Molecular Beacons: Hybridization Probes for Detection of Nucleic Acids in Homogeneous Solutions

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1. Introduction

Molecular beacons are oligonucleotide probes that can report the presence of specific nucleic acids in homogeneous solutions (Tyagi and Kramer, 1996). They are useful in situations where it is either not possible or desirable to isolate the probe-target hybrids from an excess of the hybridization probes, such as in real-time monitoring of polymerase chain reactions in sealed tubes or for the detection of RNAs within living cells. Molecular beacons are hairpin-shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid (Figure 1). They are designed in such a way that the loop portion of the molecule is a probe sequence complementary to a target nucleic acid molecule. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. Since the quencher moiety is a non-fluorescent chromophore and emits the energy that it receives from the fluorophore as heat, the probe is unable to fluoresce. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem hybrid and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence which can be detected.

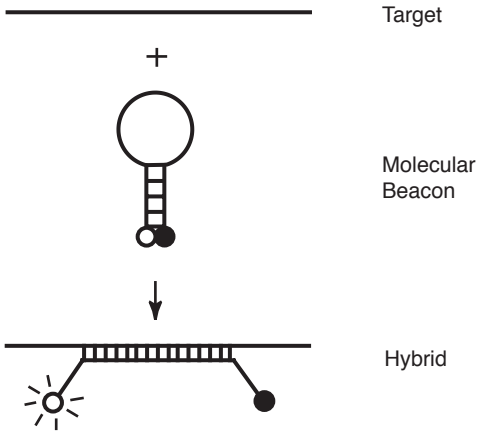

Figure 1. Operation of molecular beacons. On their own, these molecules are non-fluorescent, because the stem hybrid keeps the fluorophore close to the quencher. When the probe sequence in the loop hybridizes to its target, forming a rigid double helix, a conformational reorganization occurs that separates the quencher from the fluorophore, restoring fluorescence.

In order to detect multiple targets in the same solution, molecular beacons can be made in many different colors utilizing a broad range of fluorophores (Tyagi, Bratu, and Kramer, 1998). Dabcyl, a non-fluorescent chromophore, serves as the universal quencher for any fluorophore in molecular beacons. Owing to their stem, the recognition of targets by molecular beacons is so specific that single-nucleotide differences can be readily detected. Because of these properties, molecular beacons have been used for the detection of RNAs within living cells (Matsuo, 1998; Sokol et al., 1998), for monitoring the synthesis of specific nucleic acids in sealed reaction vessels (Tyagi, Bratu, and Kramer, 1998; Leone et al., 1998), for homogenous one-tube assays for genotyping single-nucleotide variations in DNA (Piatek et al., 1998; Kostrikis et al., 1998; Giesendorf et al., 1998, Marras, Kramer, and Tyagi, 1999) and for multiplex PCRs for the detection of four different pathogenic retroviruses (Vet et al., 1999).

2. Materials

Equipment

- High-pressure liquid chromatograph (System Gold, Beckman)
- Spectrofluorometer (QuantaMaster, Photon Technology International)
- Spectrofluorometric thermal cycler with a capacity to monitor fluorescence in real time (ABI Prism 7700, Applied Biosystems)

Buffers

- 0.1 M sodium bicarbonate, pH 8.5
- 0.2 M sodium bicarbonate, pH 9.0
- Buffer A: 0.1 M triethylammonium acetate, pH 6.5, filtered and degassed
- Buffer B: 0.1 M triethylammonium acetate in 75% acetonitrile, pH 6.5, filtered and degassed
- TE buffer: 1 mM EDTA, 10 mM Tris-HCl, pH 8.0
- Molecular beacon buffer: 1 mM MgCl2, 20 mM Tris-HCl, pH 8.0

3. Synthesis and purification of molecular beacons

The starting material for the synthesis of molecular beacons is an oligonucleotide that contains a sulfhydryl group at its 5' end and a primary amino group at its 3' end. Dabcyl is coupled to the primary amino group utilizing an amine-reactive derivative of dabcyl. The oligonucleotides that are coupled to dabcyl are then purified. The protective trityl moiety is then removed from the 5'-sulfhydryl group and a fluorophore is introduced in its place using an iodoacetamide derivative. Recently a controlled-pore glass column that introduces a dabcyl moiety at the 3' end of an oligonucleotide has become available, which enables the synthesize of a molecular beacon completely on a DNA synthesizer. The sequence of the molecular beacon used throughout this protocol is: fluorescein-5'GCG AGC TAG GAA ACA CCA AAG ATG ATA TTT GCT CGC -3'-dabcyl, where underlines identify the arm sequences.

