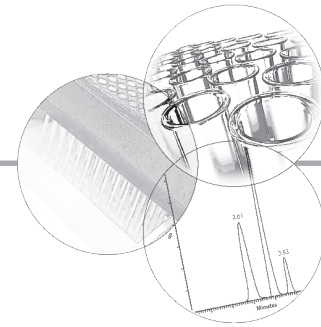

Isothermal DNA amplification in bioanalysis: strategies and applications

Isothermal DNA amplification is an alternative to PCR-based amplification for point-of-care diagnosis. Since the early 1990s, the approach has been refined into a simple, rapid and cost-effective tool by means of several distinct strategies. Input signals have been diversified from DNA to RNA, protein or small organic molecules by translating these signals into input DNA before amplification, thus allowing assays on various classes of biomolecules. *In situ* detection of single biomolecules has been achieved using an isothermal method, leveraging localized signal amplification in an intact specimen. A few pioneering studies to develop a homogenous isothermal protein assay have successfully translated structure-switching of a probe upon target binding into input DNA for isothermal amplification. In addition to the detection of specific targets, isothermal methods have made whole-genome amplification of single cells possible owing to the unbiased, linear nature of the amplification process as well as the large size of amplified products given by φ29 DNA polymerase. These applications have been devised with the four isothermal amplification strategies covered in this review: strand-displacement amplification, rolling circle amplification, helicase-dependent amplification and recombinase polymerase amplification.

DNA replication is an indispensable process in biological systems. This process produces copies of genetic instructions that are required to maintain life, thus, its strategies and components have been evolutionarily optimized. As a consequence of this optimization, the enzyme-based machinery for DNA replication has been extensively exploited for bioanalytical purposes *in vitro*. Input signals have been diversified from DNA to RNA, protein, or small molecules by translating these signals into input DNA before amplification. Currently, DNA amplification is an essential tool in not only basic research but also clinical diagnosis, forensic science, epidemiology and paleontology, among others.

PCR is one of the most widely used DNA amplification techniques, relying on thermal cycling *in vitro* [1]. The repeated heating and cooling can reach a maximum of twofold amplification in each cycle by alternating the denaturation of duplex DNA and the extension of primers that are hybridized to a denatured single-stranded DNA. With continued development of thermal cycling platforms [2-5], PCR reaction times have been greatly shortened by introducing near instantaneous temperature changes and rapid heat exchange within the sample. Currently, high-throughput is achievable with up to 1536-well plates and five multiplexing reactions in each well. In addition to sequence-specific target amplification, whole-genome amplification (WGA) – which is essential for **single cell genomics** – is possible using random or degenerated primers [6–8]. However, PCR has some fundamental limitations of use. First, the requirement of an electrically powered thermal cycler as well as an optimized experimental setup (e.g., annealing temperature, polymerization time, concentration of magnesium and number of cycles) make it difficult to execute point-of-care analysis. Second, the efficiency and specificity of exponential target amplification by PCR relies heavily on the initial few cycles. Spurious target-independent amplification driven by exogenous primers during the first few cycles is detrimental to overall PCR performance. In fact, several non-PCR based clinical kits have shown substantially better performance than PCR tests for clinical diagnosis [9,10]. Third, the amplification bias of PCR over certain loci results in the misrepresentation of unamplified genome, or incomplete coverage, after WGA [11,12]. Although recent progress in PCR-based WGA gives a better genome coverage and a lower amplification bias, more reaction steps are still required, and the amplified product size is relatively short [8]. For these reasons, the design of non-PCR based approaches for DNA amplification is in continuous demand.

Isothermal DNA amplification does not require a thermal cycler, which greatly simplifies point-of-care diagnosis. In most cases, it

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Key Terms

Single cell genomics: Culture-independent approach that enables access to the genetic material of an individual cell.

Recombinase: Catalyzes the exchange of short pieces of DNA between two long DNA strands at the target site.

MicroRNA: Short (~22 nucleotide) RNA molecule acting as a post-transcriptional regulator of gene expression. They are considered as a potential drug because aberrant expression has been implicated in numerous disease states.

is cost effective, easy-to-use and more tolerant to inhibitory components from a crude sample compared with PCR, showing equivalent or higher sensitivity and reliability in clinical diagnosis [10,13–16]. In addition to DNA, other biomolecules can be used as input signals; for example, mRNAs in a few homogeneous cells were successfully amplified with an isothermal method after converting into complementary DNA [17–19]. Proteins have also been successfully assayed with immuno-detection followed by isothermal signal amplification [20–22]. Furthermore, WGA is achievable with a linear, isothermal amplification, owing to the large size of amplifiable genomic segments (>10 Kb) by *Bacillus subtilis* bacteriophage φ29 DNA polymerase. This enzyme has extremely high processivity (up to 70 Kb within 20 min), strong proofreading, strand-displacement activity and long a half-life. Usually, micrograms of amplified genomic segments can be obtained from nanograms of initial genomic DNA within several hours [23].

In this review, we describe the strategies and highlight the recent applications of four major isothermal DNA amplification techniques, namely:

- Strand-displacement amplification (SDA);
- Rolling circle amplification (RCA);
- Helicase-dependent amplification (HDA);
- **Recombinase** polymerase amplification (RPA).

Since several reviews have previously focused on isothermal methods in diagnostic applications, we will focus more on other applications such as WGA, **microRNA** (miRNA)/protein/small-molecule assays and *in situ* target detection. This review will cover articles published between 2006 and 2010, a time period in which SDA, RCA, HDA and RPA experienced significant increases in reported use.

