The PCR Revolution: Basic Technologies and Applications

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Chapter 3

Inventing Molecular Beacons

Fred Russell Kramer, Salvatore A. E. Marras, and Sanjay Tyagi

The invention of molecular beacons followed a rather circuitous route. Our laboratory had been studying the remarkable mechanism of replication of the single-stranded genomic RNA of bacteriophage Qβ, a virus that infects *Escherichia coli*. When a few molecules of Qβ RNA are incubated in a test tube with the viral RNA-directed RNA polymerase, Qβ replicase, millions of copies of each Qβ RNA molecule are generated in only a few minutes by exponential amplification (Haruna and Spiegelman, 1965), without primers and without thermal cycling. Unfortunately, Qβ replicase is so specific for the particular sequences and structures present in Qβ RNA, that it ignores almost all other nucleic acid molecules, disappointing those who would use its extraordinary amplification characteristics to generate large amounts of any desired RNA *in vitro*. However, our laboratory discovered that if a heterologous RNA sequence is inserted into an appropriate site within MDV-1 RNA, which is a naturally occurring small RNA isolated from Qβ-infected *E. coli* (Kacian *et al.*, 1972) that possesses the sequences and structures required for replication (Nishihara and Kramer, 1983), the resulting "recombinant RNA" can be amplified exponentially by incubation with Qβ replicase (Miele *et al.*, 1983). This discovery enabled the design of recombinant RNAs that contained inserted hybridization probe sequences (Lizardi *et al.*, 1988), which were employed in the earliest real-time exponential amplification assays, and whose use, paradoxically, led to the invention of molecular beacons.

Spurred by the emergence of the pernicious infectious agent, HIV-1, which is present in as little as one in 100,000 peripheral blood mononuclear cells in infected asymptomatic individuals, we developed an assay that was designed to utilize the exponential amplification of recombinant RNA hybridization probes to measure the number of HIV-1 target molecules present in clinical samples (Lomeli *et al.*, 1989). The basic idea was to insert an HIV-1 probe sequence into the sequence of MDV-1 RNA. The resulting recombinant RNAs were bifunctional, in that they served as hybridization probes, but after washing away the RNAs that are not hybridized to target sequences, the remaining recombinant RNAs served as templates for exponential amplification by Qβ replicase. The expectation was that the large number of RNA copies that are generated from each hybridized probe would enable the detection of extremely rare targets (Chu *et al.*, 1986).

First real-time exponential amplification assays

Implicit in the use of these replicatable probes was the realization that the number of RNA molecules doubles at regular intervals (approximately every 15 seconds) as exponential amplification progresses. Consequently, the amount of time that elapses before a preselected measurable quantity of RNA is synthesized is dependent upon the number of replicatable probes that are bound to targets prior to amplification. Put mathematically, the time it takes to synthesize a predetermined number of amplicons is inversely proportional to the logarithm of the number of target molecules initially present in a sample (Kramer and Lizardi, 1989), enabling accurate measurements to be made over a wide range of initial target concentrations. This relationship applies to all exponential amplification assays, including the polymerase chain reaction; and it is the principle underlying quantitative real-time PCR. Moreover, we proposed the use of ethidium bromide (Kramer *et al.*, 1974) as a means of providing a fluorescence signal that can be measured by a simple instrument during the course of an amplification reaction to determine, in real time, the number of amplicons synthesized as the amplification progresses (Lomeli *et al.*, 1989). Thus, our laboratory was intimately familiar with the advantages of real-time assays, long before the invention of real-time PCR.

Our work was incorporated into the design of the first commercial real-time exponential amplification assays by Gene-Trak Systems (Pritchard and Stefano, 1991); and it was Gene-Trak (now a part of Abbott Laboratories) that developed the first kinetic fluorescence reader that continuously monitored the fluorescence of an intercalating dye in 96 sealed

reaction tubes, permitting precise quantification of rare nucleic acid targets in clinical samples (Shah *et al.*, 1994; Burg *et al.*, 1995).

The intrinsic problem of using amplifiable probes

Even though these early real-time exponential amplification reactions yielded precise quantitation of target amounts, and had a large dynamic range like their more modern counterparts, they suffered from a serious drawback – reactions without any target also produced a positive signal (Lomeli *et al.*, 1989). The ability to detect rare target molecules was dependent on washing away all of the amplifiable probes that were not hybridized to targets. However, we found that no matter how many wash steps were employed, and no matter whether we utilized a sophisticated method for separating probe-target hybrids from nonhybridized probes (Morrissey *et al.*, 1989), some nonhybridized probe molecules remained, and they were amplified along with the probes that were bound to targets, thereby limiting the sensitivity of the assay to approximately 10,000 target molecules.

In order to overcome this debilitating limitation, we decided to explore various designs for "smart probes," which are probes that can only be amplified if they are hybridized to their target sequence. Consequently, persistent nonhybridized probes (which, by definition, are not bound to target sequences) would not be amplified, and the resulting reactions would be extraordinarily sensitive (Kramer and Lizardi, 1989). It took us seven years to find a smart probe design that worked well. In this particular scheme, the recombinant RNA hybridization probes were cleaved into two sections (through the middle of the inserted probe sequence), with neither section possessing all of the sequences and structures required for exponential amplification. However, when these "binary probes" hybridize to adjacent positions on a target, they can be joined to each other by incubation with a template-directed RNA ligase, generating an exponentially amplifiable reporter. Persistent nonhybridized binary probes, however, are not aligned on a target, so they can not be ligated, and they do not generate a background signal. Consequently, the resulting assays were extraordinarily sensitive, and provided quantitative results for clinical samples containing as few as ten HIV-1 target molecules (Tyagi *et al.*, 1996).

Smart probes containing molecular switches

Before working on binary probes, we explored two other smart probe designs. In one design, the smart probe was not itself an amplifiable molecule. Instead, it was a small oligonucleotide

that was needed to initiate a series of steps that led to the synthesis of exponentially amplifiable reporters, but these steps could only occur if the smart probe bound to its target and changed its shape. In the other design, the smart probes were recombinant RNA molecules that could hybridize to targets and be amplified exponentially. However, they also possessed sequences that enabled them to be destroyed by incubation with *E. coli* ribonuclease III. When these probes bind to their target, they undergo a change in shape that eliminates the recognition site for ribonuclease III. The probe-target hybrids are then incubated with ribonuclease III to destroy all of the probes that are not bound to targets.

