinuous bridge amplification (Owens et al. 2018; Valk et al. 2020).

Immunoassays

Immunoassays, especially ELISA is one of the most common bioanalytical assays in biomedical research (Monsur Ali et al. 2014). ELISA has been combined with the RCA technique to lower the detection limit and obtain higher sensitivities for the assays. For instance, the presence of a target antigen immobilized on a solid support can be detected when the binding antibody is conjugated with the RCA primer resulting in primer amplification by undergoing an RCA reaction. This method is termed immunoRCA. Quantification of IgE on a glass slide was the first example of immunoRCA (Schweitzer et al. 2000). Henceforth, solid-phase-RCA approaches have been extensively used as signal amplification components on antibody microarrays for multiplexed protein analysis (Schweitzer et al. 2002; Kingsmore and Patel 2003; Li et al. 2015). A sandwich immunoRCA has been utilized to perform sensitive target detection. In this case, using an immobilized antibody, the target antigen was initially captured on a solid surface. A secondary antibody conjugated to the RCA primer is then applied to conduct the RCA reaction (Zhou et al. 2004; Lee et al. 2010; Ji et al. 2012). If the design of the RCA primer allows quadruplex sequences to be amplified, it is possible to turn the concatemeric product into a peroxidase-mimicking DNzyme reporter by adding hemin as well (M. Köster et al. 2011) (see

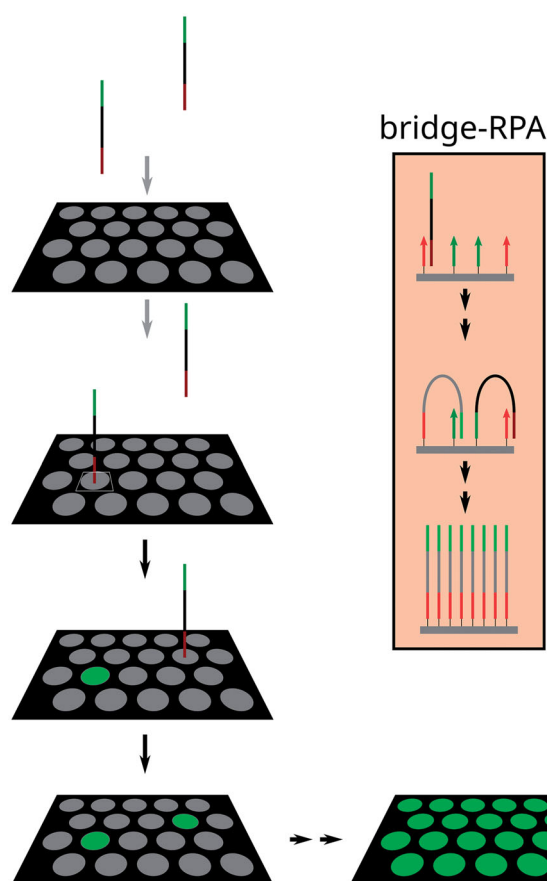


Figure 28. Kinetic exclusion cluster seeding with bridge RPA. A solid surface (black trapezoid) with a patterned array of features (gray circles) bearing a set of primers is seeded with template molecules. The concentration of templates and seeding speed is chosen to avoid the chance of having two molecules contacting the same feature at the same time. Once the template molecule is bound to a primer on a feature, a rapid RPA-based bridge amplification is initiated. The rectangular inset shows the steps of bridge RPA which allows isothermal bridge amplification to occur without denaturation steps by using a recombinase. After the primers in a feature are consumed in the amplification, further seeding by incoming molecules is (kinetically) excluded, thus ensuring monoclonal cluster generation for sequencing. After a given time, all features are occupied by amplified monoclonal sequences in high density for subsequent sequencing by synthesis (see color version of this figure at www.tandfonline.com/ibmg).