Strand-displacement amplification

In living organisms, base excision repair (a DNA repair mechanism) is initiated by cleaving the phosphodiester bond of the damaged strand. The missing part is then re-synthesized by DNA polymerase, and a DNA ligase finally seals this nicked site. A logical question to be posed is: what happens if the ligation step is missing? In this case, the nicked site is continuously exposed to DNA polymerases, resulting in strand displacement and continuous re-synthesis of the repaired strand at a linear rate if DNA polymerases have strand-displacement activities. This strand displacement mechanism inspired the development of the first generation of isothermal DNA amplification techniques, that is SDA.

■ Multiply primed strand displacement amplification

As shown in the schematic in **Figure 1A**, multiply primed SDA involves a set of target-specific primers or random primers in SDA, resulting in more efficient target amplification or sequence-nonspecific DNA amplification. The extremely strong strand displacement activity of φ29 DNA polymerase can displace a synthesized strand with average size larger than 10 Kb, permitting annealing of a new primer for strand synthesis. After initial heating to denature duplex DNA, all remaining processes are accomplished in an isothermal mode. Using random primer sets, this SDA approach was applied to amplify the human genome **(Figure 1A)** [24]. Linear amplification provided a highly uniform representation across the genome, which could not be achieved by degenerate PCR. Over a period of 8 years, this method was optimized to obtain high-quality genome replication directly from single cells [25–27], allowing genetic analysis such as genome-wide assessment of copy-number variation [28], the mapping of DNA methylation [29–31], and accurate shotgun genome sequencing [26,32]. In particular, the successful acquisition of nearly identical genomic data between an amplified genome from a single cell and a metagenome from an environmental sample suggested this method could compete with any other WGA method for single cell genomics, enabling access to the genetic materials of an individual cell [26].

■ Nicking-initiated strand-displacement amplification

Walker *et al.* devised an SDA strategy to achieve exponential amplification of duplex target DNA using a restriction enzyme and an exonuclease-deficient DNA polymerase [33], and the method was further optimized to obtain higher specificity and sensitivity [34–36]. Two key ideas are to introduce a restriction enzyme site on both ends of a target DNA before SDA cycle and to use a modified deoxynucleotide for strand synthesis **(Figure 1B)**. The nicking of only one strand is achieved with a restriction enzyme (not a nicking enzyme) by generating a hemiphosphorothioate recognition site during polymerization

