Molecular beacons: colorful analysis of nucleic acids

Jacqueline AM Vet†, Brenda JM Van der Rijt and Henk J Blom

The completion of the Humane Genome Project has resulted in an exponential rise in the demand for molecular diagnostic assays. To meet this demand, several innovative technologies have become available for performing homogeneous genetic analyses. For this type of assay, special detector probes are necessary. In 1996, Tyagi and Kramer described fluorogenic hairpin-shaped detector probes, called 'molecular beacons', which are extraordinarily specific. Since they characterize alleles by the generation of fluorescent signals, they are perfectly suited for homogeneous genetic analysis. Molecular beacons assays are simple, fast, inexpensive, sensitive, utilize a high-throughput format, enable the testing of many samples simultaneously and allow the detection of a series of different agents in the same assay tube. This review is designed to give the reader a greater understanding of the exciting applications of molecular beacons in DNA, RNA and protein studies.

Expert Rev. Mol. Diagn. 2(1), (2002)

CONTENTS

†Author for correspondence
Department of Paediatrics and Neurology, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
Tel.: +49 312 4361 4559
Fax.: +49 312 4361 8900
j.vet@ckslkn.azn.nl

KEYWORDS:
fluorescent hybridization probes, multiplex analysis, pathogen detection, real-time PCR, RNA expression analysis, SNP analysis

Molecular beacons

Molecular beacons are single-stranded oligonucleotide detector probes that form a stem-and-loop structure [1]. The loop portion of the molecule is a probe sequence that is complementary to a predetermined target sequence and the stem is formed by the annealing of complementary arm sequences that are present on either side of the probe sequence (FIGURE 1). A fluorophore is covalently attached to the end of one arm and a nonfluorescent quencher is covalently attached to the end of the other arm. In the absence of target, the stem keeps the fluorophore and the quencher in close proximity to each other, preventing fluorescence. However, when molecular beacons bind to their target they undergo a conformational change that restores the fluorescence of the internally quenched fluorophore. These probes are called 'molecular beacons' because they emit a fluorescent signal only when hybridized to target molecules.

Universal quencher for multiplex detection assays

The reason for including the hairpin stem in molecular beacons was to obtain probes that only fluoresce when bound to their targets. However, the hairpin structure also enables the use of a wide variety of differently colored fluorophores [2]. In the hairpin conformation, the fluorophore and quencher are so close to each other that the direct transfer of energy is possible. Therefore, the quencher – usually the nonfluorescent chromophore dabcyl – can quench any fluorophore (FIGURE 2). This quenching is independent of the overlap between the emission spectrum of the fluorophore with the absorption spectrum of the quencher. The mechanism of quenching is therefore different from fluorescence resonance energy transfer (FRET), since in FRET there is a relationship between spectral overlap and quenching efficiency. Using several different molecular beacons, each designed to detect a different target and each labeled with a differently colored fluorophore, multiple targets can be distinguished in the same solution. The color of the fluorescence that develops in the assay indicates which targets are present and the intensity of each color indicates how many target molecules are present.

Enhanced specificity for SNP detection

The hairpin stem of molecular beacons also enhances specificity [2,3]. When conventional

 ISSN 1473-7159

linear oligonucleotides are used as hybridization probes, the difference in stability between a single nucleotide mismatched hybrid and a perfectly complementary hybrid is rather small [4]. When a molecular beacon binds to its target sequence, the formation of the probe–target hybrid occurs at the expense of the stem hybrid. Molecular beacons can be designed in such a way that over a wide range of temperatures only perfectly complementary probe–target hybrids are sufficiently stable to force open the stem hybrid. Mismatched probe–target hybrids do not form, except at substantially lower temperatures [3,5,6]. Therefore, a relatively wide range of temperatures exists in which perfectly complementary probe–target hybrids elicit a fluorogenic response, while mismatched molecular beacons remain dark. Consequently, assays using molecular beacons robustly discriminate targets that differ from one another by as little as a single nucleotide substitution.

Real-time monitoring of PCR

As target nucleic acids are rare components of biological samples, molecular beacons are best used in conjunction with target amplification, such as for real-time detection of amplification products in PCR. Molecular beacons have been used in numerous studies to detect SNPs [5–20], DNA, RNA and pathogens [21–34]. Molecular beacons can also be used in assays that employ isothermal nucleic acid amplification schemes, such as strand displacement amplification [35], nucleic acid sequence-based amplification [36–40] and rolling circle amplification [41]. Since nonhybridized molecular beacons are dark, it is not necessary to isolate the probe–target hybrids to determine the number of amplicons synthesized during an assay. Molecular beacons are added to the assay mixture prior to carrying out amplification and fluorescence is measured in real time during PCR.

There are several platforms available for performing real-time PCR analyses. These range from ultra-rapid, air-heated thermal cyclers, where PCR is performed in glass capillaries (LightCycler, Roche Diagnostics Corporation, IN, USA), to tube-based and microtiter plate-based systems, such as the PRISM 7700 Sequence Detection system (Applied Biosystems, CA), the iCycler IQ (Bio-Rad, CA), the SmartCycler (Cepheid, CA) and the Mx4000 (Stratagene, CA). Different techniques are available to monitor real-time amplification of a gene of interest. The amplification process can be monitored using nonspecific double-stranded ds-DNA binding dyes and specific detector probes. Dyes, such as SYBR Green (Molecular Probes, OR, USA) produce enhanced fluorescence signals upon binding to dsDNA duplexes. Although dsDNA-binding dyes are a simple, fast and inexpensive way of monitoring amplicon production, the major disadvantage of the method is that the dye binds nonspecifically to all dsDNA such that primers dimers and nonspecific amplification products cannot be distinguished from the amplicon of interest. To overcome the problems encountered with the dsDNA-binding dyes different probe-based techniques have been developed, e.g., adjacent probes [42], TaqMan™ probes [43], molecular beacons [1] and Scorpion primers [44]. With adjacent probes, a donor fluorophore on one probe is brought into close proximity to an acceptor fluorophore on a second probe when both probes hybridize to adjacent regions on a target molecule. The donor fluorophore is excited by the light source of the instrument and energy is transferred from the donor to the acceptor, producing a decrease in the fluorescence of the donor and an increase in the fluorescence of the acceptor. TaqMan probes consist of a probe sequence labeled at one end with a donor fluorophore and at the other end with an acceptor fluorophore. In the unhybridized state, fluorescence is quenched by the FRET mechanism. Upon hybridization of the probe to the target sequence during PCR, the 5′-nuclease activity of Taq polymerase cleaves the probe; and the resulting separation of the fluorophore from the quencher produces an increase in the fluorescence of the donor and a decrease in the fluorescence of the acceptor. Scorpion primers consist of an oligonucleotide primer covalently linked to a molecular beacon moiety that is attached to the 5′-end of the primer by a linker that prevents the copying of the molecular beacon sequence. The molecular beacon moiety contains a probe sequence that hybridizes to a complementary sequence known to occur in the product strand that results from the extension of the scorpion primer. Just as in conventional molecular beacons, the fluorescence of a fluorophore at the 5′-end of the molecular beacon moiety is quenched by the close proximity of a quencher at the 3′-end, unless the probe sequence binds to the target sequence in the extended strand. In all these different systems – including molecular beacons – the hybridization probe sequences are chosen to be complementary to a target sequence within the expected amplicon, so they are highly unlikely to bind false amplicons or primer-dimers, thus

