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REVIEW

Isothermal Nucleic Acid Amplification Techniques and Their Use in Bioanalysis

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Abstract—Recently, there has been a rapid progress in the development of techniques for isothermal amplification of nucleic acids as an alternative to polymerase chain reaction (PCR). The advantage of these methods is that the nucleic acids amplification can be carried out at constant temperature, unlike PCR, which requires cyclic temperature changes. Moreover, isothermal amplification can be conducted directly in living cells. This review describes the principles of isothermal amplification techniques and demonstrates their high efficiency in designing new highly sensitive detection methods of nucleic acids and enzymes involved in their modifications. The data on successful application of isothermal amplification methods for the analysis of cells and biomolecules with the use of DNA/RNA aptamers are presented.

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Quantitative and qualitative determination of nucleic acids is an important problem of modern biology and medicine. This research area has been developing very rapidly since the early 1990s, and it is very likely that the number of studies on this topic will continue to grow in the next decades. Detection of DNA/RNA of pathogenic bacteria and viruses could be essential for choosing an appropriate treatment strategy. Recent studies have discovered the correlation between the risk of developing certain diseases in humans and single nucleotide polymorphisms or short insertions/deletions. It was also found that human microRNA genes are often located in the vicinity of genome regions and sites associated with cancer. The expression levels of some microRNAs in patients with chronic lymphocytic leukemia, colorectal neoplasia, Burkitt lymphoma, lung cancer, large-cell lymphomas, glioblastoma, and other diseases differ from the levels in normal tissues.

Another group, beside medical professionals, that is interested in the development of highly sensitive techniques for the analysis of nucleic acids is food chemistry specialists, as these methods allow to evaluate the quality of food products reliably and with high accuracy [1]. The methods of nucleic acid analysis have been also successfully used for a long time in forensic science [2].

Identification of nucleic acids in biological samples and directly in live organisms without prior purification is a very important task. These methods are based on hybridization, which accounts for their high selectivity. Considering that the concentration of nucleic acids in the investigated samples is often very low and its changes in various pathologies could be small, such identification methods should be highly sensitive and have low detection limits. To satisfy these requirements, current methods of DNA/RNA analysis are often used in combination with different variants of polymerase chain reaction (PCR). PCR is commonly used in practice because of its high efficiency, as it allows to synthesize up to 10^9 copies (amplicons) of the analyzed sequence. However, it has a number of drawbacks, e.g., possible non-specific hybridization resulting in the accumulation of undesired products.

Abbreviations: CHA, catalytic hairpin assembly; EASA, exonuclease III-assisted signal amplification; EXPAR, exponential amplification reaction; HCR, hybridization chain reaction; HDA, helicase-dependent amplification; ICSDP, isothermal circular strand displacement polymerization; LAMP, loop-mediated isothermal amplification; MDA, multiple displacement amplification; NASBA, nucleic acid sequence-based amplification; pWGA, primase-based whole genome amplification; RCA, rolling circle amplification; RPA, recombinase polymerase amplification; SDA, strand-displacement amplification; WGA, whole genome amplification.

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PCR with real-time product detection (quantitative PCR, qPCR) is commonly used for nucleic acid quantification [3]. The two main qPCR variants use (i) Taq DNA polymerase and linear probe (TaqMan technology) or (ii) intercalating dyes (SYBR Green, Eva Green, BOXTO, etc.), as the fluorescence of these dyes dramatically increases upon their binding to the double-stranded DNA. The linear range of qPCR is 10 to $5 \cdot 10^9$ copies of the analyzed sequence [4]; the sensitivity of this method varies significantly and depends on the structure of used primers. qPCR is also characterized with high reproducibility.

Cyclic temperature changes essential for PCR facilitate non-specific hybridization of primers and amplicons [5]. PCR cannot be used for the analysis of live cells, as it requires the melting of the DNA duplex in the course of reaction, which is achieved by DNA heating. Finally, PCR thermal cyclers are expensive.

The above limitations of PCR have stimulated the development of various platforms for the isothermal DNA/RNA detection. In this review, we present currently known isothermal amplification techniques, their advantages and drawbacks in bioanalysis.

The methods for isothermal nucleic acid amplification can be classified into two major groups: 1) techniques that increase the analytical signal by increasing the analyte concentration; 2) techniques that increase the analytical signal without changing the analyte concentration.

AMPLIFICATION TECHNIQUES THAT INCREASE ANALYTICAL SIGNAL BY INCREASING THE ANALYTE CONCENTRATION

All methods of isothermal nucleic acid amplification aiming to increase the analyte concentration use enzymes.

Figure 1a shows the principle of the **loop-mediated isothermal amplification (LAMP)** technique. This method was first described by Notomi et al. in 2000 [6]. Several primers (more often, four, but sometimes, six) complementary to different regions of the analyzed DNA are used together with DNA polymerases with pronounced strand displacement activity. LAMP is conducted at 60°C.

LAMP is initiated by hybridization of the forward inner primer with the complementary 5′-terminal fragment of the analyte DNA followed by its elongation by DNA polymerase. In the next step, forward outer primer hybridizes with the 5′-end fragment of the analyte and elongated by DNA polymerase with the synthesis of a new strand displacing the earlier synthesized sequence. After this, backward inner primer interacts with the complimentary fragment in the vicinity of the 3′-end of the newly synthesized sequence. After elongation of this primer, backward outer primer hybridizes with the synthesized DNA sequence. Elongation of this primer leads to the displacement of the earlier synthesized sequence. The ends of both synthesized sequences form the loops due to the complementary interactions. As a result, two structures with loops at the ends are synthesized, which initiates further amplification cycle with the same primers [7].

LAMP has an exponential character and generates up to 10^9 DNA copies within 15-60 min. The use of several primers ensures high specificity of the reaction. LAMP can be also used for RNA amplification using reverse transcription.

LAMP products are most often detected by electrophoresis or analysis of changes in the reaction mixture turbidity, which increases as a result of magnesium pyrophosphate formation [8]. The concentration of pyrophosphate formed in the course of LAMP could be also evaluated with the fluorescent dye calcein (fluorexon) [9]. DNA can be detected using intercalation dyes [10, 11]. Due to its simplicity, LAMP can be easily combined with microfluidic technologies to allows method automation and reduce the time of analysis and reagent consumption [11].

LAMP is widely used as a screening technique, because it can be conducted at a constant temperature and is highly efficient and specific. LAMP has been used for identification of *Mycobacterium tuberculosis*, herpes, severe acute respiratory syndrome, anthrax, human and avian influenza viruses, and other pathogens [12, 13]. LAMP was able to detect *Leptospira* DNA at a concentration as low as 200 pg/ml. The specificity of detection was 100%, as evaluated with 172 bacterial strains [12].

Beside bacterial pathogens and viruses, LAMP can be used for the detection of pathogenic protozoans, e.g., plasmodium. Using nested PCR for comparison, Poschl et al. [14] showed that the sensitivity of LAMP with *Plasmodium falciparum* was 100%. All PCR-negative samples for *P. falciparum* were also negative according to LAMP. In the diagnostics of *Plasmodium vivax*, LAMP detected 22 of 23 PCR-positives samples (96% sensitivity). All 82 PCR-negative samples were also negative according to LAMP. Hence, LAMP can be used as a reliable method for the diagnostics of *Plasmodium* species. However, in another study, LAMP-based assay produced some false positives [15]. LAMP was successfully used for identification of *Salmonella* in food products *in situ* and detection of stxA2 (Shiga toxin 2 subunit A) in *Escherichia coli* O157:H7 cells [15a, 15b].

When used for analysis of *Burkholderia mallei* and *Burkholderia pseudomallei* strains, LAMP primers failed to detect all sequences for which they were intended, but were capable to direct the synthesis of fragments of genes from heterologous strains [16]. The author suggested that these unsatisfactory results were due to the presence of GC-rich regions in the genomes of investigated bacteria and formation of secondary structures at the temperature of LAMP.