Figure 29(a)). The potential of QPA was also explored to amplify an intrinsic quadruplex sequence that can act at the same time as a DNzyme reporter. By comparison, it was found that the classical hemin aptamer sequence outperformed the QPA quadruplex sequence to yield a better product (see Figure 29(b)) (Loh et al. 2014). Yet, a very efficient means to amplify antibody-linked DNA probes is RPA in the form of RPA-ELISA for food analysis (Santiago-Felipe et al. 2014). The main advantages of the RPA-ELISA format are high speed

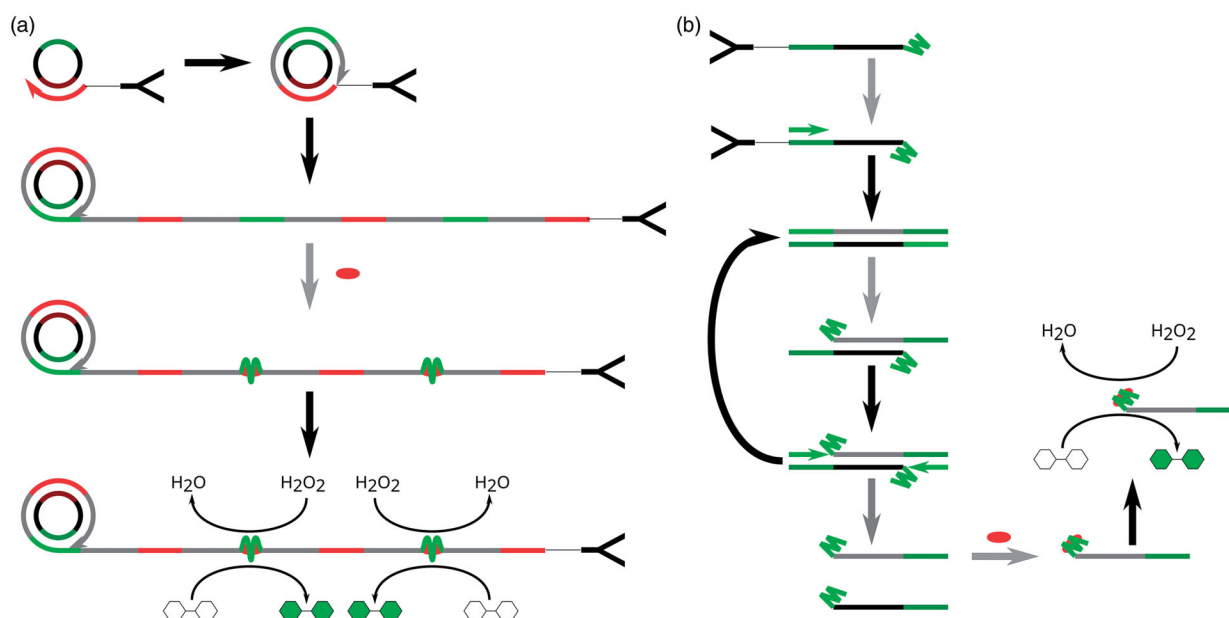


Figure 29. G-quadruplex-based detection methods. (a) RCA-based DNAzyme amplification assay. The probe comprises a binder symbolized by the Y-shaped attachment and a primer region (curved red arrow) that can be hybridized to a circular probe comprising an antisense sequence of a DNAzyme (dark green). RCA can be initiated from the primer using a strand-displacing polymerase resulting in a localized concatemer of DNAzyme sequences (light green lines). The sequences can fold into a quadruplex (light green zigzag line) and form an active DNAzyme upon addition of heme (red ellipse) that can exhibit peroxidase activity for repeated turnover of reporter molecules (dumbbell-like shapes) in the presence of peroxide. (b) QPA-based immunoassay (IQPA). The probe comprises a binder symbolized by the Y-shaped attachment and a template with two complementary QPA-primer binding sites (dark green and folded quadruplex as light green zigzag line). The template is first contacted with the QPA primer and copied to a dsDNA. Both 5' ends can re-fold into a quadruplex as a replication intermediate. The free primer binding sites can be hybridized with QPA primers to initiate exponential amplification that yield a multitude of copies comprising a 5' quadruplex. Upon addition of heme (red ellipse), the quadruplexes are able to form an enzymatically active complex that can exhibit peroxidase activity for repeated turnover of reporter molecules in the presence of peroxide (see color version of this figure at www.tandfonline.com/ibmg).

and sensitivity, which can also be adapted to lateral flow analysis (Sun et al. 2016).

RCA was applied on an ultrasensitive protein assay with a detection limit as low as 16 molecules in a 100 μ l sample (Cheng et al. 2010). RCA has also been combined with magnetic microbeads as a signal enhancement method in a sensitive self-assembled optical diffraction biosensor. The magnetic microbeads were assembled to RCA products to form diffraction gratings which would then produce distinct diffraction modes once illuminated with a laser. It was shown in this study that RCA plays a crucial role in signal amplification for optical diffraction-based sensing since there was no significant bead-binding without RCA (Lee et al. 2010).