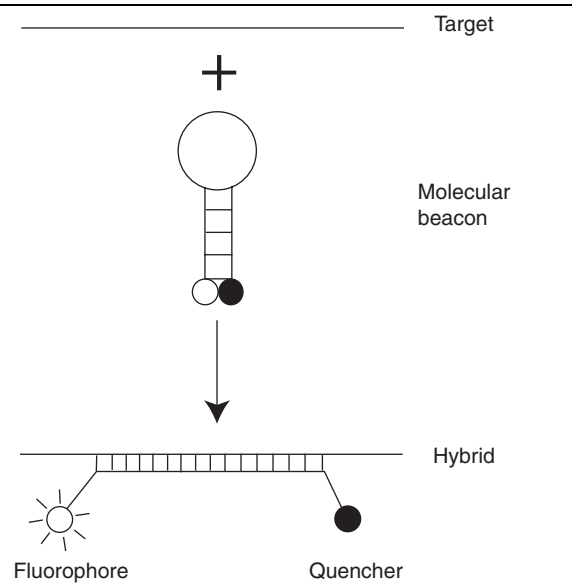

Figure 1. Principle of operation of molecular beacons [1]. Free molecular beacons are nonfluorescent because the hairpin stem keeps the fluorophore close to the quencher. When the probe sequence in the hairpin loop hybridizes to its target, forming a rigid double helix, a conformational change occurs that removes the quencher from the vicinity of the fluorophore, thereby restoring fluorescence.

enhancing the specificity of the assay. Compared to other detector probes, the use of molecular beacons in homogeneous assays has two main advantages. Firstly, they enable truly multiplex detection assays, since molecular beacons can be labeled in any desired color [32]. Moreover, the hairpin structure of molecular beacons enables the probes to be significantly more specific than corresponding linear probes [2]. Recently, Foy and Parkes reviewed the different homogenous DNA-based technologies for genetic testing in the clinical laboratory [45] and Marras reviewed the characteristics and applications of different artificial hybridization probes [46]. In the remainder of this review the design of molecular beacon assays and different diagnostic applications will be discussed.

Design of molecular beacon assays

In order to successfully monitor PCR reactions, molecular beacons should be designed so that they are able to hybridize to their targets at the annealing temperature of the PCR, whereas the free molecular beacons should stay closed and be nonfluorescent at these temperatures. This can be ensured by choosing the length of the probe sequence and the length of the arm sequences appropriately. The length of the probe sequence should be such that it will dissociate from its target above the annealing temperature of the PCR. The melting temperature of the probe target-hybrid can be predicted using the 'percent-GC' rule, which is available in most probe design programs. The prediction should be made for the probe sequence alone before adding the stem sequences. In practice, the length of the probe sequence is usually between 15–30 nucleotides.

After selecting a probe sequence, two complementary arm sequences should be added on either side of the probe sequence. In order to ensure that the molecular beacons remain closed in the absence of targets, the stem should be stable at the anneal temperature of the PCR. Since the stem forms by an intramolecular hybridization event, its melting temperature can not be predicted by the percent-GC rule. Instead, a DNA folding program (such as the Zuker folding program) should be utilized to predict its melting temperature [101]. Usually the stems are 5–7 nucleotides long. This folding program also indicates whether the chosen molecular beacon sequence will form an unwanted secondary structure, such as an extremely long stem or a structure that does not keep the fluorophore and quencher in close proximity, in which case the sequences of the arm should be changed.

Figure 2. Fluorogenic response of differently colored molecular beacons to the addition of target. A solution of each molecular beacon was placed in a pair of test tubes [2]. The molecular beacons contained (left to right) coumarin (blue), EDANS (blue-green), fluorescein (green), Lucifer (yellow), tetramethylrhodamine (orange) and Texas (red). All molecular beacons contained dabcyl as a quencher. Complementary single-stranded oligonucleotides were added to the left tube of each pair and the tubes were illuminated with a broad-wavelength ultraviolet lamp.

The primers used with molecular beacons in PCR reactions should be designed to produce a relatively short amplicon, in general less then 150 base-pairs. These shorter amplicons produce brighter fluorescent signals because molecular beacons are better able to compete with the complementary strands for binding to the target strands when the amplicons are shorter.

Molecular beacons are very versatile. The general design can be altered to fit specific applications. For instance, the shorter the loop sequence (18–21 nucleotides), the better able the molecular beacon is to discriminate SNPs in the target strand. On the other hand, longer probe sequences (25–30 nucleotides) can be used to ensure that probe–target hybrids that contain mismatches are stable at the annealing temperature of the PCR. For example, this enables detection of different subtypes of a retrovirus that may contain one or two nucleotide substitutions.

Before performing the real-time PCR experiments it is very important to characterize the molecular beacons. To check the purity of the molecular beacon preparation, the extent to which its fluorescence increases upon binding to its oligonucleotide target should be measured. Molecular beacons should have signal-to-background ratios above 20. Poor signal-to-background ratios are caused by the presence of uncoupled fluorophores in the preparation or by the presence of oligonucleotides that have a fluorophore but do not have a quencher. The molecular beacon synthesis and purification is very crucial for the production of high quality probes and subsequently, for high quality real-time PCR experiments [102].

Multiplex detection of four different pathogenic retroviruses

Multiplex molecular beacon detection assays have been described in a number of studies [2,6,29,32,47]. A study describing the multiplex detection of four different retroviruses will be discussed more in detail below.

To demonstrate the use of molecular beacons in extremely sensitive, high-throughput, clinical tests, an assay for the simultaneous detection of four retroviruses in blood samples and in tissues for transplantation has been developed [29]. A multiplex PCR assay was developed that uses four differently colored, mismatch-tolerant, molecular beacons for the simultaneous detection of amplicons generated from unique sequences found in the four different retroviruses. The assay contained four compatible sets of PCR primers that are specific for the *gag* gene of HIV-1, the *env* gene of HIV-2, the *tax* gene of HTLV-I and the *pol* gene of HTLV-II. The primers were chosen so that they would not interact with each other and their target sequences would be unique to each retrovirus, highly conserved and present in most clinical subtypes. The HIV-1-specific, fluorescein-labeled molecular beacon was designed to detect HIV-1 subtypes A, B, C, D, F and G. The HIV-2-specific, tetrachlorofluorescein-labeled molecular beacon was designed to detect HIV-2 subtypes A, D and SD. The HTLV-I-specific, tetramethylrhodamine-labeled molecular beacon was designed to detect all HTLV-I subtypes and the

HTLV-II-specific, rhodamine-labeled molecular beacon was designed to detect HTLV-II subtypes A and B. FIGURE 3 shows that the individual retroviruses could be distinguished from one another in a multiplex format. Four reactions carried out in parallel were initiated with 100,000 molecules of one of the four retroviral DNAs. Each reaction contained all four molecular beacons and all four primer pairs. The only significant fluorescence that appeared in the course of amplification reactions carried out in each assay tube was fluorescence from the molecular beacon that was complementary to the sequence of the expected amplicon (FIGURE 3). No significant fluorescence developed in a control assay that did not contain any template DNA. These results demonstrate that each molecular beacon is specific for its intended target amplicon.

