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Quencher as Leaving Group: Efficient Detection of DNA-Joining Reactions

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Recent experiments have described a number of strategies for detection of nucleic acids that involve a change in fluorescence intensity or emission wavelength.[1−3] Fluorescence-changing methods have the distinct advantage that unbound probe molecules can easily be distinguished from those bound to the desired target. Such approaches can be used either in solution or on solid supports, whereas static methods often cannot be used in solution, and typically require careful washing methods on solid supports. Approaches that rely on simple intensity variation by changes in quenching[3a,b] have the further advantage of freeing more spectral ranges so that multiple simultaneous probing can be achieved.

To date, the number of different quenching-based approaches to nucleic acid sensing is limited. Perhaps the most well-developed approach is that of "molecular beacons",[3a] which consist of hairpin-forming DNAs labeled in the stem with fluorophore and quencher. Binding to a complementary sequence results in opening of the hairpin and moving of the quencher away from the fluorophore. Beacons can be used in solution or in solid-supported approaches; however, their fluorescence change clearly depends on solution conditions (temperature, ionic strength) and so one must monitor conditions carefully. Moreover, methods that rely on hybridization alone are usually not as sequence selective as some recently developed DNA-sensing methods such as enzymatic approaches[4] or some nonenzymatic autoligation methods.[5,6] A few other intensity-changing strategies have also been described.[7]

Among the most sequence-selective DNA/RNA sensing strategies is the phosphorothioate/iodide DNA autoligation reaction.[6,8] This reaction is dependent on the presence of an exact complement to the two probes, and is more selective than T4 DNA ligase in many cases.[6] Here we show how, by replacement of iodide with a quencher acting as a leaving group, this approach can be adapted for fluorescence unquenching, resulting in a strong signal change when two DNA strands are joined.

The strategy of the quenched autoligation (QUAL) probes is simple. A dabsyl group activates the 5′ hydroxyl as a leaving group when a phosphorothioate nucleophile (from another DNA strand) attacks at the ultimate 5′ carbon. This reaction is expected to be promoted by the presence of a complementary DNA strand, which brings the two reactive groups into close proximity. To test this approach we selected thymidine to modify with the quencher/leaving group, which is conveniently available commercially as the sulfonyl chloride derivative. To ensure relatively efficient quenching we placed a standard fluorophore (a commercial fluorescein C-5-alkenyl conjugate of dU) nearby, at a distance of three nucleotides from the 5′ terminus in the quenched electrophilic probes. These 13mer probes were prepared using standard phenoxyacetyl base protection chemistry to avoid partial loss of the dabsyl group during deprotection. They were purified by HPLC and characterized by MALDI-TOF mass spectrometry. These were reacted with 7mer probes carrying a phosphorothioate group at the 3′ terminus.[9] To test for sequence selectivity in a biologically relevant context, we chose probes complementary to adjacent sites in the H-*ras* gene, such that the known C → A transversion[10] in codon 12 falls at the center of the 7mer binding site. The 7mer MUT probes were fully complementary to the C → A mutant (MUT) sequence. Target DNAs were 50nt in length and corresponded to the sequence of the H-*ras* gene sense strand.

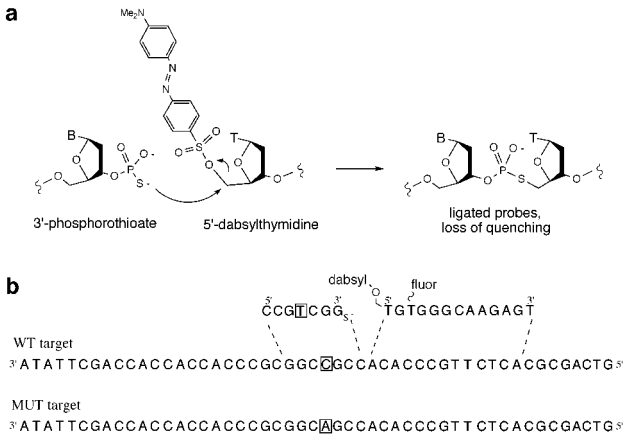

Figure 1. (a) Structure of the dabsyl-thymidine nucleotide and mechanism of dabsyl displacement. (b) Schematic showing how autoligation of two DNA strands on a complementary DNA template (the analyte) leads to displacement of "dabsylate" and loss of quenching. Sequences are as shown, and are taken from the H-*ras* protooncogene.

Reactions were initially tested in solution, following the ligation by gel electrophoresis. Results showed clearly that the autoligation reaction proceeded smoothly and with high conversion. Figure 2a shows a stained PAGE gel image, revealing ligated product when the target sequence is fully complementary, and no discernible product when it is different by one nucleotide. Conversion after 7 h is estimated at 80% in the fully complementary case. Significantly, a fluorescence image of the same gel shows (Figure 2b) that while the starting materials are only very weakly fluorescent (due to incomplete quenching by dabsyl), the product band shows robust emission, consistent with a substantial loss of quenching. A faint ligated band is also visible just beneath the major product; this we attribute to ligation by an (N − 1)mer contaminant from DNA synthesis.

