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COMMUNICATIONS

Published on Web 02/26/2002

Method for Detection of Single-Base Mismatches Using Bimolecular Beacons

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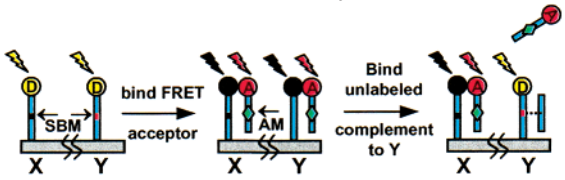
Received October 16, 2001

This paper describes a method for detection of single-base mismatches with DNA microarrays in a format that does not require labeling of the sample ("target") DNA. The method is based on disrupting fluorescence resonance energy transfer (FRET) between a fluorophore attached to an immobilized DNA strand ("probe") and a quencher-containing sequence that is complementary except for an artificial mismatch (e.g. 5-nitroindole, 3-nitropyrole, or abasic site) at the site of interrogation (Scheme 1). The analysis of a mismatch is based on differences in the amount of disruption in FRET upon hybridization of a perfectly matched DNA target and those containing mismatches. Single-base mismatches are the most common form of genetic polymorphisms. These genetic polymorphisms, commonly referred to as single nucleotide polymorphisms (SNPs), are often diagnostic of particular genetic predispositions toward disease and drug-response.[1,2] Typically, the detection of SNPs is carried out by sequencing;[3,4] however, for applications related to SNP "typing" in which the identity of the SNP-containing sequence is known, the convenience of conventional hybridization assays on microarrays offers an attractive alternative.[5] In a typical microarray-based SNP assay, genomic DNA is isolated and the regions containing the SNPs of interest are amplified and labeled with fluorescent dyes or haptens by using the polymerase chain reaction. These labeled targets are then hybridized to arrays of complementary oligonucleotide probes.[6,7] Recently, molecular beacons have been used to detect the presence of unlabeled target sequences in homogeneous solution with high discrimination; they have also been shown to be remarkably effective at detecting single base mismatches.[8,9] For use in large-scale parallel analysis using microarrays, molecular beacons must be attached to a surface. Maintaining the proper hairpin conformation for fluorescence quenching (or energy transfer) and its disruption by hybridization is nontrivial in a surface-immobilized molecular beacon.[10−12] In a molecular beacon, the FRET donor and acceptor are part of the same DNA strand; therefore, although the hybridization of the target to the loop portion is bimolecular, the disruption of the hairpin structure in the molecular beacon is unimolecular. Binding events at surfaces are limited by mass transport; consequently, for a surface-immobilized molecular beacon, the binding of the target DNA is especially inefficient relative to the disruption of FRET. In the approach presented here, both the displacement of the FRET acceptor and the hybridization of the target are bimolecular;[13,14] we therefore use the term "bimolecular beacon" to describe this approach.

To test single base mismatch discrimination using this method, we employed an oligonucleotide model system consisting of two 15-mer probe sequences (X, Y) labeled at the 5′ end with Cy3 and containing different nucleotides at position 8 (from the 5′ end); the complementary 15-mer sequence was labeled with the FRET acceptor carboxy-x-rhodamine (Rox) at the 3′ end and contained an abasic site at position 8 (from the 3′ end). Although Cy3 and Rox have been used here as the FRET donor and acceptor, respectively, any other FRET donor−acceptor pairs could also be utilized.[15] To enable attachment to the surface, the probe molecules were labeled at their 3′ end with an amine.[16] Figure 1A shows the initial fluorescence images obtained from the array by monitoring the emission of Cy3 (570 nm) and Rox (614 nm). As expected, similar (within 3%) amounts of fluorescence are observed in the Cy3 channel for both probe sequences, and little or no signal is observed in the Rox channel. Upon hybridization with the Rox-labeled sequence, there is an ∼59% decrease in fluorescence in the Cy3 channel for both probe sequences and a significant increase in fluorescence in the Rox channel (Figure 1B). This decrease in Cy3 fluorescence is due to FRET between Cy3 and Rox. In a last step, this surface−containing donor−acceptor duplexes−was exposed to a solution containing the *unlabeled*, perfectly complementary sequence to probe X. Single-base mismatch discrimination can clearly be seen in Figure 1C, which shows the fluorescence images obtained after this step. For analysis, we define the total discrimination factor between two oligonucleotides differing by one base-pair as the product of the discrimination factors in the Cy3 (donor) and Rox (acceptor) channels. For the Cy3 channel, the ratio of fluorescence intensity between match (probe X) and mismatch

Scheme 1. Biomolecular Beacon Assay for SBM Discrimination[a]



[a]SBM = single base mismatch; D = FRET donor; A = FRET acceptor; AM = artificial mismatch

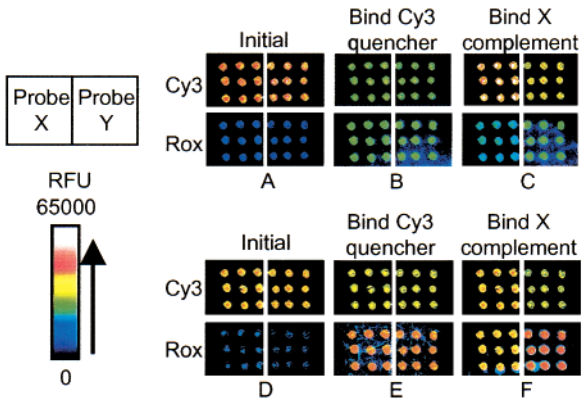


Figure 1. Fluorescence images showing discrimination of an A/C mismatch using a bimolecular beacon assay. (A) Initial images of Cy3-labeled probes. (B) Images after binding of a FRET acceptor target labeled with Rox and containing an abasic site. (C) Images after binding of an *unlabeled* target perfectly complementary to sequence X and possessing an A/C mismatch with sequence Y. Images D−F are the corresponding images for an assay using an acceptor target containing 5-nitroindole. All images were acquired with a ScanArray 5000 scanner (Packard Biosciences).[25]

