

Molecular Beacons and Other Hairpin Probes

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INTRODUCTION

Stem-loop (hairpin) DNA oligomer probes provide increased specificity of target recognition as compared with linear DNA probes. Invention of molecular beacons, a variant of stem-loop oligonucleotides, which are able to fluoresce upon hybridization but are “dark” in the absence of the target, made possible real-time monitoring of hybridization process. A variety of robust assays using diverse, structurally constrained oligonucleotides have been developed that exploit endless number of formats and detection methods.

Hairpin DNA oligonucleotides are also used as different components in many DNA amplification methods providing higher specificity and lower background. Thus, these structured probes are an indispensable tool in modern biotechnology and diagnostics.

ENHANCED SPECIFICITY OF HAIRPIN DNA PROBES

Specificity of probe/target hybridization is a crucial factor determining efficiency of most nucleic acid-based methods used in diagnostics and biotechnology. Hybridization specificity is determined as a match-versus-mismatch discrimination: binding to sites that differ from the perfectly complementary sequences even by a single base pair substitution is characterized by a substantial free-energy penalty.^[1] If the free-energy penalty is high enough, a set of conditions (so-called stringency conditions) can be found where perfect complexes will be considerably more stable than the complexes containing mismatches.

Stem-loop DNA probes are single-stranded oligonucleotides containing a sequence complementary to the target that is flanked by self-complementary target-unrelated termini. Thermodynamic analysis of hybridization characteristics of linear and stem-loop DNA probes proved that it is a general feature of structurally constrained probes to distinguish mismatches over a larger range of temperatures or other experimental parameters comparing to unstructured probes.^[2,3] Thus, stem-loop DNA probes allow for a wider window

of stringency conditions, which provide better match/mismatch discrimination.

MOLECULAR BEACONS

Molecular beacon (MB) is a stem-loop DNA oligonucleotide, which carries a fluorophore and a quencher at the 3'- and 5'-ends^[4,5] (Fig. 1A). In the absence of the target, these molecules form closed stem-loop structures in which fluorophore and quencher are in close proximity—this results in fluorescence quenching. In the presence of the DNA or RNA target, molecular beacon forms a complex with it, which brings apart the fluorophore and the quencher. Once the fluorophore and quencher are spatially separated, the fluorescence develops under illumination and quantitatively reports on the presence of the target (Fig. 1A).

Molecular beacons, being inactive in absence of the target, do not require purification of the hybridization product from the excess MB and thus provide simple, homogeneous (close-tube) format of the assay and, consequently, a possibility of real-time hybridization monitoring. This feature of MBs made them a tool of choice in many applications. They were used for detection of single nucleotide polymorphisms, in quantitative PCR, in isothermal amplification, as DNA microarray-immobilized probes and biosensors, and as antisense probes for detecting RNAs in vivo (for reviews, see Refs. [6,7]). Recent developments were directed toward simplification of the MB approach and creation of the “label-free” optical biomolecular sensors. For example, immobilization of fluorophore-labeled MBs on a gold surface allowed using the surface as a quencher, thus eliminating the need in quenching moiety in the beacon structure.^[8] Fully unlabeled stem-loop DNA probes were used as electrochemical DNA sensors capable of detecting femtomoles of DNA by electrochemical signal rather than by optical signal.^[9]

Fluorescence monitoring allows quantitative kinetic analyses of the conformation changes of molecular beacons. Therefore they have also become a useful tool in studies on conformational changes of DNA under various conditions or caused by various types of reagents

