Application strategies of peptide nucleic acids toward electrochemical nucleic acid sensors

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1.Target amplification

As we all known, the more probes and targets involved in hybridization, i.e. the larger the hybridization scale, the higher the signal intensity generated by hybridization. In most cases, the amount of probe is sufficient, but the concentration of target is too low to produce obvious hybridization signal for accurate detection. Increasing the target concentration or increasing the amount of immobilized probe under the premise of the optimal probe density is an effective method to solve this problem. Various amplification methods, such as PCR, RCA and LAMP, are the main means to increase the target concentration.

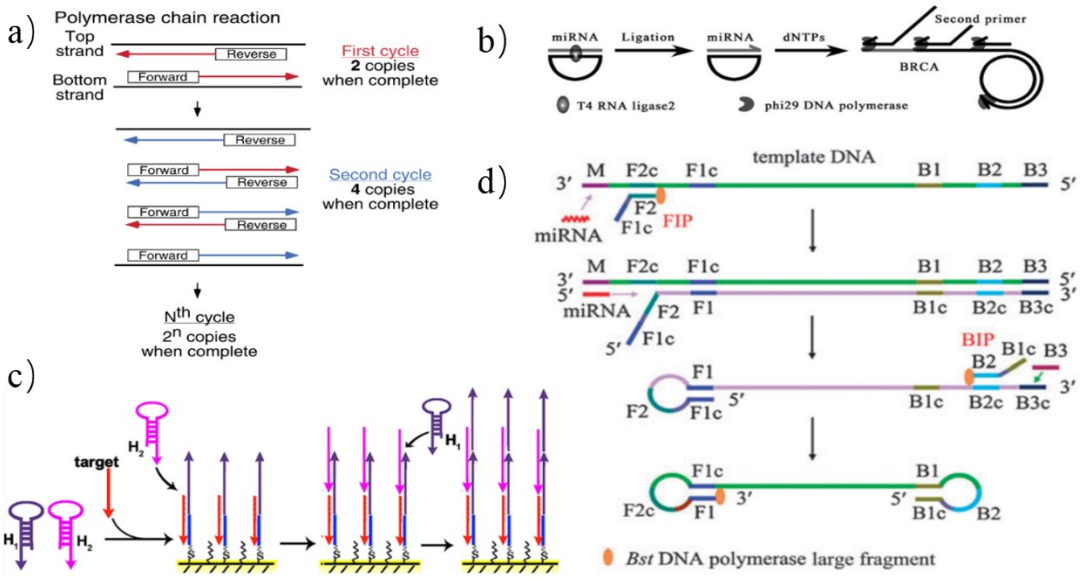

Fig.1 Target amplification. (a) Amplification process of PCR(1); (b) Illustration of the target-primed

BRCA reaction(2); (c)The Hybridization chain reaction(HCR) process(3); (d)Schematic representation of the LAMP reaction initiated by the target miRNA(4)

1.1 Polymerase chain reaction

PCR was developed by Kary Mullis in the 1980s, which allows to selectively amplify target sequence(5, 6). The typical PCR amplification process includes two main parts: thermal denaturation and annealing. After heating(95℃), the nucleic acid duplex will be dissociated into two single strands. Under the action of primers and enzymes, the disintegrated chain will be used as a template to synthesize a new sequence. Repeat the above procedures to achieve target amplification(1), and the scheme was shown in Figure 1a. The enzyme should be heat resistant.

In the past years, PCR has been used to push forward the detection of low-concentration oligonucleotides sequence, which provides an available technique for detecting trace nucleic acid markers(7). Compared with DNA probe, a unique feature of PNA sequence is that it cannot work as primer extended by DNA polymerase(8). As indicated by Nielsen et al, the binding of PNA to PCR primer site can effectively block the formation of a PCR product(9), and it was called “PCR clamping”(10, 11). Take advantage of this feature of PNA, we can selectively amplify or suppress target sequence, hence observing a different signal between target and non-target sequence(12, 13). Some researchers developed a detection method of point mutations in Ki-ras with the utilization of wild-type-specific PNA as competitors to mutation-specific primers(14, 15). The analysis time of this method was reduced from 2 days to 1 hour with respect to the time of restriction fragment length polymorphism analysis. The research of Sotlar et al. showed the addition of 0.75 μM PNA oligomer to suppress 100 ng of wild-type DNA amplification, as a result, the detection limit of c-kit mutations reached to 1:2000(16).