The common aspect of both of these designs was that they contained a "molecular switch," which is an oligonucleotide segment possessing a probe sequence embedded between two arm sequences that are complementary to each other, but that are not complementary to the target sequence (Kramer and Lizardi, 1989). In the absence of targets, molecular switches form a hairpin-shaped stem-and-loop structure, in which the arms are bound to each other to form a stem hybrid, and the probe sequence is in the single-stranded loop. In the presence of targets, the probe sequence binds to its target sequence. Because of the rigidity of double-helical nucleic acids (Shore *et al.*, 1981), the probe-target hybrid formed by the loop of the molecular switch cannot co-exist with the stem hybrid formed by the arms of the molecular switch. In effect, the molecular switch must "make a choice" to either retain the stem hybrid and not bind to the target, or to undergo a conformational reorganization in which the stem hybrid unwinds and the probe sequence in the loop binds to the target sequence to form a probe-target hybrid. Molecular switches are therefore designed to contain loop sequences that are sufficiently long (or that possess nucleotides that will form hybrids that are sufficiently strong) to favor the conformational reorganization of the molecular switch in the presence of target sequences that enables probe-target hybrids to form.

In our first design, the smart probe was a hairpin-shaped oligodeoxyribonucleotide possessing a probe sequence in its loop. When this probe binds to its target, it undergoes a conformational reorganization that unwinds its arm sequences. These probes were designed so that the unwound 3'-arm sequence could subsequently be hybridized to the 5' end of a complementary DNA strand, enabling the open arm to serve as a promoter for the synthesis of an RNA copy of the DNA strand. The idea worked as follows: The probes are hybridized to target nucleic acids present in a sample. Nonhybridized probes are then washed away (though a few persist despite vigorous washing). Template DNA is then added to the washed probe-target hybrids. The probes that are bound to targets will, of necessity, have undergone a conformational reorganization. Consequently,

their 3' arms are free to bind to the complementary DNA, forming substrates for the synthesis of RNA by incubation with DNA-directed RNA polymerase. The resulting transcripts were to be MDV-1 RNA, which could then be amplified exponentially by incubation with Qβ replicase, enabling the detection of rare target sequences. The beauty of this approach is that persistent unbound probes that are not washed away will retain their hairpin structure, so their 3' arm sequences are not available to serve as promoters, and these nonhybridized probes can not generate a background signal. The problem with this approach was that no matter what we did (and we tried many things), a small amount of MDV-1 RNA was synthesized from the template DNA by the DNA-directed RNA polymerase in the absence of targets, and even in the absence of probes possessing promoter sequences. So this approach, despite immense effort, was abandoned, and we never published our findings.

In our second design, a molecular switch, whose loop contained a probe sequence, was inserted into an appropriate site in MDV-1 RNA. The resulting recombinant RNA hybridization probes included a stem hybrid (enclosing the probe sequence) that served as a double-stranded cleavage site for RNase III (Kramer and Lizardi, 1989). The idea worked as follows: Recombinant RNAs are hybridized to target nucleic acids present in a sample. Nonhybridized recombinant RNAs are then washed away (though a few persist despite vigorous washing). The resulting probe-target hybrids are then incubated with RNase III, which ignores all probes that are bound to target sequences, because the molecular switch within the recombinant RNA hybridization probe undergoes a conformational reorganization (to enable the probe to bind to the target) that eliminates the double-stranded RNase III cleavage site. Persistent nonhybridized recombinant RNA probes, on the other hand, retain the hairpin stem enclosing the probe sequence, so those molecules are cleaved by RNase III, and are therefore unable to serve as templates for exponential amplification. The hybridized probes, on the other hand, are not cleaved, and they are amplified exponentially by incubation with Qβ replicase, generating a detectable signal. This approach also failed, as we made the discovery that the binding of recombinant RNA probe sequences to RNA target sequences creates unexpected cleavage sites for RNase III that result in the destruction of the target-bound probes (Blok and Kramer, 1997).

Misery loves company

So the situation in the summer of 1992 (when molecular beacons were invented) was that we had developed molecular diagnostic assays based on the exponential amplification of recombinant RNA hybridization probes that had the potential to be extraordinarily sensitive,

and that were amenable to being carried out rapidly, in real time, by automated instruments. However, the sensitivity of these probe-amplification assays was seriously compromised by the presence of background signals that obscured the presence of rare targets, and we had not yet developed binary probes, which eventually provided a workable solution to this problem.

We were also aware that molecular diagnostic assays based on the polymerase chain reaction, in which the targets, rather than the probes, are amplified exponentially (Saiki *et al.*, 1988), suffered from a very similar problem. The PCR primers, which are probes, in the sense that are designed to hybridize to the target sequence, can occasionally bind to non-target sequences in the sample, generating a background signal that consists of false amplicons. Moreover, primers can bind to each other, generating "primer-dimers." In either case, these background signals (just like the background signals in Qβ amplification assays) occurred in the absence of targets, and their existence obscured the presence of rare targets. It became clear that a way around this problem in PCR assays was to employ a product-recognition probe that could distinguish the intended amplicons from the false amplicons (Abbott *et al.*, 1988).

Moreover, if the products of a PCR assay are taken out of the reaction tube for further analysis (such as by gel electrophoresis or by hybridization to product-recognition probes), there is a likelihood that some of the amplicons will escape, contaminating as-yet-untested samples, thereby generating false positive signals in the contaminated samples (Kwok and Higuchi, 1989). Thus, for PCR to be of wide use in clinical diagnostic laboratories, it was essential that product-recognition probes be present in the reaction mixtures prior to the initiation of exponential amplification, and that they have the ability to generate a detectable signal in sealed reaction tubes (Holland *et al.*, 1991).

Probes that become fluorescent upon hybridization to their target

This is where molecular beacons come in. We had been spending years trying to develop extraordinarily sensitive exponential amplification assays in which amplicon synthesis was dependent on whether or not hairpin-shaped probes present in the reaction tubes hybridize to their intended target sequences. However, all of our efforts to utilize molecular switches for this purpose had failed. We were also familiar with the advantages of carrying out assays in which amplification products were measured without their removal from the reaction tube, as a few years earlier we had proposed the use of ethidium bromide as a means of generating

fluorescence signals that would enable amplicons to be measured in real time (Lomeli *et al.*, 1989). Moreover, this technique had recently been applied to PCR assays (Higuchi *et al.*, 1992, 1993). Thus, we were keenly aware that the extraordinary power of PCR (Erlich *et al.*, 1991) could only be fully realized: (a) if product-recognition probes could be included in the reaction mixture to distinguish intended amplicons from false amplicons, (b) if those probes could generate a signal indicative of the amount of probe-target hybrid present as amplification occurs, and (c) if those signals could be detected in sealed reaction tubes to avoid sample cross contamination. If all of these elements could be achieved, PCR would be transformed from a novel research tool into a practical and extraordinarily sensitive clinical diagnostic technique. It is at this point that it struck us that hairpin-shaped probes, if labeled in such a manner as to signal their change in conformation upon binding to their target, could fulfill all of these requirements.