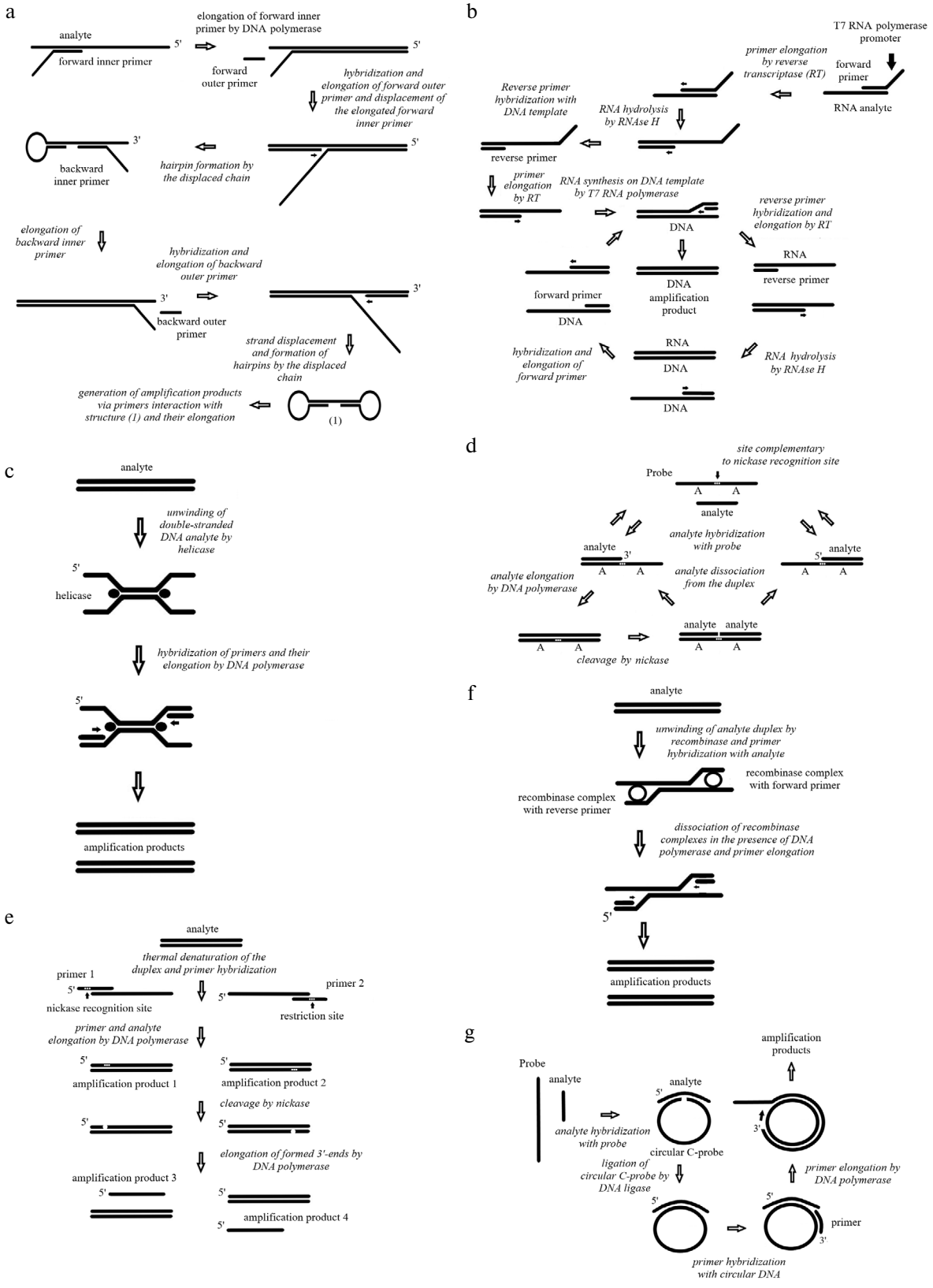

Fig. 1. Isothermal nucleic acid amplification techniques using polymerases: a) loop-mediated isothermal DNA amplification (LAMP); b) nucleic acid sequence-based amplification (NASBA); c) helicase-dependent DNA amplification (HDA); d) exponential amplification reaction (EXPAR); e) strand-displacement amplification (SDA); f) recombinase polymerase amplification (RPA); g) rolling circle amplification (RCA).

LAMP displays higher specificity in comparison with PCR, as it uses at least six primer-binding sites in the analyzed sequence. The sensitivity of LAMP was found to be an order of magnitude higher that the sensitivity of PCR. Moreover, LAMP is less sensitive to the inhibitors present in biological samples [17]. A serious drawback of this method is a high risk of contamination, often leading to the generation of false positives in negative controls [18].

Another isothermal method of nucleic acid amplification is **nucleic acid sequence-based amplification**[1] **(NASBA)**. In this method suggested by J. Compton in 1991, RNA molecules are amplified using three enzymes: avian myeloblastosis virus (AMV) reverse transcriptase, RNase H, and T7 RNA polymerase [19]. The reaction is conducted in two steps (acyclic and cyclic) (Fig. 1b).

In the first denaturation step (65°C), RNA interacts with specific primer containing T7 RNA polymerase promoter sequence. In the presence of reverse transcriptase, this enzyme synthesizes DNA on a single-stranded RNA, forming an RNA/DNA hybrid. All reactions are usually carried out at 41°C. The produced hybrid is cleaved by RNase H with the formation of a single-stranded DNA that hybridizes with the second primer. Elongation of this primer produces double-stranded DNA. Next, RNA is synthesized by T7 RNA polymerase on the DNA template. The resulting RNA molecules interact with the second primer; after elongation, the formed RNA/DNA hybrid is cleaved by RNase H. The resulting single-stranded DNA hybridizes with the first primer, which is elongated by reverse transcriptase. T7 RNA polymerase synthesizes copies of the original RNA, which initiates the next amplification cycle.

The advantage of NASBA in comparison with reverse transcription PCR is the use of the same medium for reverse transcription and the following amplification. NASBA is more sensitive and less time-consuming than PCR and can produce up to 10^9 copies of the analyte DNA. However, it should be mentioned that although exponential amplification techniques are highly efficient, they are characterized by non-specific reactions, resulting in the generation of false positives.

Immediately after its development, NASBA was used for the diagnostics of HIV infection in patients' blood serum [20]. Nowadays, this method is widely used for detecting *Salmonella* species, hepatitis viruses, papilloma viruses, and human enteroviruses. NASBA was also used for the detection of mRNAs and microRNAs [21, 22].

NASBA products are monitored by electrophoresis with ethidium bromide. Microfluidic devices and biochips using probes labeled with fluorescent dyes or peroxidase have also been employed [11, 21]. Thus, NASBA with peroxidase-like DNAzyme was used for identification of the classic swine fever virus strains [23]. NASBA has been also used in combination with the plate-based oligonucleotide analysis, e.g., for detection of the grass carp reovirus [24]. The developed technique was able to specifically detect 14 copies/μl within 5 h.

It must be mentioned that NASBA often produces false positive and false negative results. At the same time, it is less sensitive to the inhibitors present in biological samples than PCR [25].

Helicase-dependent amplification (HDA) is a PCR analogue, in which DNA duplex is dissociated by helicase instead of temperature increase (Fig. 1c) [26]. This technique uses helicase, DNA polymerase, and proteins binding single-stranded DNA. In the first step of the amplification cycle, helicase molecules attach to both ends of the double-stranded DNA, which results in the duplex dissociation. The forward and reverse primers hybridize with the single-stranded DNA released from the duplex and stabilized with the DNA-binding proteins. This is followed by primer elongation by DNA polymerase. In the end of the first HDA cycle, two DNA molecules are formed. This process is repeated multiple times to increase the analyte concentration. HDA has the exponential kinetics and can produce up to 10^7 copies of the analyte [27]. HDA is carried out either at 37°C or within the temperature of 60 to 65°C. At the lower temperature, the mismatch repair protein MutL is used, while amplification at higher temperatures does not require the presence of this protein. Decreasing the amplification temperature results in higher amount of non-specific amplification products, which can cause generation of false positives [28].

The level of the off-target background amplification in HDA is higher than in PCR [29]. In order to minimize the primer/primer duplex formation, primer modification have been suggested [30]. Another approach to minimizing the background amplification involves the use of dimethyl sulfoxide, betaine, and sorbitol, which, unfortunately, could also inhibit DNA polymerase. High-molecular-weight crowding agents, such as polyethylene glycol, have been used for the same purpose, since they increase the efficiency of polymerase reaction while simultaneously reducing primer artifacts [28].

Comparison of HDA with fluorescence detection and RT-PCR revealed that RT-PCR has higher sensitivity, and the produced concentration dependencies are better linearized. In particular, the detection limit for the *M. tuberculosis* DNA in HDA was 1 fM vs 100 aM in RT-PCR [28].