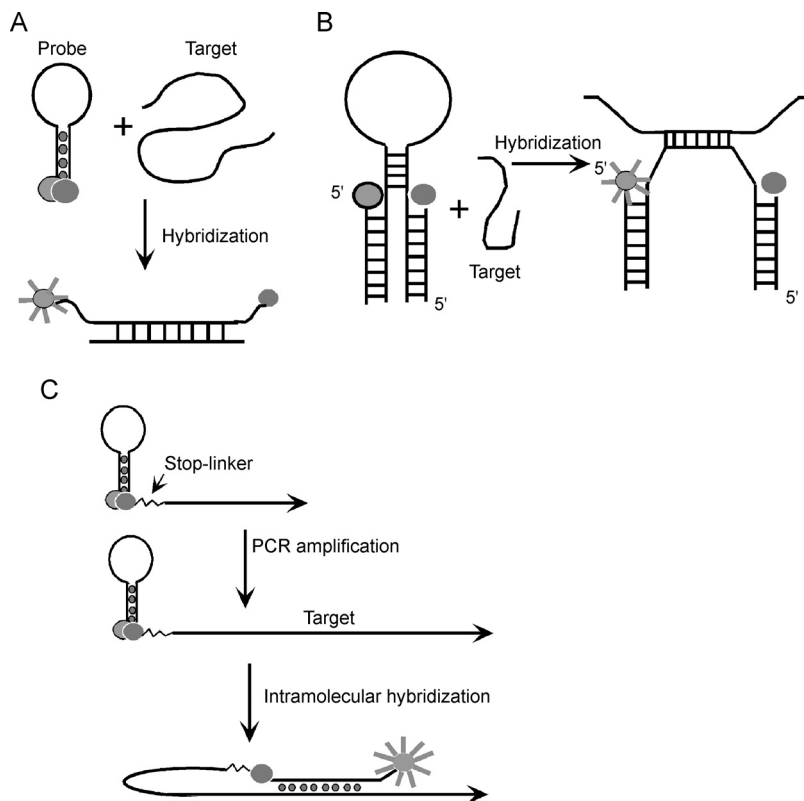


Fig. 1 Outline of different assays using molecular beacons. (A) Molecular beacon is in a closed (“dark”) form in the absence of the target, and a fluorophore (red) and a quencher (gray) are in a close proximity, which quenches fluorescence. Upon hybridization with the target, the beacon changes its conformation, which results in spatial separation of the fluorophore and quencher. As a result, bright fluorescence reveals the presence of a target. (B) Tripartite molecular beacons with universal arms and varying loop provide flexibility in design and reduced cost. (C) Schematics of the beacon-like Scorpion probe approach. Incorporation of a stem-loop-shaped probe into the PCR primer allows unimolecular target detection, which ensures faster kinetics and higher stability of the complex as compared with bimolecular reaction. (View this art in color at www.dekker.com.)

interacting with nucleic acids. These studies include real-time monitoring of DNA cleavage caused by enzymes or chemicals, protein–DNA interaction studies, and studies of various dye interactions with duplex DNA. Conformational studies of single-stranded DNAs as well as kinetic and thermodynamic characteristics of triplex formation have also been performed using molecular beacons.^[6]

Structural Variants of Molecular Beacons

There are several derivatives of “classical” MBs, which differ from the conventional stem-loop DNA oligonucleotides. First, it was shown that the linear DNA oligonucleotides without self-complementary termini (stemless) but bearing fluorophore and quencher at the 5′- and 3′- ends could work as MBs.^[10] Later, the same was reported for the peptide nucleic acid (PNA) oligomers.^[11] PNAs are DNA mimics with pseudopeptide (polyamide) backbone instead of sugar–phosphate one in DNA. Apparently, the flexibility of the sugar–phosphate and polyamide

backbones of DNA and PNA, respectively, in combination with a strong hydrophobic interaction between the fluorophore and the quencher, keeps these structures preferably in a closed form in the absence of a target.^[11,12]

Specificity, Sensitivity, and Reproducibility

Molecular beacons exhibit exceptional sequence specificity because of the favorable structure energetics. They are used in combination with different amplification technologies, such as polymerase chain reaction (PCR) and rolling circle amplification (RCA), which allows real-time amplification monitoring. Fluorescence detection is very sensitive: an increase of a signal over the background in the presence of the target up to two orders of magnitude can be achieved. Thus sensitivity of PCR and RCA in the presence of molecular beacons can be as high as single molecule in case of PCR and 10–100 molecules in case of RCA. Another important feature of MBs is the



