

# Molecular beacons: a new approach for semiautomated mutation analysis

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**Molecular beacons are oligonucleotide probes that become fluorescent upon hybridization. We designed molecular beacons to detect a point mutation in the methylenetetrahydrofolate reductase (MTHFR) gene, a mutation that has been related to an increased risk for cardiovascular disease and neural tube defects. The application of molecular beacons enables fast, semi-automated, accurate mutation detection. Moreover, the procedure is performed in a closed tube system, thereby avoiding carryover contamination. We believe these probes will find their way into nucleic acid research and diagnostics.**

Current techniques for routine detection of single base-pair mutations at the DNA level are often labor-intensive and time-consuming. This report describes a polymerase chain reaction (PCR)-based method in which novel fluorescent probes, called molecular beacons, are used. Molecular beacons are probes that consist of a stem-and-loop structure (Fig. 1) [1]. The loop portion of the molecule is a probe sequence complementary to a predetermined target DNA. The stem is formed by annealing on either side of the probe sequence two complementary arm sequences that are unrelated to the target sequence. A fluorescent moiety is attached to the end of one arm and a nonfluorescent quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, so that the fluorescence is quenched by energy transfer and the fluorophore is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the hybrid formed by the arm sequences [1]. Thus, the probe undergoes a spontaneous conformational

change that forces the arm sequences apart, thereby moving the fluorophore and the quencher away from each other and resulting in restoration of fluorescence (Fig. 1).

Because hybridization of these probes to their complementary target is highly specific, we designed molecular beacons to detect a cytosine to thymine (C677T) mutation in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene. MTHFR plays a key role in homocysteine (Hcy) metabolism. A moderate increase of Hcy in plasma has been identified as a risk factor for cardiovascular disease and neural tube defects [2–4]. MTHFR regulates the remethylation of Hcy to methionine. Recently, a mutation (C677T) in this gene has been identified that alters a conserved amino acid, alanine, to valine [5]. The mutation in the homozygous state correlates with reduced enzyme activity, redistribution of folates, and increased concentrations of total Hcy and has been related to an increased risk for cardiovascular disease and neural tube defects [5–7].

To determine the presence of the C677T mutation, we combined the sensitivity of PCR with the specificity of molecular beacons. Real-time monitoring of PCR was done by measuring the fluorescence generated by the hybridization of the molecular beacon to its perfectly matching target during PCR. The procedure is carried out in a closed tube system and fluorescence is measured through the lid, thereby avoiding carryover contamination.

In this study we want to demonstrate the ease with which molecular beacons can be applied for large-scale semiautomated mutation detection.

## Materials and Methods

**DNA.** Nucleic acids were isolated from blood obtained from a selection of control individuals at the pediatrics laboratory. All samples were analyzed for the C677T mutation by conventional PCR, *HinfI* restriction enzyme analysis, and agarose gel electrophoresis according to Frosst et al. [5] and were coded for unbiased reexamination. Extractions were carried out with the Puregene<sup>TM</sup> (Gentra Systems) DNA isolation system. DNA concen-

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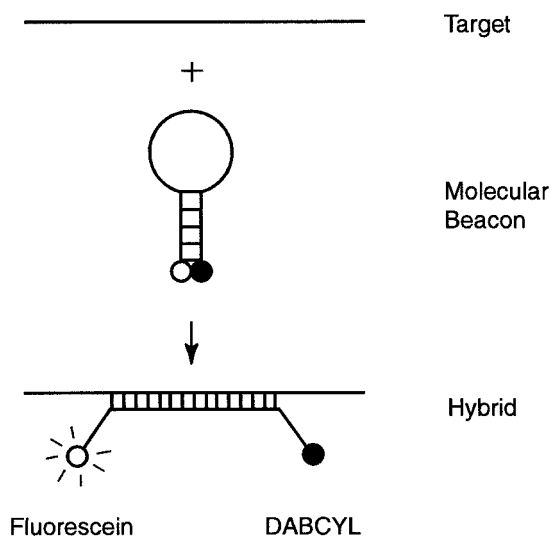


Fig. 1. Principle of operation of molecular beacons: Because the hairpin stem formed by the complementary arm sequences cannot coexist with the rigid double helix that is formed when the probe hybridizes to its target, the molecular beacon undergoes a conformational change that forces the arm sequences apart and causes the fluorophore to move away from the quencher, therefore resulting in fluorescence.