A multiwalled carbon nanotube (MWCNT)-based RCA (CRCAS) was also reported. This strategy allows *in situ* rolling circle replication of DNA primer to be conducted on the surface of MWCN. This would subsequently generate a long ssDNA where a high amount of proteins or nanoparticles could be loaded, generating a strong signal amplification ability of nano-biohybridized 3D structure. One single carbon nanotube is used as an integration platform to assess protein-protein binding,

nucleic acid hybridization, DNA replication, and enzymatic activity. The colorimetric CRCAS assay was reported to be more sensitive by 4000 fold than a conventional ELISA (Zhao et al. 2013).

Specific high sensitivity enzymatic reporter unlocking (SHERLOCK)

Point-of-care disease monitoring, pathogen detection as well as genotyping can be greatly aided by the integration of the rapid, inexpensive yet sensitive system. In an assay demonstrated to develop a portable nucleic acid detection system, an RNA-guided, RNA-targeting CRISPR effector Cas13a possesses a “collateral effect” random RNase activity upon target recognition. A rapid CRISPR-based diagnostic (CRISPR-Dx) with single-based mismatch specificity and attomolar sensitivity was achieved by combining the collateral effect with isothermal amplification. This Cas13a-based molecular detection platform, termed SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLOCKing) was utilized to detect specific strains of Zika and Dengue virus, as well as distinguishing human DNA genotype,

