

Facile SNP detection using bifunctional, cross-linking oligonucleotide probes

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ABSTRACT

A facile, sensitive method for detecting specific sequences of oligonucleotides was developed. Detection of DNA sequences with single nucleotide discrimination is achieved by combining the selectivity of hybridization with an efficient cross-linking reaction. Readily synthesized bifunctional oligonucleotide probes containing a modified pyrimidine that is capable of forming interstrand cross-links under mild oxidative conditions internally, and biotin at their 5'-termini were used to discriminate between 16-nt long sites in plasmid DNA that differ by a single nucleotide. The target sequence was detected via fluorescence spectroscopy by utilizing conjugates of avidin and horseradish peroxidase in a microtiter plate assay. The method is able to detect as little as 250 fmol of target without using PCR and exhibits single nucleotide discrimination that approaches 200:1. In principle, this method is capable of probing any target sequence containing a 2'-deoxyadenosine.

INTRODUCTION

Detecting single nucleotide polymorphisms (SNPs) is an important activity in the current post-genomic era. SNP analysis is potentially useful for predicting the likelihood of genetic disease by using nucleic acid sequence as a biomarker, and for predicting response to therapeutic agents. Given the importance of this information, a number of methods are being developed to detect SNPs (1–6). However, there are still opportunities for the development of facile, inexpensive, sensitive methods for SNP detection. Methods that report on and amplify a binding event are attractive because they can eliminate the need to PCR amplify the target (7–10). PCR free detection is also necessary for any method that is carried out in cells (11,12). We have developed a method for detecting DNA

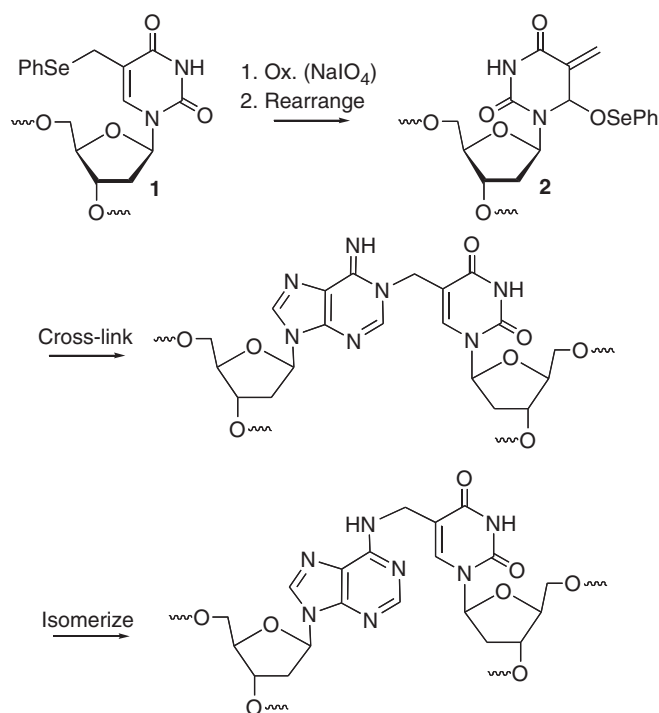
sequences that is capable of single nucleotide discrimination. The method utilizes efficient cross-linking of a modified thymidine (**1**) in a biotinylated oligonucleotide probe to an opposing dA in the target strand (Scheme 1) (13,14). The 'tagged' target is detected using a common fluorescence reporter assay that takes advantage of tight binding between biotin and avidin that is conjugated to horseradish peroxidase (HRP; Scheme 2)(15).