In order to enable measurements to be made in sealed reaction tubes, product-recognition probes need to generate a distinguishable fluorescence signal only when they become hybridized to the intended amplicons. A very promising fluorescence signal-generation technique was fluorescence resonance energy transfer (FRET), which is dependent on the distance between a "donor fluorophore" and an "acceptor fluorophore" (Stryer and Haugland, 1967). Years earlier, it had been shown that when two different probes are hybridized to a target nucleic acid at adjacent positions, one labeled with a donor fluorophore, and the other labeled with a different acceptor fluorophore, the intensity of the characteristic fluorescent color from the donor fluorophore is reduced and the intensity of the characteristic fluorescent color of the acceptor fluorophore is increased, and this only occurs when the two probes are hybridized to the target at adjacent positions (Heller and Morrison,1985; Cardullo *et al.*, 1988). This is the principle employed in "LightCycler probes" (Wittwer *et al.*, 1997). Alternatively, a pair of complementary oligonucleotides is used (Morrison *et al.*, 1989). One strand, which serves as the probe, is labeled with a donor fluorophore, and is hybridized to a complementary oligonucleotide labeled with an acceptor fluorophore. The fluorophores are linked to the oligonucleotides in such a manner that they are close to each other, decreasing the intensity of the fluorescence of the donor fluorophore. However, when target strands are present, a competition occurs, and some of the probes hybridize to the target strands instead of hybridizing to the complementary strands possessing the acceptor fluorophore. Consequently, the intensity of the fluorescence from the donor fluorophore increases, signaling the presence of the target.

These FRET techniques required two separate probe molecules, each labeled with a differently colored fluorophore. Our concept was that a hairpin-shaped probe labeled with a differently colored

fluorophore on the end of each arm sequence would accomplish the same task. When such a probe is free in solution, it forms a hairpin structure, causing the two label moieties to interact. However, when the probe hybridizes to its target, it undergoes a conformational reorganization that unwinds the arm sequences. Due to the rigidity of the resulting probe-target hybrid, the arms are kept far apart, preventing the two label moieties from interacting with each other. Consequently, the fluorescence signal from probes that are hybridized to targets would be distinguishable from the fluorescence signal from nonhybridized probes.

There was, however, one additional property that had to be included before these product-recognition probes would become true "molecular beacons." We wanted hairpin-shaped probes that were not fluorescent when free in solution, but that became fluorescent when they bind to their targets. We therefore conceived of using a pair of label moieties in which the donor was a fluorophore and the acceptor was not able to fluoresce at all. In this labeling scheme, the fluorescence of the donor would be significantly reduced when the donor was in close proximity to the nonfluorescent acceptor (i.e., the acceptor would serve as a "quencher" of the donor's fluorescence). However, when the probe is hybridized to its target, the fluorophore and the quencher would be forced apart from each other, generating a measurable fluorescence signal. That is the origin of molecular beacon probes (Tyagi and Kramer, 1996).

The use of just that sort of label pair had been known for several years in a different context. In an effort to identify the first drugs that could serve as therapeutic agents against HIV-1, Abbott Laboratories developed an assay to identify compounds that inhibit the activity of the viral protease, which is essential for the maturation of the virus. The assay was based on the preparation of a short peptide that serves as a substrate for the protease. The peptide was covalently linked to a blue-emitting fluorophore at one end (EDANS) and to a nonfluorescent quencher (dabcyl) at the other end. These two label moieties served as a well-matched FRET pair. However, because dabcyl is not a fluorescent moiety, the energy stored in the donor (EDANS) is transferred to the acceptor (dabcyl), and is then released as heat, rather than as light of a characteristic color. Due to the proximity of the dabcyl to the EDANS, there was very little fluorescence. When HIV-1 protease is incubated with this dual-labeled peptide, it cleaves the peptide, physically separating the EDANS from the suppressive presence of the nearby dabcyl, leading to a fluorescence signal. Thousands of individual assays were carried out, each possessing dual-labeled peptides and HIV-1 protease, and each containing a different test compound, in the hopes of identifying potential protease inhibitors by their ability to prevent

the generation of a fluorescence signal (Matayoshi *et al.*, 1990). EDANS and dabcyl possessed the properties that we had been looking for. We therefore decided to label our hairpin-shaped product-recognition probes with EDANS and dabcyl, thereby creating probes that were dark when free in solution, but fluorescent when hybridized to their targets.

About the same time, it was shown that small, single-stranded oligonucleotide probes can be labeled with a different fluorophore at each end, and that when those probes are free in solution, they form a random-coil configuration that brings the ends of each oligonucleotide so close to one another that the fluorophores can undergo efficient FRET. However, due to the rigidity of probe-target hybrids, when these linear probes hybridize to their target, the ends are forced apart, FRET is disrupted, and the consequent changes in the fluorescence intensity of each fluorophore signal the presence of the target (Parkhurst and Parkhurst, 1993, 1995). This is the key feature that was incorporated into linear fluorescent probes that were used in the 5'-nuclease assay (Lee *et al.*, 1993) to convert them into "TaqMan probes" (Livak *et al.*, 1995; Heid *et al.*, 1996).

Contact quenching enables multicolor probes

The first molecular beacons, which were labeled with EDANS and dabcyl, achieved a signal-to-background ratio of 25, which was an unqualified success. However, molecular beacons would have remained just a scientific curiosity if EDANS had been the only fluorophore that could have been used. Its limitations soon became apparent. It was only one fiftieth as bright as fluorescein (the most commonly used fluorophore), and its emission range coincided with the autofluorescence of the plastics used in reaction tubes. Furthermore, to realize the full promise of real-time amplification, and to detect multiple targets in the same tube, it is necessary to use a set of probes, each specific for a different target, and each possessing spectrally distinguishable fluorophores. Therefore, we explored the use of other fluorophore-quencher pairs.

For efficient FRET, not only must the acceptor and the donor be a short distance from each other, but the emission spectrum of the donor must substantially overlap the absorption spectrum of the acceptor (Haugland *et al.*, 1969). This limitation, combined with the desirability that the quencher be nonfluorescent, proved to be too restrictive, and no other appropriate FRET pairs could be found. Hoping that a partial spectral overlap might yield a useful degree of quenching, we synthesized a molecular beacon possessing fluorescein as the donor and dabcyl as the acceptor. The results were pleasantly surprising. We obtained a better quenching efficiency than we had reported for EDANS and dabcyl. Encouraged by this, we tried other fluorophores

with dabcyl, including tetramethylrhodamine and Texas red, both of whose emission spectra were farther towards the red end of the spectrum, and therefore had very little or no overlap with the absorption spectrum of dabcyl; yet both fluorophores were efficiently quenched. Indeed, when we tried a series of fluorophores whose emission spectra ranged from deep blue to far red, we obtained a uniformly high degree of quenching (signal-to-background ratios between 100 and 1000), irrespective of the degree to which their emission spectra overlapped the absorption spectrum of dabcyl (Tyagi *et al.*, 1998).

