

Stem-loop oligonucleotides: a robust tool for molecular biology and biotechnology

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The specific structural features of stem-loop (hairpin) DNA constructs provide increased specificity of target recognition. Recently, several robust assays have been developed that exploit the potential of structurally constrained oligonucleotides to hybridize with their cognate targets. Here, I review new diagnostic approaches based on the formation of stem-loop DNA oligonucleotides: molecular beacon methodology, suppression PCR approaches and the use of hairpin probes in DNA microarrays. The advantages of these techniques over existing ones for sequence-specific DNA detection, amplification and manipulation are discussed.

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Watson–Crick hybridization of complementary sequences in nucleic acids is one of the fundamental processes underlying molecular recognition *in vivo* [1]. Hybridization of a target with an oligonucleotide probe is also a major approach to nucleic acid identification and isolation *in vitro* [2]. Specificity of hybridization is, therefore, a crucial factor determining both the fidelity of the natural biological processes and efficiency of hybridization-based laboratory methods. Hybridization specificity, f , is determined as a relative factor for match-versus-mismatch discrimination: $f = \exp[-|\Delta G_{m-mmm}/RT|]$, where ΔG_{m-mmm} is the free energy penalty for binding to sites that differ from the perfectly complementary sequences by a single base pair substitution. If ΔG_{m-mmm} is ~ 4 kcal mol⁻¹ [3], hybridization specificity will be $\sim 1/100$ – $1/1000$, and it is possible to find a range of conditions (so-called stringency conditions) where perfect complexes will be substantially more stable than the complexes containing mismatches.

Naturally occurring stem-loop-forming RNA and DNA structures have important regulatory roles in cellular metabolism [4,5]. In addition to recognition by proteins, which is one of the suggested roles of stem-loop structures [6], they might exhibit some advantageous characteristics in DNA–DNA and DNA–RNA interactions compared with other nucleic acid conformations. Indeed, thermodynamic analysis of hybridization of linear and stem-loop (molecular beacon) DNA probes demonstrated significantly higher specificity of the latter [7,8]. By analyzing free-energy phase diagrams of molecular beacons in solution with matched and mismatched targets, the authors showed that structurally constrained probes can generally distinguish mismatches over a wider range

of temperatures than can unstructured probes [8]. The reason is that after dissociation from the target, unstructured probes can form more configurations than structured probes, which decreases entropy (ΔS) and, as a result, free energy of hybridization. For example, Roberts and Crothers [3] used stem-loop structures as ‘stringency clamps’ to increase the specificity of formation of triplex DNA complexes [3].

It should be mentioned, however, that secondary structures of the probes impose kinetic and thermodynamic penalties: complex formation proceeds slower (but is still fast), and results in products with lower melting temperatures and free energies [9]. Nevertheless, the specificity in mismatch discrimination remains the most important parameter in hybridization assays, and structured probes provide advantages in this respect.

The beauty of stem-loop probes lies in their ability to take on various designs in different applications. They can also be used as capture devices if the loop is immobilized on a surface and dangling ends are used for hybridization [10]. In this case, additional stacking interactions contribute to both faster kinetics and greater complex stability than seen in linear probes [10].

Several new hybridization techniques based on the formation of the stem-loop structures have been recently developed, including, molecular beacons, PCR suppression and the use of the stem-loop probes in DNA microarrays.

Molecular beacons

As originally designed, a molecular beacon is a single-stranded oligonucleotide probe containing a sequence complementary to the target that is flanked by self-complementary termini, and carries a fluorophore and a quencher at the 3'- and 5'-ends [11,12] (Fig. 1a). In the absence of the target, these molecules form closed stem-loop structures in which the fluorophore and quencher are in close proximity, which quenches the fluorescence. In the presence of the target, the molecular beacon forms a complex with its target, which dissociates the fluorophore from the quencher. Once the fluorophore and quencher are spatially separated, the fluorescence increases and quantitatively signals the presence of the target (Fig. 1a).