Real-time PCR(RT-PCR) was firstly demonstrated in the test of Higuchi and co-workers(17). After the introduction of ethidium bromide and ultraviolet, the accumulation of DNA was visualized and recorded by a video camera. RT-PCR refers to the dynamic monitoring of PCR amplification process, which is similar to the concept of “Kinetic PCR”(1). In practice, it has been used as a target DNA detection technology(18). Lillian et al. creatively proposed real-time immuno-polymerase

chain reaction (RT-iPCR) approach to enhance the detection signal of DNA(19). The detection limit is 6 aM. Firstly, biotinylated target DNA was captured by PNA probes and left on the microtiter wells surface. Then, a prepared streptavidin (STV)–DNA conjugate was added, which binds to the hybridized DNA by immunological interaction. Finally, bound streptavidin (STV)–DNA is quantified via the region of reporter DNA sequence using RT-iPCR. Compared with conventional multiplex PCR assays, RT-iPCR could be used to carry out parallel detection in one tube without mutual interference. In conventional multiplex PCR assays, competition consumption of reaction components for parallel amplification reactions impairs the reliability and sensitivity of detection(19).

For detection of miRNA, there are some limits for direct application of conventional PCR, such as extremely short lengths, significant sequence homology among family members and the lack of common sequence(20). Thus, miRNA extension and stem-looped primer method(21) have been developed to sensitively detect miRNA. The research of Chen et al.(22) indicated that stem-loop primer shows better reverse transcription efficiency and specificity than that of conventional primer. This method expands the real-time quantification method to the detection of micro molecules(such as miRNA). Compared with traditional PCR, the stem-loop primer was firstly hybridized to miRNA and then reverse transcribed with related transcriptase, subsequently the products work as the reactant of PCR.

For long sequence of PCR products, sequence amplification could improve sensitivity while the selectivity is reduced(23, 24). Therefore, possible secondary and tertiary structure within target molecular and electrostatic repulsion between probes and targets should be taken into account if the target sequence was extended before hybridization. Changing structure of targets significantly affect the hybridization between probes and targets due to the decreasing target sequence accessibility. In this case, some researchers carried the hybridization in a solution with a certain salt concentration. Therefore, how to use PCR technology scientifically is the key to design signal amplification scheme.

The power of PCR technology enables the wide application in many fields, involving biomedical research and molecular diagnostics. It has been used in detection of bacteria(25, 26), acute myeloblastic leukemia(27) and gene expression in solid tumors(28). Although PCR-based technique has obvious advantages, it also encounters the issues of easy contamination, high cost and

complicated operation(29, 30). The details of PCR technology and its application in other fields could be seen in the research of Valasek and Repa(1).

1.2 Rolling-Circle Amplification(RCA) and Loop-mediated isothermal Amplification (LAMP)

RCA is becoming more and more popular in the detection of nucleic acid, which is an isothermal enzymatic DNA replication method. A long single-strand DNA with repeating complementary units of a circular DNA template was formed under the action of DNA polymerases(31). In the mid-1990s, RCA was discovered in the replication of phage genomes(32). Recent years, RCA has been developed as a novel DNA amplification tool in signal amplification of nucleic acid detection(33-37).