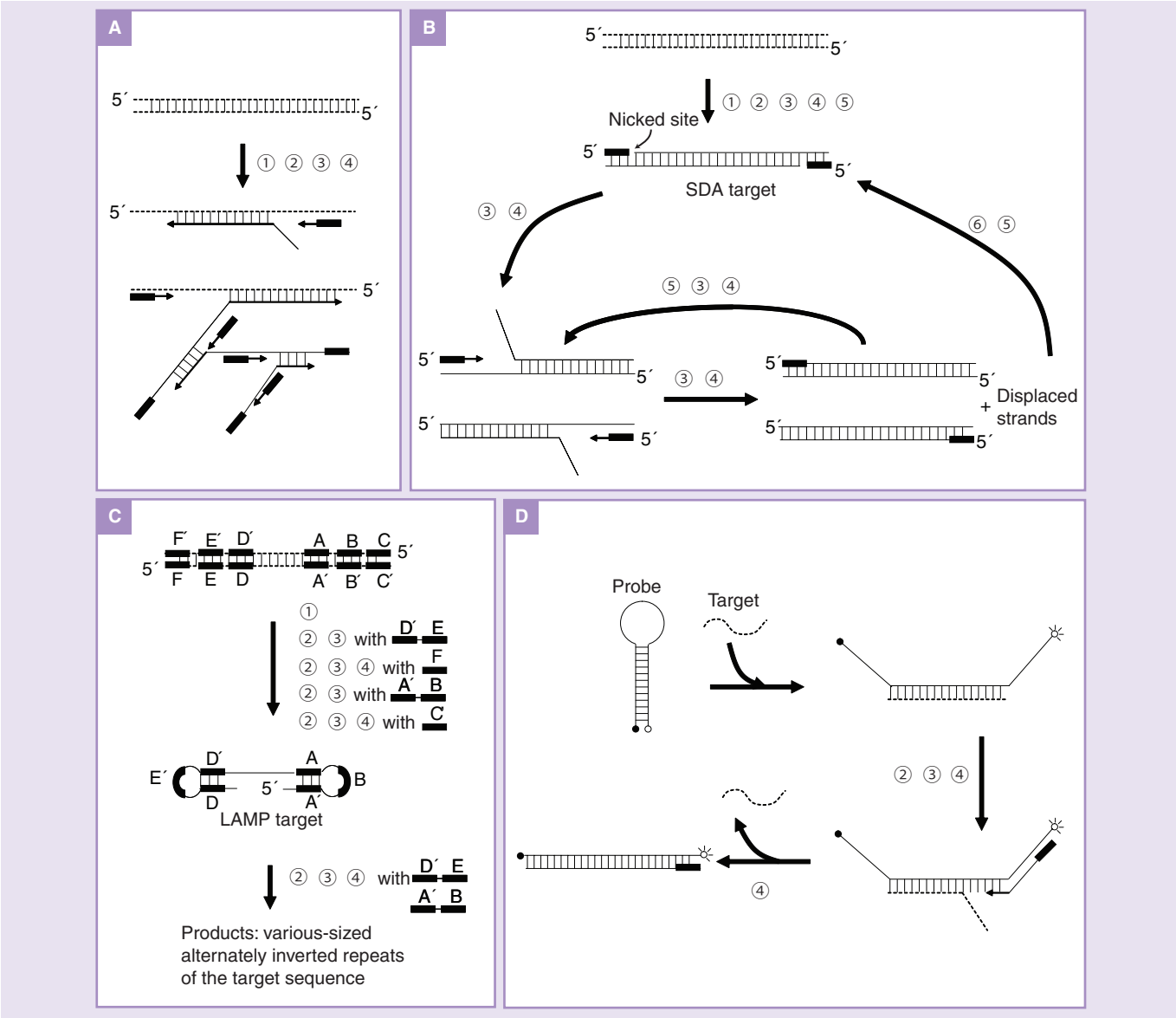

Figure 1. Strand-displacement amplification. (A–D) Initial heating (1); primer/probe annealing (2); polymerization (3); strand displacement (4); nicking (5) and annealing of displaced strands (6). The original target DNA is denoted by dotted lines, and primers are denoted by filled rectangles. Arrows indicate extended DNA strands from a primer. Filled circle: quencher; open circle: fluorophore. **(A)** Multiply primed SDA. **(B)** Nicking-initiated SDA. Only one strand is digested (nicked) at each restriction enzyme recognition site, which is located at the ends of primer sites. The annealing of displaced strands initiates the exponential cycle of this SDA. **(C)** LAMP. Five steps, including annealing of four different primers, are required to generate a loop-mediated isothermal amplification target. Once a loop-mediated isothermal amplification target is generated, exponential amplification is initiated with two inner primers (D′-E, A′-B). More detailed information with animation is available on the web [101]. **(D)** Structure-switching-triggered SDA.
SDA: Strand-displacement amplification.

reaction with dATPαS. This induces continuous replication of the same strand. Exponential amplification is achieved by the incorporation of the displaced stand into the SDA cycle. One disadvantage is the requirement of initial heating to denature a template and of a fluorescently labeled detector probe for better sensitivity, meaning that the technique is not truly isothermal or label-free.

■ Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is one of the most widely used SDA-based amplification techniques for simple and rapid pathogen detection. As of July 2010, 398 scientific articles related to LAMP had been published since the original paper was published in 2000 [37]. Amplification efficiencies greater than 10^9 can be achieved in 15–60 min with high specificity

driven by four different primers designed to recognize six distinct regions on the target sequence. **FIGURE 1C** shows the reaction scheme of LAMP. As duplex DNA is in the condition of dynamic equilibrium at 65°C, one of the LAMP primers can anneal to the complimentary sequence of double-stranded target DNA without initial heating [38,39]. Interplay between inner primers (D′-E and A′-B) – containing sequences of the sense and antisense strands of the target DNA – and outer primers (F and C′) generates a template for the LAMP reaction having a stem-loop DNA structure. Then, with the template and the two inner primers (D′-E and A′-B), the exponential phase of LAMP is initiated and eventually produces the final products containing several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Due to the inherent complexity of this approach, we refer the reader to a web-based, detailed description of LAMP, with a helpful animation [101]. Using this target-specific, extensive polymerization reaction in a small reaction volume, sensitive detection could be achieved by simply measuring the amount of pyrophosphate, a byproduct of the polymerization reaction. Visual detection of pyrophosphate is mediated by precipitation of magnesium diphosphorous tetroxide ($Mg_2P_2O_4$) or fluorescence signal through the addition of magnesium or calcein [40,41]. Recently, Fang *et al.* integrated LAMP and a detection system into one microfluidic chip and achieved a detection limit of 10 fg of *Pseudorabies* viral DNA within 1 h using only the naked eye as a detector [42]. This method was thus 100–1000-fold more sensitive than the standard PCRs for Pseudorabies viral detection. LAMP does not require special reagents or sophisticated equipments, allowing reductions in cost and complexity. In addition to the simple and rapid detection of bacterial and fungal pathogens and viruses [15], the high specificity of LAMP allows single nucleotide polymorphism (SNP) analysis by introducing a mismatched nucleotide on the 5′-end of inner primers [43–46]. However, multiplexing SNP is still challenging because of the complexity involved in designing multiple primers for LAMP.

Key Term

DNA aptamer: Functional oligonucleotide that binds to a molecular target such as a small organic molecule, a protein or a cell. Aptamers are usually selected from a random DNA library through repeated rounds of *in vitro* evolution.

■ Structure-switching-triggered strand-displacement amplification

Unlike previously described methods using primers complementary to target sequences, this method uses a primer that anneals to a probe sequence that is partially bound to the target **(FIGURE 1D)**, leading to amplification of the probe. Target binding causes a structural change of the probe, such as a hairpin dissociation, allowing a primer to bind to the probe and triggering SDA. Guo *et al.* reported such a method to detect single-stranded target DNA using a modified molecular beacon [47]. Upon recognition and hybridization with the target, the stem of the hairpin probe is opened, after which the opened probe anneals with the primer. The extension of primer displaces the target, which initiates the next cycle and results in the production of a duplex molecular beacon probe (opened hairpin), which emits a fluorescence signal at a linear amplification rate. High sensitivity and a 15 fM (1.2 amol) detection limit was achieved within several hundred seconds at 37°C owing to the low signal-to-background ratio of the molecular beacon and a small interval of time for each primer extension. Potentially, this method can be applied to detect viruses having single-stranded genomic DNA. However, the requirement of a molecular beacon as a consumable component and a relatively complex probe design may limit the use of this method. In 2010, He *et al.* used a similar strategy, but the target was a small molecule rather than single-stranded DNA [48]. This group successfully quantified 2 nM (20 fM) of cocaine in human serum in 2 h by using the binding affinity of a **DNA aptamer** to cocaine instead of target-probe hybridization. A conformational change of the DNA aptamer induced by cocaine opens and linearizes a stem structure, which allows annealing of a primer for SDA. The product is a duplex DNA aptamer that can be quantified by SYBR Green I, a fluorogenic double-stranded DNA intercalating dye. This method is noteworthy due to the successful translation of a small-molecule binding signal into a conformational change in DNA, making it feasible to assay other biomolecules other than DNA using SDA.