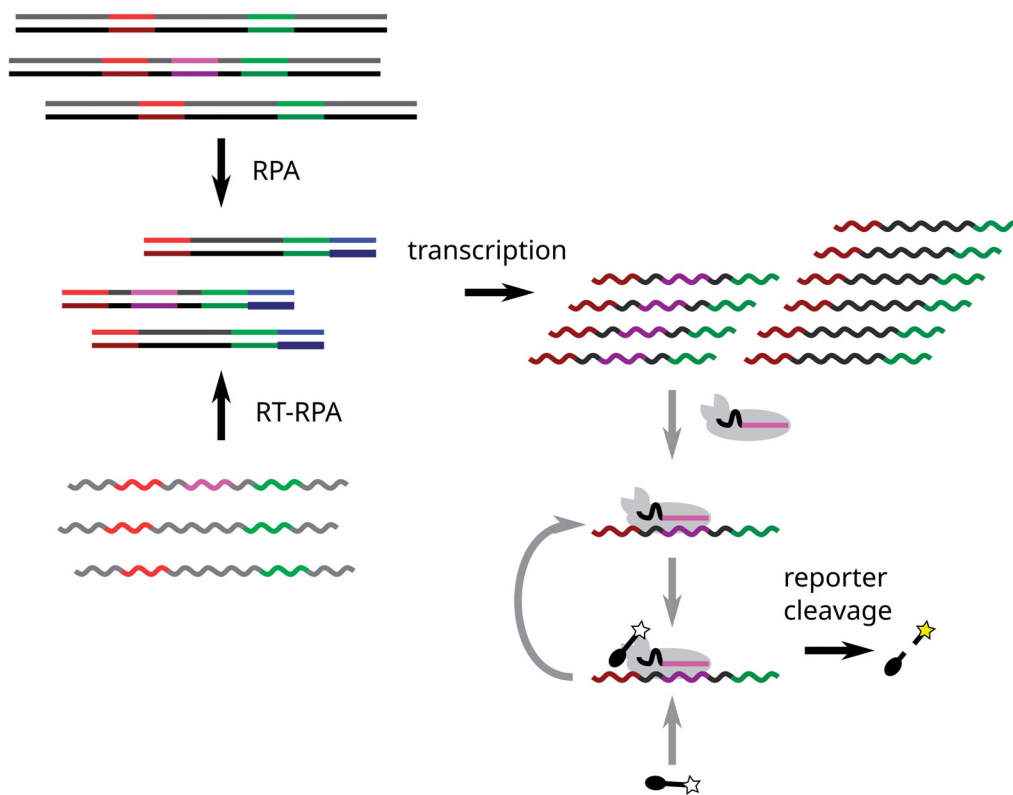


Figure 30. SHERLOCK. A multitude of sample DNA or RNA molecules are amplified by RPA or RT-RPA, respectively. Specific amplification is ensured by using selected regions and matching primers (red and green lines), wherein one primer also comprises a promoter region (blue lines). The resulting exponentially amplified dsDNA molecules therefore also comprise a terminal promoter region (blue lines). An *in vitro* transcription step of these DNA templates results in linear amplification of RNA copies. The copies bearing a specific site for detection (purple) are bound by a Cas13a/gRNA complex that becomes enzymatically activated if an exact sequence is bound by the gRNA. A quencher-dye pair reporter molecules (black ellipse and outlined star) is cleaved by the active complex to yield a fluorescent reporter (yellow star). The active complex can cleave multiple reporter molecules to further amplify for a highly sensitive and specific target detection (see color version of this figure at www.tandfonline.com/ibmg).

pathogenic bacteria, and identify tumor mutations. Of the amplification methods tested, recombinase polymerase amplification (RPA) offered the best sensitivity and was coupled with T7 transcription to convert the amplicon from DNA to RNA form for the detection of LwCas13a that followed. Hence, generally, this SHERLOCK assay is the combination of amplification by RPA, T7 RNA polymerase transcription of amplified DNA to RNA, and target RNA detection by Cas13a collateral RNA cleavage-mediated release of reporter signal (see Figure 30). Though SHERLOCK is a rapid assay, the sensitivity is of a similar level with digital droplet PCR and quantitative PCR (qPCR). RPA provides significant value since its coupling with Cas13a detection of RNA (SHERLOCK) could detect ssRNA targets of concentration as low as ~ 2 aM, providing better sensitivity than Cas13a alone (Gootenberg et al. 2017).

Extended study on SHERLOCK, coined as SHERLOCKv2, affords better sensitivity by combining Cas13 with Csm16. In this improved version, the preamplification RPA step was scaled up. LwCas13a could

produce detection signals down to 8 zM of input samples while PsmCas13b could detect 200 zM input samples of 250 μ l reactions tested (Gootenberg et al. 2018). In addition, by combining Cas nucleases of different types and organisms, it is possible to perform multiplex detection using reporter probes that are specific to the given complex. This was demonstrated for in-sample four-channel multiplexing with orthogonal Cas13 and Cas12a enzymes.

DNA nanotechnology

Isothermal amplification has also gained significant attention in the nanotechnology and nanobiotechnology field. The ability of the RCA products to construct multifunctional materials has made this amplification approach attractive since they are suitable for periodic assembly, by linking the structures at nano- and micro-scales owing to their high molecular weight (Monsur Ali et al. 2014). The amplicons comprising repetitive units that are complementary to the circular DNA template

are suitable as the building blocks for many nanostructures. These repetitive DNA sequence units act as anchoring sites for components of low molecular weight, proteins, or nanoparticles to construct periodic assemblies. From the view of material science, the engineering versatility that RCA offers makes it a fascinating tool to create DNA building blocks for highly-ordered nanostructures construction (Reiß et al. 2011; Liu et al. 2016) and new elements for utilization in drug delivery (Ouyang et al. 2013), biosensing and imaging (Kim et al. 2021), and bioelectronics (Lanier and Bermudez 2015). RCA produces long ssDNA with repeating units that have been manipulated as templates for the periodic assembly of nano species. Since these products can be customized by the use of a circular template, RCA has been exploited to produce complex DNA nanostructures such as DNA origami (Yan et al. 2015), nanotubes (Wilner et al. 2011; Hamblin et al. 2012), nanoribbons, and DNA-based metamaterials. The principles of designing RCA-based DNA origami follow a simple and common method, using the typical rules for DNA origami construction (Rothmund 2006). Concatemeric DNA amplicons generated from RCA reactions can act as a scaffold for DNA origami. Since they are composed of repetitive units, by using only a few staple strands, they can fold into vast DNA nanoribbons (Song and Fan 2016). RCA reactions synthesize scaffold ssDNA and generate long RCA amplicons before folding them into the DNA nanoribbons. Firstly, an oligonucleotide of ~ 100 nucleotides in length are circularized by a DNA ligase in a hybridization complex with a short “splint” oligonucleotide, which also functions as a primer for RCA reaction later in. Then, RCA amplicon hybridizes with three or more staple DNA strands before folding it into a 2D DNA nanoribbon. Also, the specially designed staples that link the adjacent parts of the ribbon create the possibility of folding it into the multilayer 3D constructs (Song and Fan 2016). Further steps have been made to extend the DNA origami architecture and these include the RCA-driven growth and DNA origami folding on gold nanoparticles, forming 3D gold-DNA nanoribbons (Yan et al. 2015).