MATERIALS AND METHODS

General methods

Unless otherwise specified, chemicals were purchased from Aldrich or Fisher Scientific and enzymes were obtained from New England Biolabs. Oligonucleotides were synthesized via standard automated DNA synthesis techniques using an Applied Biosystems model 394 instrument. The Pac-dA and ¹Pr-Pac-dG phosphoramidites were employed for the synthesis of oligonucleotides containing **1**. Pivaloyl anhydride/2,6-lutidine/THF (8:1:1) was used as capping reagent and 1M t-butyl-hydroperoxide in toluene was used as oxidizing reagent. The oxidation time is 40s and the capping time is 25s. Deprotection of the nucleobases and phosphate moieties as well as cleavage of the linker were carried out under mild deprotection conditions (28% aq. NH₃, room temperature, 2 h). Oligonucleotides were purified by 20% denaturing polyacrylamide gel electrophoresis and characterized by ESI-MS. Oligonucleotides **3** and **4** were subjected to additional purification by reversed-phase HPLC on a RP-C18 column (Varian, Microsorb-MV 100-5 C18 250 × 4.6 mm). Monitoring was carried out at 260 nm. The peak of interest (retention time: 38–39 min for **3** and 42–43 min for **4**) was collected using the following gradient conditions: 0–5 min 0–2% B in A, 5–15 min 2–12% B in A, 15–50 min 12–25% B in A, 50–60 min 25–80% B in A, 60–65 min 80–100% B in A, 65–80 min 100% A, at a flow rate 1.0 ml/min [A: 0.05 M (Et₃NH)OAc (pH 7.0)/MeCN 95:5; B: 0.05 M (Et₃NH)OAc (pH 7.0)/MeCN 50:50]. Radiolabeling was carried out according to the standard protocols (16).

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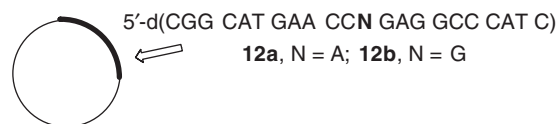
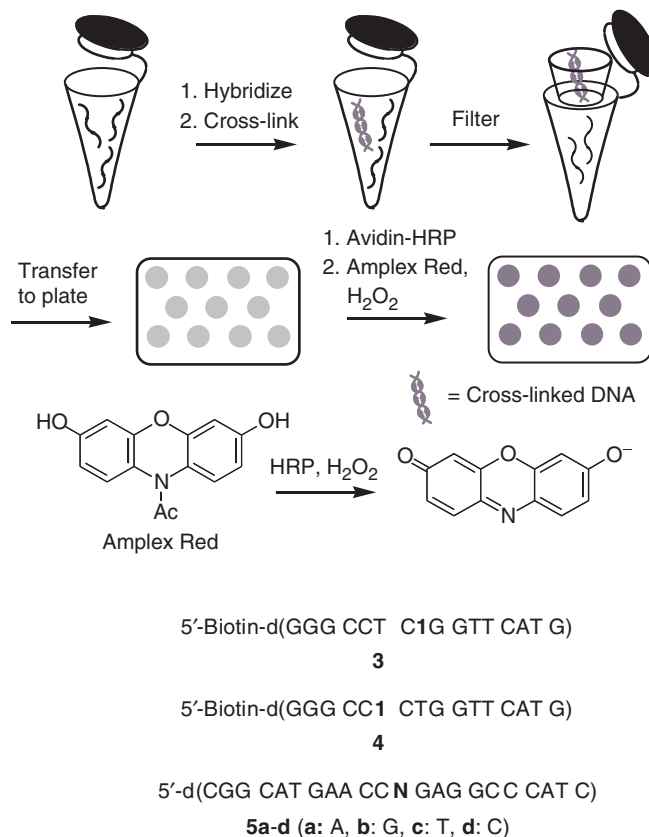


Scheme 1. Formation of interstrand cross-links via oxidation of a modified thymidine (1).

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was purchased from Amersham Pharmacia Biotech. Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Phosphor-imager equipped with ImageQuant Version 5.1 software. Fluorescence spectra were collected on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a microplate reader. Ninety-six-well flat-bottom assay plates (black) were purchased from Corning (Fisher, Cat. No. 07200509). Protamine sulfate and Tween-20 were purchased from Sigma (Cat. No. P 4020 and P 9416). SuperBlocking buffer in PBS and BlockBSA in PBS (10 \times) were purchased from Pierce (Cat. No. 37515 and 37525). ABC Elite ultra kit was purchased from Vector Laboratories (Cat. No. PK-6100). Amplex Red and Amplex Red Stop reagents were purchased from Invitrogen (Cat. No. A36006 and A33855). Cross-linking reactions were carried out in an Eppendorf Mastercycler Thermal Cycler in order to achieve maximal temperature control.