With the wide application of RCA, Ellington et al. proposed that small-molecule targets and protein could be detected with RCA method, in which the circular DNA template was formed by a ligand-dependent ligase DNAzyme(38). For miRNA sequence, it is hard to directly reverse transcription and subsequent PCR due to its extremely short sequence length. Stem-loop or locked nucleic should be added in the reverse transcription PCR-based miRNA analysis(39), which makes the monitoring process more complicated. In this case, RCA is a good alternative to PCR. In the study of Cheng et al, template miRNA was specifically ligated padlock probes by T4 RNA ligase 2, shown in Figure 1b(2). Then add the second primer to trigger the branched rolling-circle amplification (BRCA) reaction. A combinated amplification strategy of RCA and oligonucleotide functionalized nanoparticles have also been effectively applied in cancer detection. The detection limits is 10 Ramos cells mL^{-1}(40), in which the sensitivity was about 2–4 orders higher than other methods that reported in their paper. Althrough RCA and isothermal exponential amplification reaction(41) could be carried without accurate cycling control, it relies heavily on pre-ligation reaction with padlock probe. Besides, the enployment of enzyme may increase the cost and require a mild condition. And RCA required a long reaction time of about 6 hours(42).

LAMP is a new thermostatic nucleic acid amplification technique for gene diagnosis, in which four specific primers were designed for the six regions of the target gene, and the amplification was carried out under the action of strand displacement DNA polymerase. One of the outstanding advantages of LAMP is the rapid amplification rate at a constant temperature, eliminating the pre-

denaturation and temperature cycling of dsDNA procedure in PCR amplification. It was regarded as a potential nucleic acid detection strategy(43). However, since LAMP amplification is strand displacement synthesis, the target sequence length is preferably within 300 bps, it is not suitable for the sequence more than 500 bps. In the research of Sun et al.(44), they reported a one-step miRNA analysis using target-triggered LAMP mechanism. Only in the presence of miRNA, the starting material of LAMP, a double stem-loop DNA, could be synthesized. This strategy simplifies the design of probe and shortens the length of template, which contributes the efficient and sensitive detection of target. The detection limit is 100 aM. The research indicates the detection sensitivity for miRNA analysis could be remarkably improved by target-triggered LAMP strategy, which is about 1000 times higher than that of their previous LAMP-based assay(4). The scheme was shown in Figure 1d.

1.3 Hybridization chain reaction(HCR)

As researchers noted that the enzyme involved strategy often required complicated operation procedures, and the utilization of enzyme usually increases the cost and the risk of false-positive signal(45). Therefore, enzyme-free strategy, especially for target triggered hairpin assembly, has received much attention(46-48).

HCR is an in-vitro nucleic acid isothermal amplification technology, which was proposed by Dirks and co-workers(49). HCR is an enzyme-free process where the hybridization event could be triggered by targets. It was expected to lead the formation of dsDNA, enabling the signal amplification of DNA detection(49). The amplification reaction proceeds until the hairpin probes are exhausted. The number of parallel reactions is closely related to the concentration of initiators. Therefore, the average molecular weight of polymer decreases with the increased initiator concentration. The structure of the hairpin species is different from the molecular beacons(50), in which a short loop with potential energy was protected by a long stem while a short stem protects a long loop in beacon(51). In a HCR event, it is desirable to design a capture probes that is complementary to target and two or more hairpin probes. Capture probe was immobilized at the biosensor surface to specifically identify targets and capture it. Targets work as an initiator to start the reaction between hairpin probes. The HCR process was shown in Figure 1c(3).

Chen et al. have proposed HCR amplification in situ for DNA detection(3). The amplification process is an alternating assembly of two hairpin DNA triggered by the target DNA (52, 53), i.e. each target can initiate an HCR event. An outstanding advantage is that HCR could be operated at mild condition. In detection process, self assembly of hairpin probes (H_1, H_2) to form double stranded DNA. Therefore, the amplification process is easy to operate and the procedure is simple without complicate modification of electrode. Some researchers have made some improvements on the basis of HCR, they proposed hyperbranched hybridization chain reaction(HB-HCR). It changes from conventional single nanowire to multiple hyperbranched DNA polymer nanostructures during reaction, as a result, it exhibits an excellent detection capability for the exponential signal amplification in bioanalysis(54-56). The investigation of Chu et al. indicated that a scientific combination of several signal amplification methods is expected to enhance the detection ability of biosensors(57). In the study, photoelectrochemical (PEG) biosensor coupled with HCR and HB-HCR, in which the detection limit of human telomerase RNA (hTR) is 17 fM. The detection limit for CHA/HB-HCR was about 8.8 times lower than that of CHA/linear-branched HCR and 547 times lower than that of CHA. As can be seen from the practice of detection, HCR-based technology showed significant advantages in simple operation, low cost, high specificity, easy modification and room temperature testing(58).