We then monitored the same reaction in real time by following fluorescence emission at 520 nm (excitation 495 nm) using the 7mer MUT probe (Figure 2c). Monitoring the change in fluorescein emission showed a large increase in intensity (ca. 100-fold, implying a 99.0% quenching efficiency at 520 nm for the starting probe). Varying the temperature from 15 to 37 °C revealed that the rate reached a maximum near 25 °C, which corresponds to the approximate melting temperature of the probes bound to the target (data not shown). The sequence selectivity of the reaction was

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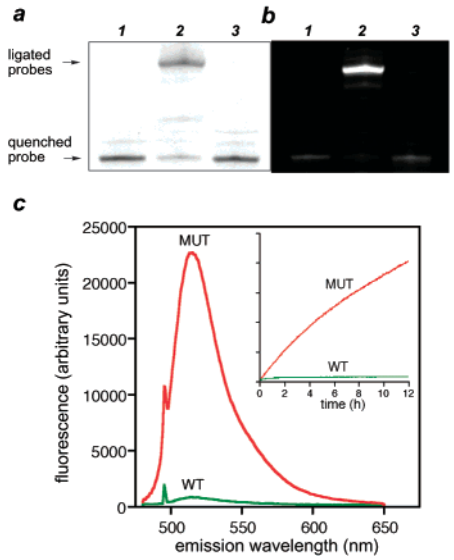

Figure 2. Analysis of dabsyl-mediated autoligations by gel electrophoresis and fluorescence. (a) Stained gel showing starting materials and ligated product: lane 1, no target; lanes 2 and 3, with MUT and WT target, respectively. Conditions: 1.3 μM dabsyl-labeled 13mer probe, 3.9 μM 7mer MUT probe and target DNA concentrations in a pH 7.0 Tris-borate buffer containing 10 mM $MgCl_2$ at 37 °C; (b) fluorescence image of the same gel, showing weak fluorescence of quenched 13mer probe and strong fluorescence of ligated product band; (c) emission spectra of reactions with MUT and WT DNA after 24 h (inset: timecourse monitored at 520 nm). Conditions: 100 nM dabsyl-labeled 13mer probe, 300 nM 7mer MUT probe and target DNA concentrations in a pH 7.0 Tris-borate buffer containing 10 mM $MgCl_2$ at 25 °C.

approximately 35-fold based on relative peak areas with WT and MUT target DNAs, resulting in a T−A pair or a T−C mismatch at the mutation site in codon 12. The overall rate of the reaction was similar to that of previously described iodide-mediated reactions.[6a,8] The 5′-dabsyl group, like 5′-iodide, has the advantage of limited reactivity (relative to tosylate[9b,11]) under DNA deprotection conditions.

Since many recent genetic analysis methods rely on probes affixed to beads, slides, and other surfaces, we then explored whether this reaction could take place on a solid support. This was tested by synthesizing a 7mer MUT probe on 90 μm diameter polystyrene beads (1000 Å pore size) using commercially available reverse (5′ → 3′) phosphoramidites, and placing a 3′ phosphorothioate moiety on the final 3′ hydroxyl group. A hexaethylene glycol linker was used to alleviate potential crowding problems near the polymer surface. Such beads then have the potential to autoligate a 13mer electrophile probe to themselves, in the presence of the correct target DNA. This is expected to result in the beads becoming fluorescent, as the dabsylate group is lost into solution and the nearby fluorescein label loses quenching.

The solid-phase autoligations were monitored by imaging under a fluorescence microscope. Results showed that the reaction proceeds on the polystyrene beads much as it does in solution (Figure 3). At the start of the reaction the beads are dark and the solution shows faint green fluorescence due to a small amount of emission from the quenched 13mer probe. As the reaction proceeds the beads become progressively brighter, reaching half-maximum after ca. 20 h.

These early experiments allow us to conclude that the use of a "dabsylate" leaving group on probes enables facile detection of DNA strand-joining reactions. The reaction retains the high sequence selectivity of the autoligation approach, and proceeds well in solution or on a solid support. Like molecular beacons, this quencher/leaving group approach results in an increase in intensity

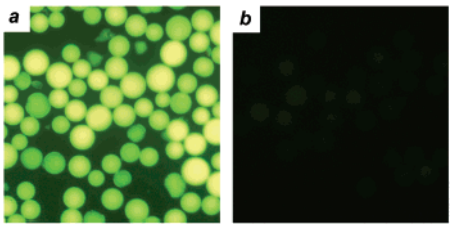

Figure 3. Dabsyl-mediated autoligation of DNAs on solid support. Photographs show beads after 24 h of incubation with (a) MUT or (b) WT target using a dabsyl/fluorescein 13mer probe at 25 °C. No washing was done. Conditions are given in the Supporting Information.

on detection of a complementary sequence, and like that method, one can in principle adapt it to multicolor detection for simultaneous (multiplex) sensing of more than one sequence.[12] It should be noted, however, that this autoligation reaction is slower than simple hybridization-based approaches, and so is not suitable for a rapid-timescale application such as real-time PCR reporting. However, the present strategy offers the significant advantage over hybridization that the signal, once generated, is permanent and does not depend on temperature or buffer conditions. Moreover, DNA autoligation reactions are more sensitive to single-nucleotide differences than are most, if not all, hybridization-based approaches.

Future work will be directed at applying this unquenching autoligation strategy to simultaneous multiple-sequence sensing, and to cellular imaging of DNAs and RNAs.

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Supporting Information Available: Details of synthesis and methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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