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10.1021/ja012374d CCC: $22.00

Table 1. Melting Temperature (T_m) Data for DNA Oligonucleotides Containing Either Natural or Artificial Mismatches

duplex (X/Q)[a]	T_m (°C)[b]	duplex (X/Q)[a]	T_m (°C)[b]
G/C	63.3	A/C	47.9
G/T	51.1	G/abasic	42.8
G/5-nitroindole	50.3		

[a] Duplex design: 5'- GGG CTA TXA CTA TAG - 3'
3'- CCC GAT AQT GAT ATC - 5'

[b] Measurements were made in a pH 7 buffer (10 mM phosphate, 1 mM EDTA, and 1 M NaCl.).

Table 2. Results of Solution Displacement Reactions[21]

	X[a] = abasic	X[a] = nitroindole
initial intensity[b]	1.0	1.0
add single-base mismatched (A/C) target[c]	2.0	1.4
add perfectly matched (A/T) target[d]	2.4	2.2

[a] Duplex design: 5' - Cy3-GGG CTA TAA CTA TAG - 3'
3' - Dab-CCC GAT A**X**T GAT ATC - 5'

[b] Data are normalized to the initial fluorescence intensity. [c] 3′-CCC GAT A**C**T GAT ATC-5′. [d] 3′-CCC GAT A**T**T GAT ATC-5′.

(probe Y) is 1.48; for the Rox channel, the ratio between probe Y and probe X is 1.95. Therefore, the total discrimination factor is 2.89 for single-base (A/C) mismatch discrimination. This level of discrimination is equal to, if not better than, the discrimination observed by Tan et al. using immobilized molecular beacons for the discrimination of a C/T mismatch.[17]

Increasing the level of single-base mismatch discrimination using bimolecular beacons requires consideration of the relative stabilities of perfectly matched duplexes, single-base mismatches, and artificial mismatches.[18] For the most effective discrimination, the stabilities of the different DNA duplexes should be (in decreasing order of stability) perfect match > artificial mismatch due to acceptor sequence > natural single-base mismatch. Table 1 shows the melting temperatures (T_m) of 15-mer duplexes containing artificial or single-base mismatches. The duplex containing the A/C mismatch is more stable than the corresponding duplex with an artificial mismatch comprising an abasic site. Therefore, although effective, the use of an abasic site is sub-optimal for single-base mismatch discrimination. The duplex containing nitroindole as an artificial mismatch is, however, more stable than the duplex with the abasic site.[19,20] Figure 1D−F shows fluorescence images from the corresponding microarray assay using nitroindole as the artificial mismatch. Clear discrimination between perfectly matched and single-base mismatched sequences can be seen in Figure 1F. From an analysis of the images we estimate a total discrimination factor of 8.5; this increased level of discrimination is consistent with the solution T_m and displacement reactions[21] presented in Tables 1 and 2.

The stability of a DNA duplex containing a mismatched base pair depends on the identity of the basepair as well as the identity of its nearest neighbors.[22,23] To a first approximation, mismatches containing G residues are the most stable of all mismatches and destabilize the duplex by ∼2.9 kcal/mol compared to ∼3.7 kcal/mol for C-containing mismatches.[24] This difference in stability is shown in Table 1 by the fact that the G/T mismatched duplex has a T_m that is 3.1 °C higher than the A/C mismatched duplex. Therefore, the discrimination of G/T mismatches represents a particularly significant challenge in SNP analysis. Figure 2A shows the results of a bimolecular beacon assay performed using a quencher sequence containing 5-nitroindole and an unlabeled target sequence perfectly complementary to probe Y and possessing a G/T mismatch with probe X. An analysis of Figure 2A reveals good discrimination in the Cy3 and Rox channels; the total discrimination observed for the G/T mismatch is 3.8. When a similar experiment was performed utilizing a quencher sequence containing an abasic site, no discrimination was observed for the G/T mismatch (Figure 2B).

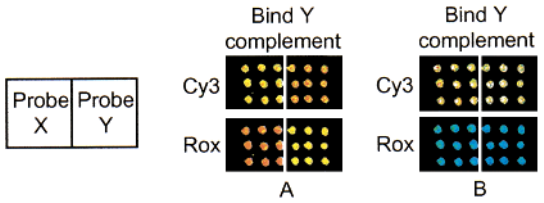

Figure 2. Fluorescence images after binding of an unlabeled target (perfectly complementary to probe Y) showing discrimination of a G/T mismatch based on a Rox labeled sequence containing (A) 5-nitroindole and (B) an abasic site.

We have described the use of FRET for detecting the presence or absence of a mismatch; the method combines aspects of conventional molecular beacons and artificial mismatches to provide an assay that is amenable to the highly parallel detection of SNPs. Although the assay utilizes the high sensitivity offered by fluorescent labels, it is convenient from the perspective of the end-user as no labeling of the target sample is required. The method is flexible in that the exact sequence of steps described is not restrictive—for example, it may be possible to immobilize the double stranded DNA duplex comprising the FRET donor and acceptor sequences or carry out the simultaneous competitive hybridization[13,14] of the target and FRET acceptor sequences. It is also anticipated that optimization of reaction conditions (e.g. hybridization temperature, salt and formamide concentration, target circulation) will lead to even further improvements in mismatch discrimination.

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JA012374D