This apparent violation of the cardinal FRET rule prompted us to reexamine the mechanism of fluorescence quenching that takes place in molecular beacons. Fluorescence is a hard-to-achieve property of a molecular moiety, and the introduction of new chemical bonds into that moiety is likely to destroy its ability to fluoresce. Furthermore, we hypothesized that when molecular beacons are in a hairpin conformation, the fluorophore and the quencher are brought so close to each other that they should be able to form chemical bonds, just as nucleotides that are present on complementary strands of a double helix form hydrogen bonds. To test this hypothesis, we compared the visible absorption spectra of molecular beacons in the presence and in the absence of target strands (i.e., we observed whether the combined absorption spectrum of a fluorophore and a quencher, which depends on which chemical bonds are present, changes when the two label moieties are brought close to one another). The results showed that all molecular beacons (even molecular beacons that possess identical fluorophores on either end) have a different visible absorption spectrum, depending upon whether they are "closed" or "open" (Tyagi *et al.*, 1998). Thus, the quenching of fluorescence in molecular beacons possessing labels on their 5' and 3' ends is not primarily due to FRET; and this realization proved to be extremely useful. Virtually any fluorophore could be used in combination with the same nonfluorescent quencher, and dabcyl was just one example of a "universal quencher" (Tyagi *et al.*, 1998). We were therefore able to design extremely sensitive, multiplex, real-time PCR assays containing sets of molecular beacon probes, each of which was specific for a different target sequence, and each of which possessed a differently colored fluorophore in combination with dabcyl (Vet *et al.*, 1999; Marras *et al.*, 1999).

As a consequence of these observations, it became clear to us that other types of probes that were designed to be used in "homogeneous" PCR assays were not limited to the selection of label pairs that interact by FRET. Any probe design that brings a label pair into intimate contact, depending on whether the probe is, or is not, hybridized to its target, can employ label pairs that interact by "contact quenching." It was subsequently confirmed that, when they are free in solution, molecular beacons form a ground state intramolecular heterodimer whose spectral qualities can

be accurately described by exciton theory (Bernacchi and Mély, 2001). The quenching moiety need not be a fluorophore or a dye; it can be any moiety that forms transient chemical bonds with a fluorophore – it can even be a guanosine nucleotide (Knemeyer *et al.*, 2000; Crockett and Wittwer, 2001). Moreover, it does not matter whether the probe design involves single oligonucleotides, such as TaqMan probes (Nasarabadi *et al.*, 1999) and molecular beacons (Marras *et al.*, 2002), or whether two separate oligonucleotides are employed, such as in strand displacement probes (Li *et al.*, 2002a) and in "molecular zippers" (Yi *et al.*, 2006). Our results spurred the introduction of novel, highly efficient "dark quenchers" with exotic names, such as "eclipse quenchers" and "black hole quenchers." And most significantly, it was shown that the use of nonfluorescent quenchers that form transient chemical bonds stabilizes the probes, serving the same function as the hairpin stem in molecular beacons, thereby yielding linear (TaqMan) probes that are almost as well quenched as molecular beacons (Johansson *et al.*, 2002; Moreira *et al.*, 2005).

Indeed, the widespread use of molecular beacons (and other dual-labeled probes) led to improvements in the materials available for their synthesis. Initially, we prepared molecular beacons by synthesizing oligonucleotides possessing a 5'-terminal sulfhydryl group and a 3'-terminal amino group. Then, in separate reactions, an iodoacetylated fluorophore was covalently linked to the 5'-terminal sulfhydryl group, and a succinimidyl ester of dabcyl was covalently linked to the 3'-terminal amino group. The introduction of controlled-pore glass columns possessing dabcyl as the starting material by Glen Research in 1997, and the parallel introduction of a variety of fluorophore-labeled phosphoramidite precursors, enabled us to greatly simplify the automated synthesis of molecular beacons (Mullah and Livak, 1999).

Real-time PCR assays performed with molecular beacons

Initially, we had no idea how to design an effective molecular beacon. Our first version had a probe sequence 40 nucleotides long and arm sequences 20 nucleotides long, based on the naïve idea that a probe-target hybrid twice as long as a stem hybrid, would drive the opening of the stem. When we tested this construct by the addition of an excess of complementary oligonucleotides, utilizing an ultraviolet view box to see if there was an increase in fluorescence, no fluorescence was detected. However, when we heated the mixture with a hairdryer, the faint blue fluorescence of EDANS appeared in the solution, indicating that, even though the probe did not respond to its target, it was well quenched in its hairpin state. Hypothesizing that the stem might be too strong to open, we tried different stem lengths, and made the astounding discovery that a stem hybrid only five basepairs long worked best, responding spontaneously to the target at room temperature. It soon became clear that we had completely missed the fact that the arm sequences, because they

are tethered to each other by the probe sequence, are much more likely to bind to each other than they would if they existed separately in solution. Ultimately, we found that molecular beacons that are designed to detect amplicons during the annealing stage of each PCR thermal cycle (which is usually between 55 ºC and 60 ºC) should preferably possess probe sequences between 18 and 25 nucleotides in length (depending on the G-C content of the target sequence) and arm sequences between 5 and 7 nucleotides in length (depending on the number of G:C basepairs that they form).

When we carried out our first real-time PCR assays with molecular beacons, no instrument was available that could simultaneously carry out thermal cycling and measure fluorescence. We therefore adopted a strategy in which a cuvette containing the reactants was cyclically transferred from a bath maintained at 95 ºC to a spectrofluorometer maintained at 37 ºC, and then to a bath maintained at 72 ºC. Several hours of "cycling" left us really tired, and only one reaction could be done per day. Later, we purchased a thermal cycler and carried out identical PCR assays containing molecular beacons, terminating each reaction after a different number of thermal cycles, and then measured the fluorescence intensity in each tube. Although the data were noisy (Tyagi and Kramer, 1996), we could see the key feature of real-time PCR: the number of thermal cycles that needed to be carried out to synthesize sufficient amplicons for the reaction to complete the exponential phase of synthesis and enter the linear phase of synthesis was inversely proportional to the logarithm of the initial number of target strands. These results were particularly gratifying, as the kinetics were the same as those that we had observed years before for the exponential synthesis of RNA by Qβ replicase (Kramer *et al.*, 1974). Later, in 1996, we formed a research relationship with Applied Biosystems, under which they provided us with the earliest real-time spectrofluorometric thermal cycler (the ABI Prism 7700). The very first PCR assays performed with this instrument yielded smooth amplification curves, enabling us to simultaneously carry out multiplex reactions with differently colored molecular beacons in sealed reaction tubes (Giesendorf *et al.*, 1998; Kostrikis *et al.*, 1998; Vet *et al.*, 1999; Marras *et al.*, 1999).