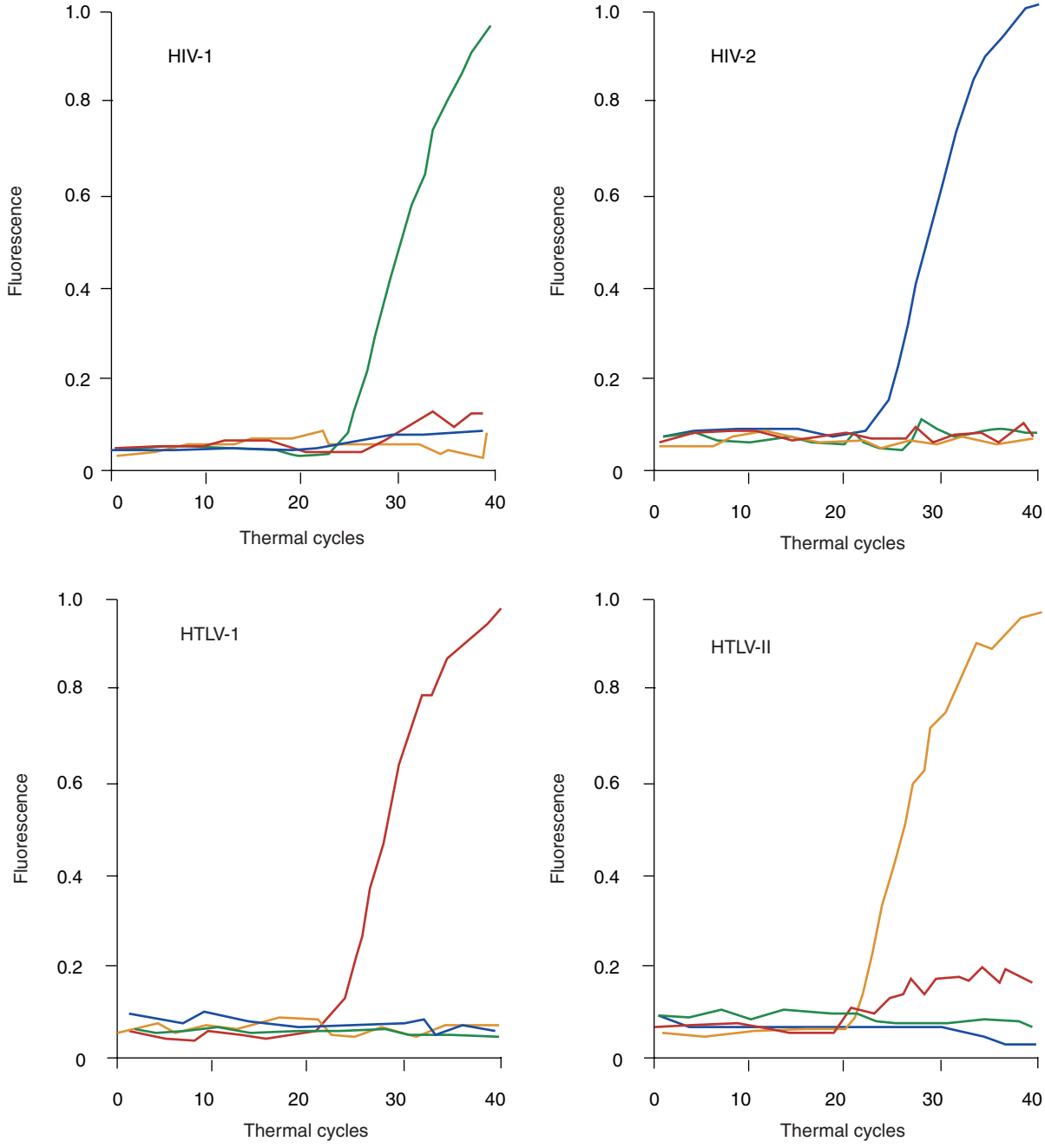

Figure 3. Real-time detection of four different retroviral DNAs in a multiplex format [39]. Four assays were carried out in sealed tubes, each initiated with a different retroviral DNA. Each reaction contained four sets of PCR primers specific for unique HIV-1, HIV-2, HTLV-I and HTLV-II nucleotide sequences and four molecular beacons, each specific for one of the four amplicons and labeled with a differently colored fluorophore. Fluorescence from the fluorescein-labeled molecular beacon (HIV-1) is plotted in red, fluorescence from the tetrachlorofluorescein-labeled molecular beacon (HIV-2) is plotted in green, fluorescence from the tetramethylrhodamine-labeled molecular beacon (HTLV-I) is plotted blue and fluorescence from the rhodamine-labeled molecular beacon (HTLV-II) is plotted in orange.

To evaluate the ability of the assay to detect a rare retroviral DNA in the presence of an abundant retroviral DNA, five reactions were initiated with 10^5 molecules of HTLV-I DNA and either 10^5, 10^4, 10^3, 10^2 or 10^1 molecules of HIV-2 DNA and a sixth reaction did not contain any template DNA. The results show that both a fluorescent signal from tetramethylrhodamine (indicative of the presence of HTLV-I amplicons) and a fluorescent signal from tetrachlorofluorescein (indicative of the presence of HIV-2 amplicons) developed in every assay, except in the control reaction, which did not contain any template DNA (FIGURE 4). The results show that the number of thermal cycles required for a significant tetramethylrhodamine signal to develop from 100,000 HTLV-I target molecules was unaffected by the number of HIV-2 target molecules and the number of thermal cycles required for a significant tetrachlorofluorescein signal to develop was indicative of the number of HIV-2 target molecules, irrespective of the presence of a relatively large number of HTLV-I target molecules.

The sensitivity of the multiplex assay was compared with the sensitivity of an extremely sensitive, conventional PCR assay in which the retroviral amplicons were analyzed by gel-electrophoresis, transferred to a membrane and identified by hybridization with a radioactively labeled probe. Serial dilutions containing 70,000, 7000, 700, 70, 7 and 0 molecules of HTLV-I DNA were used as template for both assays. Both assays were sufficiently sensitive to detect even seven molecules of HTLV-I DNA. However, the homogenous multiplex assay was much easier to perform and required only 3 h to complete, whereas the conventional assay required 3 days. Moreover, the homogenous assay could detect four different types of retroviruses and the use of a closed-tube format eliminates the risk of contaminating the laboratory with escaped amplicons.

