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# Analysis of internal (*n*–1)mer deletion sequences in synthetic oligodeoxyribonucleotides by hybridization to an immobilized probe array

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## ABSTRACT

**The purity of a drug substance can influence its toxicity and potency, so impurities must be specifically determined. In the case of synthetic oligodeoxyribonucleotide drugs, however, product complexity makes complete impurity speciation difficult. The goal of the present work was to develop a new analytical method for speciation of individual internal (*n*–1)mer impurities arising from formal nucleotide deletion in synthetic oligodeoxyribonucleotides. A complete series of oligodeoxyribonucleotide probes were designed, each complementary to an (*n*–1)mer deletion sequence of the drug in question. Glass plates were used as a solid support for individually immobilizing the entire probe array. The total mixture of internal (*n*–1) length impurities was isolated from a synthetic oligodeoxyribonucleotide by PAGE and labeled with $^{35}$S. Under stringently optimized conditions, only the perfectly sequence-matched oligodeoxyribonucleotide hybridized to each probe, while all other deletion sequences were removed by washing with buffer. The $^{35}$S signal intensity of the bound oligodeoxyribonucleotide was proportional to the concentration of each (*n*–1)mer deletion sequence in the analyte solution. This method has been applied to a number of synthetic phosphorothioate oligodeoxyribonucleotide lots and shown to be reliable for speciation and relative quantitation of the internal (*n*–1)mer deletion sequences present.**

## INTRODUCTION

During oligodeoxyribonucleotide synthesis, nucleoside monomers are typically attached to the growing chain one at a time in a repeated series of phosphoramidite coupling, oxidation or oxidative sulfurization, acyl capping and acidic detritylation reactions. The stepwise yield for each nucleoside addition is 98.6–99%, depending on internucleotide linkage type. Nucleotide deletion occurs due to failure to couple and subsequent failure to cap or to failure to detritylate or to remain detritylated (1). The resulting deletion sequences, ranging from (*n*–1)mers and (*n*–2)mers to shorter lengths, are present as impurities in the crude full-length oligodeoxyribonucleotide. Among these, (*n*–2)mer and shorter oligodeoxyribonucleotide impurities are present only at low levels and are readily removed by chromatographic purification (2). However, due to the lack of chromatographic selectivity, (*n*–1)mer impurities remain following purification, unless a low recovery of the desired (*n*)mer is accepted. After chromatographic purification at high recovery, analytical levels of (*n*–1)mer impurities may still be present in the product. These (*n*–1)mer impurities could potentially comprise all possible internal and terminal single base deletion sequences of the oligodeoxyribonucleotide. Any terminal deletion sequence present in the oligodeoxyribonucleotide drug (3) would bind to the same target mRNA as the full-length sequence with slightly lower affinity and should retain antisense activity. Therefore, it has been important to pursue speciation of individual internal base deletion sequences as part of the impurity profiling process for oligodeoxyribonucleotide drugs (4,5).

For analysis of oligodeoxyribonucleotides, chromatography and capillary electrophoresis are commonly used. Anion exchange and reverse phase HPLC can separate full-length phosphodiester oligodeoxyribonucleotides from their deletion sequences, but resolution is considerably decreased for modified oligodeoxyribonucleotides such as phosphorothioates (2). Capillary gel electrophoresis has excellent length-based resolving power for oligodeoxyribonucleotides and can separate (*n*–1)mer impurities from full-length synthetic product with acceptable resolution (6–8). However, all (*n*–1)mers migrate together and are detected as a single peak, so liquid chromatography and capillary electrophoresis cannot be used for speciation of individual internal (*n*–1)mer impurities. To date, two papers (9,10) have addressed the issue of sequence identity of individual (*n*–1)mer impurities. Electrospray ionization mass spectrometry was employed to analyze the (*n*–1)mer content in a phosphorothioate oligodeoxyribonucleotide (9). Because the mass spectrum cannot provide sequence information, it cannot distinguish the (*n*–1)mers with the same base formally deleted at different positions in the parent sequence from one another. In addition, signal-to-noise ratio is not great enough to ensure accurate quantitation. A method of cloning and sequencing has also been described for speciation of (*n*–1)mers (10). A possible problem in this method is that the plasmid and its host bacteria may have biased selection for sequence of the different inserts, so that results may differ from

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the real distribution of (*n*–1)mers. Also, hundreds of clones must be sequenced in order to get meaningful statistics reflecting the (*n*–1)mer population and these complex procedures and tedious work make the method less attractive. Finally, the method is probably limited to phosphodiester oligodeoxyribonucleotides.