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trations were measured spectrophotometrically and dilutions of 20 ng/ $\mu$ L were used for PCR amplification.

**Probes.** Molecular beacons were synthesized from oligonucleotides that contained a primary amino group at their 3' end and a sulfhydryl group protected with a trityl moiety at their 5' end. The sulfhydryl group was covalently linked to the 5' phosphate via a  $(\text{CH}_2)_6$  spacer, and the primary amino group was linked to the 3' hydroxyl moiety via a  $(\text{CH}_2)_7$  spacer. Two consecutive coupling reactions were carried out. DABCYL (Molecular Probes), the quencher, was covalently linked to the primary amino group by using an amine reactive form of DABCYL. The oligonucleotides coupled to DABCYL were purified by HPLC. The protective trityl moiety was then removed from the 5' sulfhydryl group, and the fluorophore—an iodoacetamide derivative of fluorescein (Molecular Probes)—was introduced in its place. The oligonucleotides covalently linked to the fluorophore and DABCYL were repurified by HPLC. Their structures were as follows (the underlined sequence in both molecular beacons is the probe sequence):

Wild-type beacon (SW 115): fluorescein-5'-GCG AG TGC  
GGG AGC CGA TTT CTC GC-3'-DABCYL

Mutant beacon (SW116): fluorescein-5'-GCG AG TGC  
GGG AGT CGA TTT CTC GC-3'-DABCYL

A detailed protocol for these syntheses is available on the following website: [http://www.phri.nyu.edu/molecular\\_beacons](http://www.phri.nyu.edu/molecular_beacons).

**Thermal denaturation profiles.** To determine thermal denaturation profiles of the hybrids formed by the two molecular beacons and their perfect and mismatch oligonucleotide targets, we monitored the fluorescence of a 50- $\mu$ L solution containing 125 nmol/L molecular beacon probe, 500 nmol/L oligonucleotide target, 20 mmol/L Tris-HCl, and 1 mmol/L  $\text{MgCl}_2$ , pH 8, as a function of temperature. The temperature was increased from 4° to 80 °C in 1 °C steps, with each temperature being held for 1 min. Fluorescence was monitored during each period by using a fluorescence reader with a programmed temperature control (PE/ABI 7700; Applied Biosystems).

**PCR.** Primers BPF 5'-CTGACCTGAAGCACTTGAAGG-3' and BPR1 5'-ATGTCGGTGCATGCCTTAC-3' (Eurogentec) were selected from the region flanking the C677T mutation. The product generated by these primers is 115 bp long. To the PCR mixtures, containing Taq Gold amplification buffer (Perkin-Elmer; Taqman<sup>TM</sup> PCR Core Reagent Kit), 4 mmol/L  $\text{MgCl}_2$ , each of the four nucleotides (200  $\mu$ mol final quantity), and 20 pmol of each primer in a total volume of 50  $\mu$ L per tube, was added 50–100 ng of chromosomal DNA and 15 pmol of either the wild-type or the mutant molecular beacon. One unit of AmpliTaq Gold was used per reaction. The PCR buffer contained ROX (fluorescent dye, 60 nmol final quantity; Perkin-Elmer) as reference dye for normalization of the reactions; the fluorescence of ROX is measured throughout PCR, and possible fluctuations in the ROX signal are used to correct the sample signal.