Genotyping SNPs

To test the ability to specifically identify polymorphisms in a nucleic acid population, Marras *et al.* developed a multiplex molecular beacon-PCR assay [6]. Four different target DNA templates were prepared that were identical, except that the nucleotide at one position was either adenosine, cytidine, guanosine or thymidine. One pair of PCR primers was used that generate amplicons from any of the four templates. Four differently colored molecular beacons were designed, each of which possessed a probe sequence that was perfectly complementary to the target sequence within one of the four templates. The molecular beacons designed for this assay formed perfectly complementary probe–target hybrids whose melting temperature was about 13°C higher than the melting temperature of probe–target hybrids that contain one of the three possible mismatched base-pairs. Four different PCR assays were carried out, each initiated with one of the four different target DNA templates. Each assay contained the same set of primers and a mixture of the four differently colored molecular beacons. The results show that only one of the four differently colored molecular beacons in each reaction formed probe–target hybrids during the course of the amplification (FIGURE 5). Only the molecular beacon possessing the perfectly complementary probe sequence formed a stable hybrid. Thus, the color of the fluorescence that developed in each reaction identified the variant nucleotide that was present in the target. The results of this assay indicate that molecular beacons can be designed for use in PCR reactions that are sufficiently specific to distinguish sequence differences as small as a single nucleotide substitution.

The capability of molecular beacons to detect SNPs has been confirmed in many studies. For genotyping alleles, two molecular beacons are used, one specific for the wild type allele and labeled

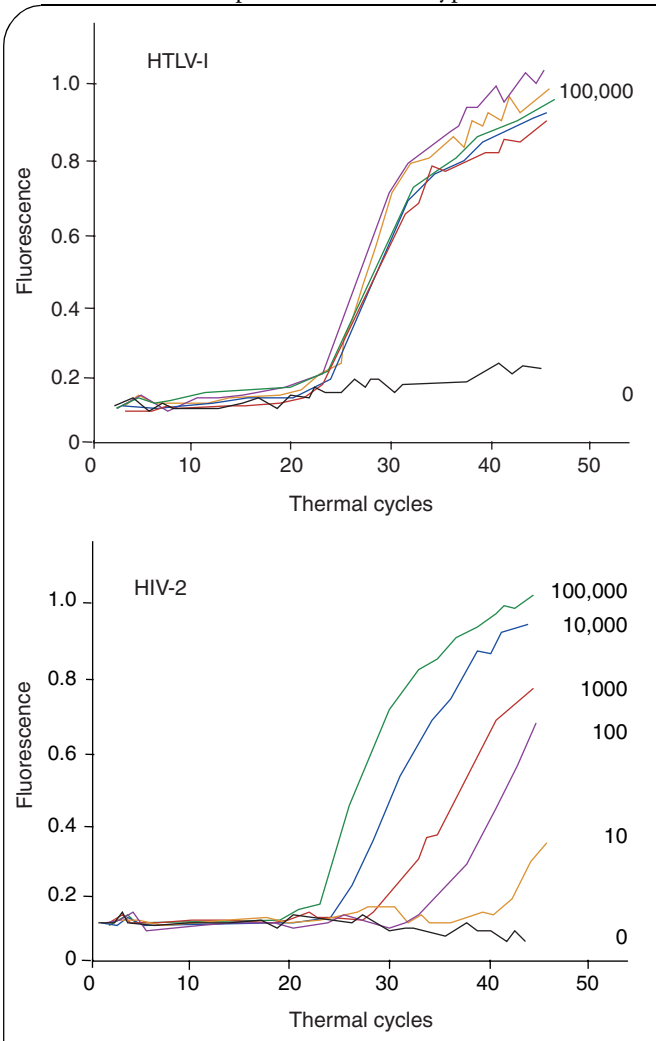

Figure 4. Detection of a rare retroviral target in the presence of an abundant retroviral target [39]. Five multiplex assays were initiated with 10^5 molecules of HTLV-I DNA and either 10^5, 10^4, 10^3, 10^2, or 10^1 molecules of HIV-2 DNA; and a sixth multiplex assay, which served as a control, did not contain any template DNA. Each assay contained four sets of PCR primers and four differently colored molecular beacons. The top panel shows the fluorescence from the tetramethylrhodamine-labeled molecular beacons, which is due to the synthesis of HTLV-I amplicons; and the bottom panel shows the fluorescence from the tetrachlorofluorescein-labeled molecular beacons, which is due to the synthesis of HIV-2 amplicons. The number of molecules of each retroviral DNA that were originally present in each assay tube is indicated to the right of each curve.

with a green fluorophore and the other specific for the mutant allele and labeled with a red fluorophore. The appearance of green fluorescence during amplification indicates homozygous wild types, red fluorescence indicates homozygous mutants and both green and red fluorescence indicates heterozygotes (FIGURE 6). Molecular beacons have been used to genotype, e.g., methylenetetrahydrofolate reductase gene mutations [5,16] human chemokine receptor mutations [8–10], Factor V Leiden mutation [7], hereditary hemochromatosis gene mutations [18], drug resistance mutations in malarian parasites [17] and drug resistance mutations in *Mycobacterium tuberculosis* [11,12,32]. Usually the mutations are detected in real time during amplification but they can also be detected after amplification [15]. In side-by-side comparisons, the specificity of molecular beacons has proven superior to probes that rely on 5′-nucleolytic cleavage activity of DNA polymerase [14]. This high specificity allows the detection of a small proportion of mutant DNA in the presence of abundant wild type DNA [13].

Self-reporting molecular beacon arrays

Today's development of molecular technologies includes the production of DNA microarrays. Current microarrays for large-scale genotyping are laboratory-based research tools. Further refinement of the techniques is necessary to produce a robust, automatable, reliable and user-friendly diagnostic tool. In different studies molecular beacons have been immobilized on solid surfaces [48–51]. Steemers *et al.* developed a randomly ordered fiberoptic gene array for rapid, parallel detection of unlabeled DNA targets with surface immobilized molecular beacons. Different molecular beacon-coated microsperes are