1.4 Target recycling amplification

Target recycling is an event-like that increased concentration of targets, in which a target generates multiple signals. Strand displacement and enzymatic cleavage are common methods to realize target recycling. Xuan et al. constructed a signal amplification sensor based on target recycling utilizing a strand displacement activity DNA polymerase(59). The 5'of hairpin DNA was labeled with PNA-ferrocene(Fc). The addition of target opens up the stem-loop of hairpin DNA, subsequently the primer at the 3'end was extended by the DNA polymerase, resulting the displacement of target and Fc-PNA. The mechanism was shown in Figure 2a. In the study of Chen et al., they use exonuclease III and hairpin DNA probes to achieve the reuse of target DNA, which is a good candidate for signal amplification(60). In the measurement, target DNA and exonuclease III were added to the tested electrode, and the target hybridize with the hairpin probe to generate a

duplex. Exo III could specifically cleave hairpin DNA/target duplex and releases the target, then the released target could hybridize with another hairpin DNA. Therefore, each target can remove multiple hairpin DNA from the electrode, instead of one target for one probe. The scheme was shown in Figure 2b.

Enzyme-catalyzed amplification of target cycles is often affected by enzyme activity. The detection is costly and possible false responses in the case of enzyme inactivation. DNAzyme can specifically recognize the sequence, which could over above disadvantages in enzyme-catalyzed amplification detection. In which, a subtle combination of lead and specific DNA enzymes has received wide interests(61). The addition of Pb^{2+} could cleave the Y structure consisting primer probe, assistant detection probe and target RNA, then the released target hybridize with other primer probes and assistant detection probes, achieving target recycling(62).

Besides, nuclease-assisted target recycling has been successfully used to detect miRNA in cancer cells(63). Nuclease can specifically cleave DNA duplexes with sequence greater than 10bp or DNA/RNA hybrid with length more than 15bp. In the absence of the target miRNAs, the short length and steric hindrance of the hairpin structures prevents nuclease from cleaving hairpin structure of probe. Therefore, nuclease can not cleave a single hairpin probe without target. In the test, the hairpin probes were labeled with MB or Fc respectively. When nuclease cleave hybrid of probe and target, current peaks at different potential positions were obtained (MB at -0.30 V and Fc at +0.36 V correspond to miRNA-141 and miRNA-21, respectively) for multiplexed detection of miRNAs. MB/Fc labels, much more than the amount of miRNA target, were removed from the sensing surface via target recycling. The detection limits are 4.2 fM for miRNA-21 and 3.0 fM for miRNA-141. The scheme was show in Figure 2c.

In addition, some researchers have designed target cycle detection methods without enzyme assistance. In the study of Bao et al., they use target miRNA to trigger the dynamic assembly of H_1 and H_2 hairpins(H_1, H_2) to form a double helix s tructure, i.e. catalyzed hairpin assembly (CHA) reaction without enzyme(64).