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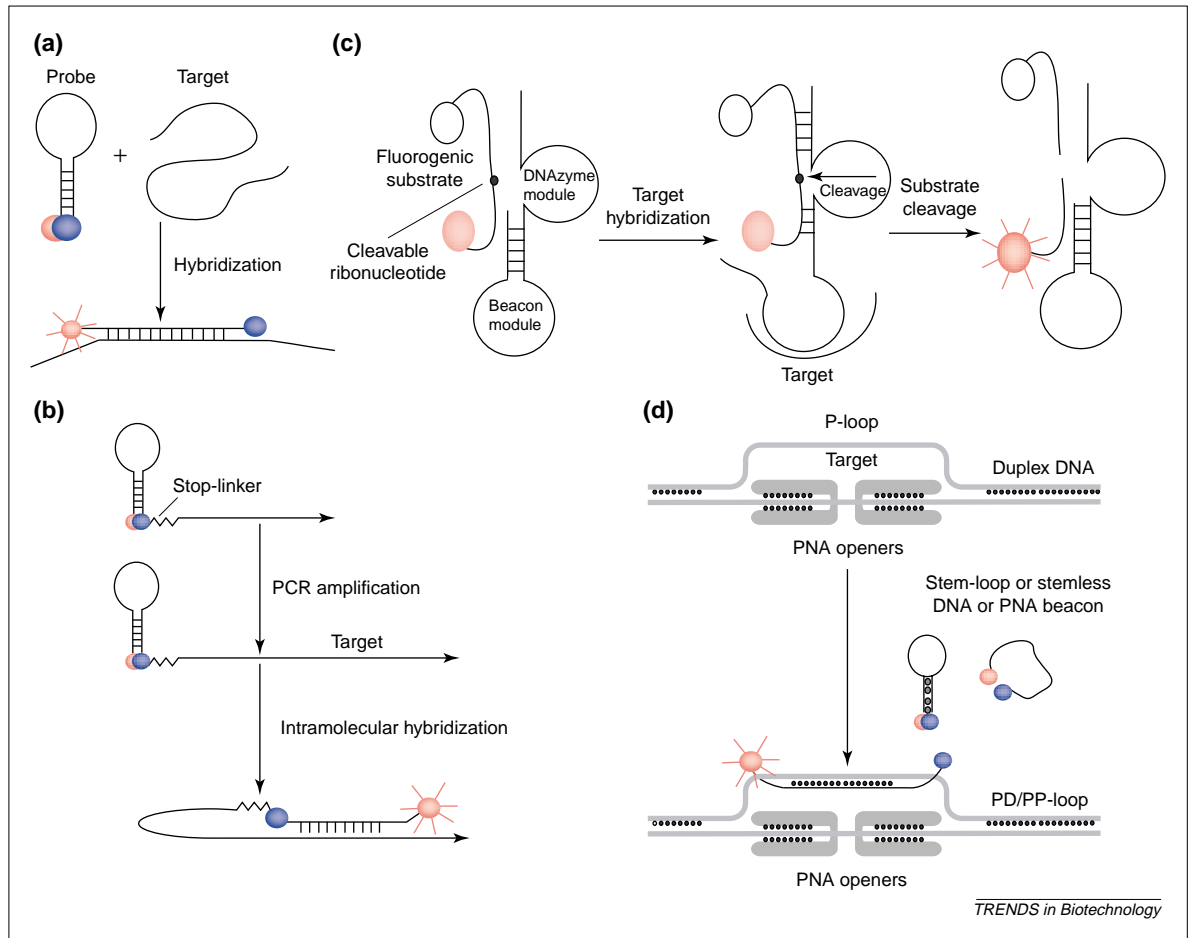


Fig. 1. Outline of different approaches using molecular beacons. (a) A stem-loop molecular beacon is in a closed form in the absence of the target, and a fluorophore (red) and a quencher (blue) are in a close proximity, which quenches fluorescence. Upon hybridization with the cognate target, the beacon changes its conformation, which results in spatial separation of the fluorophore and quencher. As a result, increasing fluorescence indicates the presence of a target. (b) In the scorpion-probe approach, a stem-loop shaped probe is incorporated into the PCR primer, allowing unimolecular target detection, which ensures faster kinetics and higher stability of the complex than the bimolecular reaction (adapted from [26]). (c) Catalytic molecular beacon approach (see text for details; adapted from [29]). (d) Combination of the molecular beacon approach with the PNA technology makes possible direct targeting of duplex DNA (adapted from [31]).

Molecular beacons have rapidly found many applications because of the simple, homogeneous (closed-tube) format of the assay and, consequently, the possibility of real-time process monitoring (reviewed in [13, 14]). Because of their superiority in mismatch discrimination, DNA molecular beacons were used to detect single nucleotide polymorphisms [15–17], in quantitative PCR [12, 18], in isothermal amplification [19], as DNA microarray-immobilized probes and biosensors [20–22], and as antisense probes for detecting RNA targets *in vivo* [23].