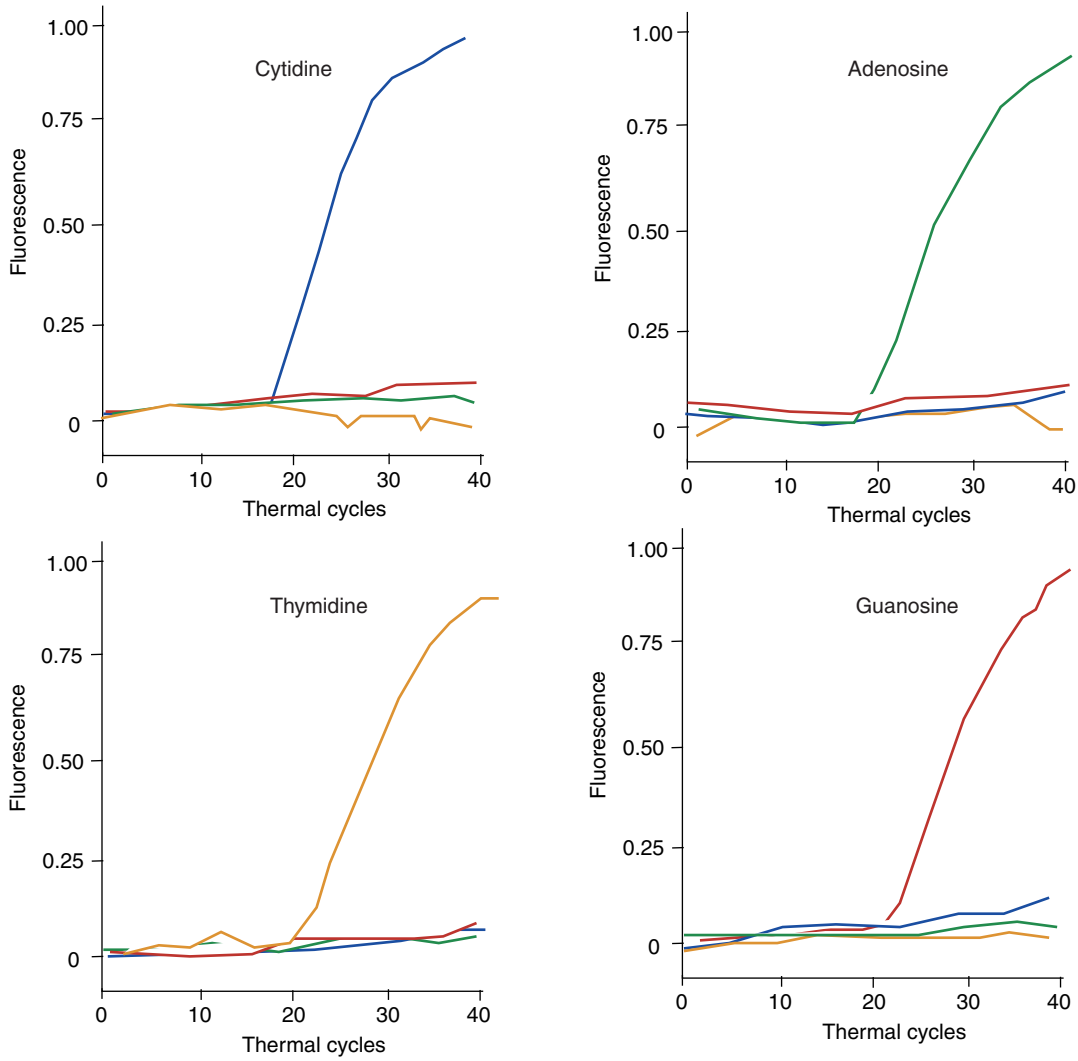

Figure 5. Multiplex detection of single-nucleotide variations in real-time polymerase chain reactions [6]. Four different molecular beacons were present in each reaction: the fluorescence intensity of the molecular beacons specific for the target sequence containing a guanosine in the variable position is plotted in blue, the fluorescence of the thymidine-specific molecular beacons is plotted in green, the fluorescence of the adenosine-specific molecular beacons is plotted in orange and the fluorescence of the cytidine-specific molecular beacon is plotted in red. The nucleoside at the site of variation in the template DNA used to initiate each amplification reaction is indicated at the top of each panel. The color of the fluorescence that developed in each reaction tube identified the variant nucleotide. No fluorescence developed in a control reaction that did not contain template DNA.

randomly distributed in an array of wells etched in a 500 µm diameter optical imaging fiber. Different genomic cystic fibrosis-related targets were detected by positional registration and fluorescence response monitoring using an optical encoding scheme and an imaging fluorescence microscope system. The advantages of this approach are that the array is self reporting and the hairpin structure of the probe ensures high specificity. In addition, the use of multiple microspheres possessing copies of each probe decreases the chance of both false-negatives and false-positives.

RNA detection in living cells

In biological studies and recent antisense research, it has been shown that in living cells it is extremely difficult to demonstrate hybridization between an antisense oligonucleotide and its mRNA target. Molecular beacons are ideal probes for use in living cells because they are dark when not hybridized and become fluorescent when they bind to their target, eliminating the need to remove nonhybridized probes. They have been introduced into living cells by liposome delivery for the visualization of human basic fibroblast growth factor mRNA in human trabecular cells of the eye [52] and by microinjection for the detection of specific mRNAs in K562 human leukemia cells [53]. In the latter study molecular beacons were designed to hybridize to an endogenous vav mRNA, which encodes an important hematopoietic cell signaling protein and to the highly expressed housekeeping gene β-actin. Both studies demonstrate that mRNA can be visualized and localized in cells. A potential drawback to using deoxyribonucleotide probes in living cells is that they can be digested by cellular nucleases.

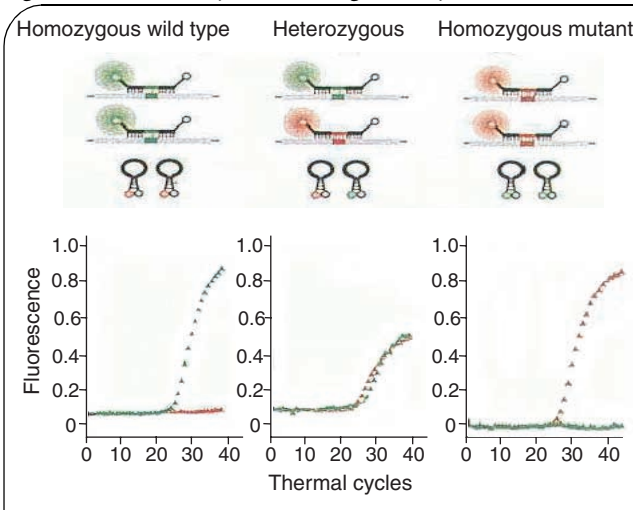

FIGURE 6. Principle of genotyping with molecular beacons [8]. with DNA from homozygous wild type individuals only the wild type molecular beacons hybridize to the amplicons, generating green fluorescence, whereas the mutant molecular beacons retain their stem-and-loop structure and do not produce a fluorescent signal. with DNA from heterozygous individuals, both molecular beacons hybridize to the amplicons, generating both green and red fluorescence. with DNA from homozygous mutant individuals, only the mutant molecular beacons hybridize to the amplicons, generating red fluorescence, whereas the wild type molecular beacons remain dark

Probes that are synthesized from 2′-O-methyl ribonucleotides have been shown to behave differently in living cells than do probes synthesized from deoxyribonucleotides, in that they cannot be cleaved by cellular nucleases and do not promote the cleavage of target RNA by cellular ribonuclease H when they form probe–target hybrids. In a recent study by Moolenaar *et al.,* 2′-O-methyl RNA molecular beacons were compared with linear 2′-O-methyl RNA probes for the visualization of RNA in living cells. Although they demonstrated an improved specificity of the 2′-O-methyl RNA molecular beacons compared to 2′-O-methyl RNA linear probes *in vitro*, they could not confirm the beneficial properties of molecular beacons in living cells. However, as has been elegantly shown in study by Sokol *et al.* when the appropriate controls are used molecular beacons enable visualization and localization of mRNA in living cells [53]. Molecular beacons could find a broad application in studying RNA processing, trafficking and folding in living cells.