Rolling circle amplification

■ Target-specific rolling circle amplification

In contrast to SDA, where one event produces a single copy of the template, RCA gives repeated copies of the template, the so-called concatamer, by employing a circularized template **(FIGURE 2A)** [49,50]. Once the polymerase reaches a primer binding site, after completing the replication of the single-stranded DNA template, it displaces the synthesized strand and 'rolls' on and

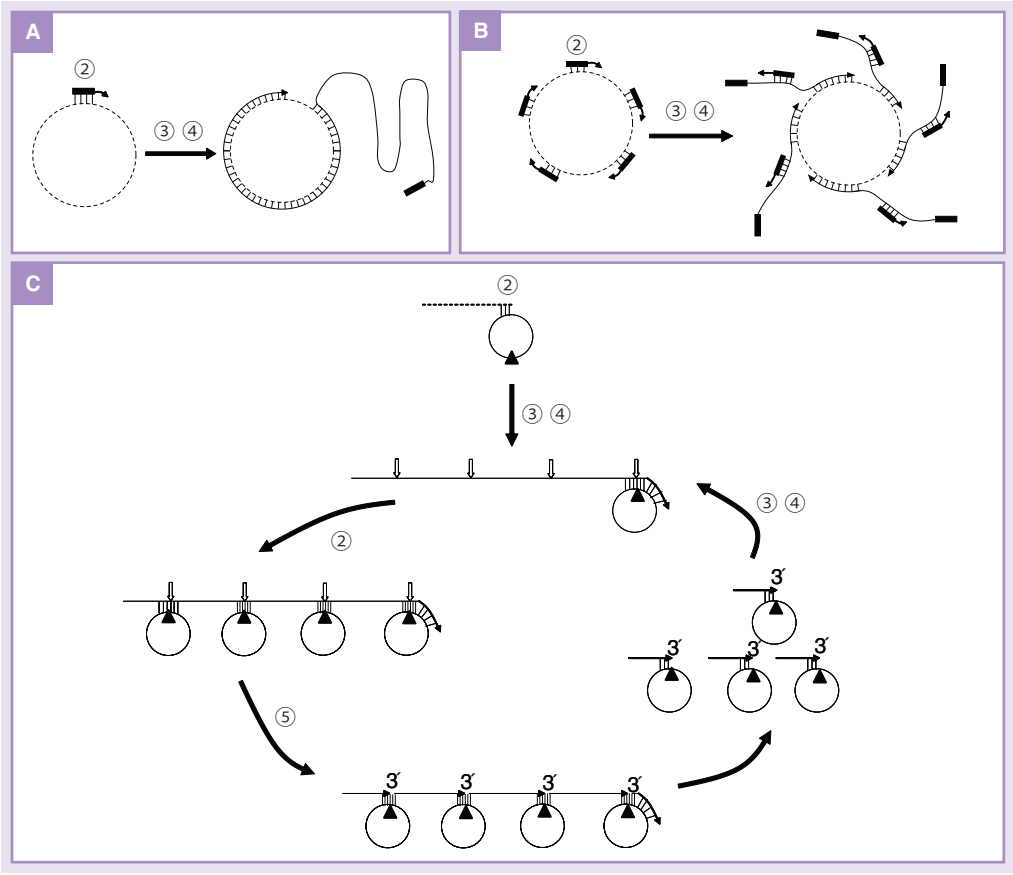

Figure 2. Rolling circle amplification. (A) Target-specific rolling circle amplification. **(B)** Multiply primed rolling circle amplification. **(C)** Primer-generation rolling circle amplification. Primer/probe annealing (2); polymerization (3); strand displacement (4) and nicking (5). Open block arrow: nicking site: filled triangle head: recognition site of a nicking enzyme.

on with DNA synthesis. Therefore, the smaller the template size is, the higher the amplification efficiency, and the lower the probability of strand breakage. Currently, target-specific RCA is used as a universal amplification platform in various assays due to its flexibility in coupling with a detection module. For this reason, we have highlighted several specific applications, as described below.

In situ rolling circle amplification for nucleic acid detection

By nature, efficient signal amplification by RCA requires a small, circular, single-stranded DNA as a template, which makes it difficult to amplify a target for detection. Instead, researchers have designed padlock probes that are circularized only if perfectly hybridized with single-stranded target DNA [51]. Padlock probes consist of two target-complementary segments connected by a linker sequence, typically carrying arbitrary sequences for detection and identification. Upon recognition of a target, the ends of the probe lie head-to-tail to the target DNA and are sealed through the action of a DNA ligase alone or in combination with a DNA polymerase. Allelic discrimination can be achieved from both padlock probe hybridization and ligation. Therefore, circularizing of a padlock probe is a strictly target-dependent action, giving high enough specificity to allow SNP analysis. Owing to high processivity of φ29 DNA polymerase, RCA products comprise hundreds of copies of the reacted probes, readily available for detection using standard hybridization probes. However, the amplification of padlock probes for highly multiplexing SNP is usually done by PCR [52]. RCA is more widely used for detection in cases where isothermal conditions are strictly required, such as *in situ* detection of biomolecules and *in vitro* protein assays. Nilsson's group is on the cutting edge in the area of *in situ* DNA/RNA genotyping. In 2004 and 2010, this group successfully

detected single-nucleotide variations in individual genomic DNA and RNA molecules, respectively **(FIGURE 3A)** [53,54]. Pretreating of fixed cells is a key step before the circularization of the padlock probe. This allows transformation of duplex genomic DNA or mRNA into single-stranded target DNA, which is accessible for padlock probe hybridization. The DNA pretreatment consists of the preparation of small sized duplex DNA by restriction digestion and subsequent degradation of one strand by 5′-3′ exonucleolysis. mRNA pretreatment consists of cDNA synthesis from mRNA by a reverse-transcription followed by mRNA degradation by RNase H digestion. In both cases, the recognition of a specific target sequence is marked