Discussion

Common features and challenges

Most of the isothermal amplification methods require an initial denaturation step for primers to anneal to their specific target and start the reaction. Denaturation can be performed chemically or by performing a brief heating step. The latter would render the method not

isothermal in a strict sense. As most polymerases would be inactivated by such an initial denaturation step, they are added after neutralization or cooling. Generally, double-stranded DNA is able to “breathe” and this property can be sequence-dependent (Frank-Kamenetskii and Prakash 2014; von Hippel 2014). Thus, with an excess of primers, specific annealing can occur over time under stringent conditions. Since this process is slow and occurs more randomly, the actual start of the amplification reaction is not fixed as it were by employing a previous denaturation step. As a consequence, such reactions can take longer until the product is formed, and quantification based solely on reaction kinetics may be difficult. Therefore, several strategies were devised to speed up the initial priming step. The subject of enhancement strategies is also further discussed in a recent review (Özay and McCalla 2021).

The use of outer or bumper primers increases the likelihood of primer annealing occurring earlier and allows the generation of a first single-stranded template for main amplification primer binding. Such outer primers were first introduced in LAMP (Notomi et al. 2000) and are frequently applied in methods based on the strand-refolding mechanism. Another means to rapidly generate a first single-stranded template for main primer binding without denaturation is to introduce a nick upstream of the template region for elongation and strand displacement. This is best achieved by a nicking enzyme selected to specifically cleave in the vicinity of at least one of the amplification primers (Korfhage et al. 2015). The most specific way to generate such a nick makes use of the Cas9/gRNA complex as described for CRISDA, yet the enzyme complex is used for a more efficient primer invasion rather than simple elongation upstream of the template (see Figure 8). A comparably elegant method to speed up primer annealing in dsDNA templates is to search for a sequence in proximity to the template which has a high propensity in “breathing” which is used in iSDA (Toley et al. 2015). An even more effective way to rapidly initiate primer binding without applying denaturing conditions is to make use of a recombinase that binds to the primers and invades the double-strand for highly specific annealing even well below typical annealing temperature. Both SIBA and RPA profit from the fast initiation reaction that makes the reaction kinetics of these methods more reproducible for quantification.

In theory, some of these initiation strategies could be combined. Yet, some of these strategies also have drawbacks. For instance, additional sets of primers like in LAMP reactions can generally lead to off-target

amplification due to unspecific annealing, but even more problematic is the primer-dependent generation of artifacts and may require more complicated mitigation strategies (Wang Y et al. 2017). Most commercially available nicking enzymes have recognition sites shorter than 7 bases and will therefore cut at many different sites in a complex DNA sample. This can generate many off-target elongation sites competing with the specific target for amplification as exemplified by random DNA amplification using Nt.CviPII with a very short recognition sequence (Chan et al. 2004).

Primers in isothermal amplifications mostly differ from those used in PCR. This is either because further functional sequences have to be added to the template such as promoters and structural motifs for refolding, or base modifications are required such as RNA for cleavage to add functionality. This means that primer oligonucleotides are often much longer in isothermal amplifications. For instance, LAMP primers comprising loop and additional binding sites often exceed a length of 40 bases and design software may not always pick ideal sequences (Lee et al. 2015). LAMP is also dependent on several sets of primers which are often argued to improve target specificity (Notomi et al. 2015). Yet this proposed specificity comes at the price of potential primer-based artifacts due to the complexity and lengths of the primer pairs. Stringent annealing is difficult to achieve in reactions without the aid of a recombinase and will ultimately depend on buffer composition and reaction temperature. Methods that rely on the Bst DNA polymerase have to use high magnesium concentrations that can lead to less specific binding of primers, thereby increasing the likelihood of artifacts even at the upper-temperature limit of the enzyme (65 °C). One way to compensate for the high melting temperatures of primers is to introduce modifications such as phosphorothioate that are known to lower them (Boczkowska et al. 2002). There may even be another benefit for such modifications in methods based on the refolding mechanism (Jung and Ellington 2016; Park et al. 2019). Another means to reduce primer-derived artifacts in isothermal amplification reactions is to use so-called SAMRS (self-avoiding molecular recognition systems) (Sharma et al. 2014; Yang et al. 2015; Wang et al. 2017). Finally, several buffer additives may be used to improve the specificity or structural flexibility of primers such as betaine and DMSO (Wang et al. 2015). More recently, a polysaccharide polymer consisting of maltotriose units (pullulan) has been shown to inhibit the nonspecific amplification of LAMP significantly (Gao et al. 2019).