Several improvements and further developments of molecular beacon technology have been reported. A multiplex nucleic acid assay simultaneously detecting four different retroviruses was developed with the use of different colored molecular beacons [12, 18]. The extreme sensitivity and specificity of each color

reaction allowed the detection of fewer than ten copies of one virus amidst a background of unrelated viruses. In another approach, the incorporation of the gold nanoparticles as a quencher instead of the commonly used 4'-[4'-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) substantially increased the sensitivity and specificity of the assay because of the superior quenching ability of gold clusters [24]. The use of metals in the molecular beacon approach opens up the possibility for creating a variety of quenchers with different properties, by changing the shape, size or composition of the metal cluster. In the future, metal-based quenchers could be adjusted to different types of fluorophores, enabling simultaneous quantitation of different targets [24]. Development of wavelength-shifting molecular beacons is another approach for multiplex target detection [25].

A scorpion probe is simultaneously a PCR primer and a molecular beacon [26]. In this approach, the PCR primer is designed to have a 5'-extension that has all the attributes of a beacon: a loop region complementary to a target flanked by the self-complementary stems, and a fluorophore and a quencher at the 5'- and 3'-ends of the extension, respectively (Fig. 1b). The stem-loop extension is linked to the PCR primer via a linker, which stops DNA polymerase from replicating the stem-loop. During PCR, when the primer is extended and the target is synthesized, the stem-loop unfolds and the

loop sequence hybridizes intramolecularly with its target, which increases fluorescence (Fig. 1b). Thus, the scorpion-primer approach uses a unimolecular mechanism of probe-target hybridization, compared with the bimolecular recognition in the traditional molecular beacon assay. This ensures faster kinetics and greater stability of the probe-target complex [27].

Another method was developed by combination of the molecular beacon approach with catalytic DNAs (DNAzymes). In this case, the PCR primer contains a sequence complementary to a DNAzyme [28]. In the course of PCR, an active amplicon is synthesized that cleaves the fluorescent beacon-shaped substrate included in the reaction mixture, increasing the fluorescence. This approach can be generalized so that one generic DNAzyme and one corresponding beacon substrate are used to detect different genomic targets.

Catalytic molecular beacons represent a next generation of molecular probes with the potential to amplify signals and, thus, to detect nucleic acid targets without PCR amplification. In this case, a DNA construct is made that combines the features of a molecular beacon and a hammerhead-type deoxyribozyme with RNase activity; these are located on two different modules [29] (Fig. 1c). In the absence of a target, the beacon module hybridizes with the deoxyribozyme module. In the presence of a target, the beacon module changes its conformation and allows the substrate to hybridize with the deoxyribozyme module. The substrate is a stemless fluorogenic oligonucleotide. The deoxyribozyme cleaves the substrate at a cleavable ribonucleotide, which results in increasing fluorescence. The substrate dissociates on cleavage, the beacon module hybridizes with the deoxyribozyme module again, and the cycle is repeated. Although the fluorescence response from the substrate turnover was several times smaller than the response caused by the opening of the conventional stem-loop molecular beacon [29], this approach initiated catalytic events. If the structure of the catalytic beacon is optimized for faster substrate turnover and the background substrate fluorescence is brought down, this approach might be able to detect DNA targets without amplification. Currently, this method is still in the development stage and is far from being applied practically.

The combination of PNA–DNA-loop (PD-loop) technology with molecular beacons offered the completely new possibility of targeting duplex DNA [30,31]. So-called PNA (peptide nucleic acid) opens locally expose a single-stranded region within duplex DNA forming a P-loop complex (Fig. 1d). A locally denatured DNA region serves as a unique target for sequence-specific binding of DNA or PNA probes forming PD- or PP-loop complexes, respectively (Fig. 1d). Recently, it was shown that stem-loop DNA and stemless PNA beacons are capable of real-time reporting on cognate targets located within the reopened double-stranded DNA sites [32,33].

Stemless beacon constructs are analogs of conventional molecular beacons, which lack the complementary stems. 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, keep these structures in a closed form in the absence of target [31,33].

Stemless beacons have one important advantage over the traditional hairpin molecular beacons: they do not contain any sequence unrelated to the target. When the stemless PNA beacons were used to target P-loops within duplex DNA, a strong match/mismatch discrimination (>20-fold) and fast kinetics were observed [33]. It is likely that in this case, the structural rigidity of the target DNA (P-loop), in combination with all the advantages of molecular beacons, ensures high rates of formation and extreme interaction specificity.

Molecular beacons have also become a useful tool in studies that focus on conformational changes of DNA targets under various conditions or those caused by various types of reagents interacting with nucleic acids. Direct fluorescence detection allows real-time quantitative monitoring of the conformation changes of a molecular beacon. These studies include real-time monitoring of DNA cleavage caused by enzymes or chemicals [34–36], protein–DNA interaction studies [37–39], and studies of various dye interactions with duplex DNA [40]. Conformational studies of single-stranded DNAs [41], as well as thermodynamic characteristics of triplex formation [42] have also been performed using the molecular beacon approach.