HDA has been used for the diagnostics of bacterial and viral infections and detections of pathogens in water and food. HDA products are usually analyzed by electrophoresis with ethidium bromide. Lateral flow devices,

[1] In our opinion, the name NASBA commonly used in English-language scientific literature provides too little information on the principle of this method; we believe it is more appropriate to call it *isothermal nucleic acid sequence-based RNA amplification*.

biochips, and electrochemical biosensors have also been used. For example, HDA in combination with the lateral flow test was used by Tang et al. [31] for identification of *Salmonella typhimurium* in water samples with the detection limit of 100 CFU/ml.

HDA with electrochemical signal detection was used for the quantitative determination of *Salmonella* species [32]. The capture probe was immobilized on the electrode surface, while the signaling primer was modified with fluorescein. The conjugate of anti-fluorescein antibodies with horseradish peroxidase was used as a detection system. The detection limit was 10 DNA copies.

HDA was also used for the DNA chip-based detection of *Phytophthora kernoviae* pathogen in plant leaves [33] with the detection limit of 10 ng/ml. A combination of HDA and plate-based enzyme-linked oligonucleotide assay for identification and quantitative determination of *Karlodinium veneficum* and *Karlodinium armiger* was suggested and used for detection of *Karlodinium* spp. in seawater [34]. The detection limit of this technique was 50 CFU/ml.

In addition to the pathogen detection, HDA has been successfully used for the development of assays for microRNAs (cancer markers). Ma et al. [35] described a sensitive fluorescent technique for determination of miR-21 microRNA with the detection limit of 12.8 fM and linearity range from 100 fM to 10 nM.

Exponential amplification reaction of nucleic acids **(EXPAR)** [36] uses a probe consisting of two identical sequences complementary to the analyzed nucleic acid connected by the nickase recognition site (Fig. 1d). During the reaction, the analyte hybridizes with one of the probe copies forming two types of duplexes. The duplexes in which the analyte is attached to the probe 5′-end cannot be elongated by DNA polymerase and dissociate relatively rapidly at the temperature of EXPAR (60°C). In the other duplex, attachment of the analyte to the 3′-end of the probe leads to its elongation by DNA polymerase. Duplex formation generates the recognition site for nickase. Hydrolysis of the duplex by nickase results in its dissociation and release of analyte copy. Nickases widely used in EXPAR are Nt.BbvCI, Nb.BbvCI, AlwI, Nt.AlwI, Nb.BssSI, Nt.BsmAI, and Nb.BtsI.

In the next cycle, the analyte is elongated by DNA polymerase and the synthesized sequence is cleaved by nickase. The following commercially available enzymes are used in EXPAR: phi29 polymerase, Klenow fragment, and polymerases Vent and Bst lacking the 3′-5′ exonuclease activity. Although 55-60°C is the optimal temperature for EXPAR, this reaction can be also performed at 37°C. In the latter case, the use of Klenow fragment is preferable [36]. Higher amplification temperatures increase the efficiency and the specificity of EXPAR.

EXPAR provides exponential accumulation of the analyte molecules. This amplification technique can be used exclusively for amplification of short oligonucleotides with the production of up to 10^8 analyte copies [27].

Unlike other amplification methods that require high primer concentrations, EXPAR uses high concentration of the probe, which is two times longer than the analyzed sequence. This is the reason for possible probe dimerization resulting in the formation of non-specific amplification products. The extent of off-target background amplification depends on the probe structure. In particular, the presence of G- and A-rich fragments in the probe could promote background amplification due to the DNA polymerase binding to purine bases. The background amplification could be also due to the formation of a hairpin structure by the probe. So far, there is no efficient approach for suppressing background amplification in EXPAR [36].

The use of EXPAR for the detection of DNA oligonucleotides was first described in 2003 [37]. Later, EXPAR has been used for improving the sensitivity of fluorescence-based quantitative detection of a fragment of the *p53* gene mRNA with the detection limit of 10 fM and the working range from 10 fM to 10 nM [38]. A combination of colorimetric assay with gold nanoparticles and EXPAR was reported by Li et al. [39] for the analysis of microRNAs. The achieved detection limit was 46 fM; the linear range was 50 fM to 10 nM. EXPAR was also successfully employed for the detection of DNA methylation and RNA mutations.

EXPAR using aptamers was applied for the detection of protein molecules (thrombin, platelet-derived growth factor, and mucin 1) [36, 40]. This method was also used for the detection of mercury cations with the detection limit of 100 pM [41].

EXPAR technique was used for highly sensitive detection of the enzymatic activity for such enzymes as telomerase (in HeLa cells) [42], methyltransferase, and uracil-DNA glycosylase [36, 43].

Strand-displacement amplification (SDA) first suggested in 1992 is based on a cyclic reaction involving polymerization, cleavage, and displacement [44]. The principle of SDA is shown in Fig. 1e. In the first step, double-stranded DNA is denatured at 95°C (all further SDA steps are conducted at 37°C), which allows primers to anneal to both daughter strands. The 5′-ends of the primers contain nickase recognition site and do not interact with the DNA analyte. Klenow fragment (lacking the exonuclease activity) catalyzes elongation of the DNA sequence from the 3′-ends resulting in the formation of duplexes with the nickase recognition sites. Enzymatic hydrolysis with nickase leads to the generation of new 3′-end sequences, which initiates polymerization reaction with simultaneous displacement of the daughter strand of the analyzed chain. This process is repeated multiple times and results in the exponential accumulation of the analyzed sequence. In the case when only one primer is

used, amplification has a linear character. The efficiency of SDA is up to 10^7 analyte copies [27].

Like other amplification techniques, SDA is used for the detection of genomic DNA. Samples with numerous components, such as human blood containing bacteria and viruses, have been successfully analyzed by SDA, which indicates the potential of this technique in medical and biological studies.

Due to its high specificity, SDA was successfully employed for detecting single-nucleotide polymorphisms. In particular, identification of point mutations by the chemiluminescence method was described by Shi et al. [45]. The use of magnetic particles in this technique allowed to significantly decrease the detection limit (to 0.1 fM).

SDA can be used for detecting large RNA molecules, such as viral RNAs, mRNAs, and rRNAs, consisting of hundreds or even thousands of nucleotides. Zhao et al. suggested a two-step colorimetric and fluorescence technique that involves RNA cleavage by DNAzyme followed by SDA [46]. This method was also applied for quantitative determination of microRNA and cancer cells [47, 48] with the detection limit of 16 fM and linear range from 16 fM to 100 nM. The detection limit for Ramos cells was 45 cells/ml; the linear range was 45 to 1000 cells/ml.

A highly sensitive SDA-based technique for the telomerase activity detection was developed by Ding et al. [49]. This method used molecular beacon with fluorescence readout and allowed to determine telomerase activity in four HeLa cells.

In recent years, aptamers have been widely used for analytical purposes. Because aptamers are DNA/RNA oligonucleotides, there have been attempts to combine aptamer-based analytical methods with nucleic acid amplification techniques. The methods based on the aptamer interaction with thrombin and cocaine have been described that demonstrated significantly higher sensitivity due to the use of SDA [50, 51]. Since a large number of aptamers to various molecules exist, the suggested approach seems very promising.

Recombinase polymerase amplification (RPA) is another method of nucleic acid isothermal amplification [27, 52]. Beginning from 2006, when Niall Armes described RPA for the first time [53], the interest to this technique has been steadily growing. The first step of the amplification cycle involves complex formation between recombinase and forward and/or reverse primer (Fig. 1f). In the presence of a sequence complementary to the primer, recombinase unwinds the duplex, thus allowing primer interaction with the analyte. This reaction is facilitated by the DNA-binding proteins. In the presence of DNA polymerase with the chain displacement activity, the recombinase complex dissociates; DNA polymerase binds to the double-stranded DNA and elongates the primer at the 3′-end. The newly synthesized duplex serves as a template for the next cycle. When two primers are used (forward and reverse), two semi-conserved duplexes are formed in the reaction, i.e., RPA has the exponential character. When one primer is used, amplification is linear.

RPA can be conducted at 22-45°C, although its optimal temperature range is 37-42°C [52]. Both single- and double-stranded DNAs, as well as methylated DNA, can be used for amplification. RPA can be performed in the presence of PCR inhibitors, such as heparin, ethanol, and hemoglobin [53], which allows to conduct amplification directly in biological samples (milk, urine, feces, pleural fluid) following thermal lysis [54]. At the same time, RPA is inhibited by some detergents (e.g., SDS and CTAB).

RPA can be conducted either in homogenous or heterogeneous media. In the case of heterogeneous amplification, one or both primers are immobilized on a solid support. Despite the high rate of homogenous amplification, heterogeneous amplification is used more widely, because in the majority of cases, it prevents the matrix effect and allows to develop more sensitive analytical techniques. It has been also reported that heterogeneous RPA reduces non-specific amplification [55].