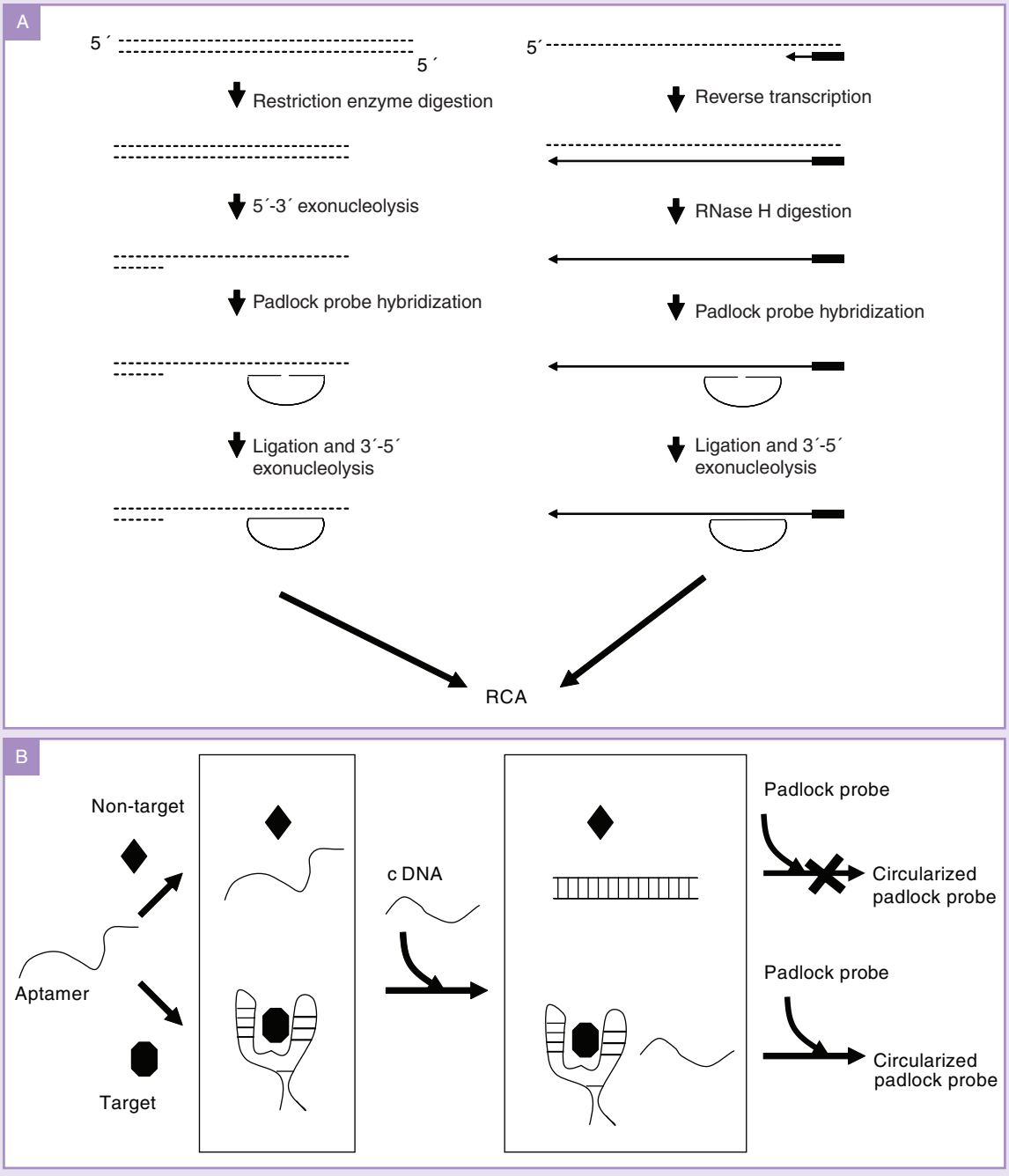

Figure 3. Preparation of single-stranded target DNA. Single-stranded DNA is used to circularize a padlock probe for **(A)** *in situ* detection of nucleic acids or **(B)** an *in vitro* homogeneous assay.
RCA: Rolling circle amplification.

by circularizing a padlock probe, and RCA from the probe anchored to target DNA produces a submicrometer-sized concatamer locally in a specimen. Introduction of stringent washing steps to the protocol allowed the entire process to be performed at 37°C. *In situ* protein localization using RCA was established by Landegren's group. Endogenous levels of protein were successfully translated into single-stranded target DNA at single-molecule resolution by hybridizing two antibody-conjugated oligonucleotides to a padlock probe. This technique also made it possible to visualize *in situ* protein–protein interactions [55] and *in situ* detection of post-translationally modified proteins [56].

miRNA detection

Quantification of miRNA, which is essential for mechanistic studies and diagnostic applications, is a challenging endeavor. Expression levels are typically very low within cells, and PCR amplification is often necessary prior to quantification. However, the small size of miRNAs (19–24 nucleotides) only allows primer annealing temperatures below 30°C, resulting in decreased specificity of PCR because of high sequence identity among miRNAs. RCA has been considered as an alternative for miRNA signal amplification [57–59] since the inherent nature of RCA is suitable for specific detection of short sequences. Recently, Cheng *et al.* developed an improved homogeneous assay for miRNA detection with T4 RNA ligase 2 and the optimized temperature for the probe circularization, showing sufficient specificity to discriminate one nucleotide difference among miRNAs, as well as high enough sensitivity to preclude the need for further miRNA purification [59].

