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Strand Displacement Amplification for Multiplex Detection of Nucleic Acids

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Abstract

The identification of various targets such as bacteria, viruses, and other cells remains a prerequisite for point-of-care diagnostics and biotechnological applications. Nucleic acids, as encoding information for all forms of life, are excellent biomarkers for detecting pathogens, hereditary diseases, and cancers. To date, many techniques have been developed to detect nucleic acids. However, most of them are based on polymerase chain reaction (PCR) technology. These methods are sensitive and robust, but they require expensive instruments and trained personnel. DNA strand displacement amplification is carried out under isothermal conditions and therefore does not need expensive instruments. It is simple, fast, sensitive, specific, and inexpensive. In this chapter, we introduce the principles, methods, and updated applications of DNA strand displacement technology in the detection of infectious diseases. We also discuss how robust, sensitive, and specific nucleic acid detection could be obtained when combined with the novel CRISPR/Cas system.

Keywords: nucleic acids, isothermal amplification, strand displacement, CRISPR/Cas, multiplex detection

1. Introduction

To date, nucleic acids have been used not only for biological studies but also as powerful biomarkers for clinical diagnosis, agriculture, forensic science, and so on. PCR is a well-known molecular biology tool for the amplification of target sequences. In PCR, DNA amplification relies on heating and cooling of nucleic acids followed by hybridization. It can efficiently amplify target sequences within a few hours in three temperature-dependent steps: initiation,

annealing, and elongation. However, PCR requires thermocyclers for reaction temperature adjustment and trained personnel, which hinders its usage in resource-limited settings; and additionally, it has mispriming and sometimes inadequate template amplification. Therefore, the development of simple and inexpensive methods for nucleic acids amplification and detection is important for on-site inspections.

Whilst several isothermal amplification platforms, such as nucleic acid sequence based amplification (NASBA) [1], strand displacement amplification (SDA) [2–5], loop-mediated isothermal amplification (LAMP) [6], rolling circle amplification (RCA) [7], recombinase polymerase amplification (RPA) [8], and Helicase dependent Isothermal DNA Amplification (HAD) [9, 10] have been developed. These amplification platforms can achieve linear or exponential dsDNA accumulation, and some of which can be purchased as kits and integrated in portable devices [5], so no expensive instruments are needed, but there are still challenges in multiplexing, primer design, and stringency in experimental design. In this chapter, we describe the development and current directions of SDA in detail. Prospects in analysis using fluorescence, colorimetry, and lateral flow biosensors are also discussed.

Moreover, CRISPR-Cas system is also currently exploited for the detection of nucleic acids. For instance, some CRISPR-Cas proteins have been found to exhibit collateral cleavage of target nucleic acids and any nonspecific single-stranded nucleic acids in the solution. Therefore, if the latter is labeled with a fluorescent or a specific antibody recognized molecule, detectable signal can be generated [11–13]. This combination of isothermal amplification and various CRISPR-Cas-based signal readouts is simple, fast, specific, and sensitive and thus can elevate the specificity of nucleic acid diagnosis. In addition, CRISPR-Cas-based diagnosis can be multiplexed to provide another convergent evolution and convenient point-of-care detection of nucleic acids in low cost.

2. Strand displacement amplification (SDA)

SDA is carried out under isothermal condition. It is inspired from normal physiological RNA transcription and DNA replication, which occurs at a constant temperature. Over the past 2 decades, SDA has been widely used as an alternative to PCR for the detection of pathogens [14, 15], hereditary diseases [16], and cancers [17–19]. Moreover, SDA amplified nucleic acids can be multiplexed and readily provide optical and visual readouts [14, 20, 21]. In a typical SDA, two pairs of primers are designed to specifically recognize two regions of a target sequence. One pair is bumper primers designed as standard PCR primers, and another pair is SDA primers, which bind immediately next to the bumper primers at the target sequences. In addition, a HincII restriction enzyme and an exonuclease deficient (exo⁻ Klenow) polymerase are added. The reaction mixture is incubated in a single constant temperature of 37°C. In this reaction, the *Hinc*II cleaves at the recognition site of the phosphorothioate (modified substrate, dATPαS) of the DNA probe, and the *exo⁻* Klenow initiates the replication of the sequence. Subsequently, the exponential reaction starts with the primer-triggered repeated cycle of nicking, extension, and strand displacement. This amplification is accelerated by additional primers franking the inner region of the target sequence, and this reaction exhibits a single

and double nicking site cycle (**Figure 1**). The final new product chain yield can reach 10^7-fold amplification within 2 h [2, 20]. However, unlike the original method of cutting double-stranded sequences using restriction enzymes, the modified SDA uses nicking endonucleases (engineered restriction enzymes) such as Nt.BsmAI, Nb.BsmI, Nb.BsrDI, Nt.BspQI, Nb.BtsI, Nt.AlwI, Nt.BbvCI, and Nt.BstNBI (**Table 1**) to enhance the site-specific cleavage accuracy of target sequences and do not require modified dNTPs such as dATPαS at the cleavage site.