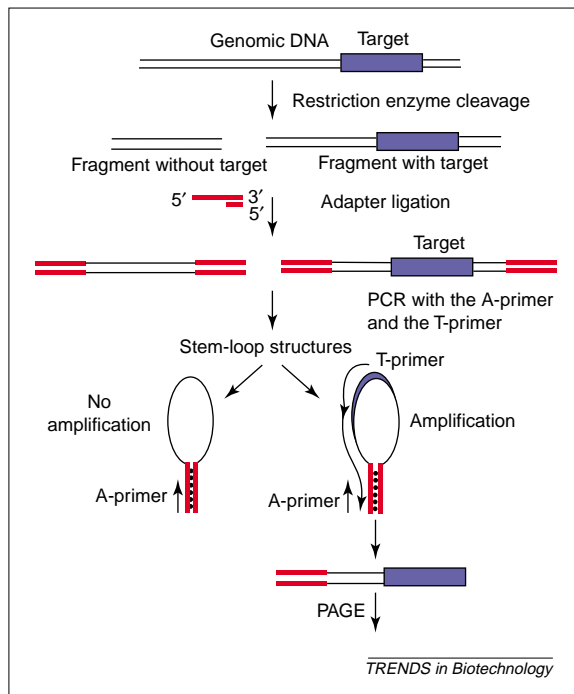
PCR suppression

Principle of PCR suppression

PCR suppression (PS) is another approach based on the formation of stem-loop DNA constructs. In this case, however, the target DNA, in contrast to the molecular beacon approach, is designed to adopt a hairpin form. In general, in all its applications, PS allows the amplification of wanted sequences and simultaneously suppresses the amplification of unwanted ones.

Stem-loop DNA constructs for PS are created by ligation of long GC-rich adapters to DNA or cDNA restriction fragments (Fig. 2) [43]. As a result, each single-stranded DNA fragment is flanked by terminal inverted repeats (i.e. by self-complementary ends). During PCR, on denaturing and annealing, the self-complementary ends of each single strand form duplex stems, converting each fragment into a large stem-loop structure. The formation of stable duplex structures at the fragment ends makes the PCR with the adapter-primer (A-primer) alone relatively inefficient, because the intramolecular annealing of the complementary termini is kinetically favored and more stable than the intermolecular annealing of shorter A-primers. This effect is therefore called PCR suppression [43].

Fig. 2. Outline of the PCR suppression principle. Genomic PCR is digested with a restriction enzyme and ligated with long GC-rich adapters. During the PCR, only the fragments containing the target are amplified efficiently (adapted from [57,58]). Abbreviation: PAGE, polyacrylamide gel electrophoresis.



In the presence of both A-primers and target-primers (T-primers, targeted at the gene-specific sequences in the single-stranded loops), however, PCR is efficient. The T-primer anneals to its target and is used by DNA polymerase to initiate DNA synthesis. The newly synthesized product has two termini, which are not complementary and, thus, cannot fold into a stem-loop structure. This fragment is, therefore, not subject to the PS effect, and is efficiently amplified. Consequently, only the fragments containing the target are exponentially amplified by PCR, while the background fragments without the target remain inert.

PS effect has already found applications in a variety of methods (reviewed in [44]). In this article, several of them will be discussed: suppression subtractive hybridization, multiplex PCR and targeted genomic differential display.

Suppression subtractive hybridization

PCR suppression was successfully used in subtractive hybridization, and resulted in an efficient and widely used method for creating subtracted cDNA libraries [45,46]. Two major problems are usually associated with cDNA subtractive hybridization: (1) large variations in the concentration of different transcripts in a RNA sample, which can range from 0.3–0.5 to 10 000 copies per cell; and (2) a high level of false positives. Application of the PS approach to subtractive hybridization allowed both issues to be addressed and resulted in an efficient method called suppression subtractive hybridization (SSH) [45,46].

In all subtractive hybridization methods, both DNAs are digested to rather short fragments and mixed in a ratio such that one DNA (driver) is vastly more abundant than the other DNA (tester). In SSH, two

different PS-driving adapters are ligated to the tester cDNA in separate aliquots and the driver cDNA remains without an adapter (Fig. 3). After two successive rounds of hybridization, only target sequences, which are present in the tester and have different ligated adapters, can be exponentially amplified with two different adapter primers. At the same time, amplification of the tester homoduplexes is suppressed by formation of stem-loop structures (Fig. 3). The important advantages of SSH are that (1) it incorporates two hybridization steps, leading to efficient normalization of cDNA concentrations, (2) requires only one subtraction round, and (3) does not require physical separation of single-stranded and double-stranded fractions. The frequency of false positives is low and >90% of clones are different [45]. As a result, SSH has become one of the most popular and efficient methods for subtractive expression studies [47–49].