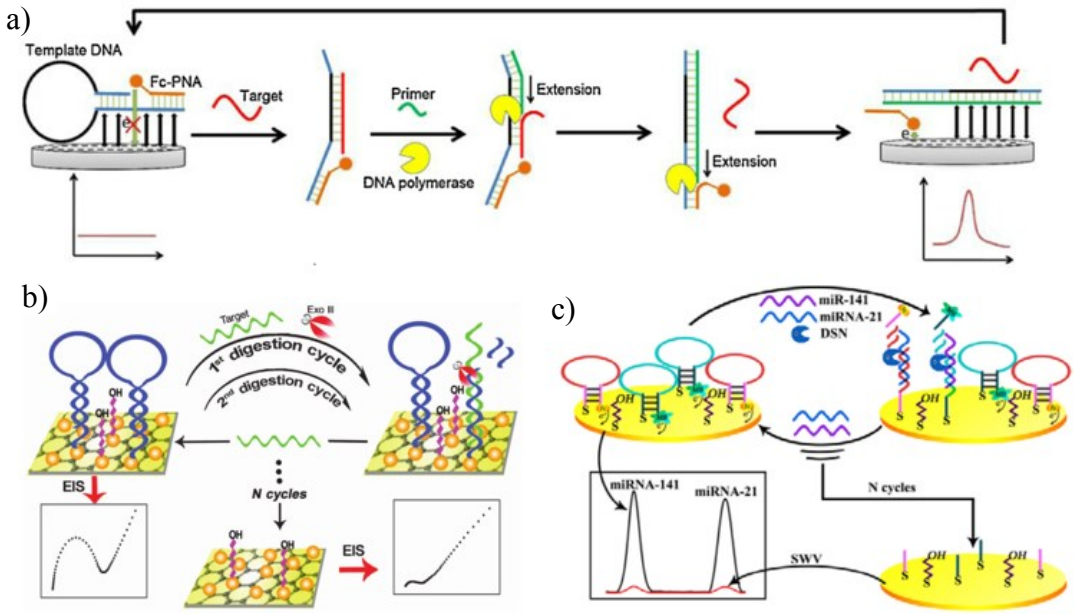

Fig.2 Target recycling amplification. (a)The mechanism of the signal-amplified electrochemical DNA sensor based on isothermal circular strand-displacement polymerization reaction(59); (b)Illustration of the enzyme-assisted target recycling for amplified EIS detection of DNA on a graphene/AuNP modified electrode(60); (c)Schematic illustration of multiplexed and amplified electrochemical detection of miRNA-141 and miRNA-21 by coupling different redox labels with DSN-assisted target recycling signal amplification(63)

References

1. Valasek MA, Repa JJ. The power of real-time PCR. Adv Physiol Educ. 2005;29(3):151-9. https://doi.org/10.1152/advan.00019.2005.

2. Cheng Y, Zhang X, Li Z, Jiao X, Wang Y, Zhang Y. Highly sensitive determination of microRNA using target-primed and branched rolling-circle amplification. Angew Chem Int Ed Engl. 2009;48(18):3268-72. https://doi.org/10.1002/anie.200805665.

3. Chen Y, Xu J, Su J, Xiang Y, Yuan R, Chai Y. In situ hybridization chain reaction amplification for universal and highly sensitive electrochemiluminescent detection of DNA. Anal Chem. 2012;84(18):7750-5. https://doi.org/10.1021/ac3012285.

4. Li C, Li Z, Jia H, Yan J. One-step ultrasensitive detection of microRNAs with loop-mediated isothermal amplification (LAMP). Chemical Communications. 2011;47(9):p.2595-7.

5. Mullis, Kary B. The Unusual Origin of the Polymerase Chain Reaction. Scientific American. 1990;262(4):56-65.

6. Saiki RK. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985;230(4732):1350.

7. Saiki R, Gelfand D, Stoffel S, Scharf S, Higuchi R, Horn G, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science. 1988;239(4839):487-91.