These endonucleases effectively create a nick for Klenow fragment or *Bst* polymerase to initiate a new strand replication and displace the downstream strand. The improved SDA exhibits an enhanced exponential amplification at 37–55°C, with 10^9-fold amplified DNA in <30 min [3]. The amplified nucleic acid products can be detected by photometry (turbidity), electrophoresis, SYBR Green's DNA-insertion dye, cross-flow text of a sequence-specific hybridization capture probe, or visual inspection of white precipitate. As shown in **Figure 1**, DNA double-strand is denatured, and primers (S1, S2, B1, and B2) bind to a specific DNA polymerase such as exo⁻ Klenow or Bst and then extend through the strand displacement activity. Upon the formation of strand containing the nicking site (linear amplification), the nicking enzyme cleave it, and the DNA polymerase primes initiate a new round of replication (1st cycle). The exponential amplification continues with a cycle of single and double-nicking, extension, and displacement cycles, producing a targeting sequence (SDA product).
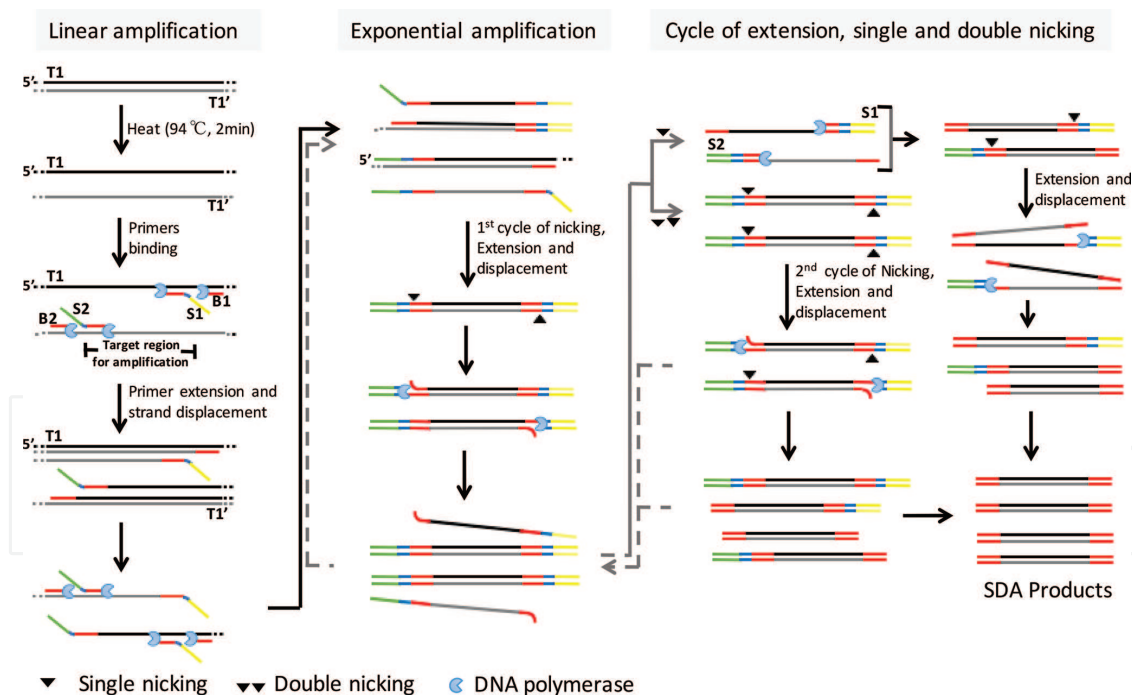

Figure 1. Schematic illustration of strand displacement isothermal amplification principle. First, double strand DNA is heat denatured for primer binding. The SDA primers (S1 and S2) contain recognition sequence for nicking endonuclease (green and yellow), a linker (blue), and sequence complementary to a target sequence (red). Bumper primers (B1 and B2) are located upstream of the SDA primers (red) and are complementary to the target sequence (black and gray). These primers bind to target sequence and extend by specific DNA polymerase such as exo⁻ Klenow or Bst with strand displacement activity. Upon formation of strands harboring a nicking site (linear amplification), the nicking enzyme nicks them, and the DNA polymerase primes a new round of replication (first cycle). Exponential amplification of the target sequence starts by cycle of single and double nicking, extension, and displacement.

Target	NEases	Template	Sample	DNA polymerase	Time (min)	Sensitivity	Detection method	Refs.
Singlex								
Salmonella spp.	Nt.BbvCI, b.Bpu10I	DNA aptamer	Bacteria strains, spiked milk	Exo⁻ Klenow, Bsm	30	1–60 CFU/ml	Lateral flow, G-quadruplex	[15, 22]
Hepatitis	Nt.BstNBI	DNA	HBV positive serum	Bst 2.0	30	2.5×10^4 copies	Electrophoresis and real-time fluorescence	[23]
E. Coli O157:H7	Nt.BbvCI	DNA aptamer	Bacteria strains, spiked milk	Exo⁻ Klenow	30	10 CFU/ml	Lateral flow	[24]
Mycobacterium (singlex)	*Hinc*II-*Eco*RI(Gl11)	DNA	DNA-spiked samples	Exo⁻ Klenow	120	<5 genome copies	Fluorescence polarization	[25]
Human papilloma virus	Nt.BsmAI	DNA	clinical samples and cell lines	Exo⁻ Klenow and phi29	30	0.1 pM–1 nM	Real-time fluorescence	[26]
HIV	BsoBI	RNA	RNA	Bst	55	250 copies	Fluorescence and flow cytometry	[27]
Bacillus cereus	-	gDNA	Bacteria strains, spiked human DNA	Exo⁻ Klenow and phi29	120	From 1 pg	NGS and real-time fluorescence	[28]
Simian Virus 40	-	DNA		Exo⁻ Klenow	40	1–250 pM	G-quadruplex	[21]
Heavy metals (e.g., Hg²⁺)	Nt.AlwI	DNA	Spiked tap water	Exo⁻ Klenow	30	2.95	Real-time fluorescence	[29]
Toxins (e.g., aflatoxin)	Nb.BbvCI	DNA	Skimmed milk sample	Exo⁻ Klenow	30	17–18 ng/kg	G-quadruplex fluorescence	[30]
VEGF₁₆₅	Nb.BbvCI	DNA aptamer	Spiked human serum	Bst	-	5–400 pg/ml	Fluorescence	[31]
Breast, prostate	Nt.AlwI	DNA	Spiked human serum	Exo⁻ Klenow	30	0.47 U/L	Real-time fluorescence, Sybr Green I	[32]
K-Ras	Nt.BbvCI	DNA	Cell lines	Exo⁻ Klenow	120	4 pM	Colorimetric and electrochemical immunosensing	[33]
MTase	Nb.BbvCI	DNA	Cell lines, spiked Dam MTase human serum	Exo⁻ Klenow	30	0.063 U/mL	G-quadruplex fluorescence	[34]