Despite the fact that RPA is fast and sensitive, high background signal often presents a problem for its implementation. In order to eliminate this disadvantage, the primer contains the site for the specific *E. coli* IV endonuclease (Nfo), which recognizes and cleaves the duplexes [52]. This primer can be used in the DNA polymerase-catalyzed elongation stage only after its cleavage by the endonuclease creating a hydroxyl group at the 3′-end. The primer can be conjugated with a fluorophore and quencher; in this case, its cleavage will be accompanied by the fluorescence signal increase. Endonuclease-mediated cleavage serves as an additional step to decrease the background signal in RPA. RPA and PCR are comparable in their efficiency, but RPA is positioned as the fastest among all other amplification techniques [54].

RPA can be used in combination with various signal readout methods for detection of different pathogens, e.g., *Yersinia pestis* [55]. In this study, the forward primer was immobilized in the wells of enzyme immunoassay microplate, and the reverse primer was modified with biotin for binding the peroxidase-streptavidin conjugate. The achieved detection limit was 0.3 fM, with the colorimetric peroxidase substrate 3,3′,5,5′-tetramethylbenzidine; the linear range was 10 fM to 10 nM.

RPA is often used in combination with electrochemical detection techniques. Using this approach, Ng et al. [56] developed an amperometric biosensor that allowed DNA detection from 1 CFU of *M. tuberculosis*. In another electrochemical method, ruthenium complex $[Ru(NH_3)_6]^{3+}$ was used as a mediator, which intercalated into the double-stranded DNA [57]. The detection limit of this technique was 11 CFU/ml.

Chemiluminescence, fluorescence, and Raman scattering have been used as alternatives to the electrochemical detection. Recently, a bandage-like wearable flexible RPA-based fluorescence sensor was developed for detecting Zika virus DNA in real time [58].

Another common amplification technique is **rolling circle amplification (RCA)** developed in 1995 [59]. This method is based on using a circular DNA (C-probe) formed by the interaction of the analyzed sequence with the single-stranded DNA probe flanked by the sequences complementary to the analyte sequence. In the process of complex formation, the 5′- and 3′-ends of the probe are brought together and then ligated to form a circular molecule (Fig. 1g).

This circular DNA hybridizes with the primer, which is elongated by DNA polymerase, resulting in the generation of a sequence consisting of numerous copies of analyte DNA.

DNA polymerases most often used in RCA are phi29 and Bst. Usually, RCA is carried out at 30-37°C [60]. Linear amplification at constant temperature takes from several hours to several days and results in the synthesis of multiple analyte copies. The efficiency of RCA is estimated as 10^3 analyte copies.

Recently, RCA modification was reported that involves addition of the second primer that hybridizes with the sequence elongated from the first primer, thus making RCA exponential [60].

RCA is widely used for the detection of bacterial and viral DNAs/RNAs and microRNAs. Moreover, RCA is highly specific due to the use of DNA ligase that catalyzes ligation only in the case of perfect complementarity of the 3′- and 5′-end sequences [61, 62]. For this reason, RCA can be used for detecting single-nucleotide polymorphisms [63].

Schopf et al. used RCA for the detection of *M. tuberculosis* genomic DNA [64]. In the first step, DNA was treated with restriction endonucleases and denatured at high temperature. Next, DNA fragments were immobilized by hybridization with capturing oligonucleotides covalently bound to the Sepharose gel particles. Following RCA, fluorescently labeled DNA fragments complementary to the tested DNA were added. The detection limit of this method was 4.3 fM for DNA and 10^4 CFU/ml for *M. tuberculosis*. RCA products can be also monitored by intercalation of the SYBR Green dye into the DNA duplex. The detection limit for the miRlet-7a microRNA was 10 fM; the linear range was 25 fM to 1 pM [65]. The use of RCA allowed visualization of the let-7 microRNA family directly in live A549 lung cancer cells [66].

DNA polymerization in RCA can be monitored by pyrophosphate accumulation in the reaction medium. In the study by Mashimo et al. [67], pyrophosphate was converted to ATP by adenylyl transferase, which was followed by ATP quantification by the bioluminescence method using luciferase. The detection limit for the model RNA analyte was 2 pM; the linear range was 2 pM to 1 nM.

RCA was used to increase the sensitivity of the methyltransferase activity assay [68]. The detection limit of the developed method was $8.1 \cdot 10^{-5}$ units/ml; the linear range was between $4 \cdot 10^{-4}$ and $1 \cdot 10^{-2}$ units/ml. RCA was also successfully used for detecting DNA methylation [69].

RCA technique can be used for increasing the analytical signal via increase in either analyte concentration or amount of detected probe.

All amplification techniques that increase of analytical signal via increase in the analyte concentration employ DNA polymerases that are susceptible to the inhibitors present in the tested samples, which might often lead to the generation of false negatives. On the other hand, the off-target background amplification caused by dimerization of primers/probes could be the cause of false positive results. These drawbacks can be minimized by using detection methods that are highly specific to the analyzed sequence.

All the amplification techniques presented above are based on the use of specific sequences. However, isothermal amplification techniques using random primers have also been developed, including **whole genome amplification (WGA)** used to increase the amount of DNA for its sequencing.

The first variant of WGA termed **multiple displacement amplification (MDA)** was suggested in 2001 [70]. This method uses random hexamer primers interacting with the circular genomes, which results in the formation of multiple replication forks. MDA uses phi29 DNA polymerase characterized by high processivity and low error frequency. The cascade of MDA reactions results in the exponential accumulation of double-stranded DNA and 10^4-fold increase of the plasmid DNA concentration within several hours.

In 2002, MDA was adapted for the amplification of linear genomes (Fig. 2a). This technique allows to synthesize ~20-30 μg of DNA with an average length of ~10 kb from 1-10 copies of human genomic DNA, which can be further used for sequencing and genotyping.

An alternative to WGA is **primase-based whole genome amplification (pWGA)**, which imitates replication of the T7 bacteriophage DNA *in vivo* [71]. Genomic DNA is unwound by the bifunctional T7 gp4 protein exhibiting the activities of helicase and primase, which is followed by the synthesis of RNA primer complementary to the single-stranded DNA (Fig. 2b). DNA is synthesized by the highly processive T7 DNA polymerase. pWGA generates 1-10 ng of human genomic DNA within 1 h at 37°C. Circular DNAs can also be used as templates. Using only 100 DNA copies, the amplification coefficient may reach 10^8. Also, pWGA does not require thermal denaturation of genomic DNA (unlike MDA).

