

Molecular Beacons in Diagnostics

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Abstract

Recent technical advances have begun to realize the potential of molecular beacons to test for diverse infections in clinical diagnostic laboratories. These include the ability to test for, and quantify, multiple pathogens in the same clinical sample, and to detect antibiotic resistant strains within hours. The design principles of molecular beacons have also spawned a variety of allied technologies.

Introduction

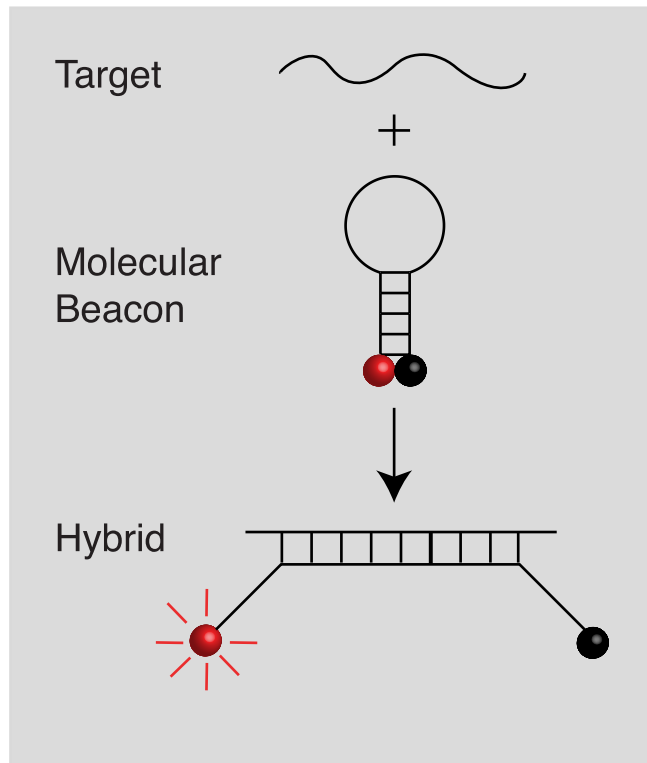
Imagine a magic reagent to which is added a droplet of body fluid from a patient. A glow appears in the tube and the color of the glow identifies the pathogen responsible for the patient's illness. Such was the promise of molecular beacons when they were first introduced 15 years ago [1]. That promise is now bearing fruit, as powerful new tests for diverse infections are being introduced into clinical diagnostic laboratories.

Molecular beacons are hairpin-shaped oligonucleotide probes that become fluorescent upon hybridization to an RNA or DNA target sequence. Their loops serve as probes and are about 15 to 25 nucleotides long. Their stems serve to bring the two ends of the molecule, which are linked to a fluorophore and a quencher, into close proximity. Although the stems are only 5 to 7 nucleotides long, they keep the labels in close proximity so that the fluorescence of the fluorophore is quenched in the free probes. However, upon binding to their target, they undergo a spontaneous conformational reorganization that removes the fluorophore from the vicinity of the quencher and restores its fluorescence (Figure 1). Molecular beacons specific for different target sequences and labeled with differently colored fluorophores can be used together to simultaneously identify and quantitate multiple targets in the same assay tube [2].

Given this fluorogenic response, the addition of the probes to nucleic acids isolated from a clinical sample containing a relatively large number of infectious agents would be sufficient to generate a detectable signal. However, human clinical samples contain only miniscule amounts of pathogen-derived nucleic acids. It is therefore necessary to first amplify an identifying target sequence from the pathogen's nucleic acid. In a typical assay, nucleic acids are extracted from the sample, and a segment of the pathogen's genome (containing the target sequence) is amplified in a polymerase chain reaction (PCR), or in a nucleic acid sequence-based amplification (NASBA) reaction, while simultaneously monitoring the fluorescence of the target-specific molecular beacons in the reaction mixture [1-3]. Since fluorescence is measured in sealed tubes, carryover contamination, which plagued earlier assays, cannot occur. Moreover, the measurement of fluorescence is in real time, as opposed to making measurements at end points, which enables quantitation over an extremely wide dynamic range of target concentrations [4].

