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Hypothesis

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A novel asymmetric-loop molecular beacon-based two-phase hybridization assay for accurate and high-throughput detection of multiple drug resistance-conferring point mutations in *Mycobacterium tuberculosis*

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

The accurate and high-throughput detection of drug resistance-related multiple point mutations remains a challenge. Although the combination of molecular beacons with bio-immobilization technology, such as microarray, is promising, its application is difficult due to the ineffective immobilization of molecular beacons on the chip surface. Here, we propose a novel asymmetric-loop molecular beacon in which the loop consists of 2 parts. One is complementary to a target, while the other is complementary to an oligonucleotide probe immobilized on the chip surface. With this novel probe, a two-phase hybridization assay can be used for simultaneously detecting multiple point mutations. This assay will have advantages, such as easy probe availability, multiplex detection, low background, and high-efficiency hybridization, and may provide a new avenue for the immobilization of molecular beacons and high-throughput detection of point mutations.

key words: ***Mycobacterium tuberculosis*** • **drug resistance** • **point mutation** • **molecular beacon**

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Background

Long-term antibiotic abuse significantly increases the development of drug-resistant mutations in *Mycobacterium tuberculosis* (TB), especially point mutations. Numerous drug resistance-conferring point mutations in TB have been identified, and they include streptomycin-resistant mutations in the rpsl (codons 43 and 88) or rrs genes, isonicotinic acid hydrazide-resistant mutations in the katG gene (codons 315 and 463), ethambutol-resistant mutations in the embB gene (codons 285, 303, 306, and 330), and rifampin-resistant mutations in the ropB gene [1,2]. Accurate and comprehensive detection of these point mutations by classical approaches is labor intensive and very difficult due to technical issues [3]. Although bio-immobilization technologies such as the microarray and microbead assay permit high-throughput detection of these point mutations, the specificity and sensitivity of these assays to detect single-base mismatched target sequence by ordinary DNA probes for hybridization remains a challenge [4–6].

Molecular beacons are a class of stem-loop DNA probes with an internally quenched fluorophore and a quencher located at the ends of stem arms [7]. The probe binds to its specific target sequence, which induces a conformational change in the probe and dissociation of the fluorophore from the quencher, leading to restoration of fluorescence. Because the loop is much longer than the stem, the perfect match between the loop and the target sequence determines the conformational change of the probe and dissociation of the stem. Well-designed molecular beacons with 1 mismatched base in their loops with the target sequences would not result in disassociation of the stem [8,9]. Accordingly, molecular beacons offer many advantages over ordinary DNA probes in detecting point mutations, such as higher sensitivity, specificity, and reusability (Figure 1). At present, molecular beacons have been used for the detection of drug resistance-related point mutations in TB [10]. However, they are mainly used in non-immobilization assays, such as fluorescence-based quantitative PCR, which cannot meet the requirements of high-throughput point mutation detection. The combination of molecular beacons with bio-immobilization assays may be a promising strategy for the high-throughput detection of point mutations [9].

However, the difficulty in effectively immobilizing molecular beacons on the chip surface restricts the wide application of molecular beacons in bio-immobilization assays. Because the 2 ends of molecular beacons are occupied by a fluorophore and a quencher, molecular beacons cannot be terminally modified for immobilization. Hence, many approaches have tried to modify the stem-arm by biotinylation, the most commonly used strategy (Figure 2A) [11]. Molecular beacons are attached on the chips through the biotin-avidin linkage, by which biotin is covalently attached to the stem arm, while avidin is covalently immobilized on the chip surface. Although this approach offers firm immobilization, it has several limitations. First, this approach usually causes a high background of fluorescent signal, which is a long-standing problem in on-chip fluorescence detection [12,13]. Second, modification of the stem-arm is expensive and has technical difficulties. Finally, the biotin-avidin linkage is not sequence-specific and not suitable for multiplex detection (Figure 2B, C). Although separation of immobilizing different probes on the chip surface before hybridization can allow multiple hybridizations simultaneously, this approach may cause low efficiency of hybridization because the efficiency of solid-liquid phase hybridization usually is lower than that of liquid-liquid phase hybridization (Figure 2D) [12]. The other changed molecule beacon is fixed on microarray by terminal amino group modification. Electrical signal coming from hybridization is detected by electrochemistry system. This way has no fluorescence FRET phenomenon, for it is only modified by fluorophore without quencher, and the electrochemistry system is complicated and expensive [14]. Paradoxically, high-efficiency liquid-liquid phase hybridization is one of the main strengths of molecular beacons. However, little is known about whether it is possible to not only preserve the advantages of free molecular beacons in solution, but also to immobilize them on the chip surface for high-throughput detection of multiple molecules.