Interstrand cross-link reaction performed with short oligonucleotides 5a–d and the ICL growth kinetics

The appropriate ^{32}P -labeled oligonucleotide and its complementary strand (1.5 eq.) were hybridized in 100 mM NaCl. NaIO_4 (5 mM) reactions of DNA duplexes (30 nM) were carried out in 10 mM potassium phosphate (pH 7.2) and 100 mM NaCl. For determination of the rate constant for interstrand cross-link formation (k_{ICL}), samples were incubated at 37°C. Aliquots were taken at the prescribed times and immediately quenched with equal volumes of 95% formamide loading buffer, heated to 80°C



Scheme 2. Sequence-selective detection of DNA via fluorescence using cross-linked DNA.

for 2 min and stored at -80°C until subjecting to 20% denaturing PAGE analysis.

Construction of site-specifically modified M13 genome (plasmid 12a or 12b containing target 5a or 5b)

M13mp7 (7240 bp, circular single-stranded plasmid, 10 pmol, 10 μl) was digested with EcoRI to produce a linearized single-stranded plasmid (7200 nt) (17). The EcoRI digest was performed at 37°C for 4 h in the presence of EcoRI (1.0 μl , 20 U), EcoRI buffer (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl_2 and 0.025% Triton X-100, pH 7.5, 2.3 μl) and H_2O (9.2 μl). To the digestion mixture, two scaffolds (15 pmol for each) were added, which are annealed to the newly formed ends of a linearized M13mp7 by heating at 90°C for 5 min, then cooling to 25°C over 2 h. In this step, the scaffolds 5'-AAA ACG ACG GCC AGT GAA TTG ATG GGC CTC-3' and 5'-GGT TCA TGC CGC ACT GAA TCA TGG

TCA TAG C-3' anneal to 20 bases of the M13mp7 vector, with 10- or 11-base overhangs to house a modified 22mer 5'-CGG CAT GAA CCA GAG GCC CAT C-3' (**5a**) or 5'-CGGCAT GAA CCG GAGGCC CAT C-3' (**5b**), leaving a 1-base gap at the site of the variable nucleotide. Meanwhile, 15 pmol of **5a** or **5b** is 5'-phosphorylated in 30 μ l containing T4-PNK buffer (10 \times , 3.0 μ l), ATP (100 mM, 0.3 μ l), 5 mM DTT and T4 PNK (0.4 μ l, 12 U). After incubating the plasmid, scaffold and probe at 37°C for 1 h, the ligation was performed by adding T4 ligase (0.5 μ l, 200 U), T4 ligase buffer (0.5 μ l), DTT (1%, 2.25 μ l), ATP (100 mM, 0.25 μ l) and H₂O (2.5 μ l) and incubation at 16°C for 12 h. T4 DNA polymerase (0.4 μ l, 3.9 U), EcoRI buffer (1.0 μ l) and H₂O (8.6 μ l) were added and incubated at 37°C for 4 h to remove the excess scaffolds. The mixture volume was increased to 100 μ l by adding water, extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, 100 μ l), and the aqueous phase passed through a home-made Sephadex-G25 Fine (Sigma-Aldrich) spin column to remove traces of phenol and salt. The M13 construct (**12a** or **12b**) was obtained with a final concentration of 18 nM (150 μ l).

Electroporation of M13 constructs (**12a** or **12b**) into GW 5100 cells

GW 5100 cells were grown in Luria Broth (LB) (10 ml) overnight at 37°C and diluted (1.0 ml in 100 ml LB buffer). The diluted cells were incubated at 37°C until OD₆₀₀ = 0.5 and then harvested by centrifugation at 5500 r.p.m. for 10 min (4°C). The cells were resuspended in 50 ml of ice-cold deionized water and centrifuged at 5500 r.p.m. for 10 min. The bacterial pellet was resuspended in glycerol/water (10%, 1.0 ml) and kept on ice until further use. For each transformation, an aliquot (100 μ l) of the cell suspension was mixed with 1 pmol of M13 construct (**12a** or **12b**) and transferred to the bottom of an ice-cold BTX disposable cuvette (2.0 mm electrode gap). Electroporation of cells was carried out in a BTX ECM 399 Generator at 36 μ F and 2.5 kV. Immediately after electroporation, LB medium (10 ml) was added and the mixture was incubated at 37°C for 1 h. An aliquot (10 μ l) of regrown cells was plated and M13 constructs (**12a** or **12b**) with modified insert gave blue plaques in the presence of isopropyl- β -D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) after incubation overnight at 37°C. A blue plaque was isolated and mixed in LB medium (1 ml). An aliquot (50 μ l) of overnight grown GW5100 cells were added and diluted to 50 ml with LB buffer. The mixture was incubated at 37°C for 7 h and centrifuged at 9500 r.p.m. for 15 min (4°C). The supernatant containing M13 progeny phage (**12a** or **12b**) was stored at 4°C for the next step.