The extraordinary specificity of molecular beacons

We compared the specificity of hairpin-shaped probes to the specificity of corresponding linear probes (Tyagi *et al.*, 1998). We prepared hairpin-shaped probes and hybridized them to target oligonucleotides that were perfectly complementary to the probe sequence in the hairpins. We then measured the stability of the resulting hybrids, as expressed by their melting temperature. We repeated these measurements, utilizing otherwise identical probes, in which the sequence of one of their arms was rearranged so that it could not form a hairpin stem. We found that both

the hairpin-shaped probes and the corresponding linear probes formed hybrids that melted apart at about the same temperature, indicating that they were equally stable. However, when we repeated the experiments with target oligonucleotides that caused there to be a mismatched basepair in the middle of each hybrid, the results revealed a fundamental and powerful property of molecular beacons: Because both hybrids now possessed a mismatched basepair, they both melted apart at a lower temperature. However, the melting temperature of the hybrids containing the probes that could form a hairpin was much lower than the melting temperature of the corresponding hybrids containing the probes that could not form a hairpin. These results demonstrated that molecular beacons are considerably more "finicky" than corresponding linear probes (Täpp *et al.*, 2000), and are thus ideal for detecting single-nucleotide polymorphisms and other mutations in PCR amplicons (Giesendorf *et al.*, 1998; Piatek *et al.*, 1998; Marras *et al.*, 1999; Mhlanga and Malmberg, 2001).

We carried out an extensive series of experiments to compare the thermodynamic attributes of probe-target hybrids formed by our hairpin-shaped probes to the attributes of probe-target hybrids formed by corresponding linear probes (Bonnet *et al.*, 1999), and the following picture emerged: Probes, just like any other molecules, are most likely to assume the most stable state possible under a given set of conditions. The presence of a mismatched basepair causes probe-target hybrids to be less stable, but has no effect on the stability of the stem hybrid that can form in hairpin-shaped probes if they are no longer hybridized to a target. When we design molecular beacons that are intended to discriminate against single-nucleotide polymorphisms in a target, we select the length (and strength) of the probe sequence and the arm sequences so that the formation of the probe-target hybrids that they will form with perfectly complementary targets is favored under the detection conditions (the annealing temperature) of the PCR assay. However, we choose the sequences of the molecular beacons so that they will form probe-target hybrids that are just a little bit more stable than the hairpins by themselves. So if a mutation is present in a target, thereby lowering the stability of the potential probe-target hybrid, the molecular beacons will prefer to remain in the nonfluorescent hairpin configuration, which is more stable than the mismatched probe-target hybrid that could be formed under those conditions. Our experiments showed that the reason that corresponding linear probes are not as useful for discriminating mutations is that, unlike probes that can form a hairpin stem, they do not have an alternative stable state to assume in the face of the destabilizing presence of a mismatched basepair in the hybrid, so they tend to remain bound to the targets despite the presence of a mismatched basepair. Thus, the discriminatory power of molecular beacons is an example of "stringency clamping" (Roberts and Crothers, 1991), which is based on the fundamental principle that the specificity of any intermolecular interaction is significantly higher if one or both of the interacting

molecules has the possibility of forming an alternative stable structure, rather than forming an intermolecular complex (Bonnet *et al.*, 1999; Broude, 2002).

Of course, linear probes (such as TaqMan probes) can also be designed so that they distinguish single-nucleotide polymorphisms. However, because there is not such a great difference in stability between a perfectly complementary hybrid formed by a linear probe and a hybrid containing a single mismatched basepair formed by the same probe, it is quite difficult to find conditions under which a set of linear probes, each specific for a different target, can all display the same degree of specificity in the same reaction tube. Molecular beacons, on the other hand, because they form alternative stable structures, rather than forming mismatched probe-target hybrids, can easily be designed so that many different molecular beacon probes, each specific for a different target, can be utilized under the same reaction conditions in a sealed reaction tube; and each molecular beacon will be so specific that it will only bind to a perfectly complementary target sequence. What this means in practice is that four different molecular beacons can be designed to distinguish four different nucleotides that can occur in the same position in an otherwise identical target sequence, and they can be used together in the same PCR assay tube, and each will only generate a fluorescence signal (in a color determined by each probe's fluorophore) when that probe binds to its perfectly complementary target, and the other three probes will not generate a signal if their target sequence is not present (Marras *et al.*, 1999). Indeed, because it is easy to design highly specific molecular beacons that only bind to complementary target sequences, even though they are used in the same reaction tube, we have been able to develop PCR screening assays that contain 15 different species-specific probes (each labeled with a distinctive combinatorial color code) that can rapidly identify a sepsis-causing bacterium that is present in a (normally sterile) blood sample (Marras *et al.*, 2008). Moreover, we are developing "molecular blood cultures" that use 35 different highly specific, color-coded molecular beacons to simultaneously screen for the presence of 35 different infectious agents in the same PCR assay tube. And finally, because of the extraordinary specificity of molecular beacons, they are ideal probes to attach to the surface of oligonucleotide hybridization arrays, since many different molecular beacons can all be designed so that they discriminate target sequences under the same set of conditions.

Molecular beacons as biosensors

When a conventional single-stranded oligonucleotide probe hybridizes to a target nucleic acid sequence, very little in the way of a physical change occurs that enables one to determine that the target is present. Molecular beacons, on the other hand, undergo a conformational reorganization

when they bind to their target that separates a fluorophore from a quencher, thereby generating a bright, easily detectable fluorescence signal of a characteristic color. Molecular beacons are therefore classic "biosensors", and they can be adapted for use in a variety of different analytical systems; they can be linked to other macromolecules in order to create multifunctional detectors; they can be linked to surfaces for use in diagnostic arrays; and they can be synthesized from unnatural nucleotides so that they can function without being destroyed in living cells. Here are a few examples:

In addition to their use in conventional PCR assays, molecular beacons are ideal for detecting amplicons in isothermal gene amplification assays, such as those that utilize nucleic acid sequence-based amplification (NASBA) (Leone *et al.*, 1998; de Baar *et al.*, 2001a,b) or rolling-circle amplification (Nilsson *et al.*, 2002; Alsmadi *et al.*, 2003). Moreover, they have been used in digital PCR assays (Vogelstein and Kinzler, 1999), LATE-PCR assays (Sanchez *et al.*, 2004; Pierce *et al.*, 2005), and for the detection of RNA during transcription (Marras *et al.*, 2004). And finally, in a new paradigm termed "multiprobe species typing," sets of differently colored "sloppy molecular beacons" possessing unusually long probe sequences have been used in screening assays, in conjunction with a determination of the thermal melting characteristics of the probe-target hybrids (Ririe *et al.*, 1997), to generate species-specific signatures that uniquely identify which infectious agent (from a long list) is present in a clinical sample (El-Hajj *et al.*, 2008).