8. Christian T, Ekkehard B, Rainer B, Burghardt W, Andreas N. Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping. Nucleic Acids Research. 1996;24(5):983-4.
9. Orum H, Nielsen PE, Egholm M, Berg RH, Buchardt O, Stanley C. Single base pair mutation analysis by PNA directed PCR clamping. Nucleic acids research. 1993;21(23):5332-6. https://doi.org/10.1093/nar/21.23.5332.
10. Murdock DG, Wallace DC. PNA-mediated PCR clamping. Applications and Methods. Methods in Molecular Biology. 2002;208:145.
11. Schuermann M, Behn M. PNA Clamping Techniques for the Determination of Oncogene Mutations. Methods Mol Biol. 2002;208:165-79.
12. Hancock DK, Schwarz FP, Song F, C WL-J, Levin BC. Design and Use of a Peptide Nucleic Acid for Detection of the Heteroplasmic Low-Frequency Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like Episodes (MELAS) Mutation in Human Mitochondrial DNA. Clinical Chemistry. 2002(12):12.
13. Michiyo U, Yui W, Ho KS, Worawan C, Yuzo K, Naotaka H, et al. High-Sensitivity Detection of the A3243G Mutation of Mitochondrial DNA by a Combination of Allele-Specific PCR and Peptide Nucleic Acid-Directed PCR Clamping. Clinical Chemistry. 2004;50(11):2045-51.
14. Chen, C.-Y. Rapid Detection of K-ras Mutations in Bile by Peptide Nucleic Acid-mediated PCR Clamping and Melting Curve Analysis: Comparison with Restriction Fragment Length Polymorphism Analysis. Clinical Chemistry. 2004;50(3):481-9.
15. Däbritz J, Hänfler J, Preston R, Stieler J, Oettle H. Däbritz J, Hänfler J, Preston R, Stieler J, Oettle HDetection of Ki-ras mutations in tissue and plasma samples of patients with pancreatic cancer using PNA-mediated PCR clamping and hybridisation probes. Br J Cancer 92(2): 405-412. British Journal of Cancer. 2005;92(2):405-12.
16. Sotlar K, Escribano L, Landt O, M?Hrle S, Herrero S, Torrelo A, et al. One-Step Detection of c-kit Point Mutations Using Peptide Nucleic Acid-Mediated Polymerase Chain Reaction Clamping and Hybridization Probes. American Journal of Pathology. 2003;162(3):737-46.
17. Higuchi R, Dollinger G, Walsh PS, Griffith R. Simultaneous Amplification and Detection of Specific DNA Sequences. Biotechnology. 1992;10(4):413-7.
18. Valasek, M. A. The power of real-time PCR. Advances in Physiology Education. 2005;29(3):151-9.
19. Roth L, Zagon J, Ehlers A, Kroh LW, Broll H. A novel approach for the detection of DNA using immobilized peptide nucleic acid (PNA) probes and signal enhancement by real-time immuno-polymerase chain reaction (RT-iPCR). Analytical and Bioanalytical Chemistry. 2009;394(2):529-37. https://doi.org/10.1007/s00216-009-2724-x.
20. Gao Z. A highly sensitive electrochemical assay for microRNA expression profiling. Analyst. 2012;137(7):1674-9. https://doi.org/10.1039/c2an15974k.
21. Rui RS, Chiang VLV. Facile means for quantifying microRNA expression by real-time PCR. Biotechniques. 2005;39(4):519.
22. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33(20):e179. https://doi.org/10.1093/nar/gni178.
23. Weiler. Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays. 1997.