Molecular beacons are added directly to the PCR. At 95 °C, the molecular beacons are denatured and have a random coil structure, allowing full fluorescence. Decrease of the temperature to 58 °C enables the formation of hairpins, which causes a drop in fluorescence. However, in the presence of chromosomal DNA target, the probe sequences of the molecular beacons bind to their complementary sequences and the fluorescence increases. During primer elongation, at 72 °C, molecular beacons are dissociated from their target. This process is repeated every cycle.

We performed the PCR in the ABI 7700 Sequence Detector (PE/Applied Biosystems), an instrument that is a combination of a thermal cycler and a laser-induced fluorescence detector and provides the instrumentation for the real-time detection of fluorescence during thermal cycling. The PCR conditions were as follows: Cycling was preceded by 10 min at 95 °C for activation of the Taq Gold DNA polymerase, followed by 40 cycles of 30 s at 95 °C, 30 s at 58 °C, another 30 s at 58 °C, and 30 s at 72 °C. In the second step of 58 °C, the fluorescence was measured.

## Results

**Determination of optimal hybridization temperature.** To explore the range of temperature in which the molecular beacons are able to discriminate between wild-type and mutant alleles, we determined the thermal denaturation

profiles of hybrids between molecular beacons and either perfect or mismatch oligonucleotide targets. Fig. 2 (top) shows the melting profiles obtained with the molecular beacon that is complementary to the wild-type target (SW 115). In the temperature interval from 56 to 61 °C the wild-type target elicits strong fluorescence, whereas the mutant target does not—thus allowing a clear discrimination between wild-type and mutant target. Fig. 2 (bottom) shows the melting profiles of the molecular beacon complementary to the mutant target (SW 116). The optimal temperature interval for this ranges from 58 to 61 °C. On the basis of these thermal denaturation profiles, we chose 58 °C as the hybridization temperature for both molecular beacons. This temperature could also be used for annealing the primers in the PCR, thus enabling hybridization of the molecular beacons during the PCR annealing step.

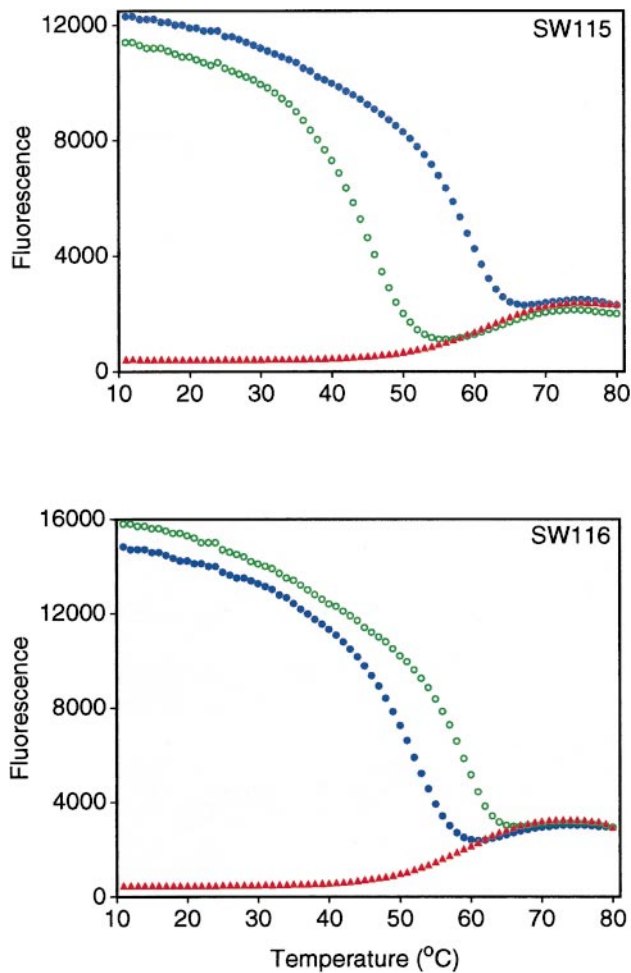


Fig. 2. Thermal denaturation profiles of the molecular beacons used in this study.