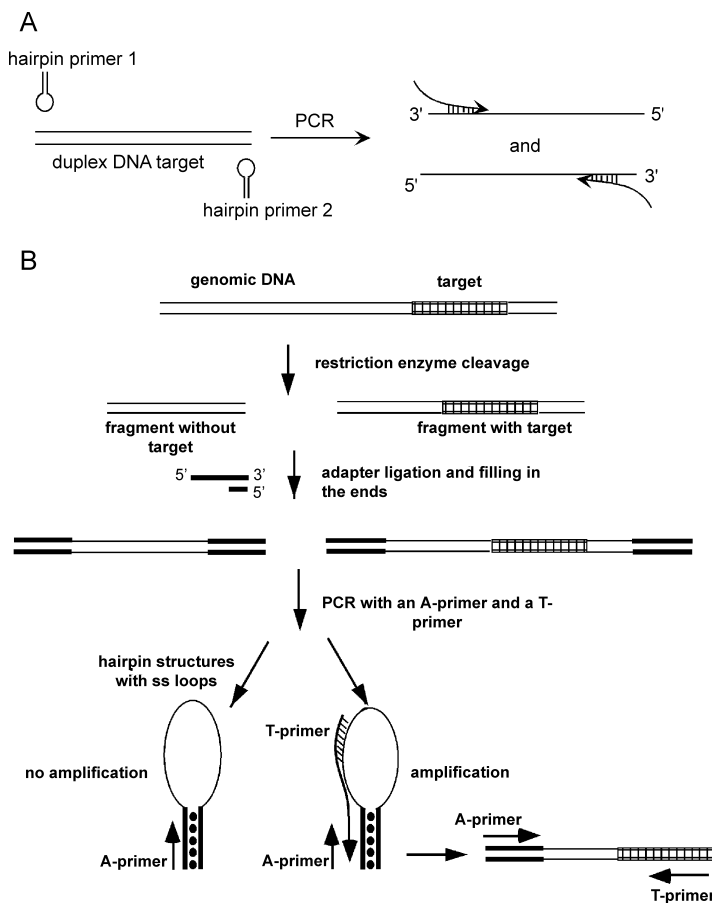


Fig. 2 Examples of application of stem-loop DNA structures in amplification methods. (A) Hairpin-shaped primers allow hot-start PCR because they anneal to the target only at high temperature upon denaturing of the stem. (B) The PCR suppression approach. Genomic PCR is digested with a restriction enzyme and ligated with specially designed adapters. During the PCR, only the DNA fragments containing the target are efficiently amplified, while the vast number of the target-unrelated fragments remains intact.

possibility of multiplex analysis by using MBs with different fluorophores.

Advantages and Limitations

The major advantage of MBs is the possibility of real-time hybridization monitoring. This provides simple close-tube format of the assay, which reduces chances of contamination and false positives. Among other advantages of hairpin probe application in biotechnology and diagnostics are an endless number of formats and detection methods.

Current limitations of MB technology include the high cost of MB synthesis and their instability in the presence of DNA polymerases with exonuclease activities. The efforts, therefore, in this respect are directed toward simplification and/or generalization of the beacon design. An example of simplification of MB design is the use of MBs immobilized on a gold surface, which serves as a quencher.^[8] Tripartite MBs with universal parts carrying fluorophore and quencher and sequence-specific oligonu-

cleotides allow flexibility in MB design^[13] (Fig. 1B). This approach also brings down the MB cost. Application of chemically modified MBs, which are not cleavable by exonucleases (e.g., PNA beacons or 2-*O*-methyl-deoxy containing MBs), is a way to overcome instability of MBs in the presence of exonucleases.^[11,14]

Combination of Molecular Beacon Approach with Other Technologies

Molecular beacons have been incorporated in many detection techniques using DNA amplification with the aim of real-time process monitoring. However, the area of MB applications is much wider and is not limited by DNA amplification methods. Catalytic MBs represent a next generation of molecular probes with the potential to amplify signals and thus to detect nucleic acid targets without PCR amplification.^[15] These MBs are DNA constructs that combine the features of molecular beacons and hammerhead-type deoxyribozymes with RNase



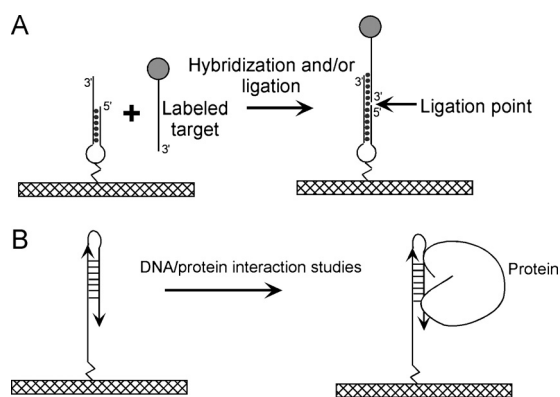


Fig. 3 Outline of two major approaches for application of immobilized stem-loop DNA oligonucleotides. (A) Sequence-specific capturing and mutation detection using immobilized stem-loop probes. (B) DNA microarrays with hairpin probes for DNA/protein interaction studies. (View this art in color at www.dekker.com.)