Molecular beacons have been covalently linked to other macromolecules in order to create bifunctional biosensors. A classic example is "amplifluor primers," which are molecular beacons linked to the 5' ends of PCR primers (Nazarenko *et al.*, 1997). After an amplifluor primer has bound to its target strand and been extended, the resulting amplicon then serves as a template for the synthesis of a complementary strand, causing the molecular beacon to open, generating a fluorescence signal that is detected at the end of the polymerization step in each PCR cycle. In a clever variant, called "LUX primers," guanosine nucleotides serve as the quenchers (Nazarenko *et al.*, 2002). And an even more intriguing combination occurs in "scorpion primers," which are molecular beacons linked to primers via a blocker group that prevents the molecular beacon from being copied during PCR (Whitcombe *et al.*, 1999). The probe sequence in the molecular beacon segment is designed to hybridize to a target sequence in the amplicon strand created by the extension of the primer segment, causing the molecular beacon to open, thereby generating a fluorescence signal that is detected at the end of the annealing step in each PCR cycle. An advantage of scorpion primers, as compared to amplifluor primers and LUX primers, is that they do not fluoresce when incorporated into false amplicons. Molecular beacons have also been covalently linked to viral peptides, enabling the probes to cross cell membranes to

light up target mRNAs in living cells (Nitin *et al.*, 2004). In addition, molecular beacons have been linked to tRNAs, in order to prevent the probes from becoming sequestered in nuclei, thereby enabling target mRNAs to be detected in the cytoplasm of living cells (Mhlanga *et al.*, 2005). And finally, molecular beacons have been linked to an additional sequence that serves as a capture probe. The resulting "tentacle probes" combine the high affinity of capture probes with the extraordinary specificity of molecular beacons (Satterfield *et al.*, 2007).

A key feature of molecular beacon probes is that they can be designed so that they cannot fluoresce when they are in their hairpin conformation, because the fluorophore and the quencher interact with each other by contact quenching to form a transient "ground-state" heterodimer that is not fluorescent (Bernacchi and Mély, 2001; Johansson *et al.*, 2002). However, the function of some molecular beacons has been enhanced by the incorporation of additional elements that interact by FRET. For example, some spectrofluorometric thermal cyclers use a blue argon ion laser to stimulate fluorescence, which efficiently excites blue and green fluorophores, but inefficiently excites orange and red fluorophores. To remedy this inefficiency, molecular beacons were designed that include a secondary fluorophore that interacts with the primary fluorophore by FRET. When these "wavelength-shifting molecular beacons" bind to their target, they undergo a conformational reorganization that enables the energy that was efficiently absorbed from the blue laser light by a primary fluorophore to be transferred by FRET to a secondary fluorophore, resulting in the generation of a bright fluorescence signal in orange or red (Tyagi *et al.*, 2000). By combining wavelength-shifting molecular beacons with conventional molecular beacons, highly multiplex clinical diagnostic PCR assays have been developed that contain differently colored probes that span the entire visible spectrum, and that are designed to be used with spectrofluorometric thermal cyclers that contain powerful monochromatic lasers (El-Hajj *et al.*, 2001). An additional advantage of wavelength-shifting molecular beacons is that they have a large Stokes shift, which means that the color of their fluorescence is very different from the color of the light that stimulates their fluorescence, thus enabling the detection of the fluorescence signal with very little interference from the stimulating light. This principle was incorporated into "dual-FRET" probes, which are pairs of molecular beacons, one possessing a donor fluorophore on its 5' end, and the other possessing an acceptor fluorophore on its 3' end, which bind to adjacent target sequences on mRNAs in living cells (Bratu *et al.*, 2003; Santangelo *et al.*, 2004). Although the fluorophore and quencher in each of the molecular beacons in the pair interact by contact quenching, nucleic acid-binding proteins in the cell occasionally unwind nonhybridized probes, and cellular nucleases occasionally cleave nonhybridized probes, creating background signals from the fluorophores on the probes. However, when a pair of dual-FRET molecular beacons are bound to adjacent positions on a target mRNA, the two fluorophores interact

by FRET, creating a unique fluorescence signal that can be distinguished from the background fluorescence.

Ideal biosensors are designed to respond to the presence of their targets, but should be unaffected by the environment in which they operate. Molecular beacons, however, are usually made of DNA or RNA, and they are often used in reaction mixtures that contain enzymes whose substrates are nucleic acids. For example, if molecular beacons composed of deoxyribonucleotides are used to monitor the synthesis of RNA from a double-stranded DNA template by a DNA-directed RNA polymerase, the molecular beacons themselves are copied, generating a background signal that mimics the synthesis of the transcripts (Marras *et al.*, 2004). It is therefore desirable for some assays to utilize molecular beacons that contain unnatural nucleotides, rendering them resistant to enzymatic activity. For example, molecular beacons have been synthesized that contain peptide nucleic acid (PNA) monomers (Ortiz *et al.*, 1998; Kuhn *et al.*, 2000; Nitin *et al.*, 2004), 2'-*O*-methyl ribonucleotides (Molenaar *et al.*, 2001; Tsourkas *et al.*, 2002), and locked nucleic acid (LNA) monomers (Wang *et al.*, 2005). By minimizing background signals, the use of these modified molecular beacons ensures that the fluorescence signal reflects the process or product that is being measured (Marras *et al.*, 2004). Molecular beacons synthesized from unnatural nucleotides, such as the 2'-*O*-methyl ribonucleotides, are of particular importance when imaging mRNA targets in living cells, otherwise the probes (and sometimes the mRNA to which the probes bind) will be destroyed by cellular nucleases (Bratu *et al.*, 2003; Tyagi and Alsmadi, 2004; Mhlanga *et al.*, 2005).

Hundreds, and even thousands, of molecular beacons, each specific for a different target sequence, can be used simultaneously in a single assay by attaching them to predetermined locations on the surface of hybridization arrays (Ramachandran *et al.*, 2004; Yao and Tan, 2004), or by attaching them to the surfaces of beads in "distributed arrays" (Steemers *et al.*, 2000), in which each bead possesses molecular beacons that identify a different nucleic acid target sequence. There are two key advantages of using molecular beacons on hybridization arrays: (a) because molecular beacons can be designed to be extraordinarily specific, all of the molecular beacons in the array function well under the same set of hybridization conditions; and (b) because each molecular beacon contains a label pair (usually a fluorophore and a quencher), there is no need to carry out preliminary reactions to label the nucleic acid mixtures that are analyzed on the arrays. Molecular beacon arrays are therefore extraordinarily specific, "self-reporting" nucleic acid analyzers, in which the intensity of the fluorescence that develops at each position on the surface of the array (or on each bead in a distributed array) is directly proportional to the abundance of that molecular beacon's target sequence in the nucleic acid mixture being analyzed.