24. Guo, Guilfoyle, Thiel, Wang. Direct fluorescence analysis of genetic polymorphisms by hybridization with oligonucleotide arrays on glass supports. Nucleic Acids Research. 1994.
25. Ballard AL, Fry NK, Chan L, Surman SB, Lee JV, Harrison TG, et al. Detection of Legionella pneumophila using a real-time PCR hybridization assay. Journal of Clinical Microbiology. 2000;38(11):4215-8.
26. Lunge VR, Miller BJ, Livak KJ, Batt C. Factors affecting the performance of 5 ' nuclease PCR assays for Listeria monocytogenes detection. Journal of Microbiological Methods. 2002;51(3):361-8.
27. Pongers-Willemse, Verhagen, Tibbe. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. Leukemia. 1998.
28. Ohnmacht GA, Wang E, Mocellin S, Abati A, Filie A, Fetsch P, et al. Short-Term Kinetics of Tumor Antigen Expression in Response to Vaccination. Journal of Immunology. 2001;167(3):1809-20.
29. Cheglakov Z, Weizmann Y, Beissenhirtz MK, Willner I. Ultrasensitive detection of DNA by the PCR-Induced generation of DNAzymes: The DNAzyme primer approach. Chemical Communications. 2006(30):3205.
30. Murakami T, Sumaoka J, Komiyama M. Nucleic acids research Volume: 37 ISSN: 1362-4962 ISO Abbreviation: Nucleic Acids Res. Publication Date: 2009 Feb. 1970.
31. Seeman NC. DNA in a material world. Nature. 2003;421(6921):427-31.
32. Fire, A., Xu, S., Q. Rolling replication of short DNA circles. Proceedings of the National Academy of Sciences. 1995.
33. Qi X, Saleha B, Devos KM, Gale MD, Anne O. L-RCA (ligation-rolling circle amplification): a general method for genotyping of single nucleotide polymorphisms (SNPs). Nucleic Acids Research. 2001(22):22.
34. Larsson C, Koch JR, Nygren A, Janssen G, Raap AK, Landegren U, et al. In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes. Nature Methods. 2004;1(3):227-32.
35. Judith P, Anona B, Varsha G, Jackie B, Giuseppe S, Phyllida R, et al. Integration of DNA ligation and rolling circle amplification for the homogeneous, end-point detection of single nucleotide polymorphisms. Nucleic Acids Research. 2002(12):12.
36. Smolina IV, Kuhn H, Lee C, Frank-Kamenetskii MD. Fluorescence-based detection of short DNA sequences under non-denaturing conditions. Bioorganic & Medicinal Chemistry. 2008;16(1):84-93.
37. Stougaard M, Lohmann JS, Zajac M, Hamilton-Dutoit S, Koch J. In situ detection of non-polyadenylated RNA molecules using Turtle Probes and target primed rolling circle PRINS. Bmc Biotechnology. 2007;7(1):69.
38. Yang L, Fung CW, Cho EJ, Ellington AD. Real-Time Rolling Circle Amplification for Protein Detection. Analytical Chemistry. 2007;79(9):3320-9.
39. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, et al. Real-time quantification of microRNAs by stem–loop RT–PCR. Nucleic Acids Research. 2005;33(20):e179.
40. Ding C, Liu H, Wang N, Wang Z. Cascade signal amplification strategy for the detection of cancer cells by rolling circle amplification and nanoparticles tagging. Chem Commun (Camb). 2012;48(41):5019-21. https://doi.org/10.1039/c2cc31390a.
41. Duan R, Zuo X, Wang S, Quan X, Chen D, Chen Z, et al. Lab in a Tube: Ultrasensitive Detection of MicroRNAs at the Single-Cell Level and in Breast Cancer Patients Using Quadratic Isothermal Amplification. Journal of the American Chemical Society. 2013;135(12):4604-7.