Multiplex PCR

PCR suppression was also used to develop a new strategy for multiplex PCR (mPCR) amplification. In mPCR, multiple DNA targets are amplified simultaneously in one tube [50]. Amplification of each target in conventional PCR requires two gene-specific primers. At a high level of multiplexing, it is often difficult to avoid primer interactions and achieve efficient and uniform target synthesis. In spite of numerous studies aimed at developing an effective strategy for mPCR [51,52] and minimizing primer–primer interactions [53], mPCR still presents a challenge. Although high multiplexing levels were achieved in some studies (e.g. [54]), they remain exceptions. Typically, a routine mPCR does not exceed 5- to 10-plex.

Suppression PCR requires one gene-specific primer per amplicon and one primer, which is common for all targets. Therefore, an n -plex PCR would require only $n+1$ primers instead of $2n$ in conventional PCR (Fig. 4a).

As expected, PS-based mPCR allowed efficient amplification of several targeted sequences [55], and only simple adjustment of conditions was necessary to amplify simultaneously 30 DNA targets of different length from different human chromosomes (Fig. 4b) [56]. Additionally, PS-based mPCR exhibited excellent specificity and provided allele-specificity in a multiplex format (Fig. 4c) [55].

Although the PS approach includes two additional steps compared with conventional PCR (digestion of genomic DNA with a restriction enzyme and ligation with the adapters), this does not create many problems. It was demonstrated that it is possible to use one restriction enzyme for multiple target amplifications, so that a single DNA sample can be used in many experiments. Thus, application of the suppression approach for mPCR seems to offer several advantages over traditional methods, such as higher levels of multiplexing, higher specificity, simpler primer design and primer cost savings.

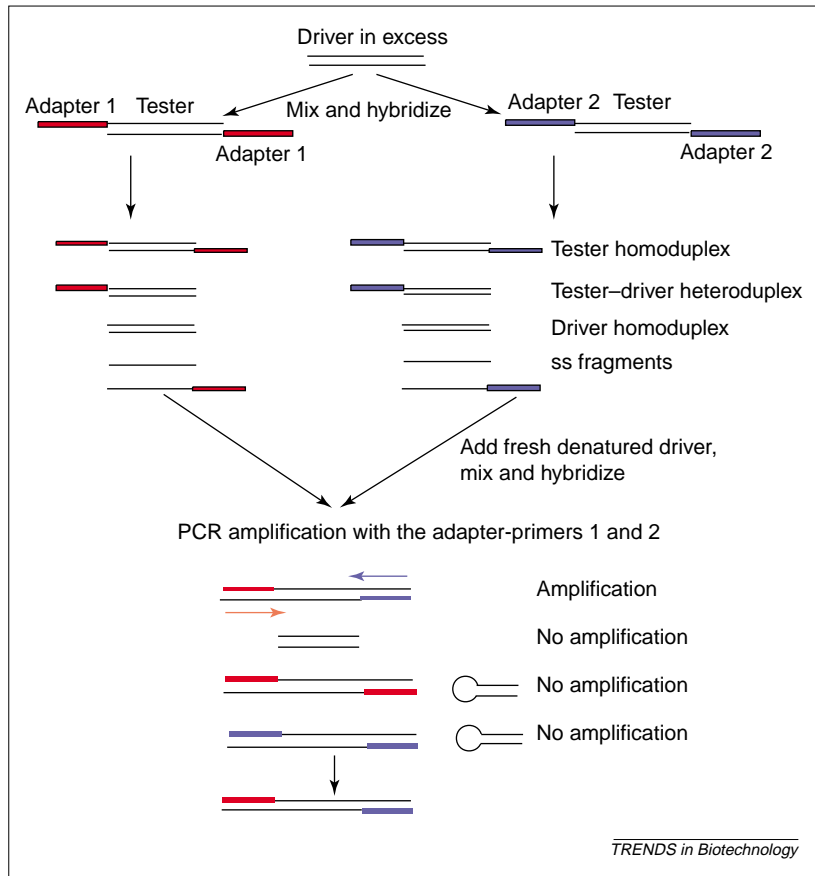


Fig. 3. Schematic outline of the suppression subtractive hybridization. mRNA is transformed into cDNA and digested with a restriction enzyme. Tester cDNA is divided into two aliquots, which are ligated with different PS-driving adapters, whereas driver cDNA is left without adapters. Excess of driver cDNA is hybridized separately with two samples of tester cDNA. Then two tester samples are mixed and a second portion of driver cDNA is added for hybridization. The hybridization mixture is then amplified by PCR with primers corresponding to ligated adapters A1 and A2 (adapted from [45]). Abbreviations: PS, PCR suppression; ss, single stranded.