Molecular beacons in protein studies

Recently, molecular beacons have been used for studying protein–DNA interactions [55–63]. Protein recognition was first realized using an *E. coli* single-stranded DNA binding protein (SSB) [57]. Using molecular beacons it was possible to detect SSB at a concentration as low as 2×10^{-10} M using a conventional spectrophotometer. A molecular beacon probe has also been used for detailed binding studies of the enzyme lactate dehydrogenase [55]. In these two studies proteins are used that bind nonspecifically to single-stranded DNA. In a study on the characterization of the interaction between αCP_2 and the untranslated region of collagen _1 (I) RNA, molecular beacons were used to examine $\_CP_2$–DNA interactions [58]. They demonstrated that molecular beacons were suitable for detecting protein–nucleotide interactions in a high-throughput format. In two studies aptamer molecular beacons have been developed for the specific detection of proteins [62,63]. Similar to regular molecular beacons, aptamer beacons form a nonfluorescent conformation when free in solution and undergo a conformational reorganization as they bind to their ligand that permits them to fluoresce brightly. In the first study, an aptamer beacon was developed to specifically detect the Tat protein of HIV. In the second study, an antithrombin aptamer was converted into an aptamer beacon by adding nucleotides to the 5′-end, which were complementary to nucleotides at the 3′-end of the aptamer. In the absence of thrombin, the aptamer beacon is in the stem-loop structure. In the presence of thrombin, the aptamer beacons form a ligand-binding structure in which the fluorophore is far away from the quencher, restoring fluorescence. Since virtually any aptamer can potentially be converted to an aptamer beacon, aptamer beacons can be sensitive tools for the detection of proteins and other chemical compounds.

Summary & conclusions

During the last few years, molecular beacons technology has proven to be widely effective for different types of genetic analysis. Multiplex assays that utilize molecular beacons have been

developed to detect and quantify DNA, RNA, infectious agents and for the discrimination of genetic alleles. Moreover, the use of molecular beacons is not limited to assays that employ PCR. They have been successfully used in assays that employ isothermal nucleic acid amplification schemes. More recently, molecular beacons have been used to detect RNAs in living cells, to analyze proteins and to develop self-reporting microarrays.

Expert opinion

Practical clinical assays should be simple, fast, inexpensive, sensitive, utilize a high-throughput format that enables the testing of many samples simultaneously and, ideally, allow the detection of a series of different agents in the same assay tube. Molecular beacons enable all of these advantageous properties. The use of a universal quencher in molecular beacons enables multiple targets to be distinguished in the same solution. The extraordinary specificity of molecular beacons enables the development of assays that robustly identify SNPs. A very important advantage of homogenous assays is that tubes remain closed during the monitoring of fluorescence, thereby eliminating the risk of carryover contamination and the generation of false-positive results. The development of a reliable molecular beacon assay is depending on the purity of the synthesized molecular beacons and the molecular beacon design. The molecular beacon preparation should be of good quality and therefore, the extent to which the fluorescence of a molecular beacon increases upon binding to its target should always be measured before a real-time PCR experiment is performed. This 'signal-to-background ratio' depends primarily on the purity of the molecular beacon preparation. The presence of uncoupled fluorophores in the molecular beacon preparation or the presence of oligonucleotides that have a fluorophore but do not have a quencher cause poor signal-to-background ratios and should not be used in the real-time PCR assay. Although the general design of molecular beacons is straightforward and easy, to design molecular beacons for optimal allele-discriminating performance at a particular temperature, some experience is needed. Therefore, it would be desirable to know how to predict the thermodynamic behaviour of a probe from its nucleotide sequence and to have a computer algorithm that can automatically select a nucleotide sequence with desired characteristics. This will be especially important in the design of high-density molecular beacon arrays, which may contain thousands of different immobilized fluorescent probes. The availability of such a computer algorithm for molecular beacon design will make the technology even more accessible for use in many different applications. The use of molecular beacons is not limited to assays that employ PCR, which require temperature cycling but can also be used in assays that employ isothermal nucleic acid amplification schemes, such as, strand displacement amplification, nucleic acid sequence-based amplification and rolling-circle amplification. These assays do not require a thermal cycler, so they can be carried out in laboratories with limited resources. No matter which amplification scheme is employed, the addition of molecular beacons to enable multiple target detection will improve the reliability, speed and ease of use of diagnostic clinical assays.

Key issues

- The extraordinary specificity of molecular beacons enables unequivocal identification of SNPs.
- The use of a universal quencher in molecular beacons enables the detection of many different targets in the same assay tube.
- Molecular beacon design is very versatile and can be altered to fit specific applications.
- Molecular beacons can be used as detector probe in homogenous genetic assays and when immobilized on a solid surface a highly-specific, self-reporting microarray can be developed.
- In addition to application in molecular diagnostic assays, molecular beacons have been successfully used to detect specific RNAs in living cells and moreover, aptamer beacons have been developed for the specific detection of proteins.

Five-year view

Although molecular beacons have been shown to be perfectly suited for multiplex homogenous genetic analysis, currently DNA chips adapt readily to the parallel format required to screen many samples for many mutations simultaneously. The microarray-based technology for analyzing SNPs is at the moment under intensive development. Improvement is needed to enhance the specificity of the arrays and moreover, to create a system for label-free detection of the hybridization signals. Since molecular beacons are much more specific than corresponding linear probes that are currently used in genotyping microarrays, molecular beacons are perfectly suited to# enhance the specificity of array-based hybridization, making the identification of the presence or absence of a mutation unequivocal. The self-reporting capacity of molecular beacons enables the detection of hybridization without labeling the targets, making the system more user-friendly, robust and suitable for diagnostic applications.

In the near future, the enormous amount of data that will be generated by RNA-expression arrays has to be confirmed by an independent technique. Although Northern blot technology is still a reliable technique, real-time PCR will be needed for the detailed investigation of candidate genes indentified by the RNA-expression arrays. Using real-time molecular beacon PCR, the expression of candidate genes can be studied easily and accurately in many different tissues or cell types to determine the clinical relevance of the pattern of differential gene expression found with the array.

Aptamer beacons are expected to become a sensitive tool for detecting proteins and other chemical compounds. Aptamers have been previously been immobilized without significant loss of activity and it should similarly be possible to immobilize multiple aptamer beacons to generate chip arrays that directly signal the presence of individual proteins. The use of aptamer beacons to create biosensors for proteomics applications is a very exiting field of research.

References

Papers of special note have been highlighted as:
- • of interest
- •• of considerable interest

1 Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnol.* 14, 303–308 (1996).