Target	NEases	Template	Sample	DNA polymerase	Time (min)	Sensitivity	Detection method	Refs.
p53	-	DNA	Mutant *p53* DNA	Exo⁻ Klenow	25	250 pmol/l	G-quadruplex fluorescence, Sybr Green I	[35]
miRNAs	Nt.AlwI	miRNA	miRNA, spiked serum	Exo⁻ Klenow	90	16 zmol	Electrophoresis and real-time fluorescence	[18]
Stem cells	Nt.BbvCI	DNA aptamer	Stem cell lines	Exo⁻ Klenow	30	19 cells	Lateral flow	[36]
Cocaine	-	DNA	Spiked human serum and complex media	Exo⁻ Klenow	60	2 nM	Electrophoresis, real-time fluorescence, SYBR Green I	[37]
GMOs and mutagens	Nt.*Bsm*AI	RNA	Rice leaves	phi29	150	0.14 fM	Electrochemiluminescence	[38]
Multiplex								
Mycobacterium species	*Hinc*II	DNA	DNA, human placental DNA	Exo⁻ Klenow	120	<5 genome copies	Fluorescence polarization	[20]
Neisseria gonorrhoeae and *Chlamydia trachomatis*	NA	DNA	Genital and urine samples	NA	60	75%	BD ProbeTec System-fluorescence reader	[14]
E. coli O157:H7 and its serotypes	BsoBI	DNA	Bovine, water and drinks	Bst polymerase	30	4.3 bacteria	Fluorescence polarization	[39]
Vibrio and *E. Coli* O157:H7	-	DNA	Bacteria strains, spiked serum	Bst 2.0	30	3.5 cells	Gel electrophoresis and lateral flow	[40]
MicroRNAs	Nt.BbvCI	miRNA	HepG2 cells, spiked lysates	Exo⁻ Klenow	90	5 pM	G-quadruplex fluorescence	[41]
S. aureus, engineered internal control sequences	Nt.BbvCI	Synthetic DNAs	DNA, simulated nasal matrix	Bst2.0WarmStart	<20	10–50 copies	Real-time fluorescence and lateral flow	[3]

NEases: nicking endonuclease, ↓: cut at 5′-3′ complementary strand, ↑: cut at 3′-5′ complementary strand, Nt: Nicking at top strand, Nb: Nicking at bottom strand, and MTase: DNA methyl transferase.

Table 1. Singlex and multiplex detection of nucleic acids using SDA-based assays.

2.1 Experimental procedures

2.1.1 Design of SDA primers

Primers are prerequisite for the initiation of nucleic acid amplification. SDA requires two pairs of primers, SDA primers (S1 and S2), and Bumper primers (B1 and B2). Bumper primers are similar to standard PCR forward and reverse primers for the identification of specific sites and amplification of a target DNA sequence. The B1 and B2 primers are 18–23 nucleotides designed based on the target strand from the 5′ end and a reverse complementary sequence of the 3′ end strand, respectively. However, the S1 and S2 primers are a pair of 5′ end-turned special primers. From 5′ to 3′, both primers contain a protecting 10–15 nucleotides, a nicking recognition sequence (~5 nucleotides), linkage sequence (~4 nucleotides), and then a 10–18 nucleotides complementary to the target sequence at the 3′ end immediately adjacent to a bumper primer (**Figure 1**).

2.1.2 Properties and choice of nicking endonuclease

Restriction endonucleases are well-known endonucleases that recognize and cleave palindromic DNA sequences. Nicking endonucleases (NEases), on the other hand, cleave one strand of a specific DNA sequence. Typically, homodimer restriction enzymes bind to two half sites of a specific palindromic sequence, that is, each monomer cut one strand. However, both the nicking endonucleases and one strand-cleaving restriction enzyme are heterodimers, allowing only single nicking or cleaving at the asymmetric recognition sequences. Through the genetic engineering of naturally occurring restriction enzymes, various nicking endonucleases, such as Nb.BbvCI and Nt.BbvCI, have been generated through modifications of the catalytic activity of the asymmetric amino acid sequences of the enzymes. BbvCI, a heterodimeric Type IIS endonuclease recognizing 7 bp of asymmetric DNA sequence, uses its R1 and R2 subunits at the catalytic sites to cleave bottom strand and top strand (..CC↓TCAGC.. and ..CCTCA↑GC..), respectively [3, 42]. The Nt.BbvCI is an engineered BbvCI with functional R2 and a missing R1 domain which can only cleave top strand (..CC↓TCAGC..) [42, 43], while Nb.BbvCI was engineered from BbvCI, with functional R1 and deficient R2, which can only cleave bottom strand (CCTCA↑GC..) [43]. However, other nicking endonucleases were generated through mutations, truncation, and domain swapping. These nicking endonucleases include bottom strand nickase such as Nt.AlwI, Nb.BsmI [44], Nb.BsrDI, Nt.BspQI [45], Nb.BtsI, and Nt.CviQII and top strand nickase such as Nt.CviPII [46–48]. For example, Nt.AlwI was engineered from the dimeric AlwI (dsDNA cleaving REase) to cleave only the top strand of the AlwI target sequence (..GGATCNNNNN↓N..). The Nt.AlwI monomer structure derives from the dimerization swapping with nonfunctional domain of Nt.BstNBI [49]. Thus, as shown in **Table 1**, the choice of nicking enzymes may depend on the preferred DNA polymerase and the desired type of reaction to achieve an efficient amplification.