Targeted genomic differential display

In targeted genomic differential display (TGDD), the PS effect is used to amplify a set of genomic DNA fragments with a single primer targeting repetitive sequences. Because of PS, the vast amount of other genomic fragments that do not have the targeted repeat remain unamplified. The PCR product consists of many fragments, each containing part of the repeat and one of the flanking regions. After resolution of the PCR products on a sequencing gel, a stable pattern of fragments specific for each DNA is obtained, with any genomic differences revealed by their different mobility (Fig. 4d) [57,58]. Single-nucleotide polymorphisms, insertions and deletions were detected when differential fragments were isolated and subjected to sequence analysis (N.E. Broude *et al.*, unpublished) [57]. In TGDD, the complexity of the displayed fragments is controlled by the 3'-anchoring of the primers, essentially in the same way as in the amplified fragment length polymorphism approach [59] and in the ordered cDNA differential display [60]. This allows non-overlapping sets of genomic fragments to be surveyed with different 3'-anchors in different reactions.

In contrast to other PCR-based display methods in which repeated sequences have been targeted [61,62], TGDD shows high specificity: >90% of the clones contained the targets [57,58]. The method demonstrated extremely good reproducibility and good discrimination because of the high polymorphism of the targeted repeats. DNA from monozygotic twins presents a unique natural system for testing the discrimination power of the display methods. Identical twins are supposed to share 100% of their genes; however, phenotypically they show considerable discordance [63,64]. Therefore, the presence of genetic and epigenetic differences in identical co-twin DNAs has long been assumed, but their detection awaited highly discriminative display methods. When trinucleotide repeats [57] and insertion sites of human endogenous retroviral long-terminal repeats [58] were analyzed in DNAs of identical twins, rare reproducible differences were detected on the background of very similar patterns. The sequence analysis of several isolated differential fragments confirmed the presence of mutations (N.E. Broude *et al.*, unpublished).

Thus, TGDD demonstrated excellent performance, with high specificity, robustness and high discrimination power. It can be adapted for the display of any type of repeats and its application is especially promising in cases where repeat instability is anticipated. Its shortcomings are common to all PCR-based methods with simultaneous amplification of a multitude of genomic fragments: (1) there is biased amplification of certain fragments, which leads to over-representation of some of the fragments and the loss of others during amplification; and (2) the complete digestion of all the DNA samples is paramount for reliable DNA comparisons.

Stem-loop DNA probes immobilized on microarrays

During the past few years, DNA microarrays have been used widely for comparative expression studies and DNA mutation analyses (reviewed in [65]). Although RNA expression studies using DNA microchips have already resulted in substantial discoveries (e.g. [66,67]), DNA mutation studies with microarrays are still being developed. The insufficient accuracy of conventional linear-probe microarrays at detecting mutations prompted development of alternative approaches (reviewed in [68]). Therefore, it is not a surprise that stem-loop DNA probes, with their enhanced fidelity in mismatch discrimination, have also found applications in DNA microarray technology.

Hairpin probes were attached to surfaces in topologically different ways, depending on the experimental design. For example, capturing probes with single-stranded overhangs were immobilized through the loop [10,69,70], whereas the molecular beacons were tethered through the 5'- or 3'-end [20,71], or through the linker attached to the stem (Fig. 5a) [72].