Molecular beacons can be attached to many different materials, including the surfaces of planar arrays, fiber-optic nucleic acid detection devices (Liu and Tan, 1999), glass beads (Steemers *et al.*, 2000; Brown *et al.*, 2000), agarose gel membranes (Wang *et al.*, 2002), and barcoded nanowires (Stoermer *et al.*, 2006). An example of a suitable linkage is the binding of molecular beacons that possess a biotin moiety to array surfaces coated with avidin or streptavidin (Ortiz *et al.*, 1998; Fang *et al.*, 1999). As an alternative to the inclusion of a quencher in each molecular beacon, the surface to which the molecular beacons are linked can sometimes serve as the quencher. For example, gold surfaces interact with fluorophores to prevent fluorescence, but when the probe sequence in the molecular beacon binds to a target sequence, the rigidity of the resulting probe-target hybrid forces the fluorophore away from the surface, generating a fluorescence signal (Dubertret *et al.*, 2001; Du *et al.*, 2003). Moreover, molecular beacons have been designed in which the fluorophore and the quencher are replaced with moieties that alter an electrical signal when the binding of the probe to its target causes an electroactive reporter on one end of the molecular beacon to lift away from the surface to which the probe is attached (Fan *et al.*, 2003). The challenge facing developers of these devices and arrays is to utilize surfaces and attachment chemistries that do not negatively affect the ability of the molecular beacons to bind to their target sequences. In addition, because it is relatively expensive to prepare many different probes that possess a covalently linked fluorophore and quencher and a third moiety for binding the probes to the surface of arrays, novel designs for molecular beacons have been explored in which the fluorophore and quencher are incorporated into "universal oligonucleotides" that are then bound to easily prepared, unlabeled, target-specific oligonucleotides to generate the probes for the arrays (Nutiu and Li, 2002; Landré *et al.*, 2005).

One of the most intriguing adaptations of the molecular beacon concept is the development of "aptamer beacons," which are oligonucleotide probes containing a fluorophore and a quencher that bind specifically to proteins, rather than to nucleic acid sequences (Yamamoto and Kumar, 2000; Hamaguchi *et al.*, 2001; Li *et al.*, 2002b), or that bind specifically to small molecules, such as cocaine (Stojanovic *et al.*, 2001). The defining feature of aptamer beacons is that they undergo a conformational reorganization when they bind to their target that changes the distance between a fluorophore and a quencher, generating a detectable signal. Some of the most innovative designs for signaling aptamers involve the use of two labeled oligonucleotides whose relationship to one another is altered by their binding to a target molecule (Nutiu and Li, 2003, 2004; Heyduk and Heyduk, 2002, 2005). Moreover, sophisticated procedures have been devised to select desired aptamers *in vitro* from large pools of oligonucleotides possessing

random sequences (Rajendran and Ellington, 2003; Nutiu and Li, 2005). And finally, "peptide beacons" have been developed, in which a peptide undergoes a conformational reorganization when it binds to its target protein, generating a detectable fluorescence signal (Oh *et al.*, 2007).

Observing the movement of mRNAs in live cells

Perhaps the most thrilling application of molecular beacons as biosensors is their use as probes to visualize mRNAs in living cells. Since molecular beacons that are not bound to mRNAs in live cells are dark and physically dispersed, their background fluorescence is low. On the other hand, when molecular beacons bind to mRNA targets, they fluoresce brightly; and if the target mRNAs are localized in particular areas in the cell, those regions are highlighted when viewed with a fluorescence microscope. Initially, the molecular beacons used in live cells were synthesized from deoxyribonucleotides (Matsuo, 1998; Sokol *et al.*, 1998; Perlette and Tan, 2000). Later, a series of modifications in the design of the molecular beacons significantly lowered fluorescence background. When it was realized that molecular beacons can be digested by cellular nucleases, and that target mRNAs that are bound to molecular beacons can be digested by cellular ribonuclease H, which cleaves RNA in DNA:RNA hybrids, the molecular beacon probes were modified by synthesizing them from unnatural 2'-*O*-methyl ribonucleotides (Molenaar *et al.*, 2001; Tsourkas *et al.*, 2002) or from peptide nucleic acids (Nitin *et al.*, 2004), which do not serve as substrates for cellular nucleases. In addition, when it was realized that cellular nucleic acid binding proteins could open molecular beacons that are not hybridized to targets, dual-FRET probes were developed (Bratu *et al.*, 2003; Santangelo *et al.*, 2004) that bind to adjacent positions on a target mRNA, generating a FRET signal that can be distinguished from the background fluorescence of each probe by itself. And finally, when it was realized that molecular beacons are rapidly sequestered in cell nuclei, the probes were tethered to the protein streptavidin, which cannot pass through pores in the nuclear membrane (Tsuji *et al.*, 2000; Tyagi and Alsmadi, 2004).

These improvements created a biosensor toolbox that enables mRNAs in living cells to be specifically lit up in a chosen fluorescent color, in order to directly observe their synthesis, movement, and localization. For example, the movement and localization of *oskar* mRNA from nurse cells to the posterior pole of developing fruit fly oocytes was observed in real time (Bratu *et al.*, 2003), demonstrating that molecular beacons enable mRNAs to be studied in much the same way that green fluorescent protein enables proteins to be studied in live cells. Utilizing molecular beacons to light up β-actin mRNA in live cultured chicken embryo fibroblasts,

which move about on glass surfaces by extending new lamellipodia (pseudopods) while withdrawing old lamellipodia, movies were taken that show β-actin mRNAs moving out of the shrinking lamellipodia and into the growing lamellipodia, where they are translated into β-actin protein, which is needed for the cell to move (Tyagi and Alsmadi, 2004). And finally, a modified gene was cloned into the genome of Chinese hamster ovary cells that could be experimentally induced to synthesize mRNAs containing a tandem array of 96 identical molecular beacon binding sites in their 3'-untranslated regions. In the presence of molecular beacons complementary to the inserted binding sites, 96 molecular beacons hybridize to each mRNA, creating a probe-target complex so bright that the movement of individual mRNA molecules could be followed in the living cells. Careful observations confirmed that, contrary to widely held beliefs, the mRNAs (each of which forms an individual complex with nuclear proteins), rather than exiting the nucleus through a nuclear pore near to the site of their synthesis, move rapidly by Brownian motion throughout the interchromatin spaces within the nucleus, and eventually exit the nucleus through whatever nuclear pore they eventually encounter (Vargas *et al.*, 2005). Sets of molecular beacons, each specific for different regions of the same primary gene transcript, and each labeled with a differently colored fluorophore, are now being used to assemble a detailed description of where in living cells mRNA splicing, maturation, transport, localization, and decay occur.