3.1. Procedure

3.1.1. Coupling of dabcyl

1. Dissolve 50-250 nanomoles of dry oligonucleotide in 500 µl of 0.1 M sodium bicarbonate, pH 8.5. Dissolve about 20 mg of dabcyl (4-(4'-dimethylaminophenylazo)benzoic acid) succinimidyl ester (Molecular Probes) in 100 µl N,N-dimethylformamide and add to a stirring solution of the oligonucleotide in 10-µl aliquots at 20-minute intervals. Continue stirring for at least 12 hours.

2. Remove particulate material by spinning the mixture in a microcentrifuge for one minute at 10,000 rpm. In order to remove unreacted dabcyl, pass the supernatant through a gel-exclusion column. Equilibrate a Sephadex G-25 column (NAP-5, Pharmacia) with buffer A, load the supernatant and elute with 1 ml buffer A. Filter the eluate through a 0.2 µm filter (Centrex MF-0.4, Schleicher & Schuell) before loading on the HPLC column.
3. Purify the oligonucleotides on a C-18 reverse-phase column (Waters), utilizing a linear elution gradient of 20 to 70% buffer B in buffer A and run for 25 minutes at a flow rate of 1 ml/min. Monitor the absorption of the elution stream at 260 nm and 491 nm. A typical chromatogram is shown in Figure 2. Collect the peak that absorbs in both wavelengths, which contains oligonucleotides with a protected sulfhydryl group at their 5'-end and dabcyl at their 3'-end (peak D).
4. Precipitate the collected material with ethanol and salt, and spin in a centrifuge for 10 minutes at 10,000 rpm, discard the supernatant, dry the pellet and dissolve it in 250 µl buffer A.

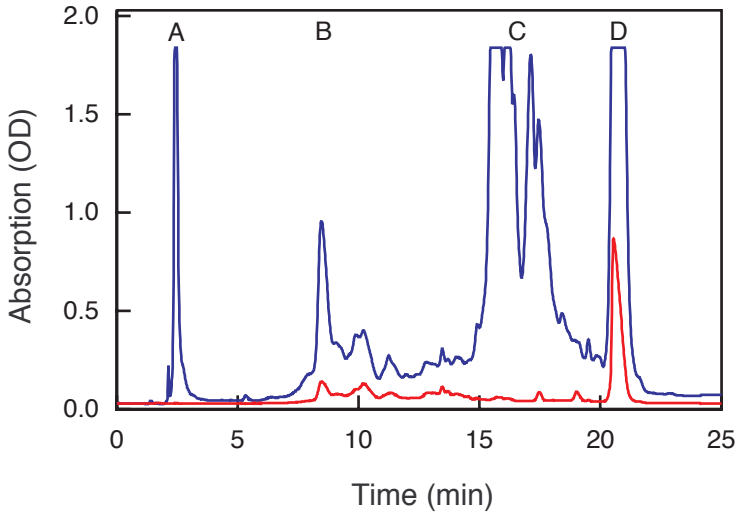

Figure 2. Chromatographic separation of oligonucleotides coupled to dabcyl. The blue line represents absorption at 260 nm and the red line represents absorption at 491 nm. The oligonucleotides in peaks A and B do not contain trityl moieties, whereas the oligonucleotides in peaks C and D are protected by trityl moieties. The oligonucleotides in peaks B and D are coupled to dabcyl, whereas the oligonucleotides in peaks A and C they are not coupled to DABCYL. Peak D should be collected.

3.1.2. Coupling of fluorophore

1. In order to remove the trityl moiety, add 10 µl of 0.15 M silver nitrate and incubate for 30 minutes. Add 15 µl of 0.15 M dithiothreitol to this mixture and shake for 5 minutes. Spin for 2 minutes at 10,000 rpm and transfer the supernatant to a new tube. Dissolve about 40 mg 5-iodoactamidofluorescein (Molecular Probes) in 250 µl of 0.2 M sodium bicarbonate, pH 9.0 and add it to the supernatant. Incubate the mixture for 90 minutes. Each of these solutions should be prepared just before use.
2. Remove excess fluorescein from the reaction mixture by gel exclusion chromatography and purify the oligonucleotides coupled to fluorescein by HPLC, following the instructions in steps 2 and 3 of the previous section. A sample chromatogram is shown in Figure 3. Collect the fractions corresponding to peak F, which absorb at wavelengths 260 nm and 491 nm and are fluorescent when observed with an ultraviolet lamp in a dark room. If a different fluorophore is coupled in place of fluorescein, its maximum absorption wavelength should be used instead of 491 nm.
3. Precipitate the collected material and dissolve the pellet in 100 µl TE buffer. Determine the absorbance at 260 nm and estimate the yield (1 OD260 = 33 µg/ml).