Another common feature to be considered in isothermal amplification methods is amplicon size. In PCR the reaction is synchronized by different temperature steps and amplicon size is mostly limited when the extension step is set too short. As the synthesis in isothermal amplification is not externally synchronized, shorter amplicons can rapidly out-compete longer ones (Fischbach, Frohme, et al. 2017) which becomes more obvious and challenging in multiplexed amplification (Doseeva et al. 2011; Mayboroda et al. 2018). Any contaminations or artifacts with a shorter amplicon size than the cognate target can easily compromise the assay. This also applies to the speed by which a functional oriA is formed or regenerated to drive the reaction. Therefore, isothermal reactions are usually designed to comprise short amplicons with limited target information and an efficient oriA mechanism in order to remain sensitive and specific.

Most of the current isothermal amplification methods employ DNA as an intermediate and final product. In order to detect RNA in a given sample, an initial reverse transcription step must be performed. Isothermal amplifications based on the Bst DNA polymerase may make use of the enhanced reverse transcriptase property of the 3.0 variant of the enzyme available from NEB. Otherwise, an initial reverse transcription step with a dedicated RT enzyme is required. A notable exception is the methods based on the promoter-based mechanism as they intrinsically comprise an RT enzyme and generate RNA intermediates along with a DNA product. As such, these methods represent a more homogeneous reaction that can be used for sensitive RNA detection.

Remedies for artifacts and contaminations

As discussed previously, artifacts and contaminations can be highly problematic in exponential isothermal amplification reactions, yet there are some approaches to avoid them more efficiently. One way to avoid premature amplification under less stringent conditions and setting a more specific starting point of the reaction is to use a “warm start” polymerase or triggering the reaction by rapidly adding or activating a key reaction component. However, the spread of non-cognate amplicons in a reaction can be contained by either using a multitude of reaction compartments or directly immobilizing amplification products. Reaction compartments can be very simply generated as water in oil emulsions or by using an array of sealed chambers. The compartments are seeded in a limited dilution step ensuring that less than one template is present in each

compartment (Tupik et al. 2019). Thus, the rare occurrence of a rapidly amplifying artifact or contamination is contained in one compartment and cannot supersede the desired amplicons in other compartments. The efficiency of artifact containment by water in oil emulsions has been demonstrated for Q β amplification which allowed large amplicons that were otherwise lost in conventional open reactions (Matsumura et al. 2016). The main advantage of such a highly parallel compartmentalized amplification is that a very exact quantitative analysis can be performed by directly counting positive compartments at the endpoint rather than monitoring the amplification kinetics of a bulk reaction. Other than compartmentalization, amplification products can be kept in place by immobilization to a surface. This has been used for next-generation sequencing, but maybe in principle employed for a more simple quantification or detection by hybridization as well. Details of these methods are discussed in the applications section above.

Carry-over contaminations can be avoided as well if the amplified product comprises a distinct modification that is otherwise not found in the template. Nucleic acids comprising such a modification can be eliminated in a previous decontamination step. The well-established principle based on the incorporation of uracil in PCR and a thermolabile uracil-DNA-glycosylase (UDG) has been applied to LAMP (Hsieh et al. 2014). However, this principle is only feasible by combining a low-temperature decontamination step with isothermal reactions that are performed at a temperature high enough to inactivate the UDG and tightly regulate a “warm start” polymerase.