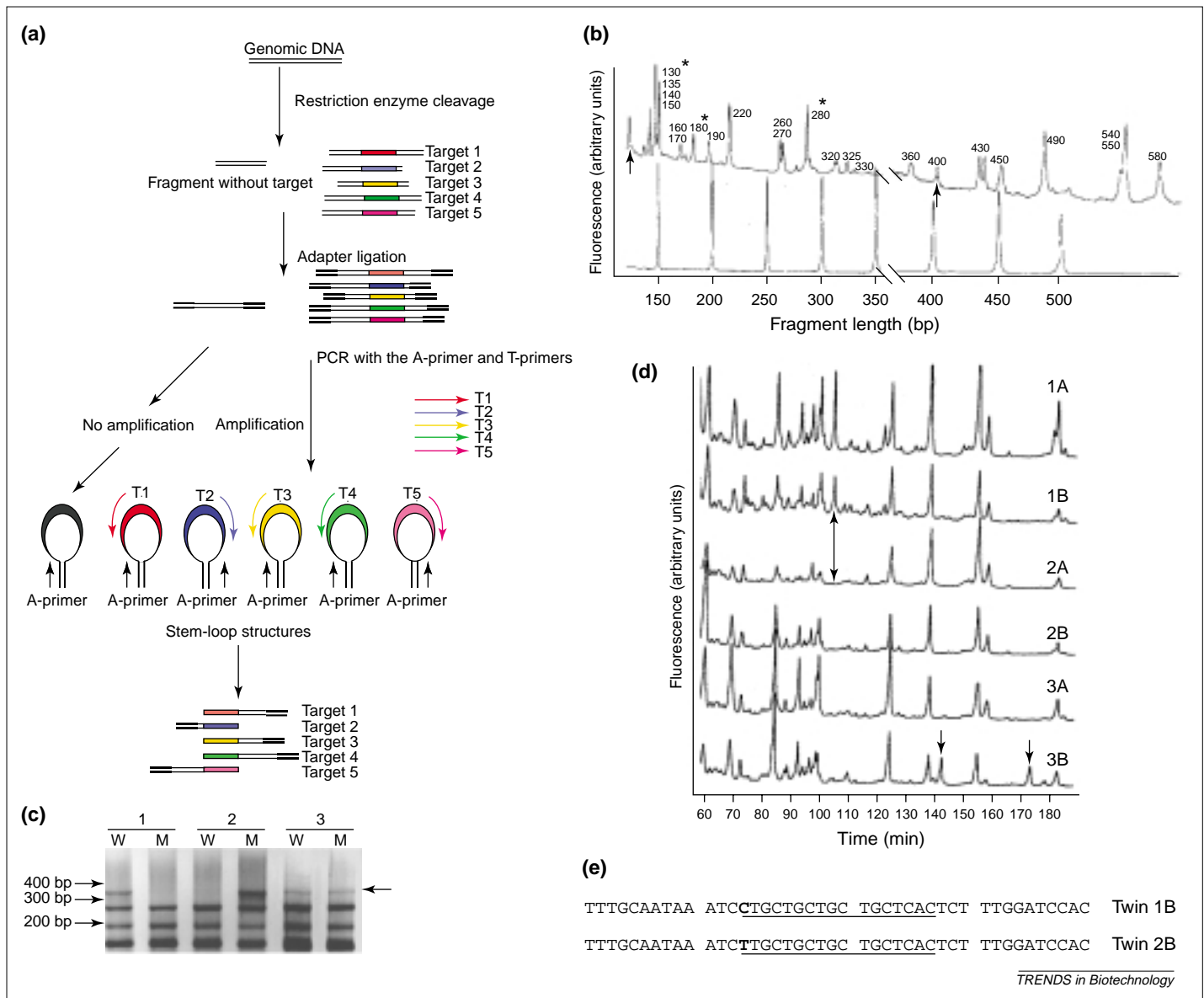


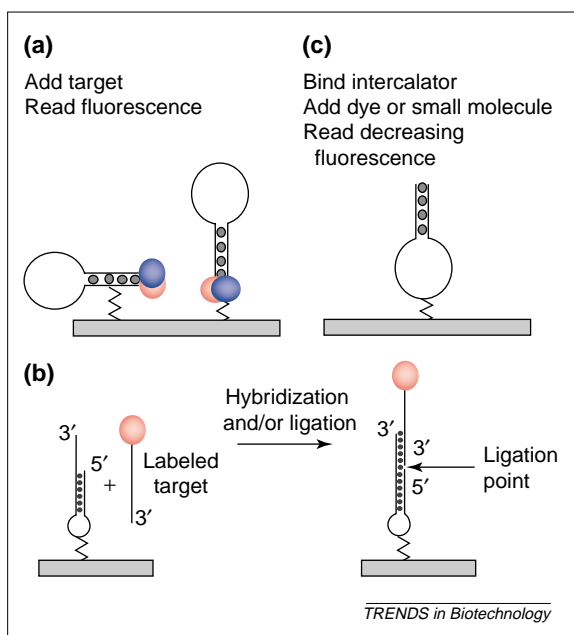
Fig. 4. Various applications of suppression PCR. (a) Schematic outline of multiplex PCR. By using multiple gene-specific primers, simultaneous amplification of multiple targets from different human chromosomes is possible. (b) Fluorescent pattern of 30-plex PCR obtained after separation of the amplicons on a high-resolution sequencing gel. Stars mark the bands containing two overlapping fragments and two unidentified peaks are indicated by arrows. The 50-bp marker is used as a standard (adapted from [56]). (c) Genotyping of $\Delta F508$ cystic fibrosis transmembrane regulator (CFTR) alleles in a 4-plex PCR. Each DNA sample was probed with a wild-type (W)- or $\Delta F508$ (M)-specific primer in combination with three other gene-specific primers. The PCR products were analyzed on a 2% agarose gel. The arrow indicates 350 bp CFTR-specific fragment (adapted from [55]). (d) Application of the suppression principle for the targeted genomic differential display (TGDD). If the T-primer is targeted at the repeat, a multitude of fragments is amplified and a DNA-specific fingerprint is obtained. The differences in patterns for different DNAs reflect single nucleotide polymorphisms, deletions, insertions and differences in methylation. Fluorescent patterns obtained with DNAs from three pairs of monozygotic co-twins (designated twins A and B). DNAs were amplified with a primer targeting CAG trinucleotide repeats and a common A-primer. Small arrows highlight differences between co-twins pair 3, and large arrow shows an inter-individual polymorphism (adapted from [57]). (e) Partial DNA sequence of a DNA fragment that was amplified in DNAs of twin pair 1 and was missing in DNAs of twin pairs 2 and 3 [large arrow in (d)]. The sequence contains a single nucleotide C/T polymorphism (bold) at the T-primer annealing site (underlined), which is responsible for the differential amplification of this fragment in DNAs from different twin pairs (adapted from [57]).

The probes were immobilized with either streptavidin–biotin binding [10,20,71], or they were covalently linked to the derivatized surfaces with different chemistries [69,70].

Immobilized DNA or DNA–PNA chimeric beacons preserve the ability to detect cognate PCR amplicons [71–73]. Hairpin DNA probes with dangling overhangs were used as sequence-specific capture devices and showed a twofold increase in hybridization rates and greater stability of the probe–target complex than the corresponding linear probes [10]. Combination of hybridization and enzymatic ligation using stem-loop DNA probes was applied for mutation detection in single-stranded DNA targets (Fig. 5b) [70].