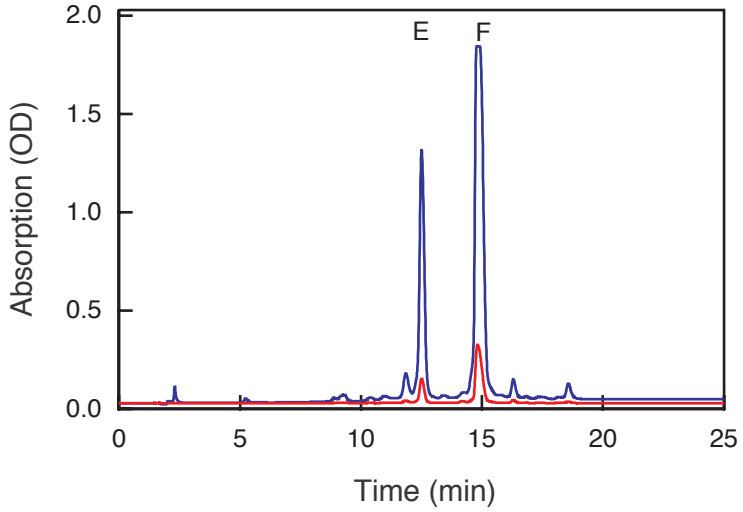

Figure 3. Chromatographic separation of oligonucleotides coupled to both dabcyl and fluorescein. The blue line represents absorption at 260 nm and the red line represents absorption at 491 nm. The oligonucleotides present in peak E are not coupled to fluorescein, whereas the oligonucleotides in peak F are coupled to fluorescein. Peak F should be collected.

3.1.3. Automated synthesis

1. Use a controlled-pore glass column to introduce dabcyl (Glen Research) at the 3' end of the oligonucleotide during automated synthesis. At the 5' end of the oligonucleotide, either a thiol or an amino modifier can be introduced for a subsequent coupling to a fluorophore, or a fluorophore can directly be introduced during automated synthesis using a phosphoramidite. The 5' modifiers and fluorophores should remained protected with a trityl moiety during the synthesis. Perform post-synthetic steps as recommended by the manufacturer of the DNA synthesizer. Dissolve the oligonucleotide in 600 µl Buffer A.
2. When the fluorophore is to be introduced manually, purify the oligonucleotide protected with a trityl moiety. Remove the trityl moiety from the purified oligonucleotide and continue with the coupling of the fluorophore, as described before.
3. When a 5' fluorophore is introduced via automated synthesis, purify the oligonucleotide protected with the trityl moiety and then remove the trityl moiety from the purified oligonucleotide. Precipitate the molecular beacon with ethanol and salt and dissolve the pellet in 100 µl TE buffer. Determine the absorbance at 260 nm and estimate the yield.

4. Characterization of molecular beacons

4.1. Procedure

4.1.1. Signal to background ratio

1. Determine the fluorescence (Fbuffer) of 200 µl of molecular beacon buffer solution using 491 nm as the excitation wavelength and 515 as the emission wavelength. If the fluorophore is not fluorescein, choose wavelengths that are optimal for the fluorophore in the molecular beacon.
2. Add 10 µl of 1 µM molecular beacon to this solution and record the new level of fluorescence (Fclose).

3. Add a two-fold molar excess of the oligonucleotide target and monitor the rise in fluorescence until it reaches a stable level (Fopen).
4. Calculate the signal-to-background ratio as (Fopen-Fbuffer)/(Fclose-Fbuffer).

4.1.2. Thermal denaturation profiles

1. Prepare two tubes containing 50 µl of 200 nM molecular beacon dissolved in 3.5 mM MgCl2 and 10 mM Tris-HCl, pH 8.0 and add the oligonucleotide target to one of the tubes at a final concentration of 400 nM.
2. Determine the fluorescence of each solution as a function of temperature using a thermal cycler with the capacity to monitor fluorescence. Decrease the temperature of these tubes from 80 °C to 10 °C in 1 °C steps, with each hold lasting one minute, while monitoring the fluorescence during each hold.