activity, which are located on two different modules.^[15] In the absence of a target, the beacon hybridizes with the deoxyribozyme module. When the target is present, the beacon changes its conformation and allows the substrate (a stemless fluorogenic oligonucleotide) to hybridize with the deoxyribozyme module. The deoxyribozyme cleaves the substrate, which results in increasing fluorescence, substrate dissociation, and beacon hybridization with the deoxyribozyme. Thus, the cycle is repeated. This approach is just in the development stage, but in the proof-of-principle experiments, it was shown that it initiated catalytic events.^[15]

The combination of PNA-based technology with molecular beacons opened the possibility of targeting duplex DNA without prior denaturation. PNA openers are short PNA oligomers, which invade duplex DNA and locally expose a single-stranded region within duplex DNA forming a P-loop complex. Locally denatured DNA region serves as a unique target for sequence-specific binding of DNA or PNA probes forming PD- or PP-loops, respectively.^[16] If the probes are DNA or PNA beacons, they are capable of real-time reporting on cognate targets located within the preopened double-stranded DNA.^[11,12]

USE OF STEM-LOOP OLIGONUCLEOTIDES IN DNA AMPLIFICATION AND MICROARRAY TECHNOLOGY

Stem-Loop Structures in DNA Amplification

Stem-loop DNA constructs are also used for different purposes in both thermocycling and isothermal DNA amplification (Fig. 2). For example, stem-loop primers

allow hot-start PCR and thus increased specificity of amplification.^[17,18] Scorpion probes are designed to serve simultaneously as a PCR primer and as a molecular beacon.^[19] During PCR, when the primer is extended and the target is synthesized, the stem loop unfolds and the loop sequence hybridizes intramolecularly with amplified target, thus developing fluorescence (Fig. 1C). The scorpion primer approach uses a unimolecular mechanism of probe–target hybridization, which ensures faster kinetics and higher stability of the probe–target complex.^[19]

Design of the large stem-loop constructs from DNA restriction fragments by ligation of special GC-rich adapters forms the basis of the PCR suppression (PS) technology (Fig. 2B). To perform suppression PCR, genomic DNA is digested with a restriction enzyme and ligated with an adapter with a high GC content.^[20,21] As a result, long self-complementary ends flank each single-stranded DNA fragment. These self-complementary termini form duplexes during each PCR annealing step, so that the fragments adopt large stem-loop structures, which makes PCR with the adapter–primer (A-primer) alone relatively inefficient. This effect is called PCR suppression (PS). However, the PCR is efficient with two primers, A-primer and target-primer (T-primer). In this case, only DNA fragments with the target are efficiently amplified on the background of all other fragments without a target. Several variants of PS approach were used in subtractive hybridization, cDNA and genomic differential displays, and in multiplex PCR (see Ref. [6] for a review). Hairpin structures were also used for elimination of PCR errors from amplified DNA.^[22] Specially designed primers forming stem loops allow also isothermal amplification of DNA; this method is designated as loop-mediated amplification (LAMP).^[23,24]

Stem-Loop Structures in DNA Microarray Technology

Hairpin DNA probes can be immobilized on the surface of microarrays and used in hybridization experiments for mutation detection (Fig. 3A)^[25] and DNA/protein interaction studies (Fig. 3B).^[26] Kinetic analysis confirmed better performance of stem-loop structures as compared with linear oligonucleotides.^[27]

CONCLUSION

Stem-loop oligonucleotide constructs are widely used in molecular biology, genomics, and diagnostic biotechnology. Two major factors are responsible for such broad applications of these DNA constructs: 1) enhanced specificity of the probe–target interaction and 2) the possibility of close-tube real-time monitoring formats.

There is no doubt that many new applications of hairpin probes will be developed in the future. Especially useful will be the methods that may allow direct detection of target molecules without prior amplification.

ACKNOWLEDGMENTS

This work was supported by the Hamilton-Thorne Biosciences via sponsored research agreement. The author thanks Vadim Demidov for critical reading of the manuscript.

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