2.1.3 Properties and choice of DNA polymerase

The combinations of nicking enzymes and DNA polymerases have great effect on the amplification efficiency. For example, among several nicking enzymes and DNA polymerases studied, Nt.BspQI coupled with Sequenase 2.0 polymerase showed a higher linear SDA amplification [47], and Nt.BstNBI coupled with Bst DNA polymerase showed a 10 times higher exponential

amplification compared to other combinatorial NEases. The authors attributed this property to the enzymatic conformation and concentration, as higher or lower concentration could be ineffective for the SDA reaction [50]. DNA polymerase such as exo⁻ Klenow and Bst (Bst 2.0, Bst 2.0 WarmStart, and Bst 3.0) have good amplification performance and are good choices for SDA [3]. Although Exo⁻ Klenow exhibits excellent performance after binding to specific nicking enzymes like Nb.BbvCI, the amplification efficiency decreases by 5–100 folds when the target nucleic acid sequence increases by 50 base pairs [47]. Bst exhibits a similar limitation. To address the limitation of Exo⁻ Klenow and Bst, newly engineered DNA polymerases such as Bst 2.0 show higher efficiency, thermal stability, salt tolerance, and greater fidelity in SDA amplification. Xu et al. showed that Bst 2.0 polymerase highly prefers Nt.BbvCI rather than other conventional nicking enzymes, and this combination showed good results for the detection of viral, bacteria, and *BRCA1* gene sequences. However, various inhibitors can affect the activity of most polymerases. Fortunately, Bst 3.0 was designed to amplify both RNA and DNA with high activity even in the presence of amplification inhibitors.

Furthermore, to encounter mispriming occurring at lower temperatures and isothermal amplification stringency, Bst 2.0 WarmStart was engineered. A specific aptamer (unique oligonucleotide sequence) targeting Bst DNA polymerase via noncovalent binding has been selected using the systematic evolution of ligands by exponential enrichment (SELEX). The Bst DNA polymerase with the bound aptamer cannot perform strand displacement unless the temperature is raised to 50°C, thereby minimizing the unwanted isothermal preamplification usually occurring at room temperature, and mispriming is therefore prevented. Another attempt for limiting undesired preamplification and spurious results is by performing all sample preparation steps in ice before starting the reaction. By this simple tuning combined with the best DNA polymerase, the detections for *C. elegans*, *E. coli*, *λ*-phage specific genes, and Hela cell genomic DNA showed rapid (~10 min) and consistent isothermal amplification [51, 52].

2.1.4 Multiplex SDA reaction

The detection of more than one target from a single sample is usually required in medical diagnosis. Multiplexing is mainly achieved using spatial separation of targets, regional separation by targeting specific sites, or label-based techniques [53]. Though spatial and regional separation methods are highly employed for on-site multiplexed detection of various targets, they still suffer from expensive apparatus and complicated procedures [54, 55]. An alternative method is target-labeling approach, which uses different molecular recognition elements (dyes, enzymes, DNA probes, beads, aptamers, etc.) to identify different targets.

Primer design and optimization are essential for multiplex SDA. Singleplex SDA requires four primers to detect one target; therefore, duplexed detection would require eight primers, and so on. Thus, the number of primers increases rapidly with the number of targets. The large number of primers increases the complexity of the multiplex amplification system and therefore decreases the stability of the system. To solve this problem, Walker et al. developed a method called "adapter-mediated duplex SDA" for simultaneous detection of *Mycobacterium species* using fewer primers and without altering amplification yield [20]. In this method, a single pair of amplification primers and adapter sequences is used, where two target strands are amplified exponentially using dual primers. The first primer is attached to one end of the second target,

while the second primer is appended to one end of the first target sequence. After amplification of the target strands by the primers, the adapter sequences start to bind to the amplified target sequences and begin extension and displacement, which results in a cascade of exponential amplification of the target sequences using the adapters rather than the primers. This method was also used for multiplexed SDA of three distinct DNA sequences of *Mycobacterium tuberculosis* and other mycobacteria [56]. Furthermore, multiplex SDA was used to amplify multiple SNPs simultaneously with molecular beacon probe-assisted fluorescent signal readout [57]. Most recently, several *BRCA* mutations were genotyped by combining SDA and mass spectrometry. Allele-specific regions were amplified and then ligated to adapters by DNA ligase, and the ligated products were SDA amplified with universal primers. The resulting fragments were analyzed and confirmed using mass spectrometry [58]. Though, this SDA method required complex equipment, it was able to detect hundreds of mutations.

2.1.5 Sample preparation

Isothermal amplification usually requires specimen isolation and culture, enzymatic treatment or genomic DNA (gDNA) extraction, and/or heat denaturation (in case of dsDNA) to obtain template DNA. Traditional sample preparation methods like genomic DNA extraction are time consuming and susceptible to contamination. Thus, crude cell lysate method is widely used, whereby cells are heat killed or enzymatically pretreated using proteinase K to expose DNA ready for amplification [59]. Cell cultures are usually heated at 92–95°C for 3–5 min and cooled to appropriate SDA temperature prior to the reaction. An up to date approach termed HUDSON (heating unextracted diagnostic samples to obliterate nucleases) combines the dual range of heating, that is, one temperature (37–50°C, 5–20 min) for nuclease inactivation, and another temperature (64–95°C, 5–20 min) for pathogen inactivation and genome exposure [60]. Though heat-assisted amplification is still frequently preferred owing to its simplicity, but it cannot differentiate nucleic acids from live and dead cells. As solution, Tong and colleagues used a set of restriction enzymes and modified nucleotides that could target different sites of target template and create a site of nicking and extension for SDA target DNA amplification [61]. However, these modified nucleotides and probes are costly, and the restriction enzymes exhibit random target digestion, which results in high background, low sensitivity, and specificity. Alternatively, heat treatment can also be abolished by using an improved SDA approach that initiates amplification at a DNA breaching site (Hoogsteen pairing), though its specificity to long templates is still of concern [3, 62].