Considerations

Before deciding upon the type of isothermal amplification reaction for a given application, it is useful to consider several parameters. Most importantly, the sample may determine which method is best employed for detection. Although less discussed in this review, complex samples comprising inhibitory components will often require a prior purification step. If the template concentration is deemed to be high, it may be possible to simply dilute the sample to reduce inhibitory components in combination with a robust polymerase. As the nucleic acid is often not available free from, but contained in viral particles or rigid cells, a lysis step is required to make the template accessible for the amplification reaction. Many commercial kits are available for the respective sample to be purified. If the template is RNA, it is important to remove and/or inhibit ubiquitous RNases and to swiftly proceed with the reaction. Highly

complex samples with a large amount of non-cognate nucleic acids also require a highly stringent amplification method to remain specific for the target. Identification of conserved sequences for target amplification can prove problematic at times when the template is more variable as often in RNA viruses due to limitations in amplicon size and/or primer length. This can in principle be addressed by the use of universal bases in primers and multiplex amplification, but this may also increase the likelihood of primer-dimer artifacts. In point-of-care settings and in the field applications, speed of amplification (rapid time to positivity) is also often a desired feature. If many assays are performed in a less dedicated area, it is also important to use homogeneous assays in order to avoid cross-contaminations that could arise by opening reaction vessels for analysis (Karthik et al. 2014). These often have the advantage to be monitored in real-time, but may require more dedicated hardware for sensitive readout (Zhang et al. 2014; Shirato 2019). A target-specific probe can avoid false-positive results, but must not interfere with the amplification, for example, by accidentally becoming a template in homogeneous assays. Final considerations for choosing a specific amplification method are costs and shelf life. The latter is often greater if the components are available in a lyophilized format (Mauk et al. 2018). Given the many different aspects and features of isothermal amplification methods and their applications discussed in this review, it should be able to provide helpful guidance as to how to design and apply the optimal method to a given application.

Outlook

PCR has dominated nucleic acid-based assays for decades since its inception. Although isothermal amplification methods have become more robust and commercialized, they have not yet taken the lead so far. Interestingly, the main competitor to isothermal amplification assays appearing now on the horizon is novel sequencing technologies (Duncan et al. 2019). Already, bedside diagnostics can be performed in a very flexible manner by highly portable devices such as the Oxford Nanopore MinION which allows sequencing and rapid identification of antibiotic resistance genes (Brinda et al. 2020) or perform multiplex pharmacogenetics assays within a few hours (Liau et al. 2019). However, in many settings, the low costs and rapid results feasible with isothermal amplification assays will still prove to be highly useful.

The current COVID-19 pandemic can provide guidance as to identify future demands and developments in nucleic acid-based diagnostics. (RT)-PCR-based assays

still take several hours to yield results which can be shortened to less than half an hour by isothermal amplification. Several different isothermal COVID-19 assays are currently under development or available in an early phase competing with conventional RT-PCR (Carter et al. 2020). Of these, the extremely fast Abbott ID NOW COVID-19 rapid nucleic acid amplification test gained notoriety by its introduction at a US Corona Taskforce Meeting on 29 March 2020 as it shortened test time to result in as little as 5 min. However, it was later shown to be less sensitive than RT-PCR, thereby causing false-negative results when viral titers are low (Smithgall et al. 2020). Sacrificing sensitivity for speed is even more problematic if samples already comprise low amounts of target molecules, for example, when the infection is in its early phase, or if the input is diluted by sample pooling in order to save time and resources. Although the ID NOW test is based on NEAR, it remains to be seen in which way it has given isothermal amplification-based test systems a bad reputation for future demands and applications. A critical review comparing recent nucleic acid-based assays for the detection of human coronaviruses has been published recently (Subsoontorn et al. 2020). Aside from mere costs and reliability of different assays, the pandemic has shown that a very rapidly increasing demand for nucleic acid-based test kits, mainly for RT-PCR, created pronounced problems in the supply chain for the required components. The resulting shortage ranges from cotton swabs to viral RNA-purification kits to enzymes, oligonucleotide synthesis services, and even otherwise unexpectedly, nucleoside triphosphates (dNTP).

In general, it is to be expected that recombinase-based assays will expand commercially (Li et al. 2018). Especially, RPA more or less ticks all boxes as it utilizes unmodified oligonucleotides that are simple to design, rapidly amplifies the target at only slightly elevated temperature without compromising the stringency, and allows a probe-based target verification. Potential drawbacks to its broad use are the current patent situation in which licenses are almost exclusively limited to Abbott and multiple unique components which may rapidly become limiting in a pandemic-like emergency. Taken together, a desired isothermal amplification method of the future would comprise all beneficial features of the RPA, but requiring less complex auxiliary factors.

Disclosure statement

Jörn Glökler holds patents based on WO 2013/167668 A1. No potential conflict of interest was reported by the other authors.

Funding

This work was supported by the Bundesministerium für Wirtschaft und Energie (BMWi) with the support code ZF4012237CR8.

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