•• **First description of molecular beacons.**

2 Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination. *Nature Biotechnol.* 16, 49–53 (1998).

•• **Describes that the presence of the hairpin stem enables the use of differently colored fluorophores and enhances specificity.**

3 Bonnet G, Tyagi S, Libchaber A, Kramer FR. Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proc. Natl Acad. Sci. USA* 96, 6171–6176 (1999).

• **A thermodynamic study of the enhanced specificity of molecular beacons.**

4 Tibanyenda N, De Bruin SH, Haasnoot CA, van der Marel GA, van Boom JH, Hilbers CW. The effect of single base-pair mismatches on the duplex stability of d(T-A-T-T-A-A-T-A-T-C-A-A-G-T-T-G).d(C-A-A-C-T-T-G-A-T-A-T-T-A-A-T-A). *Eur. J. Biochem.* 139, 19–27 (1984).

5 Giesendorf BA, Vet JA, Tyagi S, Mensink EJ, Trijbels FJ, Blom HJ. Molecular beacons: a new approach for semiautomated mutation analysis. *Clin. Chem.* 44, 482–486 (1998).

•• **Describes the first application of molecular beacons for SNP analysis.**

6 Marras SAE, Kramer FR, Tyagi S. Multiplex detection of single-nucleotide variations using molecular beacons. *Genet. Anal.* 14, 151–156 (1999).

•• **Describes a multiplex PCR for simultaneous detection of four different single nucleotide variations.**

7 Chenn A, Dorfman DM, Walsh CA, Winkelman JW. Detection of Factor V Leiden mutation with molecular beacons. *Am. J. Clin. Pathol.* 112, 1 (1999).

8 Kostrikis LG, Tyagi S, Mhlanga MM, Ho DD, Kramer FR. Spectral genotyping of human alleles. *Science* 279, 1228–1229 (1998).

9 Kostrikis LG, Huang Y, Moore JP *et al.* A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promotor mutation. *Nature Med.* 4, 350–353 (1998).

10 Gonzalez E, Bamshad M, Sato N *et al.* Race-specific HIV-1 disease-modifying effects associated with CCR5 haplotypes. *Proc. Natl Acad. Sci. USA* 96, 12004–12009 (1999).

11 Piatek AS, Tyagi S, Pol AC *et al.* Molecular beacon sequence analysis for detecting drug resistance in Mycobacterium tuberculosis. *Nature Biotechnol.* 16, 359–363 (1998).

• **Describes the visual detection of SNPs.**

12 Rhee JT, Piatek AS, Small PM *et al.* Molecular epidemiologic evaluation of transmissibility and virulence of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 37, 1764–1770 (1999).

13 Szuhai K, Ouweland JM, Dirks RW *et al.* Simultaneous A8344G heteroplasmy and mitochondrial DNA copy number quantification in myoclonus epilepsy and ragged-red fibers (MERRF) syndrome by a multiplex molecular beacon based real-time fluorescence PCR. *Nucleic Acids Res.* 29, E13 (2001).

14 Tapp I, Malmberg L, Rennel E, Wik M, Syvanen AC. Homogeneous scoring of single-nucleotide polymorphisms: comparison of the 5′-nuclease TaqMan assay and molecular beacon probes. *Biotechniques* 28, 732–738 (2000).

15 Vogelstein B, Kinzler KW. Digital PCR. *Proc. Natl Acad. Sci. USA* 96, 9236–9241(1999).

16 Smit ML, Giesendorf BA, Vet JA, Trijbels FJ, Blom HJ. Semiautomated DNA mutation analysis using a robotic workstation and molecular beacons. *Clin. Chem.* 47, 739–744 (2001).

17 Durand R, Eslahpazire J, Jafari S *et al.* Use of molecular beacons to detect an antifolate resistance-associated mutation in *Plasmodium falciparum*. *Antimicrob. Agents Chemother.* 44, 3461–3464 (2000).

18 Hu X, Belachew B, Chen L *et al.* Fluorescence-based single-tube assays to rapidly detect human gene mutations. *Strategies* 13, 71–73 (2000).

19 Chen W, Martinez G, Mulchandani A. Molecular beacons: a real-time polymerase chain reaction assay for detecting *Salmonella*. *Anal. Biochem.* 280, 166–172 (2000).

20 Clayton SJ, Scott FM, Walker J *et al.* K-ras point mutation detection in lung cancer: comparison of two approaches to somatic mutation detection using ARMS allele-specific amplification. *Clin. Chem.* 46, 1929–1938 (2000).

21 Gao W, Tyagi S, Kramer FR, Goldman E. Messenger RNA release from ribosomes during 5′-translational blockage by consecutive low-usage arginine but not leucine codons in *Escherichia coli*. *Mol. Microbiol.* 25, 707–716 (1997).

22 Lewin SR, Vesanen M, Kostrikis L *et al.* Use of real-time PCR and molecular beacons to detect virus replication in human immunodeficiency virus Type 1-infected individuals on prolonged effective antiretroviral therapy. *J. Virol.* 73, 6099–6103 (1999).

23 Manganelli R, Dubnau E, Tyagi S, Kramer FR, Smith I. Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 31, 715–724 (1999).

24 Metzner KJ, Jin X, Lee FV *et al.* Effects of *in vivo* CD8(+) T-cell depletion on virus replication in rhesus macaques immunized with a live, attenuated simian immunodeficiency virus vaccine. *J. Exp. Med.* 191, 1921–1931 (2000).

25 Park S, Wong M, Marras SA *et al.* Rapid identification of *Candida dubliniensis* using a species-specific molecular beacon. *J. Clin. Microbiol.* 38, 2829–2836 (2000).

26 Shih IM, Zhou W, Goodman SN, Lengauer C, Kinzler KW, Vogelstein B. Evidence that genetic instability occurs at an early stage of colorectal tumorigenesis. *Cancer Res.* 61, 818–822 (2001).

27 Steuerwald N, Cohen J, Herrera RJ, Brenner CA. Analysis of gene expression in single oocytes and embryos by real-time rapid cycle fluorescence monitored RT-PCR. *Mol. Hum. Reprod.* 5, 1034–1039 (1999).

28 Van Schie RC, Marras SA, Conroy JM, Nowak NJ, Catanese JJ, de Jong PJ. Semiautomated clone verification by real-time PCR using molecular beacons. *Biotechniques* 29, 1296–1306 (2000).

29 Vet JA, Majithia AR, Marras SAE *et al.* Multiplex detection of four pathogenic retroviruses using molecular beacons. *Proc. Natl Acad. Sci. USA* 96, 6394–6399 (1999).

•• **Describes a multiplex assay that enables the rapid and reliable screening of donated blood and transplantable tissues for the presence of four different retroviruses.**

30 Xiao G, Chicas A, Olivier M *et al.* A DNA damage signal is required for p53 to activate gadd45. *Cancer Res.* 60, 1711–1719 (2000).