Recent advances

Soon after the introduction of molecular beacons and other target-specific fluorogenic probes, such as TaqMan probes [1,5], spectrofluorometric thermal cyclers became available that could perform PCR assays while simultaneously monitoring fluorescence changes. In addition, automated nucleic acid extraction protocols were

Figure 1. Principle of operation of molecular beacons

In the free probes, a fluorophore attached at one of the molecule (red ball) is held close to a quencher (black ball) attached at the other end of the molecule, so that the probe is not fluorescent. The interaction of the sequence in the loop with the target separates the fluorophore and quencher and causes the fluorophore to become fluorescent.

introduced. With the availability of these resources, the development of nucleic acid-based assays for pathogens became relatively simple, and there are now a large number of tests available for the detection of human pathogens, and food and environmental contaminants (Table 1).

Most of the assays listed in the table are designed to detect an individual pathogenic species. However, it is often desirable to determine the type of pathogens that are present in a clinical sample, for example, does the sample contain Gram-negative bacteria, Gram-positive bacteria, or a fungal species [6]? These tests would be even more powerful if they could determine which species (from a selected list) is present, and indeed, investigators have recently developed tests that do this. For example, in a single assay, Chakravorty and his colleagues [7] were able to identify which pathogenic or commensal bacterial species was present in a blood sample from a list of 111 species in 64 different genera. Similarly, El-Hajj and her colleagues [8] have developed an assay that distinguishes

27 different species of *Mycobacteria*. These assays include several molecular beacons labeled with differently colored fluorophores in the same tube. Current instruments are able to distinguish up to seven fluorescent colors in the same tube, which suggests a limit of seven targets. So, to achieve this high level of multiplexing, the assays not only distinguish the fluorescence of each differently colored molecular beacon, they measure the characteristic stability of the probe-target hybrids that each molecular beacon forms by raising the temperature to see when each molecular beacon dissociates from the target (causing a marked decrease in fluorescence). This analysis identifies the target by indicating the relationship of the probe sequence to the target sequence. If the probe is a good match to the target, the probe-target hybrid is quite stable, and this stability is reflected in a high melting temperature, whereas, if the probe is not a good match to the target, the probe-target hybrid is less stable and melts apart at a lower temperature.

Some of these tests can also determine whether the specific pathogen has acquired drug resistance. For example, an assay developed by Alland and colleagues [9-11] for *Mycobacterium tuberculosis* detects the bacterial RNA polymerase gene with several different molecular beacons that are so specific that the presence of any mutation in the target region causes one of the differently colored molecular beacons not to bind to the target (indicating that the commonly used antibiotic rifampicin will not be able to inhibit the activity of the encoded RNA polymerase). This PCR assay takes only two hours, whereas traditional culture-based tests take at least two weeks.

In a number of clinical situations, it is necessary to detect hundreds or thousands of targets at the same time. For example, such situations occur when the mutations responsible for drug resistance are scattered over long sequence stretches, or can be present on multiple genes. To probe for a large number of mutations, new assay formats are under development that simultaneously screen genomes with thousands of molecular beacons. In these formats, different molecular beacons are immobilized at different locations of a planar array [12], on the surface of beads trapped in microfluidic chambers [13], or on different locations of nanowires [14]. The locations that become fluorescent indicate which mutations are present.