(Top) Wild-type beacon (SW 115) is added to the wild-type oligonucleotide target (blue closed dots) and the mutant oligonucleotide target (green open dots). The red triangles are obtained from the wild-type beacon without target. (Bottom) Mutant beacon (SW 116) is added to the mutant oligonucleotide target (green open dots) and the wild-type oligonucleotide target (blue closed dots). The red triangles are obtained from the mutant beacon without target.

*Real-time PCR analysis.* Nucleic acids isolated from blood of 45 individuals were examined for the presence of the C677T mutation by PCR with allele-specific molecular beacons. Each sample was analyzed with both the wild-type and the mutant beacon. Fluorescence was measured

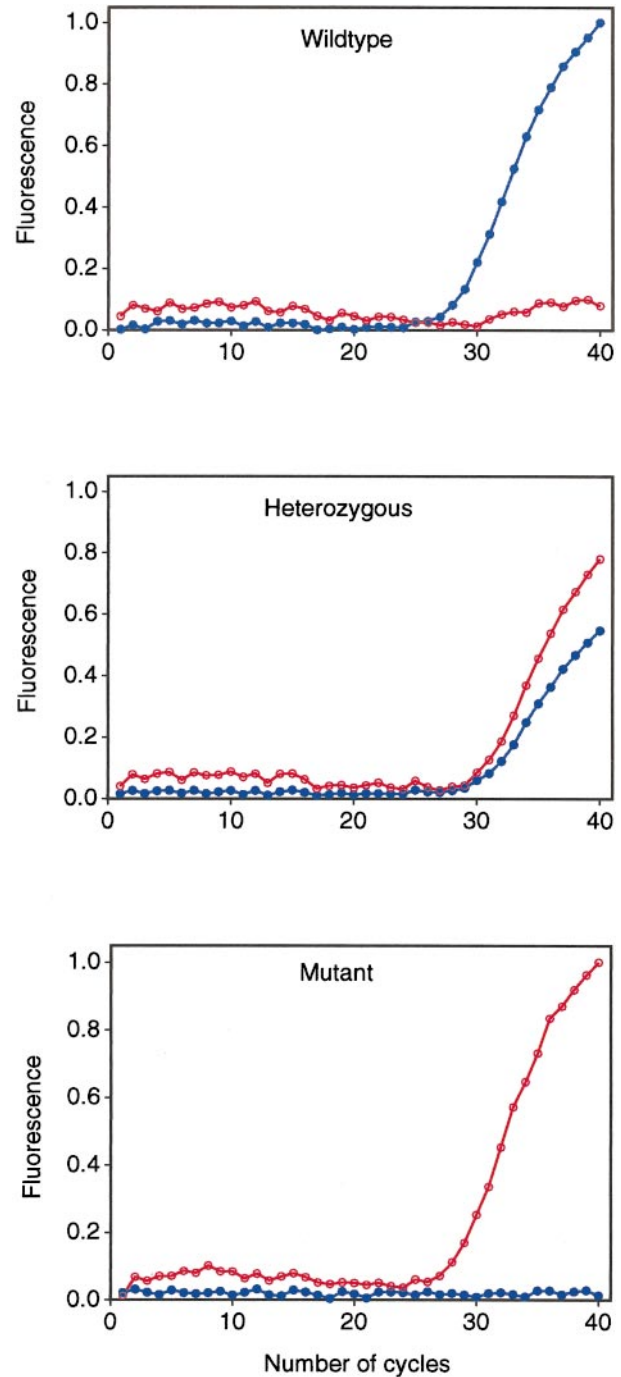


Fig. 3. Results obtained after PCR with molecular beacons for (top) a homozygous normal individual, (middle) a heterozygous individual, and (bottom) a person homozygous for the C677T mutation.