To overcome contamination and detect live targets accurately, gDNA extraction free isothermal amplification methods have been adopted, which use extracellular compartment recognizing molecules such as aptamers. We reported ultrasensitive aptamer-based biosensors for the detection of live pathogens including *E. coli* and *S. enteritidis* [15, 24, 36]. In these methods, a dual aptamer system recognizing two extracellular membrane components are used. One bacterium-targeting aptamer is modified with biotin in order to react with streptavidin-coated magnetic beads during positive selection, while another bacterium-targeting aptamer is used as a template for SDA amplification. The bacteria-aptamer-magnetic bead complex is enriched, amplified, and applied to lateral flow strip for visual detection (**Figure 2**). This method sensitivity to pathogens is 10 *cfu* of live cells, therefore reduces false-positive results.
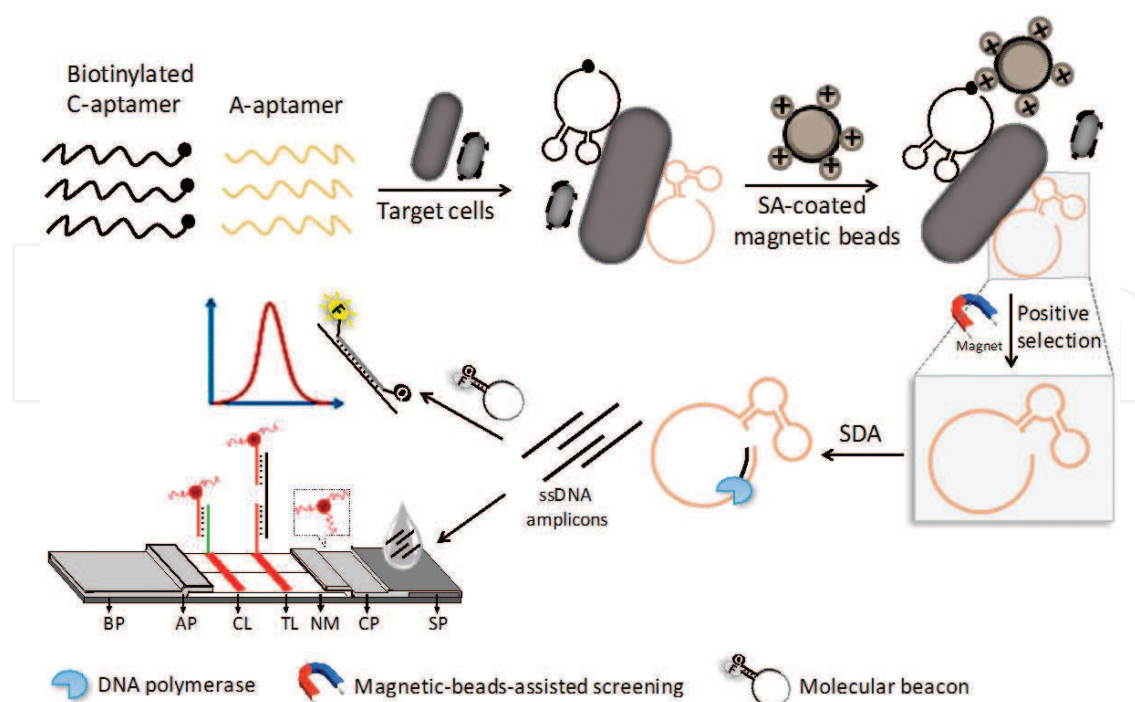
Figure 2. Conceptual illustration of SDA and aptamer-based nucleic acid biosensor. Two target-specific aptamers termed biotinylated capture aptamers (C-aptamer) and amplifying aptamers (A-aptamer) bind to target cells, for the enrichment of targeted cells and strand displacement amplification (SDA), respectively. After the binding reaction, streptavidin (SA)-coated magnetic beads are added to form cell-magnetic bead-aptamer complexes that are collected for SDA amplification. Finally, the single strand amplicons are loaded onto the lateral flow strip for visual readout or hybridized to fluorophore-probe after displacement of quencher-labeled probe for fluorescent detection.

3. SDA product analysis

Detection of amplified product is critical in isothermal amplification of nucleic acids, which includes quantification of the final amplification product and monitoring of the product during the process of amplification. Hereafter, we introduce a variety of methods for the detection of nucleic acids, which include intercalating fluorescent dyes, fluorescent probes, lateral flow biosensors, and CRISPR/Cas system.

3.1 Fluorescent dye-based analysis

Fluorescent gel staining dyes such as SYBR Green and ethidium bromide are commonly used for monitoring and quantitation of nucleic acids. SYBR Green I can intercalate into dsDNA, which emits light under UV-light. SYBR Green II is supersensitive to RNAs and ssDNA; however, it fluoresces in the presence of dsDNA or ssDNA [50]. Although SYBR Green is the most sensitive dyes employed to monitor nucleic acid amplification progress, high concentration of SYBR Green can inhibit SDA amplification [47]. Thus, other dyes such as Picogreen, Ribogreen, and Oligogreen have been developed. These versatile fluorescent dyes are specific, sensitive, and rapid for a broad spectrum of applications such as nucleic acid typing, amplification, and purification, Band-shift assays as well as DNA-damage assays. Interestingly, they are not interfered by free nucleotides and proteins. They are much more specific than

UV-based absorbance (A_{260}) and much easier to use than laborious radioisotope labeling and silver staining. They have been widely applied to quantitatively detect the early onset of diseases with window periods such as virus, oncogenes, etc. Furthermore, DNA dyes such as EMA (ethidium monoazide bromide azide) and PMA (propidium monoazide bromide azide) have been extensively used to distinguish nucleic acids of live and dead cells [63, 64]. These dyes can penetrate damaged cells and bind to DNA with little effect on live cells endowed from their intact cell membranes [65, 66]. However, their off-target binding and background result in spurious amplicon staining [47, 50] and are limited to single-strand conformation polymorphism (not at the level of single nucleotide polymorphism) [67].