42. Yongqiang, Cheng, Xian, Zhang, Zhengping, Li, et al. Highly Sensitive Determination of microRNA Using Target-Primed and Branched Rolling-Circle Amplification. Angewandte Chemie. 2009.
43. Tomita N, Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nature Protocols. 2008;3(5):877-82.
44. Sun Y, Tian H, Liu C, Sun Y, Li Z, Sun Y, et al. One-Step Detection of MicroRNA with High Sensitivity and Specificity via Target-triggered Loop-Mediated Isothermal Amplification (TT-LAMP). Chemical Communications. 2017;53(80).
45. Liu S, Cheng C, Gong H, Wang L. Programmable Mg(2+)-dependent DNAzyme switch by the catalytic hairpin DNA assembly for dual-signal amplification toward homogeneous analysis of protein and DNA. Chem Commun (Camb). 2015;51(34):7364-7. https://doi.org/10.1039/c5cc01649e.
46. Jung C, Ellington AD. Diagnostic Applications of Nucleic Acid Circuits. Accounts of Chemical Research. 2014;47(6):1825-35.
47. Scida K, Li B, Ellington AD, Crooks RM. DNA Detection Using Origami Paper Analytical Devices. Analytical Chemistry. 2013;85(20):9713-20.
48. Fu B, Cao J, Jiang W, Wang L. A novel enzyme-free and label-free fluorescence aptasensor for amplified detection of adenosine. Biosensors & Bioelectronics. 2013;44C(1):52-6.
49. Dirks RM, Pierce NA. Triggered amplification by hybridization chain reaction. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(43):15275-8.
50. Tyagi S, Kramer FR. Molecular Beacons: Probes that Fluoresce upon Hybridization. Nature Biotechnology. 1996;14(3):303-8.
51. Dirks RM, Pierce NA. Triggered amplification by hybridization chain reaction. Proc Natl Acad Sci U S A. 2004;101(43):15275-8. https://doi.org/10.1073/pnas.0407024101.
52. Ju H. A plasmonic colorimetric strategy for biosensing through enzyme guided growth of silver nanoparticles on gold nanostars. Biosensors & Bioelectronics.78:267-73.
53. Pierce NA. Triggered amplification by hybridization chain reaction. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(43):15275-8.
54. Labean TH. An autonomously self-assembling dendritic DNA nanostructure for target DNA detection. Biotechnology Journal. 2013;8(2):221-7.
55. Fenselau C. Unexpected Trypsin Cleavage at Ubiquitinated Lysines. Analytical Chemistry.87(16):8144-8.
56. Hsing IM. Triggering Hairpin-Free Chain-Branching Growth of Fluorescent DNA Dendrimers for Nonlinear Hybridization Chain Reaction. Journal of the American Chemical Society.136(28):9810-3.
57. Chu Y, Deng AP, Wang W, Zhu JJ. Concatenated Catalytic Hairpin Assembly/Hyperbranched Hybridization Chain Reaction Based Enzyme-Free Signal Amplification for the Sensitive Photoelectrochemical Detection of Human Telomerase RNA. Anal Chem. 2019;91(5):3619-27. https://doi.org/10.1021/acs.analchem.8b05610.
58. Li S, Li G, Du Z, Zhu L, Tian J, Luo Y, et al. The ultra-sensitive visual biosensor based on thermostatic triple step functional nucleic acid cascade amplification for detecting Zn(2). Food Chem. 2019;290:95-100. https://doi.org/10.1016/j.foodchem.2019.03.134.
59. Xuan F, Luo X, Hsing IM. Sensitive immobilization-free electrochemical DNA sensor based on isothermal circular strand displacement polymerization reaction. Biosensors & Bioelectronics. 2012;35(1):230-4. https://doi.org/10.1016/j.bios.2012.02.054.
60. Chen Y, Jiang B, Xiang Y, Chai Y, Yuan R. Target recycling amplification for sensitive and label-

free impedimetric genosensing based on hairpin DNA and graphene/Au nanocomposites. Chem Commun (Camb). 2011;47(48):12798-800. https://doi.org/10.1039/c1cc14902d.
61. Fu T, Zhao XH, Bai HR, Zhao ZL, Hu R, Kong RM, et al. A superquenched DNAzyme-perylene complex: a convenient, universal and low-background strategy for fluorescence catalytic biosensors. Chemical Communications. 2013;49(59):6644-6.
62. Zhang P, Wu X, Yuan R, Chai Y. An "off-on" electrochemiluminescent biosensor based on DNAzyme-assisted target recycling and rolling circle amplifications for ultrasensitive detection of microRNA. Anal Chem. 2015;87(6):3202-7. https://doi.org/10.1021/ac504455z.
63. Yang C, Dou B, Shi K, Chai Y, Xiang Y, Yuan R. Multiplexed and amplified electronic sensor for the detection of microRNAs from cancer cells. Anal Chem. 2014;86(23):11913-8. https://doi.org/10.1021/ac503860d.
64. Bao J, Hou C, Zhao Y, Geng X, Samalo M, Yang H, et al. An enzyme-free sensitive electrochemical microRNA-16 biosensor by applying a multiple signal amplification strategy based on Au/PPy-rGO nanocomposite as a substrate. Talanta. 2019;196:329-36. https://doi.org/10.1016/j.talanta.2018.12.082.