4.2. Results

An example of a hybridization reaction performed for the determination of the signal-to-background ratio is shown in Figure 4. The signal-to-background ratio in this example was 190. Usually the ratio ranges from 30 to 200. An example of a thermal denaturation profile is shown in Figure 5. The probe-target hybrid denatures at 58 °C and the stem of the molecular beacon denatures at 64 °C. In the range of 10 °C to 50 °C, the free probe has very little fluorescence, whereas the target-bound form is fluorescent. The sequence of the molecular beacon used in both experiments was shown in the previous section.

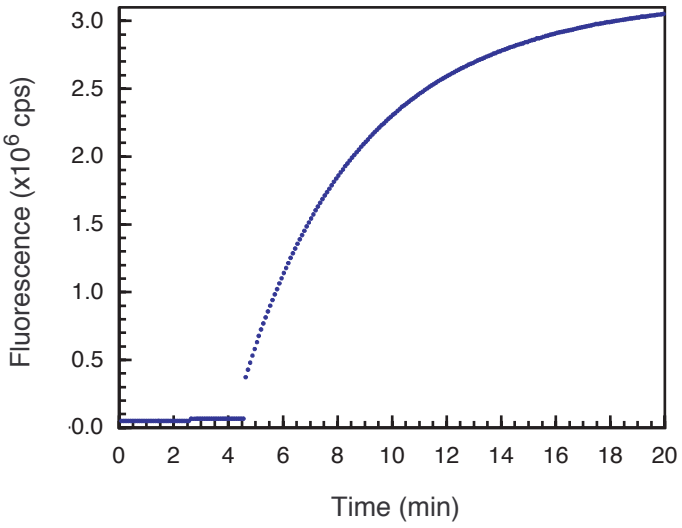

Figure 4. The spontaneous fluorogenic response of molecular beacons to the addition of target. The first segment of the data is due to the fluorescence of the buffer, the second segment is due to the fluorescence of the buffer containing the molecular beacons, and the third segment shows the increase in fluorescence that occurs upon the addition of target oligonucleotides.

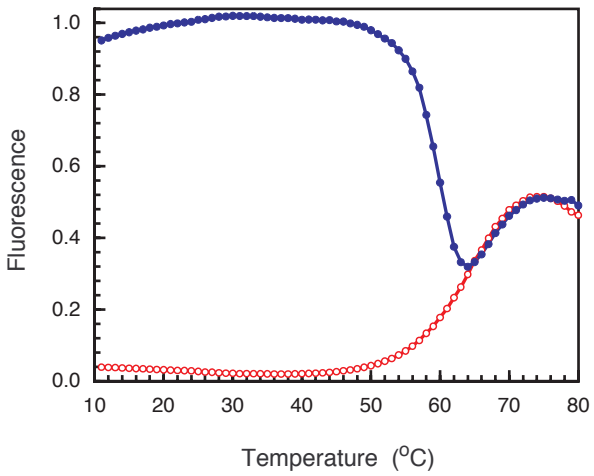

Figure 5. Thermal denaturation profiles of a molecular beacon (open circles) and the hybrid formed between the molecular beacons and its oligonucleotide target (filled circles). The profiles indicate that this molecular beacon can be used below 50 °C.

5. Real-time monitoring of polymerase chain reactions

Utilize molecular beacons that are complementary to a sequence in the middle of the expected amplicon. The length of their arm sequences should be chosen so that a stem is formed at the annealing temperature of the polymerase chain reaction. The length of the loop sequence should be chosen so that the probe-target hybrid is stable at the annealing temperature. Whether a molecular beacon actually exhibits these design features is determined by obtaining thermal denaturation profiles, as detailed in the previous section. Molecular beacons with appropriate thermal denaturation characteristics are included in each reaction at a concentration similar to the concentration of the primers. During the denaturation step, the molecular beacons assume a random-coil configuration and fluoresce. As the temperature is lowered to allow annealing of the primers, stem hybrids form rapidly, preventing fluorescence. However, at the annealing temperature, molecular beacons also bind to the amplicons and generate fluorescence. When the temperature is raised to allow primer extension, the molecular beacons dissociate from their targets and do not interfere with polymerization. A new hybridization takes place in the annealing step of every cycle, and the intensity of the resulting fluorescence indicates the amount of accumulated amplicon. In the procedure below, the synthesis of an 84-nucleotide-long amplicon is monitored with the same molecular beacon whose synthesis and characterization was described in previous sections.