31 Zhang L, Lewin SR, Markowitz M *et al.* Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. *J. Exp. Med.* 190, 725–732 (1999).

32 El Hajj HH, Marras SAE, Tyagi S *et al.* Detection of rifampin resistance in *Mycobacterium tuberculosis* in a single tube with molecular beacons. *J. Clin. Microbiol.* 39, 4131–4137 (2001).

33 Jordens JZ, Lanham S, Pickett MA, Amarasekara S, Abeywickrema I, Watt PJ. Amplification with molecular beacon primers and reverse line blotting for the detection and typing of human papillomaviruses. *J. Virol. Methods* 89, 29–37 (2000).

34 Kaboev OK, Luchkina LA, Tret'iakov AN, Bahrmand AR. PCR hot start using primers with the structure of molecular beacons (hairpin-like structure). *Nucleic Acids Res.* 28, E94 (2000).

35 Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP. Strand displacement amplification-an isothermal, *in vitro* DNA amplification technique. *Nucleic Acids Res.* 20, 1691–1696 (1992).

36 De Baar MP, van Dooren MW, de Rooij E *et al.* A single rapid real-time monitored isothermal RNA amplification assay for quantification of Human Immunodeficiency Virus Type 1 isolates from groups M, N and O. *J. Clin. Microbiol.* 39, 1378–1384 (2001).

37 De Baar MP, Timmermans EC, Bakker M, de Rooij E, van Gemen B, Goudsmit J. One tube real-time isothermal amplification assay to identify and distinguish Human Immunodeficiency Virus Type 1 subtypes A, B and C and circulating recombinant forms AE and AG. *J. Clin. Microbiol.* 39, 1895–1902 (2001).

38 De Ronde A, van Dooren M, van Der Hoek L *et al.* Establishment of new transmissible and drug-sensitive Human Immunodeficiency Virus Type 1 wild-types due to transmission of nucleoside analogue-resistant virus. *J. Virol.* 75, 595–602 (2001).

39 Klerks MM, Leone GO, Verbeek M, van den Heuvel JF, Schoen CD. Development of a multiplex AmpliDet RNA for the simultaneous detection of Potato leafroll virus and Potato virus Y in potato tubers. *J. Virol. Methods* 93, 115–125 (2001).

40 Leone G, van Schijndel H, van Gemen B, Kramer FR, Schoen CD. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Res.* 26, 2150–2155 (1998).

41 Lizardi PM, Huang X, Zhu Z, Bray-Ward P, Thomas DC, Ward DC. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nature Genet.* 19, 225–232 (1998).

42 Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. *Biotechniques* 22, 130–131 (1997).

43 Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz KA. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.* 4, 357–362 (1995).

44 Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. *Nat. Biotechnol.* 17, 804–807 (1999).

45 Foy CA, Parkes HC. Emerging homogeneous DNA-based technologies in the clinical laboratory. *Clin. Chem.* 47(6), 990–1000 (2001).

46 Marras SAE. Artifical hybridization probes. In: *Artificial DNA.* Fields HA, Khudyakov YE (Eds.), CRC Publishing, Kansas City, MO, USA (In Press).

47 Tyagi S, Marras SAE, Kramer FR. Wavelength-shifting molecular beacons. *Nature Biotechnol.* 18, 1191–1196 (2000).

48 Brown LJ, Cummins J, Hamilton A, Brown T. Molecular beacons attached to glass beads fluoresce upon hybridisation to target DNA. *Chem. Comm.* 621–622 (2000).

49 Liu X, Farmerie W, Schuster S, Tan W. Molecular beacons for DNA biosensors with micrometer to submicrometer dimensions. *Anal. Biochem.* 283, 56–63 (2000).

50 Liu X, Tan W. A fiber-optic evanescent wave DNA biosensor based on novel molecular beacons. *Anal. Chem.* 71, 5054–5059 (1999).

51 Steemers FJ, Ferguson JA, Walt DR. Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. *Nature Biotechnol.* 18, 91–94 (2000).

•• **First demonstration of a microarray capable of detecting unlabeled DNA targets.**

52 Matsuo T. *In situ* visualization of mRNA for basic fibroblast growth factor in living cells. *Biochim. Biophys. Acta* 1379, 178–184 (1998).

53 Sokol DL, Zhang X, Lu P, Gewirtz AM. Real time detection of DNA, RNA hybridization in living cells. *Proc. Natl Acad. Sci. USA* 95, 11538–11543 (1998).

54 Moolenaar C, Marras SA, Slats JCM *et al.* Linear 2′O-methyl RNA probes for the visualization of RNA in living cells. *Nucleic Acids Res.* 29, E89 (2001).

55 Fang X, Li JJ, Tan W. Using molecular beacons to probe molecular interactions between lactate dehydrogenase and single-stranded DNA. *Anal. Chem.* 72, 3280–3285 (2000).

56 Li JJ, Geyer R, Tan W. Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA. *Nucleic Acids Res.* 28, E52 (2000).

57 Li JJ, Fang XH, Schuster SM, Tan WH. Molecular beacons: a novel approach to detect protein-DNA interactions. *Angew. Chem. Int. Ed.* 39, 1049–1052 (2000).

58 Lindquist JN, Kauschke SG, Stefanovic B, Burchardt ER, Brenner DA. Characterization of the interaction between αCP(2) and the 3′- untranslated region of collagen α1(I) mRNA. *Nucleic Acids Res.* 28, 4306–4316 (2000).

59 Strouse RJ, Hakki FZ, Wang SC, DeFusco AW, Garrett JL, Schenerman MA. Using molecular beacons to quantify low levels of Type I endonuclease activity. *Biopharm.* 13, 40–47 (2000).

60 Tan W, Fang X, Li J, Liu X. Molecular beacons: a novel DNA probe for nucleic acid and protein studies. *Chemistry* 6, 1107–1111 (2000).

61 Tung CH, Mahmood U, Bredow S, Weissleder R. *In vivo* imaging of proteolytic enzyme activity using a novel molecular reporter. *C#ncer Res.* 60, 4953–4958 (2000).

62 Yamamoto R, Baba T, Kumar PK. Molecular beacon aptamer fluoresces in the presence of Tat protein of HIV-1. *Genes*

Cells 5, 389–396 (2000).

63 Hamaguchi N, Ellington A, Stanton M. Aptamer beacons for the direct detection of proteins. *Anal. Biochem.* 294, 126–13 (2001).

Websites

101 http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi

102 www.molecular-beacons.org/

For further reading see:

- www.molecular-beacons.org/
- http://bioinfo.math.rpi.edu/~mfold/dna/form1.cgi

Affiliations

- *Jacqueline AM Vet,*
  University Hospital Nijmegen,
  Department of Paediatrics and Neurology,
  P.O. Box 9101, 6500 HB Nijmegen,
  Tel.: +49 312 4361 4559
  Fax.: +49 312 4361 8900
  j.vet@ckslkn.azn.nl
- *Brenda JM Van der Rijt*
- *Henk J Blom*