The purpose of the present work was to develop a new method for identification and quantitation of the internal (*n*–1)mer deletion sequence impurities in synthetic oligodeoxyribonucleotides. The (*n*–1)-length mixture is isolated from full-length oligodeoxyribonucleotide by polyacrylamide slab gel electrophoresis and radiolabeled with $^{35}$S at the 5′-end. A series of oligodeoxyribonucleotide probes are designed, each complementary to a unique internal (*n*–1)mer deletion sequence. The probes are individually covalently bound to a glass plate support in a rectangular array. Each probe forms a perfect Watson–Crick match with a unique (*n*–1) deletion sequence and is a mismatch for all other (*n*–1)mer deletion sequences in the synthetic product. A mixture comprising the target (*n*–1)mer oligodeoxyribonucleotides is placed in contact with the immobilized probes under stringent hybridization conditions. Hybridization of perfectly matched (*n*–1) oligodeoxyribonucleotide is preferentially achieved while mismatched (*n*–1) oligodeoxyribonucleotides hybridize poorly and are subsequently removed by a washing step. The intensity of the radiation at the position of each probe is proportional to the sample concentration of each complementary (*n*–1) deletion sequence. Four clinical supply production lots of a 21mer phosphorothioate oligodeoxyribonucleotide have been analyzed by the method with good results, demonstrating reliability for the identification and relative quantitation of the internal (*n*–1) deletion sequences in synthetic oligodeoxyribonucleotides.

## MATERIALS AND METHODS

T4 nucleotidyl kinase was purchased from New England Biolabs (Beverly, MA). 3-Aminopropyltrimethoxysilane and 1,4-phenylene diisothiocyanate were purchased from Aldrich Chemical (Milwaukee, WI). [α-$^{35}$S]dATP, deoxynucleotidyl transferase and [γ-$^{35}$S]dATP were purchased from Amersham Life Science (Arlington Heights, IL). Microscope slides, autoradiographic film and MicroSpin™ G-25 columns were purchased from Fisher Scientific (Pittsburgh, PA). All oligodeoxyribonucleotide probes and oligodeoxyribonucleotide analytes were synthesized on Applied Biosystems automated DNA synthesizers and purified in the dimethoxytrityl-on mode by reverse phase HPLC.

### Glass surface chemistry

1-[3″-(Trimethoxysilyl)propyl]-1′-(4″-isocyanatophenyl)thiourea, the reagent used for treatment of the glass surface, was synthesized at Isis using 3-aminopropyltrimethoxysilane and 1,4-phenylene diisothiocyanate as reactants. 1,4-Phenylene diisothiocyanate (5.0 g, 26 mmol) was dissolved in 50 ml of dichloromethane at 45–50°C. A solution of 3-aminopropyl trimethoxysilane (4.1 ml, 22.9 mmol) in 30 ml of dichloromethane was added slowly to the 1,4-phenylene diisothiocyanate solution with stirring at 45–50°C for 4 h. The reaction mixture was stirred for another 3 h at the same temperature. The solvent was then evaporated under reduced pressure. The resulting white solid (8.4 g) was recrystallized from cyclohexane/hexane (1:3) containing 2–3% ethyl acetate to obtain 4.5 g (yield 53%) of the desired mono-thiourea product as white crystals [silica gel TLC: $R_f$ 0.55 (1:1 hexanes:ethyl acetate). $^1$H NMR ($CDCl_3$) δ: 0.61 (t, 2H, $J$ = 7.8 Hz), 1.60–1.80 (m, 2H), 3.49 (s, 9H), 3.52–3.65 (m, 2H), 6.40 (b, 1H), 7.22 (s, 4H), 8.52 (b, 1H). $^{13}$C NMR ($CDCl_3$) δ: 6.4, 22.1, 47.4, 50.6, 125.8, 127.1, 129.5, 135.7 (N=C=S), 180.4 (C=S)].

Surface modification of glass microscope slides was achieved in two ways. (i) Treatment with 1% aminopropyltrimethoxysilane in 95% aqueous acetone for 2 min, washing with acetone several times and drying followed by treatment with 0.2% 1,4-phenylene diisothiocyanate in 1:9 pyridine/dimethyl formamide (v/v) for 2 h (11). (ii) Treatment with 2% 1-[3″-(trimethoxysilyl)propyl]-1′-(4″-isocyanatophenyl)thiourea in 95% acetone (19 ml acetone and 1 ml 0.1 M NaOH) for 10 min. After surface modification, the glass slides were washed with methanol and acetone and stored in a vacuum desiccator at 4°C.