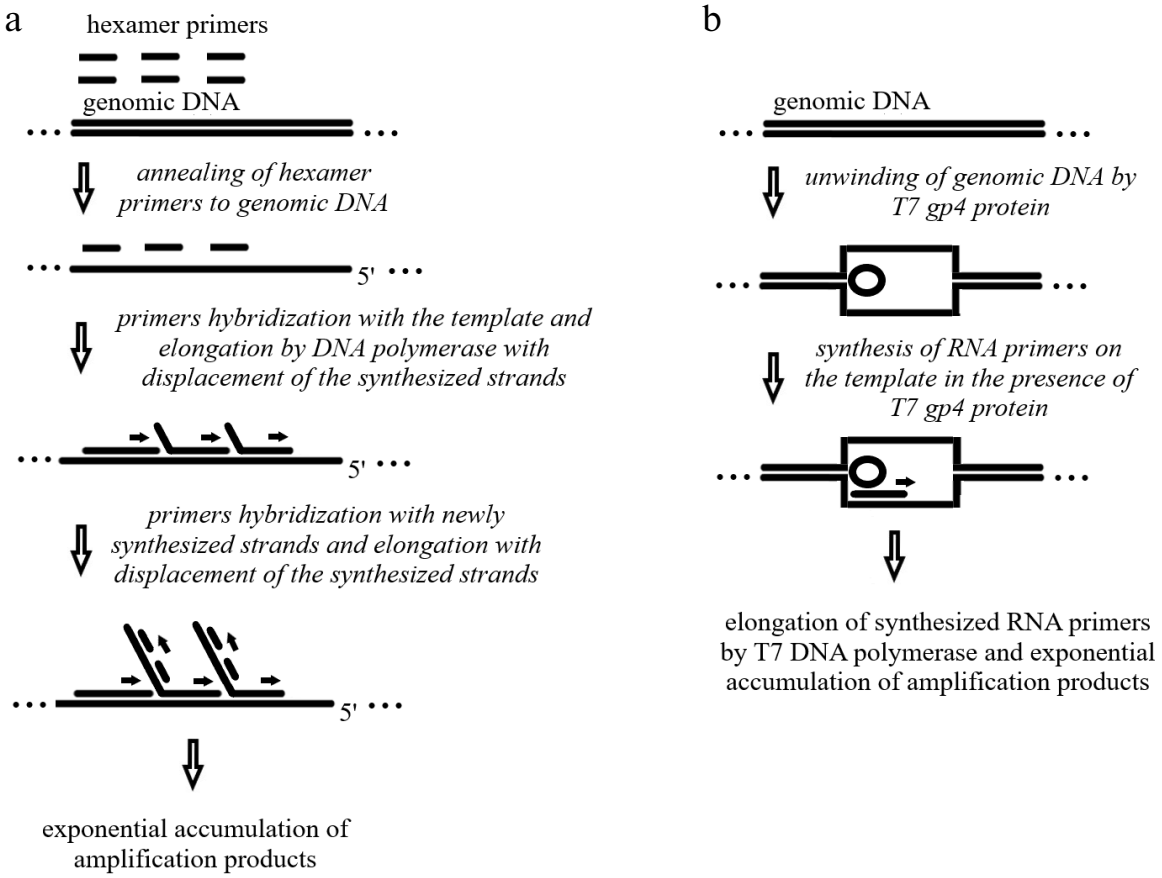

Fig. 2. WGA techniques: a) MDA; b) pWGA.

AMPLIFICATION TECHNIQUES THAT PROVIDE INCREASE IN THE ANALYTICAL SIGNAL WITHOUT INCREASING ANALYTE CONCENTRATION

Isothermal amplification techniques that provide an increase in the analytical signal without changing the analyte concentration usually have linear amplification kinetics. Some of them use enzymes; other do not, which makes these methods less expensive and eliminates the drawbacks typical for enzymatic assays. Both types of methods will be presented below, as all of them have their own advantages and disadvantages and can be used for development of analytical procedures.

One of the isothermal amplification techniques that does not change the analyte concentration is **exonuclease III-assisted signal amplification (EASA)** [7, 72]. Exonuclease III catalyzes step-wise deletion of mononucleotides from the 3′-hydroxylated ends of double-stranded DNAs by hydrolyzing the phosphodiester bond [73], i.e., displays the non-specific 3′-5′ exonuclease activity. The substrates for this enzyme are DNA molecules with blunt or recessed 3′-ends. The substrates with 3′-overhanging sequences of four or more nucleotides are not cleaved by exonuclease III.

In 2010, Plaxco et al. [74] described the principle of EASA for the first time (Fig. 3). This method involves hybridization of the analyzed nucleic acid with a DNA probe that forms a double-stranded structure with blunt ends. This allows stepwise removal of mononucleotides from the probe 3′-end by exonuclease III. As a result of enzymatic hydrolysis, the analyte molecule is released and can interact with another probe, which initiates the next amplification cycle. Hence, one analyte molecule could generate a large number of molecules formed by the probe hydrolysis. If the probe is modified with a label, the recorded analytical signal will be amplified in the course of reaction. EASA is usually carried out at 25 or 37°C.

Later, similar amplification techniques were developed using T7 and λ exonucleases [75], which, unlike exonuclease III, cleave mononucleotides from the 5′-end of the probe (but not from the 3′-end).

In the pioneer study [74], EASA was used for quantification of a model DNA oligonucleotide using molecular beacon containing fluorescence dye at the 5′-end and quencher on one of the nucleotides located in the inner

region of the beacon sequence. The probe forms a hairpin structure by self-hybridization, in which the overhanging end is resistant to hydrolysis by exonuclease III. The fluorophore in the resulting hairpin structure is close to the quencher, thus producing very weak fluorescence signal. In the presence of DNA analyte, the hairpin structure opens with the formation of a double stranded structure with the blunt 3′-end, which allows removal of mononucleotides by exonuclease III and subsequent fluorophore release accompanied by the increase in fluorescence. Simultaneously, released analyte interacts with another beacon molecule, thus initiating the next EASA cycle. Therefore, EASA has facilitated the development of a simple fluorescence technique for detection of DNA analytes.

Later, numerous studies have been published [76-80], where EASA was used to increase the sensitivity of nucleic acid assays employing fluorescence, electrochemical, colorimetric, and chemiluminescence readouts.

The EASA method for quantitative determination of mercury ions with the detection limit of 1 pM Hg^{2+} and linear range from 10 pM to 100 nM was developed based on Hg^{2+} interaction with thymine [79]. A combination of EASA and aptamers has allowed to develop the detection methods for ATP, lysozyme, and thrombin.

Some publications on the nucleic acid detection have reported the detection limits at the femto- and even attomolar levels. Such low detection limits for EASA should be considered with caution, because the authors of the pioneer study [74] noticed that exonuclease III exhibits considerable catalytic activity toward single-stranded DNA sequences, which should result in the enzymatic hydrolysis of the analyzed sequence. The existence of this side activity was also mentioned by other authors [80, 81]. Moreover, we demonstrated that exonuclease III was able to hydrolyze DNA molecules with the G-quadruplex structure (unpublished data). Other exonucleases also catalyze such side reactions. This fact significantly limits further development of amplification techniques using these enzymes.

EASA was used to increase the sensitivity of several enzymatic assays (e.g., for T4 polynucleotide kinase, methyltransferase, and telomerase [82-84]) that employ either exonuclease III or λ exonuclease.

One of the novel isothermal amplification techniques is the **method with formation of Y-junction probes** [7]. These structures are formed from two partially complementary probes (usually 4-6 bp) that do not form duplexes because of only partial complementarity. At the same time, they can form stable Y-shaped complexes in the presence of analyzed sequence that is partially complementary to both probes (Fig. 4a). Such complexes also contain a restriction endonuclease site. Addition of the respective endonuclease to the reaction mixture results in the enzymatic cleavage of the duplex with the following dissociation of the Y-shaped structure and release of the analyzed sequence, which is used for the formation of a new Y-shaped complex. The formed Y-shaped structure participates in the next amplification cycle, eventually leading to the formation of multiple fragments of the hydrolyzed probes. The availability of a large number of restriction endonucleases (~3500) facilitates the design of various Y-shaped probes with different restriction endonuclease sites. Sintim et al. [85] demonstrated that the probe architecture significantly affects the rate of enzymatic hydrolysis of the Y-shaped probes, which should be considered in their design. Nickases have been successfully used by some researchers instead of restriction endonucleases for the hydrolysis of Y-junction probes [86]. The amplification reaction in this method is conducted at 25-37°C.

As follows for Fig. 4a, amplification technique with the use of Y-shaped probes can be employed for the detection of DNA, RNA, and analytes of other chemical

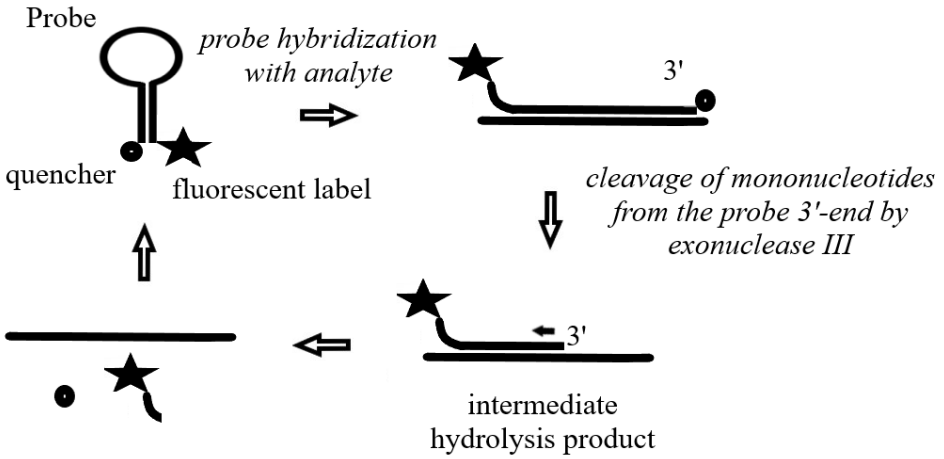

Fig. 3. Quantitative DNA oligonucleotide assay using EASA technique.