In vitro assays

The development of several RCA-based biomolecule assays has recently been reported. The early version of an RCA-based protein assay was immuno-RCA, which is similar to enzyme-amplified immunoassay (EAIA), except that a detection antibody is linked to an oligonucleotide instead of an enzyme. Quantitative immuno-PCR, which also uses an oligonucleotide for signal amplification, typically leads to a 10- to 1000-fold increase in sensitivity, but relatively low reproducibility compared with an analogous EAIA [60]. Recent studies on immuno-RCA showed an equivalent sensitivity to immuno-PCR and a wider dynamic range using fluorescence or electrochemical detection. Zhou *et al.* could detect as low as 10 fM (0.2 amol) PDGF with a detection range of four orders of magnitude by electrochemical detection from enzymatic silver deposition [20]. Cheng *et al.* achieved subattomolar VEGF detection (16 molecules) and a wider detection range of five orders of magnitude using quantum-dot tags on multiple RCA products generated from a single protein detection [21]. Ou *et al.* showed 80 ag/ml (8 ag) prostate-specific antigen detection with a detection range of three orders of magnitude by bead-based florescence signal enrichment [22]. These recent achievements support the argument that RCA-based protein assays, with linear amplification coupled to various sensitive detection tools, can be used effectively as alternative platforms to immuno-PCR and EAIA, having equivalent sensitivity and dynamic range. In addition to the improvement of such heterogeneous assays, coupling of RCA to the structure-switching of aptamers upon target binding (structure-switching-triggered RCA) allows homogeneous and isothermal measurement of target proteins, without incorporating any washing steps in the assays [61–63]. Such homogeneous assays greatly simplify protocols, eliminating complex processes for interface preparation such as a conjugation of a detection antibody to an oligonucleotide. Wu *et al.* designed a single-stranded cDNA as a sensing molecule to quantify aptamer–target complexes and to serve as a target for the padlock probe-based recognition and ligation **(FIGURE 3B)**. In the absence of target, hybridization of aptamer to cDNA depletes the population of cDNA. Since cDNA serves as the template for padlock probe circularization, the absence of target blocks RCA. Upon target binding, the change in aptamer structure allows hybridization of the free cDNA to a padlock probe, initiating RCA. Thus, the amount of RCA product is strictly proportional to target concentration. With this method, 6.8 pM (1.36 amol) of PDGF was detected, and the linear dynamic range was three orders of magnitude [63].

■ Multiply primed rolling circle amplification

The number of primers that anneal to a circular target DNA is linearly correlated with amplification efficiency of RCA. In addition, introducing primers that include the sequence of the target DNA yields even higher amplification efficiency because SDA is initiated on RCA

Key Term

Primase: Catalyzes the synthesis of a short RNA segment, which is used as a primer during DNA replication. T7 gp4 primase, used for helicase-dependent amplification-based whole genome amplification, has recognition sites that likely result in slightly higher amplification bias compared with strand displacement amplification-based whole genome amplification.

products **(Figure 2B)**. Multiply primed RCA utilizes φ29 DNA polymerase with an exonuclease-resistant random primer [23,64], and this method has been shown to have great value for the analysis of small circular DNA genomes. Although small circular DNA molecules are rare in higher organisms, they are abundant as genomes in several microorganisms such as viruses and bacteriophages, and as plasmids in bacteria. In particular, this technique has enabled the identification of uncultured novel viruses [65] and of phage genomes carrying lethal mutations that cannot be cloned [66]. Furthermore, *in vitro*, cell-free translation of proteins with RCA products supported that genes were accurately amplified enough to perform a cellular process [67].

■ Primer-generation rolling circle amplification

In 2009, Murakami *et al.* proposed a simple RCA scheme to amplify an input signal exponentially **(Figure 2C)** [68]. The group recycled a portion of the RCA product population to generate primers by chopping the RCA products with a nicking enzyme. This not only led to exponential signal amplification, but also diminished artifacts such as primer dimerization or nonspecific amplification because exogenous primers were not added. This amplification method was achievable simply by mixing circular, single-stranded DNA probe, DNA polymerase, and nicking enzyme at 60°C. Under optimized conditions, the method could detect as low as approximately 60 molecules of genomic DNA from *Listeria monocytogenes* and showed a large dynamic range of seven orders of magnitude. Thus, primer-generation RCA can provide a powerful molecular diagnostic assay as long as the target sequence includes nicking sites.