### Immobilization and hybridization

For the purpose of measuring efficiency of probe immobilization on the plate, the probe was first labeled with $^{35}$S at the 3′-end {52 µl distilled water, 10 µl 20 pmol/µl oligodeoxyribonucleotide probe, 10 µl [α-$^{35}$S]dATP, 20 µl 5× reaction buffer, 8 µl terminal deoxynucleotidyl transferase (5 U/µl)} at 37°C for 1 h. The labeled oligodeoxyribonucleotide solution was then purified on a MicroSpin™ G-25 column. The probe solution, 0.5 pmol/µl in 0.001 N NaOH, was used for immobilization and by using a template for positioning 2 µl of the solution was loaded onto the modified glass surface in a circle of ~2 mm diameter. The glass slide was then incubated at 37°C for 2 h in a sealed box, with atmospheric moisture level maintained by equilibration against an open container of water. The glass slides were then washed with 1% ammonia and water. The oligodeoxyribonucleotide used for the hybridization efficiency study (to unlabeled bound probes) was labeled with $^{35}$S {8 µl deionized water, 10 µl 20 pmol/µl olignucleotides, 4 µl kinase buffer, 10 µl [γ-$^{35}$S]dATP and 8 µl T4 nucleotidyl kinase (10 U/µl)} at 37°C for 2 h. The labeled oligodeoxyribonucleotide solution was then purified on a MicroSpin™ G-25 column. The oligodeoxyribonucleotide hybridization solution was then diluted to 0.5 pmol/µl in 3× SSPE buffer (SSPE: 10 mM $Na_2HPO_4$, 150 mM NaCl, 1 mM EDTA, pH 7.4) and 0.5% SDS. Two microliters of the solution was loaded onto the glass slides to cover the immobilized probe, the glass slides were then placed into a preheated box containing water to maintain the moisture and incubated for hybridization at the desired temperature for 3 h. The glass slides were then washed twice with SSPE buffer for 20 min on a shaker. Hybridized labeled oligodeoxyribonucleotides were detected by autoradiography and quantified by densitometry.

## RESULTS AND DISCUSSION

### Design of the probe array

The probe array comprises four parts: (i) a glass plate solid support, (ii) a linker, (iii) a spacer and (iv) the affinity unit. The linker and spacer provide a bridge between the solid support and affinity unit so as not to significantly alter or reduce Watson–Crick binding capacity or affinity. All bound probes share a common linker and spacer structure, differing only in the oligodeoxyribonucleotide affinity portion.

Glass microscopic slides were selected as a solid support for the probe array from among a large number of possible materials

(12–23), due to the simplicity and convenience of probe attachment and array capacity for large scale analysis.

An aminohexyl chain [$NH_2(CH_2)_6$] was selected as the linker arm from among a range of possible chemistries. An oligodeoxyribonucleotide chain, oligo(dT), was used as the spacer arm, with thymidylic acid chosen for the stability it confers on the oligomer. This chemical similarity of the spacer and probe provides the potential benefit of placing the probe oligodeoxyribonucleotide in a chemical environment and conformation favoring hybridization to the analyte.

The oligodeoxyribonucleotide probe must specifically bind sequence-matched (*n*–1)mers and discriminate against others. Probe length was considered in the range 8–20 bases. The shorter the length, the higher the power of discrimination for base mismatches or bulges. The longer the probe length, the higher the melting temperature of the Watson–Crick duplex and hence the higher stability of the double helix. A probe length of 12 nt was chosen for the present work. The design of the probe array is dependent on the internal (*n*–1)mer sequences. For example, for the single base deletion sequences of the synthetic phosphorothioate oligodeoxyribonucleotide d(GCGTTTGCTCTTCTTCTTGCG), there are 14 possible different internal (*n*–1) deletion sequences (Table 1).

**Table 1.** Internal (*n*–1)mer deletion sequences of a phosphorothioate oligodeoxyribonucleotide, where ^ represents the missing base

| Number | Sequence | Degeneracy |
|---|---|---|
| D1 | d(GCGTTTGCTCTTCTTCTTG^G) | 1 |
| D2 | d(GCGTTTGCTCTTCTTCTT^CG) | 1 |
| D3 | d(GCGTTTGCTCTTCTTCT^GCG) | 2 |
| | d(GCGTTTGCTCTTCTTC^TGCG) | |
| D4 | d(GCGTTTGCTCTTCTT^TTGCG) | 1 |
| D5 | d(GCGTTTGCTCTTCT^CTTGCG) | 2 |
| | d(GCGTTTGCTCTTC^TCTTGCG) | |
| D6 | d(GCGTTTGCTCTT^TTCTTGCG) | 1 |
| D7 | d(GCGTTTGCTCT^CTTCTTGCG) | 2 |
| | d(GCGTTTGCTC^TCTTCTTGCG) | |
| D8 | d(GCGTTTGCT^TTCTTCTTGCG) | 1 |
| D9 | d(GCGTTTGC^CTTCTTCTTGCG) | 1 |
| D10 | d(GCGTTTG^TCTTCTTCTTGCG) | 1 |
| D11 | d(GCGTTT^CTCTTCTTCTTGCG) | 1 |
| D12 | d(GCGTT^GCTCTTCTTCTTGCG) | 3 |
| | d(GCGT^TGCTCTTCTTCTTGCG) | |
| | d(GCG^TTGCTCTTCTTCTTGCG) | |
| D13 | d(GC^TTTGCTCTTCTTCTTGCG) | 1 |
| D14 | d(G^GTTTGCTCTTCTTCTTGCG) | 1 |

In order to speciate and quantify the (*n*–1)mers in this product, 14 different 12mer probes were designed, each complementary to a portion of a possible failure sequence. For example, probe P9 [3′-CAAACGGAAGAAT$_{15}$$(CH_2)_6NH_2$-5′], with a hybridization length of 12 bases, forms a perfect match with phosphorothioate oligodeoxyribonucleotide deletion sequence D9, in which the thirteenth nucleotide from the 3′-terminus (thymidine) is missing, and will have mismatches of from 1 to 4 bp for all the other formal (*n*–1)mer deletion sequences. Some mismatch duplexes may achieve a higher number of matched base pairs by forming a bulged structure. For example, D2–D4 have the same base pairs and bulge as (*n*–1)mer D1, as their sequences differ in the unpaired 3′-sequence.

