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A new homogeneous assay system for specific nucleic acid sequences: poly-dA and poly-A detection

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Homogeneous assay systems for specific nucleic acid sequences are performed in a single reaction phase requiring no washing steps to eliminate unbound probe (1). Current homogeneous fluorescence-based assay geometries generally involve the interaction of two or more separately labelled molecules for DNA/RNA target detection (2,3,4,5). In the presence of target, the labels are close to each other and can affect the fluorescence intensity via collisional or resonance energy transfer mechanisms.

This report describes a single molecule, homogeneous assay system that has the advantages of simplicity and rapidity. The UniFluor probe is a two-hairpin oligonucleotide that when in contact with specific sequences, changes its conformation with resultant increase in fluorescence. The probe's fluorescence increases because of an induced separation of conjugated fluorochromes with subsequent release from a geometry which quenches fluorescence (see Fig. 1).

The major components of the probe are described as follows: The 'target sequence' is any single-stranded sequence of either RNA or DNA nucleotides that is to be detected. The 'specificity sequence' is the Watson and Crick complement of the 'target sequence'. 'Loop 1' and 'Loop 2' are sequences of generally seven nucleotides that are not complementary to either target or probe sequence(s). The 'competitive arms' are, for the most part, complementary to the opposing 'specificity sequence'. The intramolecular base pairing causes the probe to form a two-hairpin structure that brings the Q1, Q2, and Q3 fluorescent/quenching groups into close proximity. Base pair mismatches are introduced into the 'competitive arms' such that the 'specificity sequence' will favor binding to the 'target sequence'. Q1, Q2, and Q3 are locations which may be bound to molecules that have the property of changing their aggregate fluorescence properties when in close proximity to each other; therefore, when the UniFluor probe interacts with a 'target sequence', the 'competitive arms' are displaced, increasing the distance between Q groups, resulting in a detectable change in fluorescence intensity. P1 is the location of another reporter group that is not significantly affected by the interaction between the Q1, Q2, and Q3 group locations and can be placed on either 'loop 1' or 'loop 2' or both, or on some other distant site for tracking the overall amount of probe present in a reaction. The UniFluor probe presented in this report arbitrarily uses fluorescein for all three of the Q group locations and does not employ the P tracking group. The probable mechanism of fluorescein–fluorescein quenching is the formation of a relatively nonfluorescent dimer or trimer (6).

The probe is created in a conventional DNA/RNA synthesizer, conjugated with fluorescein, and normally reconstituted in a neutral pH buffer (see Fig. 2 for specific structure). The reactivity of the molecule is increased by 20–30% by purification on a polyacrylamide gel. The UniFluor probe studied in this report is directed against poly-A or poly-dA targets (see Fig. 3). UniFluor poly-dA T_m is 43.3 C as determined by standard thermodynamic analysis of melting curves (7). The specific target tested is a poly-dA oligonucleotide and the irrelevant targets were Random II and H4 specific oligonucleotides (see Fig. 3).

The specificity and kinetic characteristics of the UniFluor poly-dA probe are shown in Fig 4. The probe shows little or no detectable percent increase in fluorescence to irrelevant targets

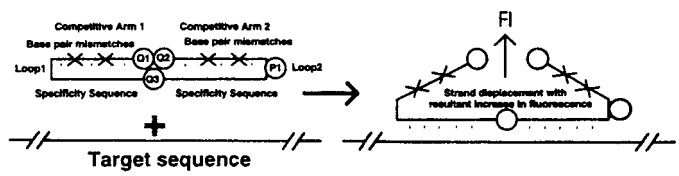

Figure 1. General structure of the UniFluor probe.

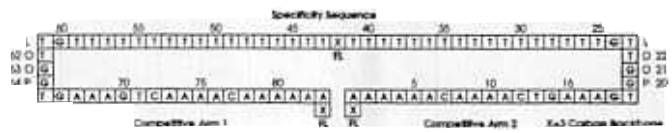

Figure 2. UniFluor Poly-dA structure.

Poly-dA (50-nt)

5'-d(AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA)-3'

Random II (51-nt)

5'-d(ATTGGCCAGGTTTGGTTGCAGTATGGCTACCGGAGGCCATCGGTATGACGT)-3'

H4 (50-nt)

5'-d(CCATGGATGTGGTCTACGCGCTCAAGCGCCAGGGCCGCACCCTCTACGGT)-3'

Figure 3. Target sequences.