Preparative isolation of plasmids (**12a** or **12b**) in GW 5100 cells

An overnight culture of GW5100 cells (~0.2 μ l) was grown in 2 \times YT (10 ml) with orbital shaking (250 r.p.m.) at 37°C. An aliquot (1 ml) of this stock was added to 2 \times YT

(500 ml) and grown at 37°C for 2 h. The M13 progeny phage (**12a** or **12b**) (2 ml) (from last step) was then added and grown for 8 h at 37°C. The cells were cooled on ice for 10 min and then centrifuged at 9500 r.p.m., 4°C, for 15 min. The supernatant (500 ml) was transferred to a beaker containing 125 ml of 20% PEG-800 and NaCl (2.5 M) and stirred at 25°C for 5 min. The mixture was transferred to centrifuge tubes, stored at 4°C for 24 h and spun at 9500 r.p.m., 4°C, for 18 min. The supernatant was decanted and the pellet was resuspended in TE buffer (pH 8.0, 8.0 ml), which was extracted four times with phenol/chloroform/isoamyl alcohol (25:24:1, 3 ml). The aqueous layer was loaded onto a hydroxyapatite column (2 g + 30 ml H₂O), which was first washed with H₂O (10 ml) and then TE buffer pH 8.0 (5.0 ml). DNA was eluted using 10 ml of elution buffer (0.07 M K₂HPO₄, 0.07 M KH₂PO₄) and further purified using a Centricon cartridge (100 000 NMWL) following the manufacturer's protocol. Isolated plasmids containing the appropriate inserts (**12a** or **12b**) were verified by direct sequencing (see Supplementary Data).

Interstrand cross-linking using plasmid DNA target

Targets **12a** and **12b** (1.5 pmol) were hybridized with probe (**3** or **4**, 2 eq.) in 10 mM potassium phosphate (pH 7.2) and 100 mM NaCl by heating at 70°C for 5 min and then cooling to 4°C over 2 h. NaIO₄ (5 mM) reactions of DNA duplexes (30 nM) were carried out in 10 mM potassium phosphate (pH 7.2) and 100 mM NaCl at 25°C for 6 h (direct detection) or 45°C for 5 h (indirect detection).

Detection of mismatches via fluorescence spectroscopy

(i) The cross-linked samples were dissolved in 0.5 ml with PPF buffer (100 mM potassium phosphate pH 4.7 and 50% formamide). The sample was then heated at 80°C for 10 min and centrifuged in the Centricon cartridge (100 000 NMWL) for 10 min at 5000 r.c.f. The retentates were dissolved in 0.5 ml of the hot PPF buffer (preheated at 70°C) and centrifuged. This procedure was repeated. The retentates were dissolved in 0.5 ml of TE buffer (10 mM Tris, 1.0 mM EDTA, pH 7.5) and centrifuged (2 \times). The final retentate was dissolved in 200 μ l of TE buffer and quantified by UV-measurement. (ii) A 96-well black flat-bottom plate was incubated with 0.1% protamine (200 μ l) for 20 h at 4°C, followed by washing with water (3 \times 250 μ l). Plasmid DNA (300 fmol, 200 μ l) in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) was incubated in the pretreated assay plate in an incubator at 37°C for 2 h without shaking. After washing with TPBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄ and 0.1% Tween-20, pH 7.5, 10 \times 250 μ l), the plate was incubated with BSA/SuperBlock solution (1 ml/10 ml) (2 \times 250 μ l) for 15 min at room temperature with shaking. The plate was then washed with TPBS buffer (5 \times 250 μ l), followed by incubation with ABC ultra solution (50 times dilution, Vector lab) for 30 min at room temperature. After washing with TPBS (10 \times 250 μ l), the plate was incubated with Amplex Ultra Red substrate solution (100 μ l, 0.1 mM Amplex Ultra Red and 0.015%