Two additional considerations were taken into account in design of the probes: (i) to maximize (*n*–1)mer discrimination, the mismatch location was placed as near to the center of the probe as possible; (ii) GC contents among probes were as similar as possible in order to obtain similar duplex stabilities. The probes used are listed in Table 2.

**Table 2.** Probes for internal (*n*–1)mer deletion sequences in a phosphorothiate oligodeoxyribonucleotide

| Number | Sequence |
|---|---|
| P1 | 3′-AGAAGAAGAACC-$T_{15}(CH_2)_6NH_2$-5′ |
| P2 | 3′-AGAAGAAGAAGC-$T_{15}(CH_2)_6NH_2$-5′ |
| P3 | 3′-AGAAGAAGACGC-$T_{15}(CH_2)_6NH_2$-5′ |
| P4 | 3′-AGAAGAAAACGC-$T_{15}(CH_2)_6NH_2$-5′ |
| P5 | 3′-GAGAAGAGAACG-$T_{15}(CH_2)_6NH_2$-5′ |
| P6 | 3′-CGAGAAAAGAAC-$T_{15}(CH_2)_6NH_2$-5′ |
| P7 | 3′-AACGAGAGAAGA-$T_{15}(CH_2)_6NH_2$-5′ |
| P8 | 3′-AAACGAAAGAAG-$T_{15}(CH_2)_6NH_2$-5′ |
| P9 | 3′-CAAACGGAAGAA-$T_{15}(CH_2)_6NH_2$-5′ |
| P10 | 3′-GCAAACAGAAGA-$T_{15}(CH_2)_6NH_2$-5′ |
| P11 | 3′-CGCAAAGAGAAG-$T_{15}(CH_2)_6NH_2$-5′ |
| P12 | 3′-CGCAACGAGAAG-$T_{15}(CH_2)_6NH_2$-5′ |
| P13 | 3′-CGAAACGAGAAG-$T_{15}(CH_2)_6NH_2$-5′ |
| P14 | 3′-CCAAACGAGAAG-$T_{15}(CH_2)_6NH_2$-5′ |

## Immobilization

The glass surface of microscopic slides is covalently modified to provide a linker arm with an amino-reactive terminus. Modification was achieved by either treating the glass surface with 1-[3′′-(trimethoxysilyl)propyl]-1′-(4′′-isocyanatophenyl)thiourea or with 3-aminopropyltrimethoxysilane and 1,4-phenylene diisothiocyanate sequentially, providing an amino-reactive phenylisothiocyanate group fixed to the glass surface. The former is a more convenient one step reaction while the latter gives slightly better capture efficiency.

Under mildly alkaline conditions, primary amines at the 5′-terminus of each probe react with isothiocyanates on the glass surface to form a thiourea adduct. Immobilization is effective in the range pH 9–11. No net reaction occurs in 0.01–0.1 N NaOH because hydroxide reacts with isothiocyanate to form an unstable N-substituted thiocarbamic acid, which decomposes to the starting amine. Control experiments were performed using an oligodeoxyribonucleotide without a 5′-end amine group on the derivatized glass slide and using probe to the underivatized glass slide. The probe didn't bind to the unmodified glass surface and other oligodeoxyribonucleotides lacking the 5′-end aminohexyl group didn't bind to the modified glass surface, confirming the

reaction between the 5′-end primary amine and the isothiocyanate group on the glass surface.

The kinetics of probe immobilization were determined at room temperature at 0.5, 1, 2, 3, 4 and 5 h. As a comparison, the same experiment was performed using an underivatized glass slide and no radioactive signals were detected, indicating that background immobilization does not occur on an unmodified glass surface. For surface-modified glass, radioactive signal intensity increases with probe immobilization time, indicating that more probe molecules are being immobilized onto the glass surface. However, the rate of increase is low after 1 h. The immobilization efficiency was ~95% at 37°C for 2 h.

## Hybridization

Formation of an oligodeoxyribonucleotide double helix is reversible. Hybridization in this assay depends on analyte ionic strength, analyte and probe base composition, double helix length, concentration of the probe and target oligodeoxyribonucleotide and the concentration of helix-destabilizing agents. The number and position of the mismatches affect the stability of a duplex containing mismatched bases. For oligodeoxyribonucleotides, $T_m$ decreases by ~5°C for every mismatched base pair. The greater the number of mismatches, the easier the sequence discrimination between perfectly and imperfectly matched oligodeoxyribonucleotides. A centered position for the mismatch provides the greatest differentiation. Hybridization stringency can be adjusted by salt concentration, concentration of destabilizing agents such as SDS and/or formamide and/or by changing the temperature. Discrimination can be also achieved by post-hybridization washes.