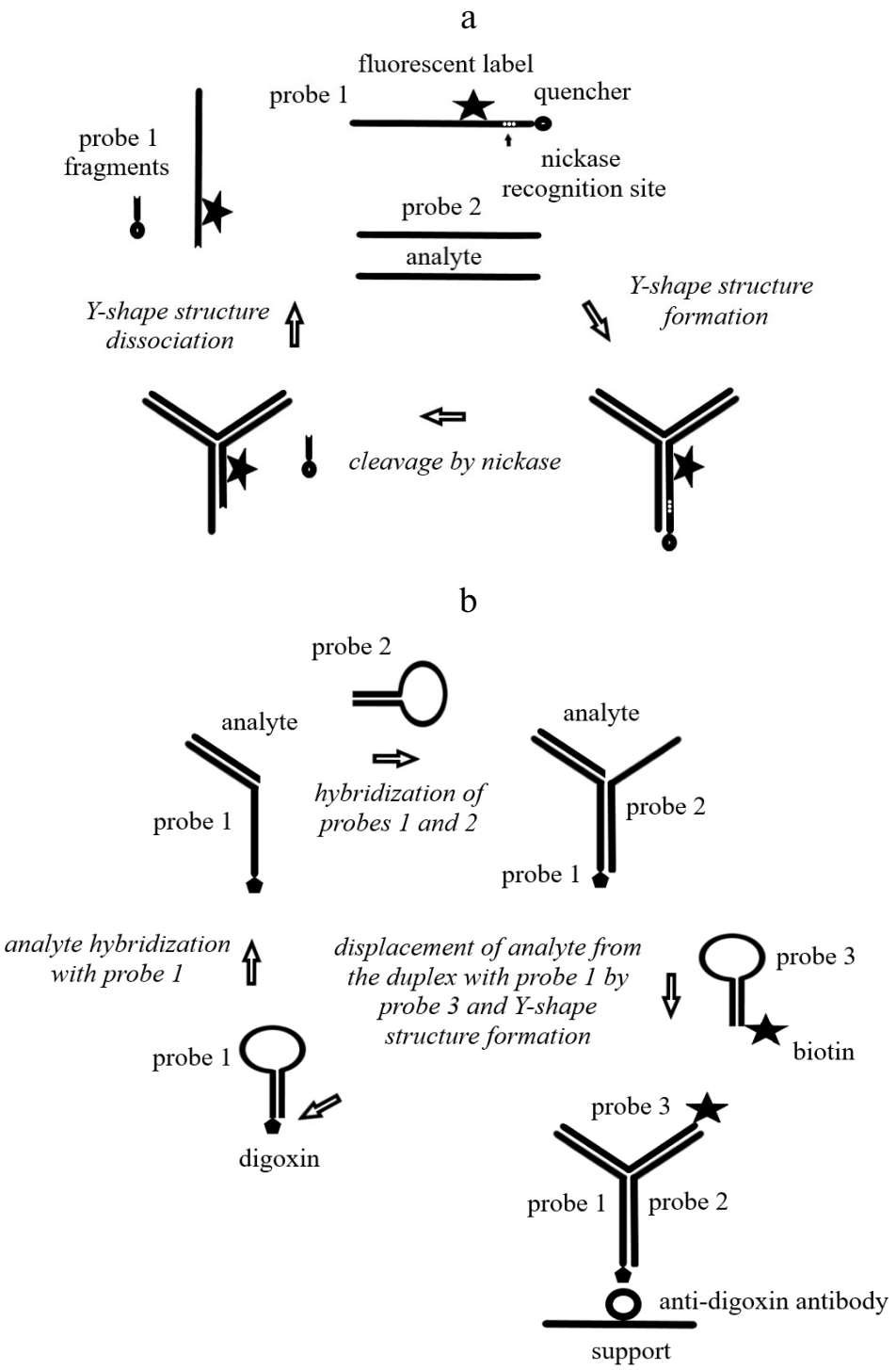

Fig. 4. Isothermal amplification with the formation of Y-shaped structures: a) method based on the formation and following enzymatic cleavage of Y-shaped probe; b) enzyme-free method.

nature (such as antibiotics) using aptamers as recognition elements [87].

The electrochemical method for determination of a 28-mer DNA model nucleotide using Y-junction probes was developed in [88]. In this study, Y-probes were cleaved with exonuclease HaeIII. To monitor Y-probe cleavage, one of its sequences immobilized on the electrode surface was conjugated with methylene blue. Because methylene blue was located at a distance from the electrode surface, it was not electrochemically oxi-

dized. As a result of probe hydrolysis, methylene blue acquired the ability to migrate towards the electrode surface, where it was oxidized, thus increasing the recorded current that was proportional to the analyte concentration. The detection limit of this assay was 14 pM.

The use of Y-junction-based method in combination with lateral flow device with colorimetric readout for detection of miR-16 microRNA was described in [89]. In this study, the amplification reaction was conducted in a homogeneous medium. The Y-junction was formed by the interaction of the molecular beacon, assistant DNA oligonucleotide, and miR-16. The molecular beacon in the composition of the formed Y-junction was cleaved into two fragments by Nt.BbvCI endonuclease, which enabled interaction of the assistant oligonucleotide and miR-16 with the next molecular beacon molecule, after which the process repeated again. The concentration of the beacon fragments was determined with the lateral flow biosensor. The detection limit of this method for miR-16 was 0.1 pM; the linear range was 0.1 pM to 10 nM.

A modification of the Y-junction amplification technique that did not require the use of the enzyme was suggested in [90]. In the first step, the analyte molecule formed via complementary interactions a complex with two hairpin structures, one of which was conjugated with digoxin (Fig. 4b). The hairpins were incapable of hybridization in the absence of the analyte. Next, the Y-junction was formed upon addition of the third biotin-labeled hairpin that replaced the analyte molecule in the complex. The released analyte formed a complex with hairpins 1 and 2, thus initiating the next amplification cycle. As a result, the final concentration of the produced Y-junctions significantly exceeded the analyte concentration. The concentration of the formed Y-junction containing digoxin and biotin was monitored electrochemically. For this purpose, Y-junctions were immobilized on the electrode surface via interaction with the attached anti-digoxin antibodies, followed by addition of 3,3′,5,5′-tetramethylbenzidine and horseradish peroxidase conjugates with streptavidin, which enabled to record electrical current proportional to the concentration of Y-junctions. The detection limit for the model DNA oligonucleotide was 10.9 aM; the linear range of the assay was 100 aM to 1 μM.

Another amplification method employing nickases is based on the use of specific duplexes [91] (Fig. 5). The analyzed sequence forms a specific duplex with a DNA probe simultaneously creating a site recognized by one of the nickases. The probe is modified with a label, thus enabling evaluation of the extent of probe hydrolysis by the enzyme. Next, specific nickase cleaves the probe, which results in the duplex dissociation. The released analyte molecule interacts with the next probe, and the process repeats multiple times. As a result, one analyte molecule can generate of a large number of probe fragments, which leads to the increase in the recorded signal. The reaction should be conducted at rather high temperatures to ensure fast dissociation of the cleaved probe, but the temperature is limited by the thermal stability of nickase. The temperature optimum of the reaction with Nt.AlwI nickase was 58°C [91].

Depending on the label incorporated into the probe, different method of signal recording can be applied. For example, Lin et al. [92] used a hairpin DNA probe with a heme-binding aptamer in one of the chains of the hairpin stem. The duplex formed by the probe with the analyzed DNA sequence (19-mer oligonucleotide of the *p53* gene) contained the Nt.BstNBI nickase cleavage site. Enzymatic hydrolysis of the duplex released the heme-binding aptamer, and addition of heme to the reaction mixture resulted in the formation of catalytically active peroxidase-like DNAzyme, whose activity was monitored by oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) in the presence of hydrogen peroxide. The analyte released from the duplex interacted with the next probe molecule. The detection limit for the *p53* gene fragment was 1 pM; the working range was 1 to 100 pM.

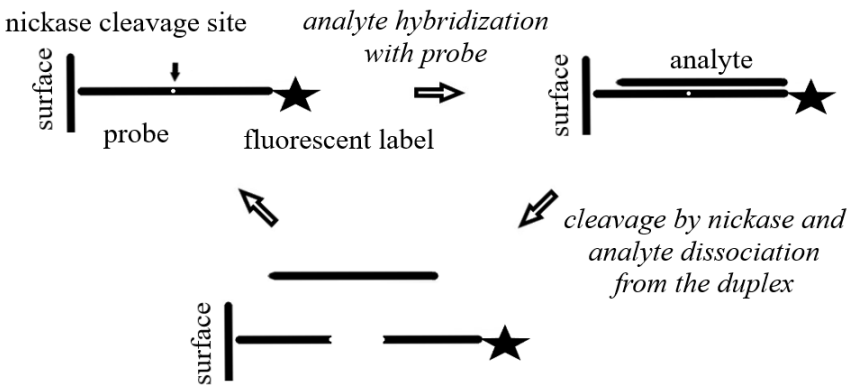

Fig. 5. Amplification based on the formation of DNA duplexes with their subsequent enzymatic hydrolysis by nickase.