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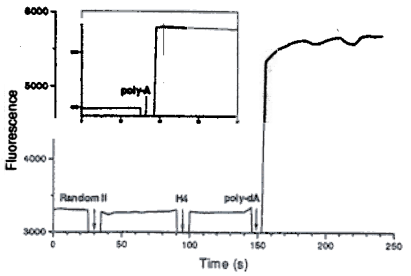

Figure 4. Specificity and kinetics of the UniFluor probe. UniFluor poly-dA probe was diluted to a final concentration of 50 nM in 0.5 ml HHM. The probe solution was added to a polystyrene disposable cuvette (10 mm×4 mm). When the UniFluor probe was challenged with its specific target, poly-dA, the fluorescence increased approximately two-fold over a period of 30 seconds. A similar reaction was observed with poly-A target (see Fig. 4 inset). There were no apparent responses to the addition of Random II and H4 irrelevant target controls.

at room temperature (n = 10; mean = 0.4%, std dev = 0.5%). When specific poly-dA target is added to the probe, fluorescence increases rapidly, stabilizing in approximately 30 seconds (n = 14, mean = 79.3%, std dev = 6.2%). A similar response is seen for poly-A target. The sensitivity of the probe at room temperature is between 10 and 40 fmoles of target in an approximate 200 mm^3 sensing volume. The probe is currently limited to detection systems rather than target quantification because of the relatively small signal intensity dynamic range.

Although this report focuses on a simple poly-A target sequence, the probe is capable of detecting specific sequences and thus may have application for high-speed pathogen detection systems. UniFluor probe's fast room temperature kinetics and simple homogeneous assay format make it a potential candidate for detecting specific intracellular pools of mRNA or mRNA extracts.

Synthesis: Oligonucleotide probes were synthesized on an Applied Biosystems, Inc. (Foster City, CA) Model 392 DNA/RNA synthesizer using cyanoethylphosphoramidites (Fisher Scientific, Pittsburgh, PA). Aminoalkyl-derivatized oligonucleotides were prepared using 3'-amine-on controlled-pore glass columns for incorporation of the 3' primary amine (CPG Inc., Fairfield, NJ), and Uni-Link Aminomodifier phosphoramidites (Clontech, Inc., Palo Alto, CA) were incorporated at base position 42 and the 5' terminus. Synthesized oligonucleotides were deblocked in 20–22% NH_4OH (Fisher Scientific), evaporated to dryness on a Savant Speed-Vac SC100 (Savant Instruments, Inc), desalted on a NAP-5 molecular sieve column (Pharmacia, Piscataway, NJ) in 0.1 M Bicine buffer containing 0.5 M NaCl, pH 8.2 (Sigma Chemical Co., St Louis, MO) and quantified by absorbance at 260 nm.

Conjugation: The conjugation method was adapted from a previously described method (8). Succinimidyl fluorescein (Molecular Probes, Eugene, OR) was prepared at 10 mg ml^{-1} in dimethylformamide. A 30-fold molar excess of succinimidyl fluorescein was added to 50–70 nmoles of the NAP-5 eluate and incubated at 55–C for 1 hour followed by addition of a 5-fold molar excess of glycine (relative to succinimidyl fluorescein) and incubated for 15 min at room temperature. A NAP-25 (Pharmacia) molecular sieve column was equilibrated in 0.01 M phosphate-buffered saline, pH 7.2 (PBS) containing 1% bovine serum albumin (Sigma), followed by washing of the column with PBS without albumin. The labelling reaction mixture was applied to the column and the labelled oligonucleotide was collected in the void volume. Label incorporation was estimated from the A_{260}/A_{493} ratio using an extinction coefficient for the fluorescein moiety of 66 $L \cdot mmole^{-1} \cdot cm^{-1}$. The extinction coefficient of the oligonucleotide was estimated from the base content using absorptivity values of 15.4 (A), 7.3 (C), 11.7 (G), and 8.8 (T) $L \cdot mmole^{-1} \cdot cm^{-1}$.

Probe buffers: Hank's Balanced Salt Solution without Ca^{++}, Mg^{++}, or Phenol red (Gibco Laboratories, Grand Island, NY) was buffered with Hepes Buffer Solution (Gibco Laboratories) at a final concentration of 0.02 M, pH 7.2 (HH Buffer). Optimal probe reactivity was observed with 0.67×HH Buffer + 4 mM $MgCl_2$ (HHM buffer).

Purification: Further purification of 10 nmoles of the labelled probe was accomplished by preparative-scale native 10% polyacrylamide gel electrophoresis (100 V, 25 mA, 2 hours) in 1×TBE buffer (9). Four fluorescent bands were seen under UV illumination, one of which was identified, as the uncoupled free glycyl–succinimidyl fluorescein. The remaining three bands were independently excised from the gel and eluted into 1 ml DEPC-treated water (10) overnight at room temperature; gel slices were eluted overnight a second time into 1 ml PBS. Oligonucleotide concentration and fluorescein coupling efficiency were determined spectrophotometrically as described. Fluorescence characteristics of each eluate were evaluated on a SPEX Fluorometer (Model 212, Edison, NJ) with 492 +/– 2 nm excitation and 530 +/– 2 nm emission. Eluates were diluted to a concentration of approximately 100 nM into 0.67× HH Buffer and pipetted into 10 mm×10 mm×45 mm polystyrene disposable cuvettes (VWR Scientific, Boston, MA). fluorescence intensity was measured at ambient temperature, after which the cuvette was warmed to 45°C for 10 min in a water bath and the fluorescence intensity at 45°C was measured. Eluates which corresponded in both electrophoretic mobility and fluorescence signal increased were pooled; the pool demonstrating the greatest increase in fluorescence signal intensity when heated was further characterized.

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