Fluorescence was measured every cycle and, after normalization, plotted vs the cycle number. In each panel, the blue closed dots are obtained from the wild-type beacon, the red open dots from the mutant beacon.

during every cycle of the PCR. To compare the multiple amplification curves, we used the relation  $(F - F_{\min}) / (F_{\max} - F_{\min})$  to normalize the fluorescence values for each molecular beacon.

Figure 3 (top) shows results obtained from a normal individual. The fluorescent signal is generated by the molecular beacon complementary to the wild-type allele, whereas the mutant beacon does not generate a signal. In the middle panel, which shows results obtained from a heterozygous person, both beacons generate a fluorescent signal. The bottom panel illustrates amplification of DNA obtained from a homozygous mutant individual: The fluorescent signal is generated by the beacon complementary to the mutant allele, whereas the wild-type beacon does not generate a signal. Negative control samples, containing no template, also did not generate any fluorescence (data not shown).

Analysis of the samples indicated that 8 were homozygous mutants, 17 were heterozygous individuals, and 20 were wild-type persons. This is illustrated by Fig. 4, a plot of threshold cycle (Ct) values of both molecular beacons, derived from every sample. Ct is the thermal cycle at which fluorescence exceeds a defined value above the background fluorescence. Samples that show no detectable increase in fluorescence throughout the reaction (threshold cycles  $>40$ ) are considered negative. Heterozygotes, homozygote mutants, and wild-type samples cluster in three different groups. Plotting the Ct values demonstrates an overview and control of the complete data set because samples that do not cluster in one of the three groups are easily identified. Results obtained by conventional screening of the C677T mutation, which included PCR, restriction enzyme analysis, and agarose

gel electrophoresis, were in complete agreement with results generated by molecular beacons (data not shown).

### Discussion

This report describes the first application of molecular beacons for single-basepair mutation analysis. The present method, a closed-tube PCR/hybridization assay, is a simple and fast procedure that is fully automated. Depending on the PCR cycling times, 48 samples can be completely analyzed in  $\sim 2$  h with both molecular beacons, because restriction enzyme analysis and gel electrophoresis are no longer required. The method is also highly reproducible and sensitive and generates unambiguous data. One of the most important advantages is that tubes remain closed during monitoring of fluorescence, thereby eliminating the risk of carryover contamination and the generation of false-positive results. In general, this technique can be applied for the diagnosis of known mutations in diseases, e.g., in cancer or cardiovascular disease. The high throughput of this method also enables setting up large-scale studies designed to establish the risk associated with certain genetic defects.

The molecular beacons in the present study were designed to detect a C-to-T transition in the MTHFR gene (resulting in an alanine to valine substitution). The wild-type beacon carries a C at the putative mutation site, whereas the mutant beacon carries a T at this position. Although thymine is able to form a relatively stable basepair with guanosine [8], indicating the possible occurrence of cross-hybridization, both beacons discriminate well between wild-type and mutant target alleles, at the predetermined hybridization temperature of  $58^\circ\text{C}$ , and do not generate fluorescence with their respective