It should be emphasized that arrays with stem-loop DNA probes are still at the early development stage. One of a few examples of practical use of the immobilized molecular beacons is a miniaturized array technology that combines fiber-optic technique and microsphere-attached differentially labeled molecular beacons [20]. An optical encoding scheme and fluorescent microscope recording allowed parallel detection of several cystic fibrosis transmembrane regulator mutations

Fig. 5. Schematic outline of different applications of the stem-loop shaped DNA probes in microarray technology. (a) Molecular beacons [20,71–73], (b) sequence-specific capturing and mutation detection [10,70], and (c) fluorescent intercalator displacement [40,74].



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in unlabeled DNA [20]. Another example of practical use of stem-loop DNA arrays is a high-throughput quantitative analysis of DNA–ligand interactions based on a competitive displacement of prebound ethidium bromide (Fig. 5c) [40,74]. This method allows the screening of a large library of compounds against a single DNA sequence to identify high-affinity binders, or the screening of a single compound against an array of different DNA probes to determine sequence specificity.

Concluding remarks

The methods discussed in this article do not cover all applications of hairpin DNA probes. For example, the use of stem-loop probes as primers for DNA amplification has been omitted. Nevertheless, it is clear that stem-loop DNA constructs already have a wide range of applications and touch many aspects of structural and molecular biology, genomics and biotechnology. Two major factors that are responsible for such broad applications of these DNA constructs are: (1) enhanced specificity of the probe–target interaction; and (2) the possibility of simple close-tube formats and real-time reaction monitoring with molecular beacons. There is no doubt that in the future, new applications will be developed. For example, a recent study has demonstrated the remote electronic control over hybridization of a molecular beacon by radio-frequency magnetic heating of a metal nanocrystal covalently linked to DNA [75]. Methods that allow direct detection of unamplified target molecules will be especially insightful. Thus, the sensitivity of future assays is crucial. Techniques that combine DNA hairpin probes with other molecular devices (e.g. ribozymes and deoxyribozymes, PD-loops, inorganic nanoparticles) and technological developments (e.g. microarrays, advanced optic techniques) promise to become sensitive and robust, high-throughput research and diagnostic methods. Additionally, small molecule–DNA interactions, protein–DNA interactions, nanotechnology and biosensors are also fields that should make extensive use of the remarkable advantages of the stem-loop DNA constructs.

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