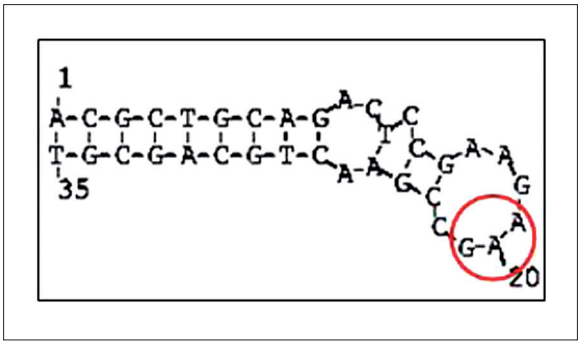

Figure 1. Predicted secondary structure of a molecular beacon. The secondary structure of a molecular beacon designed for detection of streptomycin-resistant point mutation at codon 43 (AAG, red circle) in the rpsl gene was predicted using DNAsis software. The loop is composed of 17 bases, and the stem is composed of 7 base pairs. As the GC/AT ratios are 9:8 in the loop and 6:3 in the stem, the binding energy of the loop to the target is significantly higher than that of the stem, ensuring dissociation of the stem to turn on the fluorescence when binding to the target. If a point mutation occurs at codon AAG, the binding energy should be calculated separately for the sequences flanking the mutation site. The GC/AT ratio is 4:4 in the left loop and 5:5 in the right loop, which is lower than that in the stem (6:3). In theory, the dissociation of the stem will not occur and there will be no fluorescence. Thus, the presence of the point mutation can be effectively identified by the absence of fluorescence.

Hypothesis

Given the unique structural features of molecular beacons, we designed a novel asymmetric-loop molecular beacon in which the loop consists of 2 parts. One (segment A, adjacent to the fluorophore-labeled arm of the stem) is complementary to a target and the other (segment B, adjacent to the quencher-labeled arm of the stem) is complementary to an oligonucleotide sequence immobilized on the chip surface (Figure 3A). Hence, the molecular beacon attaches to the chip surface through liquid-solid hybridization with the immobilized oligonucleotide sequence. The segment A sequence in the loop of the molecular beacon can hybridize to its specific target simultaneously or sequentially. Because the loop of the molecular beacon contains 2 different segments of sequences, this probe is designated as the asymmetric-loop molecular beacon.

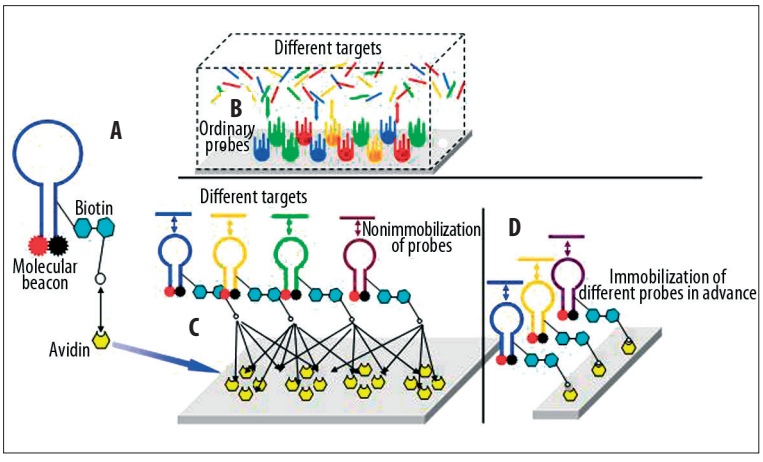

Figure 2. Outline of the working principle and disadvantages of biotinylated molecular beacon-based chip assay. Biotin (cyan) is covalently linked to the stem arm, while avidin (yellow) is covalently immobilized on the chip surface (**A**). Ordinary probe-based chip assay permits high-throughput detection of targets (**B**), whereas the biotinylated molecular beacon-based chip assay is not suitable for multiplex detection as the biotin-avidin linkage is not sequence-specific (**C**). Although this problem can be overcome by separately immobilizing different probes on the chip surface in advance (**D**), the hybridization efficiency becomes low.