Helicase-dependent amplification

As the first step in any isothermal DNA amplification method, various forms of signal (DNA, RNA and protein) should be in some way translated into single-stranded DNA, which then serves as the input signal for amplification. For example, pretreatment of cells with endo- and exo-nucleases or with reverse transcriptase and RNase H will convert double-stranded genomic DNA or mRNA, respectively, into single-stranded target DNA for *in situ* RCA. In the *in vitro* assays, an analyte-dependent conformational change of a probe makes the amount of single-stranded target DNA represents the amount of an analyte. In LAMP, a dynamic equilibrium between double-stranded and single-stranded DNA is used for initial primer annealing. In contrast to these aforementioned approaches, Kong and coworkers generated single-stranded DNA by reconstituting DNA replication fork mechanisms *in vitro*. This approach accomplished enzymatic unwinding of duplex DNA using a helicase, then used the resultant single-stranded DNA as an input signal for isothermal DNA amplification. The method is referred to as helicase-dependant amplification (HDA) [16]. Duplex DNAs were unwound by a DNA helicase in the presence of ATP, and the displaced DNA strands were coated and stabilized by single-stranded DNA-binding proteins. Importantly, DNA polymerase retains accessibility to the protein-coated single-stranded DNA. Therefore, the initial heat denaturation and subsequent thermal cycling steps were not necessary with HDA, providing an even further simplified isothermal DNA amplification method.

■ Sequence-specific helicase-dependent amplification

Sequence-specific HDA employs essentially the same reaction scheme as PCR, excluding the heat denaturation of double-stranded DNA **(Figure 4A)**. This characteristic of HDA allows exponential amplification to proceed much faster than in PCR due to the simultaneous and continuous DNA unwinding and polymerization. At the same time, sequence-specific HDA preserves all the advantages of PCR, such as high sensitivity, relatively simple primer design, reaction scheme and multiplex detection. Currently, this method is mainly used for pathogen detection in clinical diagnosis because it is simple, rapid and user-friendly [13,18,69–72]. The sequence-specific HDA method has been further developed to allow multiplex detection of pathogens by microarray [71]. Sequence-specific HDA can also adopt PCR-based strategies (e.g., quantitative-PCR and reverse transcription-PCR), thus simplifying the approaches. For example, the signal from quantitatve HDA could be monitored in real-time using a real-time quantitative-PCR instrument as a detector without thermal cycling [70]. In fact, such an instrument was not necessary and a simple plate reader with an isothermal heating block was shown to be sufficient for quantitatve HDA. RNA detection by reverse transcription-HDA

was also achieved in one tube by simply adding a reverse transcriptase to an established HDA reaction mixture [18].

■ Primase-based helicase-dependent amplification for whole genome amplification

An HDA approach has also been used for WGA. Unlike other isothermal WGA techniques, **primase**-based HDA is based upon *in vitro* reconstitution of the naturally existing cellular DNA replication machinery of bacteriophage T7, which means that neither initial heating nor exogenous random primers are required **(FIGURE 4B)** [73]. This is achieved by using a primase to synthesize primers on-site, generating multiple initiation sites. Because a polymerization reaction occurs in both directions after unwinding duplex DNA, amplification is exponential, which is not achieved by other WGA techniques. In addition, DNA unwinding is faster than DNA polymerization, providing multiple initiation sites and a total reaction time that is much shorter than other WGA methods (<1 h). Typically, microgram quantities of amplified genomic segments (mostly > 3 Kb) are yielded from a few nanograms of initial genomic DNA, reaching up to 3×10^3-fold amplification after 1h at 37°C. However, this method showed slightly higher amplification bias than the most widely used SDA based WGA kit, the GenomiPhi V2 kit, when analyzing amplification efficiency over 20 loci of human genomic DNA, a result likely due to biased sites for primer synthesis. The major advantages of primase-based HDA are the removal of requirements for initial heating and exogenous primers.

Recombinase polymerase amplification

■ Sequence-specific recombinase polymerase amplification

Regardless of the amplification method, a key step in selective amplification of target DNA is primer/probe annealing, which is usually kinetically driven using high concentrations of the primer/probe. For this purpose, the annealing temperature (~65°C) is preset in all of the previously described isothermal methods (SDA, RCA and HDA). In 2006, Piepenburg *et al.* revolutionized the strategy of primer annealing [74]. The isothermal temperature was lowered to 37°C by combining recombinase with primers to scan duplex DNA and facilitate strand exchange at target sites. After this enzyme-aided target searching, recombinase is disassembled and leaves the 3′-end of the primer accessible to DNA polymerase. The reaction scheme is similar to PCR and HDA, and targets are amplified exponentially. By contrast, neither initial heating for DNA denaturation