H₂O₂ in 50 mM sodium phosphate buffer, pH 7.5) for 30 min at room temperature with shaking, followed by addition of 20 μ l Amplex Ultra Red Stop solution (Invitrogen). Sodium phosphate buffer (180 μ l, 50 mM, pH 7.5) was added into each well to achieve the final volume (300 μ l). The fluorescence of each well in the plate was then determined using a fluorescence plate reader [excitation: 568 nm, emission: 581 nm, PMT voltage 550 (when using indirect probe **4**), 520 (when using direct probe **3**), slit width 10 nm].

RESULTS AND DISCUSSION

Experimental design

Hybridization methods distinguish between fully matched and single nucleotide mismatched sequences by taking advantage of higher melting temperatures of duplexes containing the former. The utilization of hybridization methods are complicated by conflicting properties of duplex DNA. Differences in melting temperatures of fully matched versus single nucleotide mismatched duplexes are inversely proportional to duplex length. However, the overall stability of duplexes increases with increasing length. Duplex stability is important because one needs to remove the mismatched probes, typically by washing, without disrupting the fully matched complexes. Alternative backbones that form more stable complexes with DNA targets, such as peptide nucleic acids (PNAs) can help overcome these conflicting properties (7). We anticipated that the recently discovered interstrand cross-linking reaction (Scheme 1) induced upon mild oxidation of the modified thymidine (**1**) would enable us to utilize short oligonucleotides to selectively target nucleic acid sequences.

Detection using HRP conjugated to avidin is attractive because it is sensitive, easy to carry out and large numbers of scientists are familiar with it. As described below, the ability to covalently link the probe and target is crucial for employing this detection method (Scheme 2). Sandwich assays that utilize biotin binding to an avidin-horseradish peroxidase (avidin-HRP) conjugate detect as little as 25 fmol of DNA (15,18). The detection limit of the assay is dependent upon the ability to achieve low background fluorescence levels by effectively separating unbound avidin-HRP from biotinylated target. We anticipated that a biotinylated oligonucleotide probe covalently linked to target DNA (Scheme 1) would withstand rigorous conditions for removing excess probe and avidin-HRP (Scheme 2).

The cross-linking reaction is useful in the presence of a variety of flanking sequences

The cross-linking reaction between **1** and an opposing dA upon mild oxidation by NaIO₄ proceeds through the rearranged methide (**2**, Scheme 1). In order for this reaction to be useful as part of a probe to specifically detect a complementary sequence, it must proceed comparably regardless of flanking sequence. Cross-linking was examined in 4 of the 16 possible flanking sequences at 37°C (Table 1). The rate constants spanned <3-fold range.

Table 1. Interstrand cross-link (ICL) yields from **1** as a function of flanking sequence

5'-d(AGA TGG ANI NAG GTA C) 3'-d(TCT ACC TN'A N'TC CAT G)			
N:N'	k (s ⁻¹) ^a	$t_{1/2}$ (min)	Yield ICL (%)
T:A	$1.2 \pm 0.3 \times 10^{-3}$	9.4 ± 0.6	33.8 ± 1.4
A:T	$5.1 \pm 0.2 \times 10^{-4}$	22.6 ± 0.3	24.9 ± 0.5
C:G	$1.5 \pm 0.2 \times 10^{-3}$	7.7 ± 0.6	34.8 ± 0.5
G:C	$6.4 \pm 0.4 \times 10^{-4}$	18.0 ± 0.6	18.6 ± 1.2

^aAverage of three independent experiments each carried out in triplicate.

Furthermore, cross-linking proceeded more rapidly when **1** was flanked by pyrimidines than by larger purines. We propose that the greater size of the purines and π -stacking energy increases the barrier to interconversion between the *anti* and *syn* forms of the methide like electrophile (**2**, Scheme 1). Overall, the kinetic experiments indicate that the cross-linking reaction is not strongly biased by flanking sequence and will be generally useful for detecting oligonucleotide sequences.