*Buffer concentration*. Hybridization has been tested in different concentrations of SSPE buffer. Probe P11 was loaded onto a glass slide using a template; the reference solution without probe was also loaded as a control. The $^{35}$S-labeled target oligodeoxyribonucleotide, D11, which has a perfect 12 base match with probe P11, was prepared in 0.5% SDS and 1, 2, 3, 4 and 5× SSPE buffer and loaded onto each row. The results indicate that hybridization and non-specific binding increase with increasing buffer concentration, with 3× SSPE being optimal for the best signal to background ratio.

*SDS concentration*. SDS concentration has been tested for its effect on hybridization. Probe P11 was immobilized on a glass slide using a template; the reference solution without probe was also loaded as a control. $^{35}$S-labeled oligodeoxyribonucleotide D11 was prepared in 3× SSPE buffer and 0, 0.1, 0.25, 0.5, 0.75 and 1.0% SDS and loaded onto each row. The obtained autoradiography images indicate that SDS can significantly reduce non-specific binding. Without SDS, the target oligodeoxyribonucleotide non-specifically binds the glass surface. With 0.1–1.0% SDS, oligodeoxyribonucleotide D11 hybridizes specifically to probe P11; no signals were obtained in the control group. On the other hand, high concentrations of SDS slightly decreased hybridization between the probe and target oligodeoxyribonucleotide. A concentration of 0.5% SDS was selected for hybridization.

*Spacer length*. Spacer length was tested using 0, 5, 10, 15, 30 and 45mer poly(T) 2′-deoxynucleotides. The hybridization signal was poor when the spacer length was <10 nt and increased with lengths up to 15 nt, whereas no improvement was observed with spacer lengths >15 nt. On the other hand, the impurity levels and costs of probes increase with spacer length. For these reasons, $T_{15}$ was selected as the spacer arm.

*Background suppression*. Hybridization conditions were further optimized for reduction of background caused by non-specific binding of labeled oligodeoxyribonucleotide to the modified glass surface. This interaction is expected to be relatively non-sequence dependent, therefore background signal may be reduced by incubation of the immobilized plate with an unlabeled oligodeoxyribonucleotide with a sequence different from that of the analyte, prior to hybridization. A 100-fold higher concentration (relative to target oligodeoxyribonucleotide) of cold non-specific oligodeoxyribonucleotide in the hybridization buffer was applied to the area where the probe was attached, then incubated for 1 h at 30°C. The glass plate was then washed once with hybridization buffer. $^{35}$S-labeled target oligodeoxyribonucleotide D11, which has a perfect 12 base match with probe P11, was prepared in 0.5% SDS and 3× SSPE buffer and loaded onto the glass surface where the probe was immobilized and allowed to hybridize for 3 h at 30°C. The results, compared with those of the control group without pretreatment with unlabeled oligodeoxyribonucleotide, indicate that a 3- to 4-fold reduction in background is obtained by pretreatment with unlabeled non-specific oligodeoxyribonucleotide prior to hybridization of analytes.

*Hybridization time*. Hybridization was performed for time intervals ranging from 0.5 to 5 h, demonstrating that the longer the hybridization time, the greater the signal obtained in 3 h and no obvious signal increase was observed afterwards. Three hours was selected as a compromise between signal intensity and time expended.

*Temperature*. Selection of the optimum hybridization temperature is very important to selective assay of the level of oligodeoxyribonucleotide analyte fully complementary to the probe sequence. The temperature should be high enough to melt duplexes formed by partially mismatched oligodeoxyribonucleotides, especially those with a 1 bp mismatch at the duplex terminus, but not so high as to significantly impair binding of the perfectly matched oligodeoxyribonucleotide. The difference in stability between a perfectly matched duplex and a terminal base mismatched duplex is so small that the terminal base mismatched duplex interferes with determination of the target oligodeoxyribonucleotide at mild hybridization temperatures. For example, the D3–P14 duplex has only a C-C mismatch at the 5′-terminus, compared with the D14–P14 perfect match. D3 interferes with determination of D14 by probe P14. $^{35}$S-labeled D3 and D14 were hybridized to probe P14 at 30, 35, 40, 45 and 50°C, respectively, for 3 h. The autoradiogram obtained is shown in Figure 1. Net signal intensity decreases with increasing temperature, but the rate of decrease is much faster for mismatched D3 than for matched D14. Therefore, the ratio of hybridized D14 to hybridized D3 increases with increasing temperature up to 45°C, then, as the temperature reaches 50°C, sequence-matched D14 also melts.