3.2 Fluorescent probe-based analysis

To increase specificity, fluorescent oligo probes such as TaqMan probes and molecular beacons have been used to monitor and quantify nucleic acid amplification products [68–70]. A fluorescent probe consists of a fluorophore and a quencher covalently attached at 5′ and 3′ ends of a DNA probe sequence. There are two main types of fluorescent oligo probes, TaqMan probes and molecular beacons. In TaqMan probes, the fluorescent light of the fluorophore (e.g., FAM) is absorbed by the quencher (e.g., TAMRA) before amplification; therefore, no fluorescence can be detected. During amplification, the fluorescent probe hybridizes complementary to the target sequence, and the DNA polymerase degrades the probe via its 5′-3′ exonuclease activity. As a result, the fluorescent reporter and the quencher are separated, and the fluorescent reporter is then detected [71]. This technology has been widely used in real time PCR for medical diagnosis.

TaqMan probes depend on probe hybridization, polymerase extension, and cleavage of the probes. Molecular beacons, on the other hand, do not require polymerase extension and cleavage activity. Molecular beacons comprise modified stem ends with fluorescent and quencher molecules, a hairpin loop probe sequence (~20–25 bases) and complementary stem sequences (~4–6 base pairs). Before hybridization with the target sequence, the fluorophore on one end of the molecular beacon is quenched by the quencher on the other end of the beacon as the two ends are close together. When the probe hybridizes with the target nucleic acid sequence, the molecular beacon sequence becomes linear. As a result, the fluorophore and the quencher are separated, and the fluorescence is then detected. For example, the putative molecular beacon probes with fluorophore (5-(2′-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS)) and quencher (4-(4′-dimethylaminophenylazo) benzoic acid (DABCYL)) changes conformation and fluoresces spontaneously upon perfect complementarity with the target nucleic acid (**Figure 2)** [72]. This approach is best used to detect ssDNA products produced from SDA. It can also been multiplexed using multicolored molecular beacons for different targets [69].

Organic dyes such as rhodamine are conventionally used in fluorescent oligo probes. However, organic dyes have low quantum yield and are easily photo bleached. Other fluorophores such as quantum dots (QDs), silver nanoclusters, upconversion nanoparticles, and corresponding quenchers such as gold nanoparticles and carbon nanomaterials have been used to replace organic fluorophores and quenchers [73] for nuclei acid as well as protein detection [74, 75]. These nanomaterials have high quantum yield and photostability. In addition, they can be simultaneously excited using one wavelength during multiplex detection of various targets.

They are promising substitutes of organic dyes in the detection of nucleic acid detection using fluorescent oligo probes.

Tavares et al. reported an on-chip immobilization of QDs as energy donors in FRET and Cy3-labeled dsDNA target as a receiver for transduction of nucleic acid hybridization, which resulted in rapid quantitative determination of nucleic acid at the fmol level within 7 min after target introduction [76]. Silver nanoclusters possess much higher photostability and fluorescence than organic fluorophores and QDs, which have been used to detect influenza specific nucleic acids. Upon hybridization, these DNA-silver nanocluster probes fluoresce up to 500-fold when placed near G-rich nucleic acid targets and exhibited high signal to background ratio [77]. This finding was promising; however, it was elusive how fluorescence increased upon G-rich target detection. It is speculated that upon target binding guanines, G-quadruplex structures may be formed to yield reddish nanoclusters, or serve as electron donors since guanines have lowest oxidation potential compared with other nucleotides [78], or otherwise reduce oxidized nanoclusters and render them reddish [79, 80].

3.3 Lateral flow biosensor

Lateral flow biosensor is the most commonly used technology for the point of care testing [81–84]. A test strip consists of four parts: a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorption pad. This method uses fiber chromatography material as a solid phase to allow capillary flow of sample solution, followed by the reaction between the analyte in the sample and the target recognition molecules fixed on the nitrocellulose membrane [9] (**Figure 2**). Color development can be obtained through enzymatic reaction, or visually detectable materials such as gold nanoparticles.

For the detection of nucleic acids, traditional lateral flow biosensor has been modified and termed nucleic acid lateral flow biosensor. In nucleic acid lateral flow biosensors, antibodies or antigens are replaced with probe DNAs that are fixed on the test zone and control zone to capture specific targets via nucleic acid hybridization. Based on SDA and aptamers, we developed a nucleic acid lateral flow biosensor to detect as low as 1 cfu/ml of pathogens, 1 ppm heavy metals, SNPs, and stem cells [15, 24] (**Figure 2**). This nucleic acid biosensor consists of (i) a specific capture probe, complementary to one part of the target nucleic acid and conjugated on gold nanoparticles, (ii) a target-hybridizing probe, immobilized on the nitrocellulose membrane test zone to capture amplified target sequence, and (iii) a specific nucleic acid probe on control zone that can hybridize with nanoparticle labeled probe. The hybridization on test line occurs at the presence of target, while in the absence of target sequence, the test zone does not show up. The appearance of the control zone shows the assay works properly. This method is fast, specific, sensitive, and cost effective. With different targeting aptamers and corresponding test zones on the test strip, multiplexing assay can be developed to detect multiple pathogens [84].