Practical Applications

Molecular beacons are primarily employed as highly specific amplicon detection probes in homogeneous, real-time, multiplex gene amplification assays. In addition to applications in basic research, molecular beacons are used for the detection of infectious agents in food, in donated blood, and in agricultural, veterinary, and environmental samples. Molecular beacons are also used in forensics and paternity testing -- and they are even used for the detection of DNA markers added to products to prevent counterfeiting (Wolfrum and Josten, 2005). An extensive list of publications describing the many applications of molecular beacons is available at http://www.molecular-beacons.org.

By far, the most significant applications for molecular beacons occur in the field of human *in vitro* diagnostics. Hundreds of different PCR assays have been designed (and many have been commercialized). There are assays for the detection of specific genes (Pierce *et al.*, 2000) and specific mRNAs (Dracheva *et al.*, 2001; Lai *et al.*, 2002); there are assays for the detection of mutations that cause genetic diseases (Giesendorf *et al.*, 1998; Szuhai *et al.*, 2001a; Smit *et al.*, 2001; Frei *et al.*, 2002; Rice *et al.*, 2002; Pierce et al., 2003; Orrù *et al.*, 2005); there are assays

that identify somatic mutations associated with cancer (Shih *et al.*, 2001; Martínez-López *et al.*, 2004; Yang *et al.*, 2005), and that provide guidance for determining prognosis and appropriate treatment (Span *et al.*, 2003; Zhao *et al.*, 2007); and most importantly, there are assays that identify and quantitate an extraordinarily wide range of different infectious agents in clinical samples (Abravaya *et al.*, 2003).

PCR assays have been developed for the detection of viruses, including pathogenic retroviruses (Vet *et al.*, 1999; Lewin *et al.*, 1999), adenoviruses (Poddar, 1999, 2000; Claas *et al.*, 2005), papillomaviruses (Szuhai *et al.*, 2001b; Takács *et al.*, 2008), cytomegaloviruses (Yeo *et al.*, 2005), respiratory viruses (Templeton *et al.*, 2004; O'Shea and Cane, 2004), and hepatitis viruses (Yang *et al.*, 2002; Sum *et al.*, 2004; Waltz *et al.*, 2005). PCR assays have also been developed for the detection of pathogenic bacteria, including *Mycobacterium tuberculosis* (Piatek *et al.*, 1998; Li *et al.*, 2000; El-Hajj *et al.*, 2001), *Salmonella* (Chen *et al.*, 2000), *Bordetella pertussis* (Poddar and Le, 2001), *Shigella dysenteriae* and *E. coli* O157:H7 (Fortin *et al.*, 2001; Bélanger *et al.*, 2002), *Clostridium difficile* (Bélanger *et al.*, 2003), *Vibrio cholerae* (Gubala and Proll, 2006), antibiotic-resistant *Staphylococcus aureus* strains (Elsayed *et al.*, 2003; Sinsimer *et al.*, 2005), bacterial bioterrorism agents *Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, and *Francisella tularensis* (Varma-Basil *et al.*, 2004), *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (Abravaya *et al.*, 2003), and bacteria that cause pneumonia (Templeton *et al.*, 2003; Morozumi *et al.*, 2006; Gullsby *et al.*, 2008). And lastly, PCR assays have been developed for the detection of pathogenic fungi such as *Candida dubliensis* (Park *et al.*, 2000) and *Aspergillus fumigatus* (Balashov *et al.*, 2005), and for the detection of pathogenic protozoa that cause malaria (Durand *et al.*, 2000; 2002) and dysentery (Roy *et al.*, 2005).

NASBA assays are particularly amenable to the use of molecular beacons (Leone *et al.*, 1998), because the vast majority of the amplicons produced by this isothermal exponential amplification process are single-stranded "plus" RNAs, rather than the complementary "plus and minus" DNA strands synthesized during PCR. Therefore, unlike the situation that occurs during PCR, there is no competition between the molecular beacon probes and the minus strands for binding to the target (plus) strands, and a significantly greater portion of the target strands are lit up by the molecular beacons. Assays that utilize LATE-PCR, in which far more plus strands are synthesized than minus strands (Sanchez *et al.*, 2004; Pierce *et al.*, 2005), have a similar advantage.

NASBA assays containing molecular beacons can distinguish single-nucleotide polymorphisms in human DNA (Berard *et al.*, 2004), and can identify mutant mRNAs associated with cancer

(Fradet *et al.*, 2004). NASBA assays have been developed for the detection of viruses, including HIV-1 (van Beuningen *et al.*, 2001; de Baar *et al.*, 2001a,b; Weusten *et al.*, 2002), West Nile virus and St. Louis encephalitis virus (Lanciotti and Kerst, 2001), herpesvirus (Polstra *et al.*, 2002), cytomegalovirus (Greijer *et al.*, 2002), hepatitis viruses (Yates *et al.*, 2001; Abd el-Galil *et al.*, 2005), respiratory viruses (Hibitts *et al.*, 2003, Moore *et al.*, 2004; Deiman *et al.*, 2007), enteroviruses (Landry *et al.*, 2005; Capaul and Gorgievski-Hrisoho, 2005), SARS virus (Keightley *et al.*, 2005), and papillomaviruses (Molden *et al.*, 2007). NASBA assays have also been developed for the detection of pathogenic bacteria, including bacteria that cause pneumonia (Loens *et al.*, 2003, 2006, 2008), *Vibrio cholerae* (Fykse *et al.*, 2007), and bacteria that contaminate food (Gore *et al.*, 2003; Rodríguez-Lázaro *et al.*, 2004; Churruca *et al.*, 2007; Nadal *et al.*, 2007). And lastly, NASBA assays have been developed for the detection of *Plasmodium falciparum* (Schneider *et al.*, 2005).

We take particular pride in assays that have received the approval of regulatory agencies for medical diagnostic use, including a NASBA assay used throughout the developing world for quantitating viral load in people infected with HIV-1 (van Beuningen *et al.*, 2001; de Mendoza *et al.*, 2005), and a widely used PCR assay for detecting the presence of methicillin-resistant *S. aureus* in people entering hospitals (Huletsky *et al.*, 2004; Warren *et al.*, 2004), whose use has curtailed the spread of nosocomial infections (Paule *et al.*, 2007; Robicsek *et al.*, 2008).

The significance of gene amplification assays that use molecular beacons is illustrated by the widespread availability of a PCR assay that detects the presence of group B *Streptococci* in samples taken from pregnant women (Davies *et al.*, 2004). Babies born to infected mothers can develop meningitis, which can cause blindness, deafness, and death. However, by performing this assay on women entering the hospital to give birth, those infected are treated with antibiotics that cross the placenta, preventing the development of meningitis (Goodrich and Miller, 2007). It is thus particularly gratifying to see that what began as a basic research program to explore the mechanism by which bacteriophage Qβ RNA is amplified exponentially has led (circuitously) to the invention of simple, elegant, and extraordinarily specific biosensors that enhance the use of exponential amplification assays for beneficial medical purposes.

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