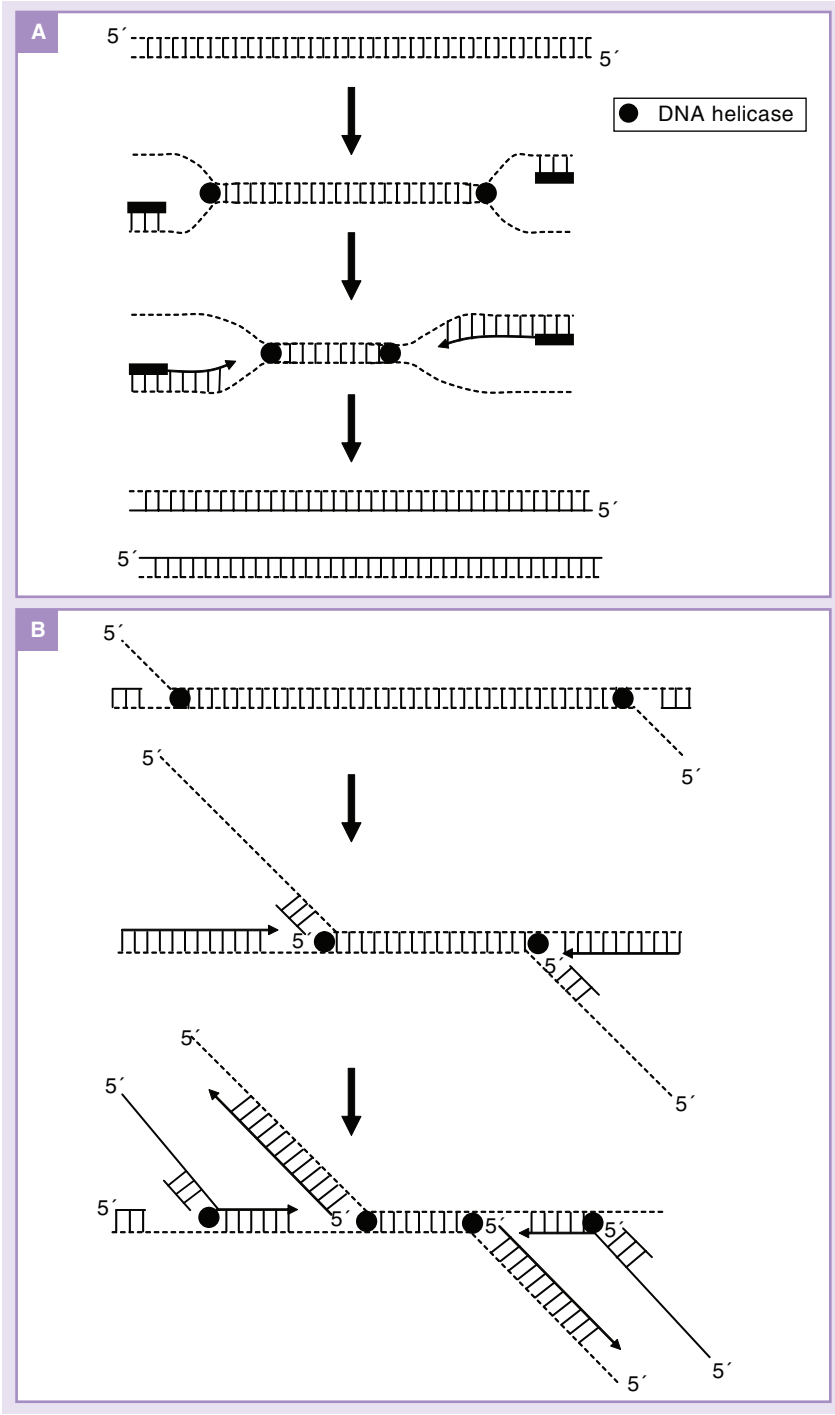

Figure 4. Helicase-dependent amplification. (A) Target-specific helicase-dependent amplification and **(B)** primase-based HDA.

nor high reaction temperature is required, thus making DNA quantitation potentially accessible in a point-of-care environment. This method allowed detection of the *Bacillus subtilis SpoB* locus from 100 copies of *B. subtillis* DNA using SYBR Green I within 30 min. However, unlike SDA, RCA or HDA, the approach may not be adequate for unbiased whole genome amplification when short random primers are used, since the length of primers should be no less than 28 nucleotides.

Future perspective

Although isothermal DNA amplification is capable of eliminating the need for a thermal cycler in point-of-care analysis, the development of simple devices such as microfluidic chips is still in demand [75]. Such devices are preferably integrated chips that can combine sample preparation, signal amplification and detection, thus reducing user interaction, time and cost of analysis [76]. Such attempts have been successful in a few recent cases with isothermal amplification approaches [42,77,78]. With respect to WGA, adequate sensitivity was achieved for gene cloning from a single cell. However, amplification bias should be further reduced. Complete coverage of a single cell's genome can promote delineation of that cell from closely related cells; for example, distinguishing one cancer cell from other cancer cells. With high quality amplification of a genome, comparative genomic hybridization microarrays can be performed, and cancer cell lineage can be traceable by reconstructing a phylogenetic tree from copy number aberration data [79]. With respect to bioassays, the aforementioned capabilities of translating various input signals into DNA have allowed isothermal DNA amplification to become a more flexible platform for signal amplification. Since homogeneous assays are exceptionally valuable in terms of simplicity and cost–effectiveness, one particularly promising approach would be the development of an aptamer that can switch isothermal DNA amplification on and off upon target binding.

Financial & competing interests disclosure

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Executive summary

- Isothermal DNA amplification techniques are simple, rapid and cost effective with equivalent specificity and sensitivity to PCR, enabling point-of-care diagnostics such as pathogen detection, single nucleotide polymorphism analysis, biomarker detection and so forth. Future work in this area should focus on microfluidic adaptations to these techniques for integrated sample preparation and reduced cost and time of analyses.
- Effective amplification of large genomic segments by isothermal methods results in unbiased whole genome amplification from a few homogeneous cells, thus serving as high-quality single-cell genetic material. This material can then be used for archiving and downstream analysis such as genotyping, comparative genomic hybridization and single cell genomics.
- Immunoassays coupled to isothermal DNA amplification have improved sensitivities compared with traditional enzyme-amplified immunoassay and improved dynamic range and reproducibility compared with immuno-PCR.
- This review also highlighted unique recent applications of isothermal DNA amplification for bioanalysis:
  - *In situ* signal amplification;
  - MicroRNA quantification;
  - Homogeneous isothermal assays.
- These isothermal approaches made possible:
  - The detection of biomolecules at single-molecule resolution in a cell;
  - The quantitation of lowly expressed small RNAs at single nucleotide resolution without further purification from total RNA;
  - The establishment of simple protocols to assay biomolecules with amplified signal, requiring no washing or separation steps.

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- ▪ of interest
- ▪▪ of considerable interest

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