Analysis of single nucleotide discrimination by gel electrophoresis

With this strategy in mind, bifunctional oligonucleotide probes (**3**, **4**) equipped with biotin at their 5'-termini and **1** at an internal site were synthesized using commercially available reagents and the previously reported requisite phosphoramidite for introducing **1** (13,14,19). The oligonucleotides were prepared using standard solid-phase oligonucleotide synthesis methods. The probes were chosen to be 16-nt long in order to target unique sequences in the human genome. The specific sequences were designed to target codon 248 in exon 7 of the p53 gene. The p53 gene is often mutated in human cancer, and there is a high frequency of G \rightarrow A transitions at codon 248 (the following website catalogues p53 mutations: <http://p53.free.fr>). The 'direct' probe (**3**) was designed to utilize **1** to cross-link ('tag') and to discriminate between matched (dA) and mismatched sequences. Denaturing polyacrylamide gel electrophoresis (PAGE) was used to test the ability of the biotinylated cross-linking probes to discriminate against single nucleotide mismatches. The chemoselectivity of the cross-linking reaction was exploited when using the direct probe (**3**) to distinguish between a target having an opposing dA (**5a**) or dG (**5b**). The methide intermediate produced upon oxidation of **1** reacts with the N1-position of dA (Scheme 1), which is more nucleophilic than the corresponding nitrogen in dG (14). Indeed, cross-linked product was observed in $47.2 \pm 0.8\%$ when **3** was hybridized with the matched (**5a**) sequence (**6**) and treated with NaIO₄ at 25°C. No product was detected when dG was opposite **1**, despite the fact that the T_m (**7**, Table 2) was $\sim 20^\circ\text{C}$ higher than the reaction temperature. Carrying out the incubation at 40°C decreased the yield of cross-link in the matched duplex (**6**) to 34.7%, but still no product was observed when **1** was opposite dG (**7**). The lower cross-link yield may be due to decreased residence time of the duplex, but we also cannot

Table 2. Duplex melting temperatures

5'-Biotin-d(GGG CCT C1G GTT CAT G) 3'-d(CTA CCC GGA GNC CAA GTA CGG C)		
Duplex	N	T_m (°C)
6	A	49.8 ± 0.6
7	G	45.6 ± 0.3

5'-Biotin-d(GGG CC1 CTG GTT CAT G) 3'-d(CTA CCC GGA GNC CAA GTA CGG C)		
Duplex	N	T_m (°C)
8	A	49.8 ± 0.1
9	G	45.0 ± 0.6
10	T	43.2 ± 0.3
11	C	39.5 ± 0.5

Table 3. Interstrand cross-link (ICL) yields from **1** as a function of temperature

Temp. (°C)	% Yield ICL		Selectivity ^b
	8 ^a	9 ^a	
25	47.2 ± 0.8	44.5 ± 0.4	1.1
40	34.7 ± 0.9	11.0 ± 0.3	3.2
43	26.3 ± 1.7	2.3 ± 0.2	11.4
44	23.7 ± 1.0	1.8 ± 0.3	13.2
45	21.4 ± 0.3	0.9 ± 0.1	23.8
46	8.9 ± 0.5	0.1 ± 0.1	89.0

^aAverage of three independent samples.^bRatio of average ICL yield in **8**:**9**.

eliminate other effects, such as changes in the relative reaction rates of the methide with dA and water.

If this was the only manner in which **1** could be used to probe DNA sequence, the method would be limited to detecting sequences in which dA was the target. Consequently, 'indirect' probe **4** was prepared in order to demonstrate that **1** could in principle be used to detect any DNA sequence containing a dA. The incubation temperature was critical when using the indirect probe (**4**) where duplex thermal stability (Table 2) was expected to be the primary determinant for achieving selectivity. The matched duplex (**8**) containing the indirect probe (**4**) melts more than 4°C higher than that of any mismatch, indicating that the presence of the nonnative nucleotide (**1**) does not eliminate the differences in thermal stabilities of matched and mismatched duplexes. Using the T_m 's as a guide we carried out the hybridization/cross-linking reactions using the indirect probe at the approximate T_m of the most stable mismatch (**9**, T:G, 45°C, Table 2). Higher cross-linking yields for the matched sequence (**8**) were obtained at temperatures lower than the T_m of the T:G mismatch (**9**), but at a cost in selectivity (Table 3). Increasing the reaction temperature above the T_m of **9** improved selectivity, but also resulted in a significant decrease in cross-linking yield for the matched (**8**, T:A) sequence (Table 3). The most satisfactory combination of