In the absence of a target, the stem hybrid will not dissociate and the probes will be washed off. When the novel probe encounters a target at the appropriate temperature, segment A of its loop hybridizes to the target sequence, leading to a conformational change of the probe. As a result, the stem hybrid of the probe dissociates to expose segment B, which in turn hybridizes to the oligonucleotide sequence immobilized on the chip surface, resulting in a fluorescent signal (Figure 3B). Because hybridizations occur in both the liquid-liquid (segment A and targets) and liquid-solid (segment B and immobilized oligonucleotides) phases in a single test, this novel assay is designated as a two-phase hybridization assay.

In this project, FAM-DABCLYE will be tagged on the end of molecule beacon (the maximum excitation wavelength of FAM is 480 nm). The main multiple drug resistance mutant gene points in mycobacterium tuberculosis include [15]: streptomycin-resistant mutations in the rpsl gene (codons 43 and 88) or rrs genes, isonicotinic acid hydrazide-resistant mutations in the katG gene (codons 315 and 463), ethambutol-resistant mutations in the embB gene (codons 285, 303, 306, and 330), rifampin-resistant mutations in the ropB gene (codons 526, 531), pyrazinamide-resistant mutations in the pncA gene, ethionamide-resistant mutations in the katG gene, fluoroquinolones-resistant mutations in the gyrA gene (codon 94), and aminoglycosides-resistant mutations in 2 mutations (A1400G, A1401G).

The main study process includes: 1) The above-mentioned main drug resistance points will be found in NCBI, and relative amplification primers will be designed and synthesized; 2) Asymmetric-loop molecular beacons and oligonucleotide probes aiming at these drug resistance mutant gene points will be individually designed and synthesized; 3) Microarray modified by oligonucleotide probes of the above points will be made and hybridization solution and instruments will be prepared; 4) PCR of these points will be amplified, and PCR products will be purified; 5) Hybridization between PCR products and oligonucleotide probes on microarray will be completed; 6) Microarray eluted will be scanned by microarray scanner, and the results will be recorded and saved; and 7) Clinical drug resistance samples come from culture will be amplified by PCR and PCR products will be detected by microarray and compared with sequencing.

Evaluation of the Hypothesis

The asymmetric-loop molecular beacon-based two-phase hybridization assay is feasible because it has the following features. First, asymmetric-loop molecular beacons retain the stem-loop structure of ordinary molecular beacons without requirement for further modification. In comparison with arm-modified molecular beacons, the synthesis of asymmetric-loop molecular beacons is easier, with little technical difficulty. Second, hybridization with asymmetric-loop molecular beacons has low background because only molecular beacons hybridizing to its target sequence can bind to oligonucleotides immobilized on chips, while free probes will be washed off. Thus the difference in the intensity between positive and negative fluorescent signals is huge, which should be much higher than that of biotin-avidin-based immobilized molecular beacons (Figure 3B). Furthermore, the sequence-specific hybridization between segment B of the probe and the immobilized oligonucleotides permits high-throughput detection of target molecules (Figure 3C). In addition, the asymmetric-loop molecular beacon-based two-phase hybridization assay retains the advantages of liquid-liquid phase hybridization between probes and targets, which should be highly efficient. Finally, the asymmetric-loop molecular beacon-based two-phase hybridization assay may also be used in magnetic bead- and microbead-based assays (Figure 3D).

The asymmetric loop molecular beacon-based two-phase hybridization assay can overcome the difficulty of immobilizing molecular beacons in a relatively simple way. This assay has the advantages of low fluorescence background, high-throughput detection, and high-efficiency hybridization, and holds great promise for accurate, comprehensive, and simultaneous detection of many drug resistance-related point mutations in TB (Figure 4).