*Unlabeled mismatch suppression*. Although stringent temperature selection can enhance selectivity of (*n*–1)mer identification, end base mismatched D3 still exhibits considerable hybridization at 45°C, hence interfering with determination of perfectly matched D14. This interference can be suppressed, however, by adding a relatively large, fixed quantity of unlabeled D3 to the analyte

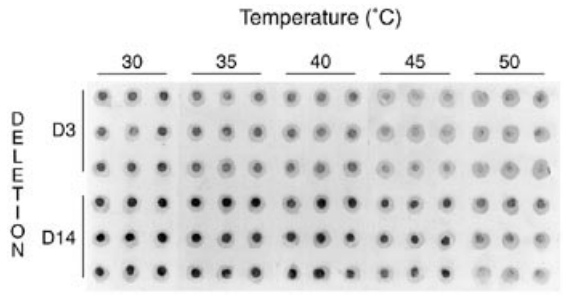


**Figure 1.** Hybridization temperature. D3 and D14 were labeled with $^{35}S$, diluted to 0.5 pmol/µl in hybridization buffer, nine replicates loaded (2 µl each) on slides where probe P14 was immobilized and hybridized for 3 h at temperatures of 30, 35, 40, 45 and 50°C.

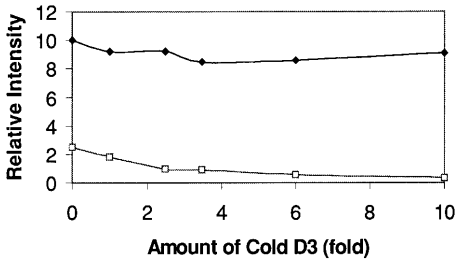


**Figure 2.** Unlabeled mismatch suppression. D3 and D14 were labeled with $^{35}S$, diluted to 0.5 pmol/µl in hybridization buffer containing 0- to 10-fold cold D3 and hybridized in tripicate (2 µl each) on slides where probe P14 was immobilized. ◆, $^{35}S$-labeled D14; □, $^{35}S$-labeled D3.

mixture. Figure 2 shows the effect of adding unlabeled D3 in the concentration range 0–10 pmol/µl on the hybridization of D14 and mismatch sequence D3 to probe P14. Little influence of added unlabeled D3 was observed on the level of perfectly matched duplex D14–P14, while a considerable reduction in the signal intensity from labeled D3 resulted for the mismatched duplex D3–P14. Assay interference was reduced from 25 to 5% by using unlabeled D3 as a suppressor.

*Probe concentration.* Probe concentration is an important factor for assay selectivity and must be optimized for specific hybridization of the corresponding target oligodeoxyribonucleotide. Different probe concentrations (0.01–7 pmol/µl) were tested for hybridization with 0.5 pmol/µl target oligodeoxyribonucleotide. Relative intensity increases with probe concentration in the 0.01–0.1 pmol/µl range and remains almost constant in the 0.1–7 pmol/µl range. Figure 3 shows relative signal intensities obtained at 0.01–2 pmol/µl probe concentrations. A probe concentration of 0.5 pmol/µl is preferred for optimal assay selectivity and signal intensity without sacrifice of the linearity. A large excess of probe may result in the binding of mismatched oligodeoxyribonucleotide.

*Target oligodeoxyribonucleotide concentration.* Different concentrations of target oligodeoxyribonucleotide (0.05–2 pmol/µl D3) were tested for hybridization to 0.1, 0.25 and 0.5 pmol/µl probe P3 (Fig. 4). A linear relationship between the signal intensity and the target oligodeoxyribonucleotide concentration was observed in the range 0.05–0.5 pmol/µl oligodeoxyribonucleotide concentration at 0.5 pmol/µl probe concentration. For the lower probe concentrations, the signal remains roughly the same when oligodeoxyribonucleotide concentration becomes high enough to saturate the probe.

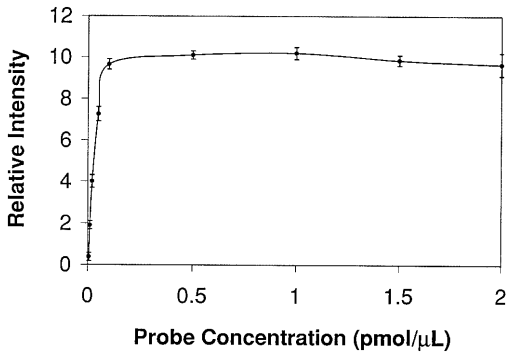


**Figure 3.** Probe concentration. P14 at concentrations of 0.01–7 pmol/µl was immobilized onto modified glass slides at 37°C for 2 h. $^{35}S$-labeled D14 (0.5 pmol/µl) was hybridized to P14 at 30°C for 3 h. Only the data for the 0.01–2 pmol/µl probe concentrations are shown.

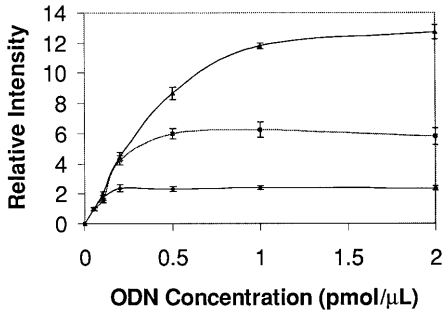


**Figure 4.** The concentration of target oligodeoxyribonucleotide. P3 was immobilized onto modified glass slides at concentrations of 0.1, 0.25 and 0.5 pmol/µl. $^{35}S$-labeled D3 was hybridized to P3 in 2 µl solutions of different concentrations (0.05–2 pmol/µl). ◆, 0.1 pmol/µl P3; ■, 0.25 pmol/µl P3; ▲, 0.5 pmol/µl P3.