Table 4. Interstrand cross-link (ICL) yields from **4** as a function of opposing nucleotide at 45°C

Duplex	N	Yield ICL ^a (%)	Selectivity ^b
8	A	21.4 ± 0.3	–
9	G	0.9 ± 0.1	23.8
10	T	0.3 ± 0.1	71.3
11	C	0.1 ± 0.1	214.0

^aAverage of three independent samples.^bRatio of ICL yield in **8**:respective duplex.

cross-link yield (Table 3) and selectivity was obtained by carrying out the reaction at the T_m of the most stable mismatch (Table 2). Consequently, the selectivity of the cross-linking reaction involving **1** opposite dA was screened (**8–11**) at 45°C (Table 4). As expected, there was a correlation between the ICL yield and T_m . The matched cross-link yield was lower than that observed for the direct probe (**3**) which was measured at lower temperatures. The decreased yield using **4** may affect the selectivity observed in the fluorescence experiments (see below).

Fluorescence detection of single nucleotide discrimination in plasmid DNA

Having established the sequence selectivity of the tagging reaction, the utility of the SNP method was demonstrated using a fluorescence assay in microtiter plates (Scheme 2). Single-stranded plasmids (**12a**, **12b**) containing **5a** or **5b** were prepared from M13mp7. The plasmid was linearized by cleaving a hairpin site with EcoR1 and then recircularized with the appropriate insert. Cells were transfected with the circular plasmids via electroporation and recovered by standard methods following overnight growth.

The assay conditions were optimized using the direct probe (**3**). Plasmids were hybridized with a 2-fold molar excess of the direct or indirect probes. Following addition of NaIO₄ (5 mM) to initiate the cross-linking reaction, the samples were incubated with the direct probe at 25°C for 6 h. Removal of the excess biotinylated probe (**3**), crucial for achieving sufficiently low background fluorescence levels needed for sensitive detection, was enabled by the cross-linking reaction. The dehybridized (80°C) mixture was filtered through a membrane, which was then washed with warm buffered formamide solution (2×), followed by pH 7.5 buffer (2×) (20). The biotinylated plasmid was then transferred to the well of an activated microtiter plate, conjugated to avidin-HRP and reacted with Amplex Red and H₂O₂. The emission (581 nm) was measured for the matched (opposing dA, **12a**) and mismatched (opposing dG, **12b**) samples (Figure 1). The fluorescence was measured as a function of plasmid loading in the microtiter plate well and reached a maximum at 250 fmol (Figure 1). Subsequent experiments were carried out using 300 fmol of plasmid. Background measurements (in triplicate) were made for samples in which plasmid was hybridized with probe but not oxidized. The reported fluorescence signals (Figure 2) were determined by subtracting the average

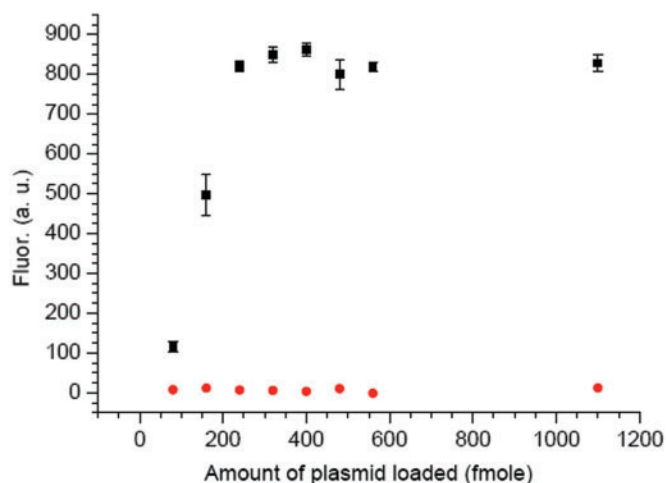


Figure 1. Fluorescence intensity versus amount of plasmid loaded (plasmid **12a**, black square; plasmid **12b**, red circle).