This amplification technique was also used in combination with the electrochemical method of nucleic acid detection. Chen et al. [93] reported generation of a hairpin DNA probe with covalently bound ferrocene, which was immobilized on the surface of a gold electrode. Following enzymatic cleavage of the site formed by the probe interaction with the analyte, the fragments of the ferrocene-modified probe were released from the electrode surface. The detection limit for the DNA analyzed with this electrochemical biosensor was 68 aM; the linear range was 0.1 to 100 fM.

Recently, the use of the Nt.AlwI nickase-based amplification technique for quantitative analysis of mercury cations was reported [94]. Using formation of the thymine–Hg^{2+}–thymine complex as an indicator reaction, Vijayan et al. have developed an assay with the detection limit of 0.14 nM that can be used for determination of mercury in drinking water. Aptamer-based nickase-mediated amplification methods have been also designed for determination of lysozyme, carcinoembryonic- and prostate-specific antigens, and mucine-16 in the human blood serum [95].

This type of amplification is characterized by a high specificity. All the more surprising is the fact that the number of publications on this amplification method is much smaller than for other isothermal amplification techniques.

The new **isothermal circular strand-displacement polymerization (ICSDP)** method was suggested in 2009 [96] (Fig. 6). This technique uses a hairpin DNA probe, short primer, and DNA polymerase. The probe is modified with a label allowing evaluation of the probe concentration. The probe 5′-end forming the stem and a fragment of the loop are complementary to the analyzed sequence, which leads to their specific interaction resulting in the duplex formation and opening of the hairpin structure. The primer (usually, 8-nucleotide long) is complementary to the 3′-end of the probe stem. In the absence of the analyte, the primer does not interact with the probe, while in the case of duplex formation, the primer binds to the released 3′-end of the probe.

The binding of the primer initiates its elongation in the presence of DNA polymerase and deoxynucleoside 5′-triphosphates. The synthesized sequence displaces the analyte, resulting in its release from the duplex and hybridization with another probe molecule, which initiates the next polymerization/displacement cycle. Hence, the analyte molecule acts as a trigger of polymerization reaction. The accumulation of the duplex consisting of the probe and the synthesized sequence occurs in parallel with the probe consumption; the concentration of the duplex can be measured by various physicochemical methods. Therefore, ICSDP (commonly carried out at 37°C) provides signal amplification at a constant analyte concentration.

Guo et al. [96] used a molecular beacon modified with fluorescein (label) and DABCYL (quencher) as a probe. The polymerization reaction was catalyzed by the Klenow fragment. The detection limit of the developed ICSDP assay for the model 26-mer DNA oligonucleotide was 6.4 fM.

A similar principle was used for developing an assay for the miR-210 microRNA [97] with the detection limit of 50 pM. However, the linear range of this method was rather narrow (from 330 pM to 1.66 nM). The developed assay was used for the analysis of miR-210 in the transfected K562 cells.

ICSDP was also used for the development of heterogeneous techniques for the nucleic acid determination. Thus, Gao et al. [98] immobilized a ferrocene-modified

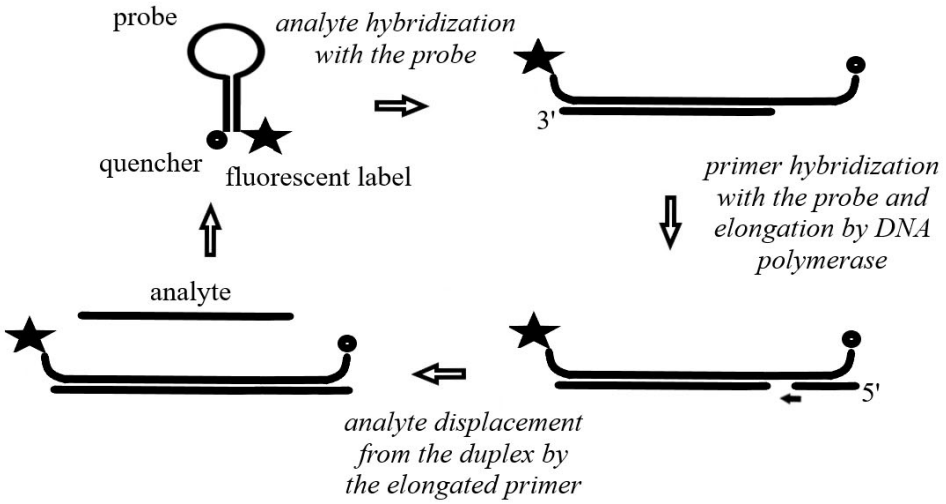

Fig. 6. Isothermal circular-strand-displacement polymerization (ICSDP).

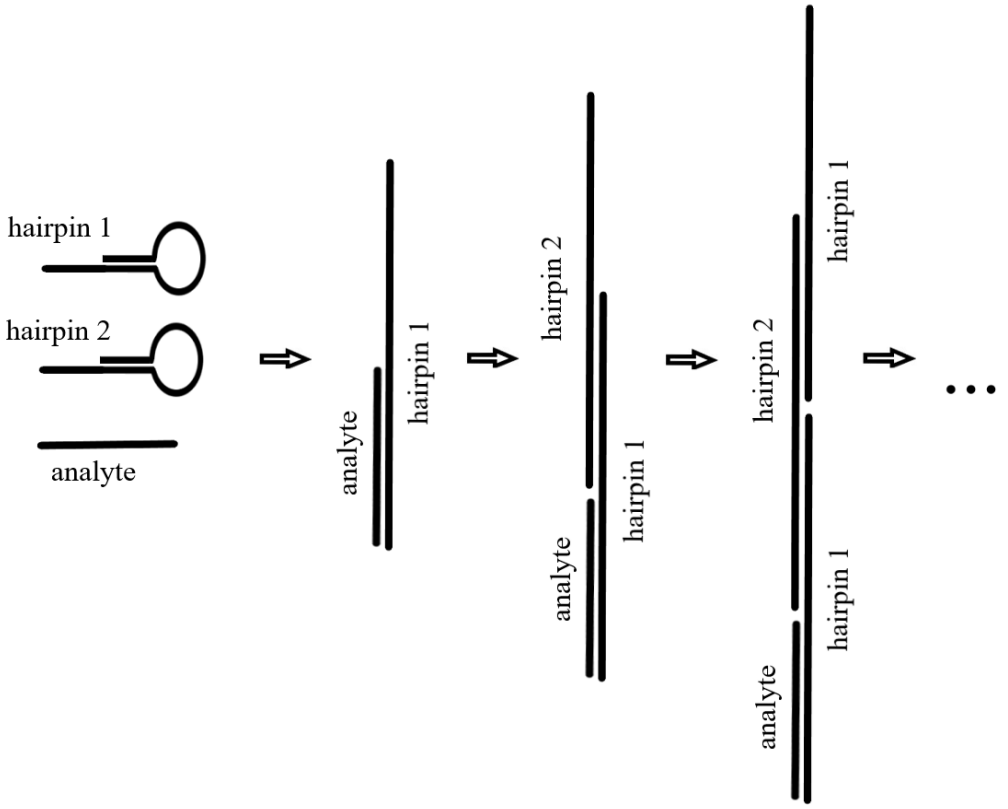

Fig. 7. Enzyme-free isothermal amplification technique that uses hybridization chain reaction (HCR).

hairpin sequence on the surface of gold electrode. In the presence of analyte, this hairpin opened exposing a methylene blue-modified fragment complementary to the primer sequence. After hybridization, the primer was elongated by DNA polymerase with the displacement activity. The detection limit for the model DNA analyte (31 nucleotides) with the developed electrochemical biosensor was 28 fM; the working concentration range was 100 fM to 10 nM. A similar approach was used for the detection of the *mecA* gene in methicillin-resistant strains of *Staphylococcus aureus* [99].

It must be mentioned that because of the use of DNA polymerase, ICSDP can be sensitive to DNA polymerase inhibitors, which could lead to false negative results. At the same time, the background amplification caused by the non-specific hybridization could lead to false positives.