5.1. Procedure

1. Set up six 50 µl reactions so that each contains a different number of targets, 0.34 µM molecular beacon, 1 µM of each primer, 2.5 units of Amplitaq Gold DNA polymerase (Perkin Elmer), 0.25 mM of each deoxyribonucleotide, 3.5 mM MgCl2, 50 mM KCl, and 10 mM Tris-HCl, pH 8.0.
2. Program the thermal cycler to incubate the tubes at 95 °C for 10 min to activate the Amplitaq Gold DNA polymerase, followed by 40 cycles of 30 sec at 95 °C, 60 sec at 50 °C and 30 sec at 72 °C. Monitor fluorescence during the 50 °C annealing steps.

5.2. Results

Figure 6 shows the level of fluorescence as a function of the number of temperature cycles completed. The level of fluorescence is proportional to the amount of amplicons present in each cycle. The reaction that did not contain any template, did not show any rise in fluorescence. The number of temperature cycles required before the fluorescence signal becomes detectable over the background is inversely proportional to the logarithm of the initial number of template molecules. This relationship is true over a wide range of template concentrations.

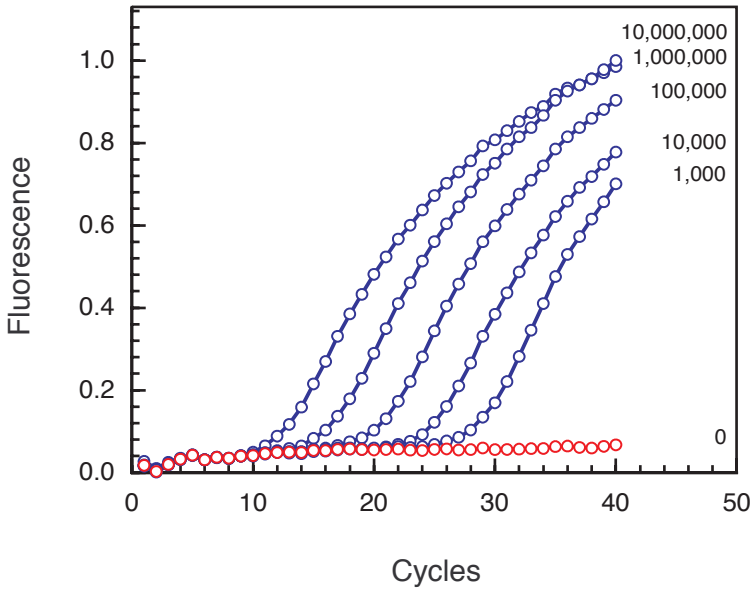

Figure 6. Real-time measurements of amplicon concentrations during polymerase chain reactions containing molecular beacons in sealed tubes. Six polymerase chain reactions, each initiated with a different number of template molecules (as indicated in the figure), were performed in a fluorometric thermal cycler. Fluorescence was measured during the annealing stage of each temperature cycle. The amount of fluorescence in each cycle is proportional to the amplicon concentration.

6. Troubleshooting

Low yield in coupling reactions.

- Check the pH of the buffers used in the coupling reactions and use fresh dyes. The reactive dyes should be stored at -20 °C in the presence of a desiccant.

Low signal-to-background ratio.

- The most likely reason is contamination by either free fluorophores or oligonucleotides that contain the fluorophore but not the quencher. The fluorophores can be removed by passage through a Sephadex column. In order to ensure that every molecule contains a quencher, repeat the purification of oligonucleotides that are protected by a trityl moiety and labeled with DABCYL prior to coupling with the fluorophore.
- The assay medium may contain insufficient salt. There should be at least 1 mM MgCl2 in the solution in order to ensure that stem hybrids form.

- The molecular beacon may fold into an alternate conformation that results in a sub-population that is not quenched well. Change the stem sequence (and probe sequence, if necessary) to eliminate that possibility. Incomplete restoration of fluorescence at low temperatures.
- If the stem of a molecular beacon is too strong, at low temperatures it may remain closed while the probe is bound to the target. This may happen inadvertently if the probe sequence can participate in the formation of a hairpin that results in a stem longer and stronger than originally designed. Change the sequence at the edges of the probe and the stem sequence to avoid this problem.

7. References

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