## Washing

The purpose of washing is to remove mismatched oligodeoxyribonucleotides from the probe and labeled oligodeoxyribonucleotide non-specifically bound to the modified glass surface. Experimentation shows that elution with 2× SSPE buffer can enlarge the difference in radioisotope signal intensity between oligodeoxyribonucleotides of matched and mismatched sequences. The greater the washing time, the weaker the background signal, but the signal from perfectly matched oligodeoxyribonucleotide will also decrease if washing times reach 40 min. Twenty minutes was selected for washing.

## Sample analysis

Under optimized conditions, each probe of the array is selective for determination of a specific (*n*–1) deletion sequence. Figure 5 shows an example. Single (*n*–1) oligodeoxyribonucleotide species were loaded onto the probe array and the perfectly matched probe produces the greatest signal, while mismatched probes give either no or weak signals. It must be pointed out that

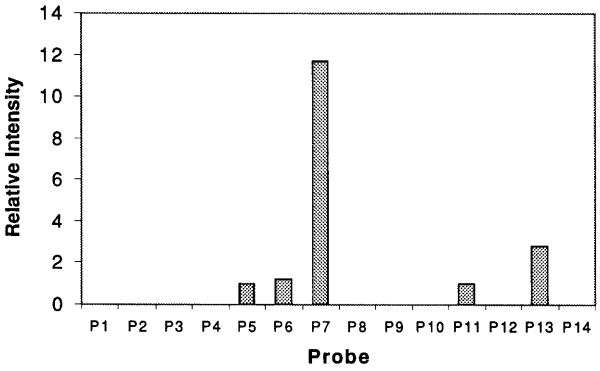


**Figure 5.** Selectivity of the probe array for a single (*n*–1)mer species. $^{35}$S-labeled D7 (0.5 pmol/μl) was hybridized to the probe array at 45°C for 3 h.

selectivity is even better than this for actual sample analysis, because the sample (*n*–1)mer is composed of multiple species that compete for binding to the probe.

**Table 3.** Percentage of individual (*n*–1)mer deletion sequences in a phosphorothioate oligodeoxyribonucleotide

| Deletion number | Individual lot A | B | C | D | Lot-to-lot (average ± SD) |
|---|---|---|---|---|---|
| D1 | 5.11 | 4.79 | 5.32 | 4.79 | 5.00 ± 0.26 |
| D2 | 3.68 | 2.95 | 4.79 | 4.11 | 3.88 ± 0.77 |
| D3 | 11.58 | 12.79 | 12.16 | 13.05 | 12.39 ± 0.66 |
| D4 | 5.32 | 4.05 | 6.53 | 5.42 | 5.33 ± 1.01 |
| D5 | 14.32 | 11.84 | 10.05 | 10.68 | 11.72 ± 1.88 |
| D6 | 4.05 | 3.42 | 5.32 | 4.74 | 4.38 ± 0.82 |
| D7 | 12.26 | 11.21 | 11.95 | 8.42 | 10.96 ± 1.75 |
| D8 | 3.05 | 5.16 | 5.05 | 4.00 | 4.32 ± 0.99 |
| D9 | 5.32 | 5.89 | 5.74 | 5.00 | 5.49 ± 0.41 |
| D10 | 5.00 | 5.16 | 5.47 | 6.68 | 5.58 ± 0.76 |
| D11 | 5.58 | 4.32 | 6.21 | 4.74 | 5.21 ± 0.85 |
| D12 | 14.00 | 15.74 | 12.05 | 16.21 | 14.50 ± 1.89 |
| D13 | 6.26 | 4.53 | 5.79 | 4.95 | 5.38 ± 0.79 |
| D14 | 4.47 | 8.26 | 3.53 | 7.16 | 5.86 ± 2.22 |

Artificial samples were made by randomly adding variable amounts (0–0.4 pmol/μl) of each (*n*–1)mer deletion sequence to form several (*n*–1)mer populations. A large fraction of the full-length oligodeoxyribonucleotide was included in two of the samples to test its interference with the determination of each component. The mixtures were then labeled with $^{35}$S at the 5′-end. The probe array was immobilized on the same glass slide at 37°C for 2 h using 2 μl solution for each probe at a concentration of 0.5 pmol/μl and was pre-hybridized with 2 μl of mismatched oligodeoxyribonucleotide at a concentration of 5 pmol/μl. The hybridization solution of the sample was prepared by mixing 80 μl of the $^{35}$S-labeled sample with 15 μl of 20× SSPE buffer and 5 μl of 10% SDS. The solution of labeled (*n*–1)mers was presented to the probe array in a 2 μl volume for each probe. The glass slide was incubated at 45°C for 3 h and then washed with