Unlike the isothermal nucleic acid amplification techniques described above, **hybridization chain reaction (HCR)** does not used enzymes. This method was developed by Dirks and Pierce in 2004 [100]. HCR is based on the formation of a double-stranded DNA as a result of interaction between two hairpin sequences, which is initiated by the addition of DNA/RNA analyte (Fig. 7). The structures of hairpins 1 and 2 are designed in such a way that the complementary interactions between them are kinetically obstructed. The duplex formation between hairpin 1 and analyzed sequence results in the exposure of the hairpin 1 stem, which is not participating in the duplex formation, which allows this single-stranded fragment to interact with hairpin 2. This leads to the duplex formation between the two hairpins and simultaneous exposure of the uninvolved stem of hairpin 2, which interacts with another hairpin 1 molecule. Introduction of labels in the hairpin structure or dyes that can intercalate into the duplex makes it possible to monitor the formed DNA. Therefore, HCR produces double-stranded DNA with nicks in each chain, and the length of this molecule is determined by the amount of hairpin structures in the reaction mixture. Usually, HCR is carried out at 25 or 37°C.

In order to enhance amplification, new methods have been developed that use hairpins with two or more loops, which allows production of branched double-stranded DNAs [101]. A modification of the HCR technique named hyperbranched HCR that uses four hairpins and two additional single-stranded DNAs has been suggested. The amplification kinetics in the hyperbranched HCR is exponential [102]. Therefore, the analyte in the HCR can initiate formation of DNA duplexes with nicks in both chains; the increase in the analytical signal intensity is achieved due to a large number of incorporated labels in the structure of the formed DNA.

Bioanalytical applications of HCR often use biotin-labeled hairpins (one or two). For example, in the study

by Yang et al. [103], model DNA analyte initiated HCR resulting in the formation of biotin-containing duplex, as one of the used hairpins was modified with biotin. Due to the presence of biotin, this duplex was capable of interacting with streptavidin adsorbed on magnetic particles. Because not all biotin molecules were involved in the duplex immobilization on magnetic particles, the remaining biotin residues was used for the reaction with the avidin–glucose oxidase conjugate. Glucose oxidation by glucose oxidase resulted in the formation of hydrogen peroxide that corroded the surface of silver nanoparticles, which was recorded with the surface plasmon resonance technique. The detection limit for the used analyte was 6 fM; the linear range was 10 fM to 100 pM.

HCR was used in the design of electrochemical biosensor for determination of microRNAs in the lysates of HUVEC, HK-2, HeLa, and MCF-7 cells [104]. The reached detection limit for the Hsa-miR-17-5p microRNA with a voltamperometric readout was 2 aM; the linear range was 100 aM to 100 pM.

It was demonstrated that HCR could be effectively used for visualization of intracellular RNAs. Thus, Wu et al. [105] suggested a fluorescence method for evaluation of mRNA expression in a picomolar concentration range. It should be also mentioned that this technique does not require the use of reverse transcription.

HCR application for the analysis of mercury ions, ATP, and proteins has been also reported. Guo et al. [106] used α-fetoprotein and prostate-specific antigen as model biomarkers. In the presence of analytes, immune complexes were formed on the electrode surface. The secondary antibodies were covalently bound to the DNA oligonucleotide that initiated HCR. The detection limit of this method with voltamperometric readout was 0.25 pg/ml for α-fetoprotein and 0.17 pg/ml for the prostate-specific antigen.

HCR was successfully used for identification of tumor cells [107]. In this case, the method was based on the interaction between aptamers and specific tumor markers located at the tumor cell surface.

Another technique that does not require the use of enzymes is **catalytic hairpin assembly (CHA)** (Fig. 8). CHA is based on the use of two oligonucleotide hairpins [108]. The first hairpin forms a duplex with the analyte via complementary interactions. The hairpin 1 sequence complementary to the sequence of the hairpin 2 tail becomes available for the interaction. The structure of the used hairpin probes is modeled in such a way that they are complementary to each other, but their interactions are hindered kinetically.

Newly accessible sequence interacts with hairpin 2, which is accompanied by the analyte displacement from the original duplex and initiates the next amplification cycle. Hence, one analyte molecule can initiate formation of a large number of duplexes. It is very important in the CHA to prevent the interaction between the hairpins in the absence of analyte, because this can increase the background signal, thus decreasing the method sensitivity. Although CHA is similar to HCR in the absence of need for the enzyme, the increase in the intensity of the analytical signal in CHA is achieved via multiple participation of the analyte molecule in the indicator reaction.

At present, the majority of studies using CHA aim at the development of methods for the analysis of microRNAs, which are considered as promising biomarkers in the diagnostics of oncological disorders [109]. CHA products can be monitored using various physicochemical methods. For example, Zhang et al. [110] used electrophoresis for the CHA-mediated detection of miR-21 microRNA with the detection limit of 10 pM. CHA with electrochemical detection was used by Shuai et al. [111]. In this study, hairpin 1 was immobilized on the electrode surface and hairpin 2 was modified by the biotin/streptavidin conjugate with alkaline phosphatase. The detection limit of this method for miR-21 was 50 aM; the linear range was 0.1 fM to 100 pM.

Jiang et al. [112] employed homogenous fluorescence assay for the detection of miR-let-7a microRNA using molecular beacon and CHA for signal amplification with the detection limit of 1 pM. The linear range of the method was 1 pM to 2 nM.

It must be mentioned that some authors use a combination of two different amplification techniques in order to further increase the assay sensitivity. These com-

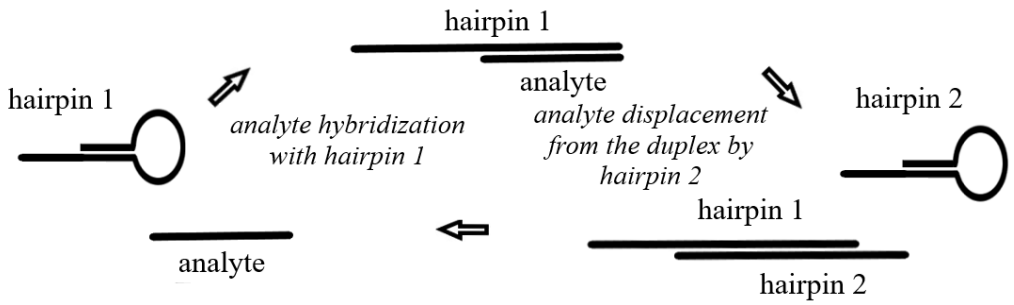

Fig. 8. Enzyme-free isothermal amplification technique using catalytic hairpin assembly (CHA) approach.

binations were termed cascade amplification methods [24]. For example, Dong et al. [113] and Xu et al. [114] used nickase-assisted amplification technique in combination with CHA and RCA, respectively. A combination of RCA and EASA was employed for the identification of genetically modified soybean (MON89788) DNA with the detection limit of 45 aM [115]. In order to increase the sensitivity of RCA, it was combined with CHA; however, the detection limit in this case was insufficient (100 fM) [116].

A fluorescence biosensor developed for the detection of bisphenol A was used in a combination of Y-junction method and EASA [117]. The detection limit for bisphenol A was 5 fM; the linear range was 10 fM to 10 nM. Sun et al. [118] reported the method for adenosine determination based on HCR and EASA. In some cases, the same technique was applied several times to amplify various intermediate compounds [119].

In this review, we presented the data on the techniques for isothermal amplification of nucleic acids that have been developed as an alternative to PCR. Nowadays, these methods are widely and successfully used in bioanalysis to improve detection and to increase the sensitivity of quantitative determination of various compounds, such as nucleic acids and other substances (proteins, enzymes, antibiotics, narcotics, etc.), whose detection is based on the use of DNA/RNA aptamers. The majority of methods involving nucleic acid isothermal amplification are highly sensitive; hence, many analytes can be detected at femto- and picomolar concentrations. Some described methods were able to detect analyzed compounds at the attomolar concentrations. Such high sensitivity is sufficient for identification of virtually any analyte of interest in biological samples.

As the same time, there is a significant number of issues that should be addressed in future research, mostly related to the generation of false positive and false negative results in the analysis of biological samples. Another important issue that should be resolved is that the detection limits of the methods developed by different research groups using the same amplification and detection techniques can differ by several orders of magnitude. The reasons for such discrepancies must be identified in the nearest future. It should be mentioned that many of the described methods with low detection limits have low sensitivity, which prevents their use for the determination of analytes, whose concentration differs only slightly in norm and pathology (e.g., miR-21 microRNA) [120]. The publications on the use of analytical amplification techniques for quantification of biomarkers in biological samples are scarce; in most studies, model analytes in buffer solutions were analyzed. All of the above indicates the need for continuing research on the applicability and improvement of bioanalytical techniques based on isothermal amplification, as well as development for practical applications of highly sensitive, accurate, and selective assays on their basis.

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