It is not always necessary to amplify nucleic acid to detect a pathogen. Molecular beacons complementary to species-specific regions of ribosomal RNA can be used to identify bacterial and fungal pathogens by *in situ* hybridization. In novel "molecular blood culture" assays, pathogens are grown for a short period and then

Table 1. Molecular beacon-based assays for pathogens

Organism	Amplification Scheme	References
Human Pathogens		
HIV	NASBA, PCR	[4,19,20]
HTLV	PCR	[4,21]
Oncogenic HPV	NASBA, PCR	[22-24]
Mycobacterium tuberculosis	PCR	[9-11,25]
hMPV	NASBA	[26]
RSV	NASBA, PCR	[27-29]
Enterovirus	NASBA	[30,31]
Influenza virus	NASBA, PCR	[32,33]
Entamoeba histolytica	PCR	[34]
Methicillin-resistant Staphylococcus	PCR	[35-37]
Hepatitis B	NASBA, PCR	[38,39]
HPIV	NASBA	[40]
West Nile virus	NASBA, PCR	[41,42]
Candida dubliniensis	PCR	[43]
Scedosporium	PCR	[44]
Pan-bacteria	NASBA, PCR, melting analysis	[6,7]
Pan-fungi	NASBA, PCR	[6]
27 Mycobacterial species	PCR, melting analysis	[8]
6 Pneumonia-causing agents	PCR	[45]
Chlamydomydia pneumoniae	NASBA	[46]
Multiple bacteria in blood stream	PCR	[47]
Lyme disease spirochetes	PCR	[48]
Plasmodium	NASBA, PCR	[49,50]
Food Pathogens		
Salmonella	PCR	[51]
Escherichia coli	NASBA	[52]
Listeria	NASBA	[53]
8 food pathogens	PCR, melting analysis	[54]
Environmental Pathogens		
Salmonella	PCR	[55]
Escherichia coli	PCR	[56]
Baylisascaris procyonis	PCR	[57]
Vibrio cholerae	NASBA, PCR	[58,59]
Bacillus anthracis	PCR	[60]

Abbreviations: hMPV, human metapneumovirus; HPIV, human parainfluenza virus; RSV, respiratory syncytial virus.

identified by *in situ* hybridization with molecular beacons, followed by imaging [15].

In addition to their main attribute (that they generate signals without having to separate probe target-hybrids from excess probes, thereby enabling sealed-tube assays), molecular beacons also introduced several design principles that have inspired the development of other kinds of probes. Among these are the use of non-fluorescent dyes as quenchers of fluorescence, the use of hairpins as a means of keeping fluorophores in close contact with quenchers, and the use of hairpins to increase the specificity of hybridization. A small industry of non-fluorescent quenchers, often called "dark" quenchers, with names such as Eclipse, Iowa Black, BlackBerry, and Black Hole Quenchers has been spawned [16]. Dark quenchers afford a larger extent of multiplexing, since they do not crowd the available spectrum by emitting light. A number of novel probe formats that utilize hairpins as their central element have also been introduced. For example, Amplifluor Primers contain a 5' hairpin that possesses a

fluorophore and a quencher in close contact, but once the resulting amplicon (a piece of DNA that has been synthesized using amplification techniques) is copied, the fluorophore and quencher are separated, generating a bright fluorescence signal [17]. In another variant, called Scorpion Primers, a molecular beacon is attached at the 5' end of the primer, but in this case, it cannot be copied. However, the molecular beacon is designed to bind to a segment of the amplified DNA. Since the probe and the target are now in the same molecule, the molecular beacon can come around and bind to the target. This intramolecular interaction happens more readily than the intermolecular binding of a normal molecular beacon [18].

Future directions

Recent years have seen a wave of technical advances that have begun to realize the potential of molecular beacons to test for diverse human pathogens in clinical diagnostic laboratories, and also food and environmental contaminants. These include new techniques with the ability to test for, and quantify, multiple pathogens in the same

clinical sample, and to detect antibiotic resistant strains within hours.

As molecular beacons and allied technologies are adopted in the clinical diagnostics industry, physicians will be able to diagnose diseases with greater precision than ever before.

Abbreviations

NASBA, nucleic acid sequence-based amplification; PCR, polymerase chain reaction.

Competing interest

The authors are inventors of molecular beacons technology, which is patented and licensed non-exclusively to more than 70 licensees, and the authors receive a portion of the resulting licensing income, both personally, and as unrestricted funds that support ongoing research in their laboratory.

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