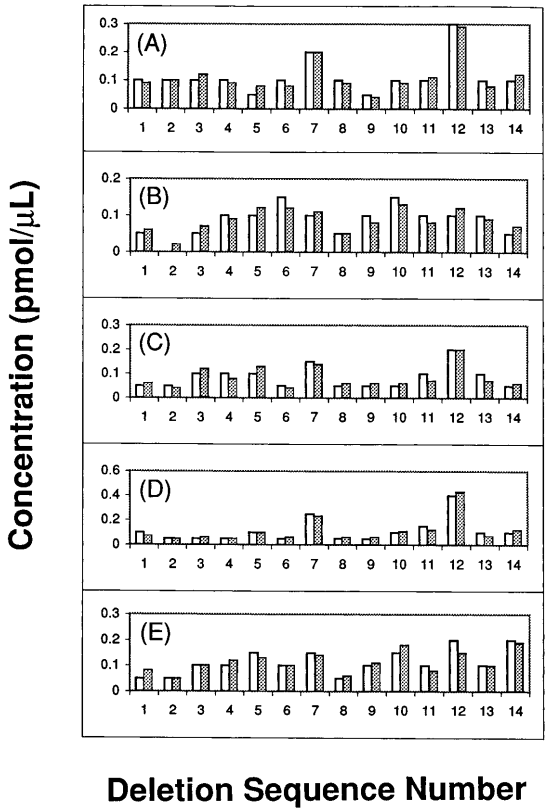


**Figure 6.** Synthesized sample analysis. Five artificial samples (**A**–**E**) were prepared with different variable amount (0–0.4 pmol/μl) of individual (*n*–1)mer components (samples C and D contained 0.5 pmol/μl of the parent full-length oligodeoxyribonucleotide). The experimental results analyzed by the proposed method were then compared with the amount added in each (*n*–1)mer population. □, amount added; ■, amount found.

2× SSPE for 20 min. Three separate slides were used for the sample analysis. The intensity of radioactive signal obtained at the position of each probe was compared with that of the standard to obtain the internal (*n*–1)mer deletion sequence profile, which was also obtained on different glass slides. The experimental results were then compared with the theoretical values (Fig. 6). Good agreements were obtained for all of the authentic samples and the full-length oligodeoxyribonucleotide did not interfere with the determination of each individual (*n*–1)mer component.

Four lots of a 21mer phosphorothioate oligodeoxyribonucleotide were used for the (*n*–1)mer speciation (lots A and B were synthesized at the 3–4 g scale on a Milligen 8800 synthesizer using controlled pore glass support and lots C and D were synthesized at large scale on a Pharmacia OligoProcess synthesizer using polystyrene as the solid support). The (*n*–1)mer mixture was isolated from full-length oligodeoxyribonucleotide by preparative PAGE. Good separation of the (*n*–1)mer mixture from full-length oligodeoxyribonucleotide was achieved. The (*n*–1)mer band was cut from the gel, then (*n*–1)mers were extracted and labeled with $^{35}$S at the 5′-ends for the analysis. Table 3 shows the percentage of individual (*n*–1) impurities for each lot, the average results and lot-to-lot variation. The results demonstrate that: (i) no single (*n*–1)mer species is dominant in the mixture (D3, D5, D7 and D12 have higher concentrations simply because of their degeneracy;

see Table 1), the levels of each individual internal (*n*–1)mer species being equivalent and consistent with their degeneracy in the parent sequence; (ii) lot-to-lot variation in the internal (*n*–1)mer profile is small, the impurity profile obtained being similar for all lots regardless of the solid support used, type of synthesizer or the scale of the synthesis. The occurrence of nucleotide deletions is shown to be neither base dependent nor position dependent. It is anticipated that these findings will prove general and applicable to other oligodeoxyribonucleotides made by phosphoramidite coupling.

## CONCLUSION

We have described a method for the direct analysis of internal (*n*–1)mer deletion impurities in a synthetic oligodeoxyribonucleotide by solid support-based hybridization. The probe array is made of 14 covalently bound oligodeoxyribonucleotide probes, each specific for binding a unique (*n*–1)mer deletion sequence. Interference by other (*n*–1)mer deletion sequences can be greatly suppressed by adding unlabeled mismatched oligodeoxyribonucleotide to the hybridization solution. Temperature is a very effective parameter for enhancement of the selectivity. Under the optimized conditions, each probe in the probe array specifically binds its complementary (*n*–1)mer species through Watson–Crick hybridization. Other deletion sequences hybridize either weakly or not at all to the probe and are easily removed, resulting in minimal interference of the other (*n*–1)mer deletion sequences. The method provides reliable speciation, relative quantitation and, in combination with a CGE impurity profile (6), quantitation of the internal (*n*–1)mer deletion sequences in synthetic oligodeoxyribonucleotides. Although $^{35}S$ was used in this manuscript, $^{32}P$ with storage phosphor technology or fluorescence detection would be even better to enhance the linearity of signal.

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