3.4 CRISPR-Cas-based analysis of nucleic acids

CRISPR-Cas is known to endow bacteria and archaea adaptive immunity against foreign nucleic acids using mobile genetic elements [85]. CRISPR-Cas proteins cleave invading DNA

and generate spacer nucleotides known as protospacer. The protospacer integrates into genome near the protospacer adjacent motif (PAM) region required as memory for future interrogation and cleavage of same invader depending on spacer-phage similarity. For the targeting, CRISPR-Cas9 requires a gRNA (guide RNA) composed of *tracr*RNA (trans-activating) and crRNA, or a chimeric sgRNA (single guide RNA). The RNA-guided cleavage is mediated by RuvC (a member of RNase H family) and HNH catalytic domains at the site of gRNA-target sequence base-pairing. In this mechanism, Cas9-gRNA complex recognizes a G-rich PAM region of the target sequence followed by blunt end cleavage. However, some Cas enzymes such as Cas9 and Cas12a, purified from *Francisella tularensis novicida* and *Streptococcus pyogenes*, exhibit nonspecific RNA-independent DNA cleavage in the presence of $Mn2^+$ [86], suggesting the significant role of several mediators including Cas RuvC nuclease domain. On the basis of gRNAs, more literatures indicated that Cas12a, Cas13a, and Cas13b enzyme effectors require a mature crRNA for self-assembly and processing and ribonucleoprotein surveillance-dependent nuclease or DNA for interference activity [87, 88]. Moreover, the Cas12a, Cas13a, and Cas13b enzymes do not require a dual functional crRNA-TrancRNA as for Cas9 [89, 90].

Basically, CRISPR-Cas systems are categorized into three main types (type I, type II, and type III) and 12 subclasses based on the genetic and structural differences [91]. From these classes, Type II CRISPR-Cas is widely used for genome-editing applications. Currently, researchers have exploited the CRISPR-Cas system in diagnostics. Two CRISPR-Cas-based diagnostic systems termed DETECTR (DNA Endonuclease Targeted CRISPR *Trans* Reporter) and SHERLOCK (Shorthand for Specific High Sensitivity Reporter unLOCKing) have been developed as a new platform for real time detection of nucleic acid based on Type II Cas13a and Cas12a, respectively. These enzymes exhibit collateral cleavage of nucleic acid targets and nontarget single strands in vicinity. For instance, Cas13, an RNA-targeting CRISPR-associated type VI-A protein, cleaves an ssRNA at a non-G PFS (protospacer flanking site) of target sequence in a gRNA-independent manner via its endonuclease HPN domain [12]. On the contrary of Cas13a and distinct from Cas9, Cas12a (cpf1) is a CRISPR-Cas family enzyme that possess a unique RNA-guided DNase activity [88]. It targets at 5′ T-rich PAM region (TTTN) by cleaving ~18 nucleotides on the DNA strand opposite to the gRNA complementary strand by leaving 5 nucleotide staggered cuts on both 5′-ends of the target sequence [92]. Cas12a can collaterally cleave both targeted dsDNA and a nontarget ssDNA in vicinity. Through the integration of a fluorescently labeled ssDNA reporter, a detectable signal can be obtained after cleavage. However, the cleavage of reporter nucleic acids is motif dependent and requires 41–44 nucleotide crRNA to recognize a 5′ T-rich PAM of the target sequence, while Cas9 requires ~100 nucleotide gRNA at the 3′G-rich PAM target site. Nevertheless, Cas12a is unable to *trans*-cleave an ssRNA reporter and a targeted ssRNA sequence [11], suggesting that it exhibits only a DNA-activated DNase activity. In contrast, Cas13 enzymes (e.g., CcaCas13b and LwaCas13b) from some bacteria strains have shown a random enriched motif cleavage with U-dependent nucleotide preference, while some other Cas13 enzymes (e.g., PsmCas13b and AsCas12a) strongly preferred A-nucleotides and A-T dinucleotide across the motif, respectively [11]. Nevertheless, other CRISPR-Cas13a/b exhibited dinucleotide preference collateral cleavage activity. This activity can be enhanced with optimized target concentration, buffer, and crRNAs. Therefore, irrespective of the target

efficiency of the CRISPR/Cas system, it is consent that a single-guided RNA-Cas enzyme complex recognition of target nucleic acid and reaction conditions is required to initiate cleavage of both target and a nearby nontarget (reporter sequence).

Various diagnostic applications necessitate detection of one or more targets, and therefore with tremendous propensity of both platforms, CRISPR-Cas enzymes can detect a single or multiple targets in complex liquid biopsy samples. Various samples suspected with Zika, dengue, and human papilloma viruses and bacteria as well as SNP and mutation discrimination have been developed. Their multiplex detection relies on reprogrammable crRNA tailing specific target sequence and enriched multiple motif fluorescent reporters. For example, Gootenberg et al. showed that isothermally amplified four different target nucleic acids could be detected simultaneously by LwaCas13a, PsmCas13b, CcaCas13b, and AsCas12a, with leveraged dinucleotide motifs harboring FAM, TEX, Cy5, and HEX quenched fluorescent reporters, respectively. After, the reporter is cleaved by Cas enzymes; the read-out can be achieved by high specific detection of four different quenched fluorescent reporters or using lateral flow biosensor analysis with specific antibodies against fluorescein-biotin reporters at conjugate pad and protein A as second antibody immobilized at the control line (**Figure 3**). Thus, these enzymes are intriguing for broad spectrum diagnostic applications (**Figure 3,** left). Moreover, the combination of isothermal amplification and CRISPR-Cas system for amplification and signal readout, respectively, revealed an amplified signal detection of 8zM in a 250 µl reaction volume. It should be noted that isothermal preamplification of target nucleic acids is crucial to achieve that robust sensitivity with Cas12 and Cas13 enzymes [13]. This approach could be adopted, however with the most minimized cost. More interestingly, it is simple, fast, specific, sensitive, and can be multiplexed. Thus, it is convenient in minimally instrumented fields for point-of-care detection of nucleic acids.
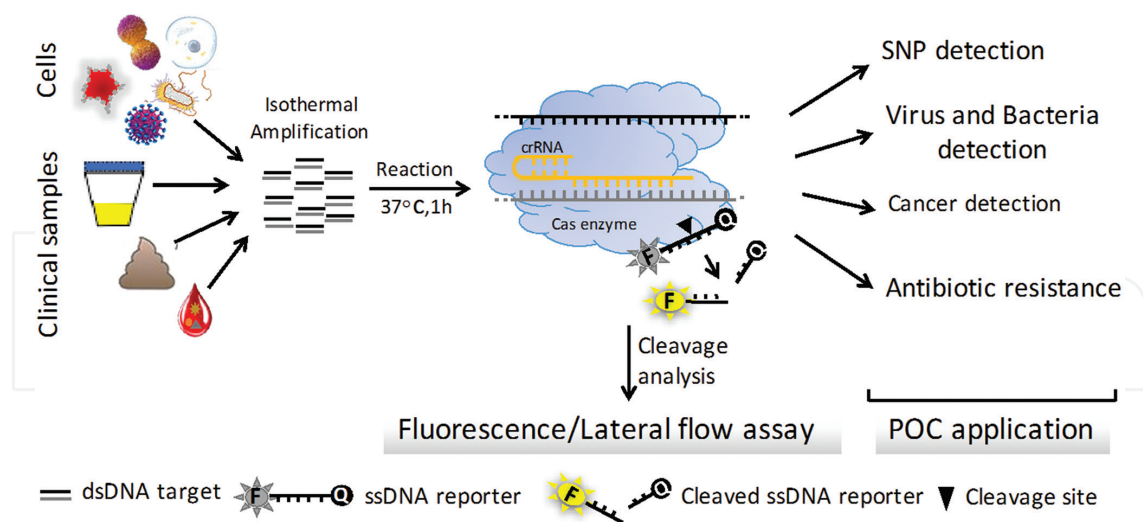