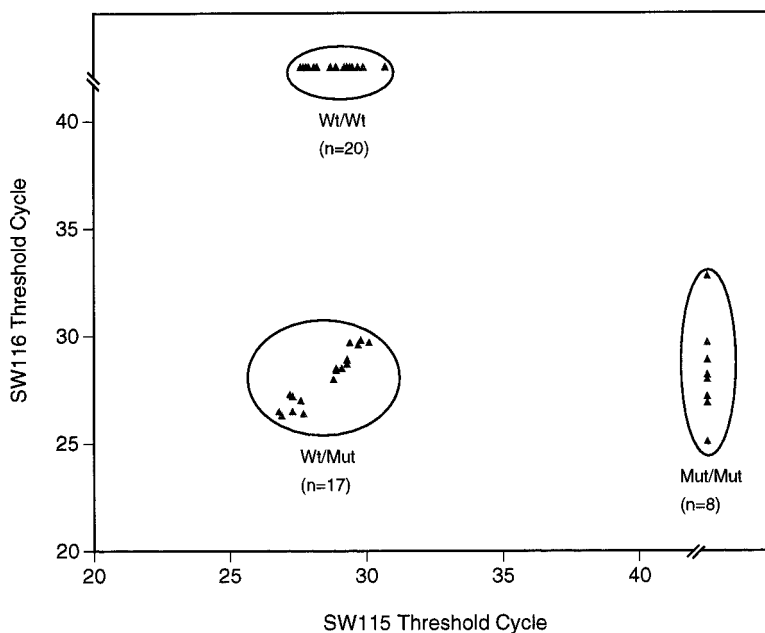


Fig. 4. Graphical representation of threshold-cycle values of SW-115 (wild-type molecular beacon, x) vs SW-116 (mutant molecular beacon, y) for all 45 samples analyzed.

mismatch targets. This demonstrates the high specificity of interaction of molecular beacons with their target. Compared with linear probes, e.g., the TaqMan probes [9], molecular beacons exhibit a much greater specificity in the interaction with their targets, which is attributable to the presence of their stem [10]. Hybrids formed between molecular beacons and mismatch targets dissociate at a much lower temperature than hybrids formed between linear probes and mismatch targets. Thus a wider temperature range is observed between melting of the perfect and mismatch hybrid. As a result, one can use a common temperature to detect several mutations by means of molecular beacons.

Although in this report molecular beacons are applied for point-mutation analysis, any type of mutation can be detected by these probes. Furthermore, the use of several molecular beacons, aimed at different targets, and each coupled to a different fluorophore, should enable simultaneous detection of multiple mutations [10]. In case the number of mutations under investigation is large, e.g.,  $>4$ , the use of many molecular beacons of different colors in the same solution is impractical. However, this can be overcome by covalent linkage of the beacons to a solid support (e.g., a reaction vessel, dipstick, or chip). This will also eliminate the need to use different fluorescent labels and will allow the extensive survey of an amplified region.

Multiplex PCR will provide the simultaneous survey of several different genes. The pattern of fluorescence that develops should indicate which mutations are present.

In conclusion, the present data show that molecular beacons are excellently suitable for mutation detection. This report is one of the first of the many applications of these promising molecules that we expect will follow in the future.

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### References

1. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol* 1996;14:303-8.
2. Malinow MR. Homocysteine and arterial occlusive diseases [Review]. *J Intern Med* 1994;236:603-17.
3. Boushey CJ, Beresford SAA, Omenn GS, Motulsky AG. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* 1995;274:1049-57.
4. Steegers-Theunissen R, Boers GHJ, Trijbels JMF, Finkelstein JD, Blom HJ, Thomas CM, et al. Maternal hyperhomocysteinemia: a risk factor for neural-tube defects? *Metabolism* 1994;43:1475-80.
5. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995;10:111-3.
6. Kluijtmans LAJ, Van denHeuvel LPW, Boers GHI, Frosst P, Van Oost BA, DenHeijer M, et al. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am J Hum Genet* 1996;58:35-41.
7. VanderPut NJM, Steegers-Theunissen RPM, Frosst P, Trijbels JMF, Eskes TKAB, Van denHeuvel LPW. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet* 1995;346:1070-1.
8. Ikuta S, Takagi K, Wallace RB, Itakura K. Dissociation kinetics of 19 base-paired oligonucleotide DNA duplexes containing different single mismatched base pairs. *Nucleic Acids Res* 1987;15:797-811.
9. Livak KJ, Flood SJA, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* 1995;4:357-62.
10. Tyagi S, Bratu D, Kramer FR. Multicolor molecular beacons for allele discrimination. *Nat Biotechnol* 1998;16:49-53.