Conclusions

We propose a novel asymmetric-loop molecular beacon-based two-phase hybridization assay for accurate and high-throughput detection of multiple drug resistance-conferring point mutations. Possessing the advantages of easy probe availability, multiplex detection, low background,

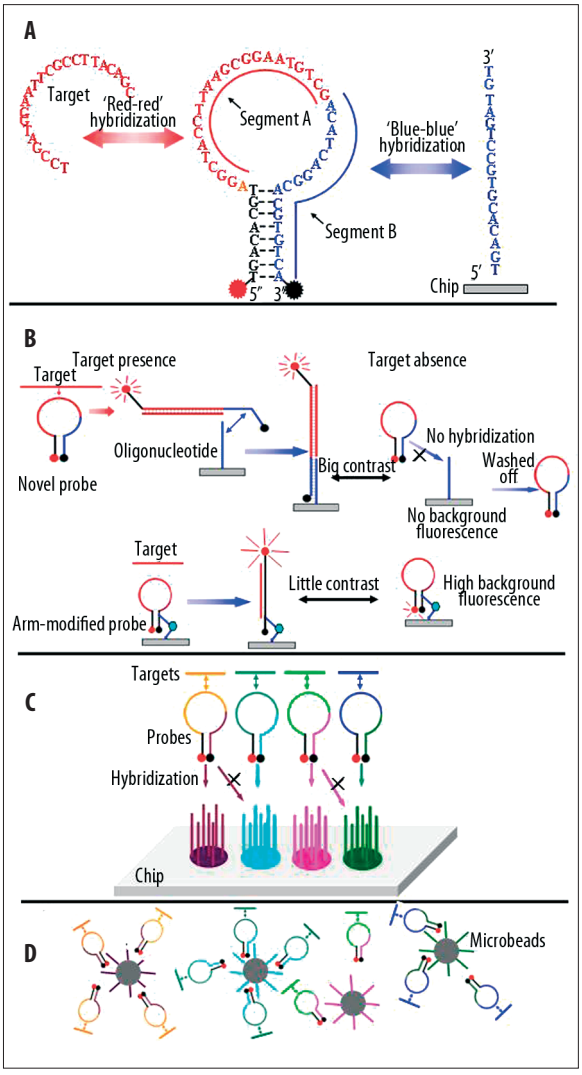

Figure 3. Outline of the working principle and advantages of nonsymmetric-loop molecular beacon-based two-phase hybridization assay. (**A**) Asymmetric-loop molecular beacons retain the stem-loop structure of ordinary molecular beacons. (**B**) Compared with arm-modified molecular beacons, asymmetric-loop molecular beacons generate very low background. (**C**) The asymmetric-loop molecular beacon-based two-phase hybridization assay permits high-throughput detection of target molecules. (**D**) Asymmetric-loop molecular beacons can also be used in microbead-based assay.

and high-efficiency hybridization, this novel assay may provide a new avenue for immobilization of molecular beacons and high-throughput detection of point mutations such as main multiple drug resistance mutant gene points in mycobacterium tuberculosis.

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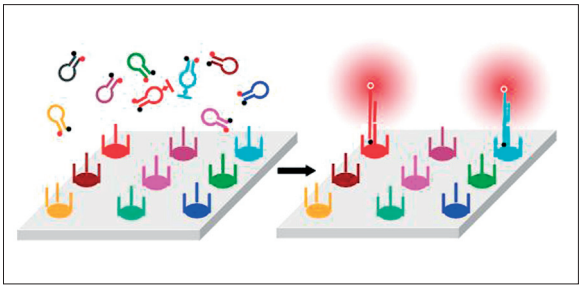

Figure 4. Schematic illustration of the use of novel molecular beacon-based two-phase hybridization assay for high-throughput detection of drug resistance-related point mutations in *Mycobacterium tuberculosis*. In the presence of targets, only the probes that can form probe-target hybrids (red and cyan) can hybridize with oligonucleotides immobilized on the chip surface, whereas free probes are washed off.

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