Figure 3. Workflow of nucleic acid detection with the CRISPR-Cas system. Nucleic acids are obtained from samples by proteinase K treatment or heat treatment. The nucleic acids are isothermally amplified using recombinase polymerase amplification at 37°C for 10 min. A one-pot reaction comprising amplicons, Cas protein, a designed crRNA for specific DNA/RNA target spotting, and reporters is prepared. To detect the presence of target nucleic acid, a fluorophore molecule (yellow star) and a quenching molecule (circle) are used. When Cas protein slices its nucleic acid target, and any ssDNA reporter nearby, the quenching molecule frees from the fluorophore, letting it fluoresce. The fluorescence can be detected directly or the reaction mixture can be applied to lateral flow assay.

4. Discussion, conclusion, and future perspectives

The development of new methodologies in nucleic acid amplification is of great importance in point of care diagnosis for research and public health. The SDA and its colleagues such as RPA, LAMP, etc. have proved reliable application for this purpose. Nevertheless, SDA has poorly achieved longer sequence amplification and a comparable amplification as for LAMP toward turbidity measurement, but under stringent conditions, possesses higher sensitivity, specificity, and cost-efficiency, and can be multiplexed and reprogrammed for various targets [58, 93–96]. But also, other isothermal amplification methods such as LAMP have hardly shown multiplexing capacity [97–99] and are solely dye dependent for colorimetric detection and quantification, which are target independent and nonspecific. Up to now, no perfect method can overcome all shortcomings. However, probe-based lateral flow assay in combination with SDA could provide robust multiplexing detection higher than their peer antibodies [84]. For example, our group [15, 24, 36] and other researchers [3, 100, 101] demonstrated that SDA can be used to amplify short target recognition sequences, for example, aptamers, after target binding and then integrated with lateral flow biosensors for analysis. Therefore, preheat denaturation of templates, background signals, and unspecificity encountered during the use of long nucleic acid templates or the presence of DNA contaminants are prevented [102, 103]. Furthermore, the lab-on chip approach, consisting of on-chip fixed multiple analytes, could eliminate partially primer-dimerization frequently observed during multiplexed isothermal amplification [104].

DNA fluorescent probes were widely applied for singlex and multiplex detection of isothermal amplified nucleic acid targets. Notwithstanding, comparing with fluorescent probe detection of nucleic acids and other PCR-based techniques, CRISPR-Cas-based analysis of isothermal amplified products is fast, sensitive, and specific, allowing its on-site implementation. This technique is reprogrammable for the ultrasensitive and specific detection of various targets and is amenable to multiplexing by using their targeting Cas proteins and fluorescent labels [11, 13, 60]. Nevertheless, further studies are still needed to overcome some drawbacks of this technology. For example, the cost of enzymes and anti-fluorophore reporters still needs to be reduced. Moreover, the operability needs to be improved because the usage of more enzymes in one reaction requires complicated optimization for one-pot concurrent catalysis. In the lateral flow assay, we propose the combination of CRISPR-Cas with DNA capture probe technique for the cleaved and noncleaved reporters' detection, in order to replace the immunoassay approaches which are prone to cross reactivity. Furthermore, the sensitivity of this emerging diagnosis depends solely on target (s) nucleic acid (s) amplification. Thus, alternatively, one can develop an intrinsic signal amplification of reporters' signal by either cationic and fluorescent conjugated polymers or direct inhibition of fluorescent loss using fluorophore encapsulation. For signal amplification, the CRISPR system might be promoted by using other fluorescent-intensity-based nanostructures (e.g., quantum dots and silver nanostructures) and up conversion nanomaterials alongside with robust quenchers such as gold nanoparticles [105] and carbon nanomaterials (graphene oxides and single-walled carbon nanotubes) [106]. Additionally, the use of G-quadruplex-mediated catalysis for colorimetric detection of isothermal amplified amplicons or cleaved-CRISPR reporters could eliminate the

need of more enzymes and thus allow conveniently the one-pot reaction toward simplified, cost-effective, specific, and sensitive on-site detection of nucleic acids.

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Conflict of interest

The authors declare no competing financial interest.

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