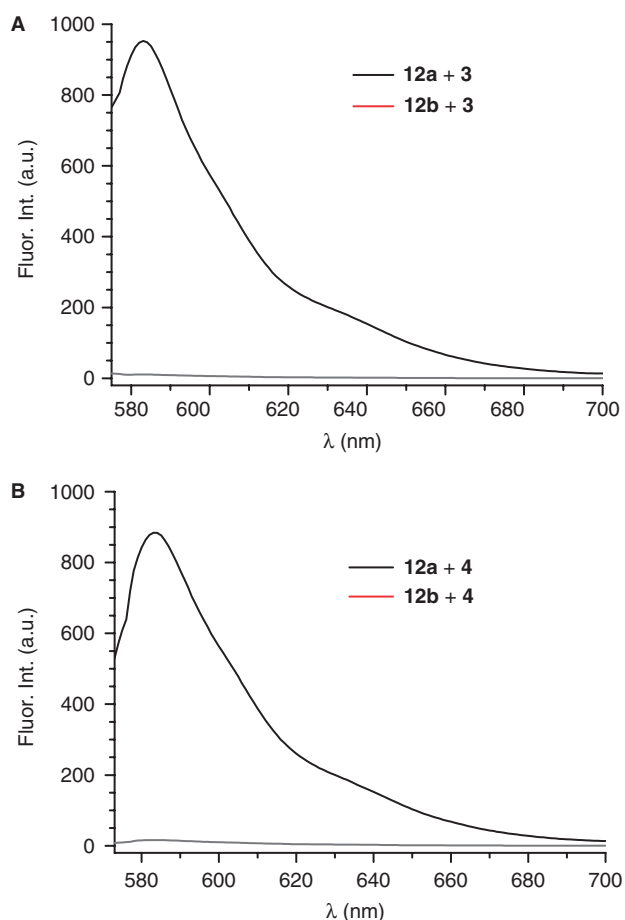


Figure 2. Selective DNA detection in plasmids (**12a**, **12b**) via fluorescence using bifunctional oligonucleotides. (A) Using direct probe **3**. (B) Using indirect probe **4**. Signal for matched pairs are in black; mismatched are in red.

background signal from the average signal obtained from samples in the presence of NaIO_4 . Most importantly, the discrimination for the matched versus mismatched sequence was 193:1 (Figure 2A, Table 5).

Table 5. Fluorescence detection of DNA in single stranded plasmids

Plasmid	Probe	Fluor. Int. ^a	Selectivity ^b
12a	3	964 ± 4	–
12b	3	5 ± 3	193
12a	4	849 ± 9	–
12b	4	19 ± 4	45

^aAverage of three independent samples.

^bRatio of average fluorescence intensity in matched versus mismatched plasmid.

The ability of the indirect probe (**4**) to discriminate between plasmids containing a matching (dA, **12a**) or mismatching (dG, **12b**) target sequences was determined using the procedure optimized for the direct probe with the exception that the reaction was carried out at the previously optimized temperature (Table 3, 45°C). The fluorescence intensities (Figure 2B, Table 5) correlated well with the cross-linking yields measured by denaturing PAGE (Table 4). For instance, the overall intensity for the matched sequence was lower than that observed using the direct probe (**3**). In addition, the signal from the mismatched target was slightly greater than when the direct probe was used. Of the two effects the signal from the mismatched indirect probe (**4** and **12b**) accounted for most of the reduction in selectivity when using the indirect probe. Nonetheless, the indirect probe (**4**) showed 45-fold selectivity for the matched versus mismatched target sequence.

CONCLUSION

We have developed a facile method for detecting DNA sequences capable of single nucleotide discrimination that detects target DNA at nanomolar concentration. By relying upon DNA interstrand cross-linking to dA by a modified thymidine, oligonucleotides (16 nt) whose complexes would otherwise be too unstable can be used (7). In addition, cross-linking to the target enables us to detect and amplify a specific sequence using biotin-avidin binding. The bifunctional probes are, in principle, capable of probing any sequence that contains a dA and show excellent selectivity of plasmid targets using short oligonucleotides, which along with eliminating PCR as a step in this process, makes it a rapid and inexpensive DNA detection method.

SUPPLEMENTARY DATA

Supplementary Data are available are NAR Online.

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Conflict of interest statement. None declared.

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