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The production of PCR products with 5′ single-stranded tails using primers that incorporate novel phosphoramidite intermediates*

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ABSTRACT

We have prepared several novel phosphoramidites and have synthesised oligonucleotides incorporating them internally. The presence of these residues in an oligonucleotide template presents an impassible barrier to primed synthesis by *Taq* DNA polymerase. When extended as polymerase chain reaction products, these oligonucleotides no longer serve as templates for the polymerase beyond the insertion sites of the modified intermediates, thereby producing single-stranded tails on amplification products. These tails can then be used for solid phase capture and colourimetric detection of PCR products.

INTRODUCTION

The polymerase chain reaction (PCR) (1,2) has revolutionised genetic analysis by increasing speed, simplicity and sensitivity relative to filter bound hybridisation assays. As reported by several groups, the first step in many non-gel assays for PCR products is the solid phase capture of denatured amplified DNA (3–8). Colourimetric detection of amplified DNA has also been described (see refs 7–10 for examples). Denaturation of PCR products has usually been required hitherto for affinity hybridisation and detection. One consequence of this denaturation is competition for the capture or detection hybridisation sequence by the opposing strand of amplified product, so reducing sensitivity. A further consequence is a limitation of the number of amplification products that can be analysed simultaneously. This is imposed by the number of heat stable hapten complexes that are available for the immobilisation of PCR products to solid supports.

One method of overcoming these problems is the provision of single-stranded PCR products, either by asymmetric PCR (11) or by selective chemical or enzymatic degradation of one of the strands of a segment of conventionally amplified DNA (12). However, asymmetric PCR itself suffers from several limitations and selective degradation involves the user in post-PCR manipulations which would be less amenable to a high throughput screening regimen.

A more attractive alternative would be the production *in situ* of amplified DNA with single stranded termini. A complementary sequence covalently bound to a solid phase (eg the well of a microtitre plate), could then be used to capture the PCR product, thus providing an extra level of detection specificity. The efficiency achieved by using standard PCR protocols would also be maintained. In addition, where multiple loci are to be examined by PCR each could now be captured independently by virtue of a single-stranded tail unique to each amplification target. A single stranded tail at the other end of each amplification product may then be detected by a common signalling primer.

We report here the use of novel oligonucleotide synthesis reagents in the generation of primers with non-amplifiable tails. We demonstrate how the non-amplifiable, single-stranded tails may be used in a sandwich hybridisation format and discuss their utility in the diagnosis of infectious or inherited diseases.

MATERIALS AND METHODS

Protected thymidine methylphosphonamidite was purchased from Applied Biosystems Inc. Deprotection of oligonucleotides incorporating this reagent was performed according to the suppliers recommendations.

All solvents were analytical grade. Anhydrous pyridine was prepared by distillation from potassium hydroxide and acetonitrile was purified by distillation from calcium hydride under nitrogen. Tetrahydrofuran was distilled from sodium/benzophenone under

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nitrogen and anhydrous dichloromethane was prepared with 4A molecular sieve.

N,N-diisopropylethylamine, 2-cyanoethoxy-N,N-diisopropylchlorophosphine and chloro-N,N-diisopropylmethoxyphosphine were distilled immediately prior to use.

1H-NMR were recorded on a Brucker 200AM (200MHz) or a Brucker 250AC (250mHz) spectrometer. CI (ammonia source) mass spectra were recorded on a VG 70−250 spectrometer.

Tlc was on silica-gel 60F254 (Merck) and products were visualised using UV absorption and spraying with a solution of ammonium molybdate (5%) in aqueous sulphuric acid (20%), followed by heating.

Preparation of 1-[β-D-ribofuranosyl-3′-5′-(1,1,3,3-tetraisopropyldisiloxyl)] naphthalene {1}

1-(β-D-Ribofuranosyl)naphthalene (1.0g, 3.8mmole) prepared as described (13) was stirred in dry pyridine (15ml) for 2 hours at room temperature with 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (1.6g, 5mmole). Dichloromethane (450ml) was added and the solution washed sequentially with equal volumes of a saturated solution of sodium hydrogen carbonate and saturated brine. The crude product, (2.6g) was obtained after drying the organic solution over magnesium sulphate and removal of the solvent under reduced pressure. The product, 1-[β-D-ribofuranosyl-3′-5′-(1,1,3,3-tetraisopropyldisiloxyl)] naphthalene (0.8g, 1.6 mmole, 42%) was isolated as a colourless oil by flash chromatography on silica gel (Merck Art 9385) eluted with dichloromethane.

Found: $(M+NH_4)$ 520, (M+H) 503. $C_{27}H_{42}O_5Si_2$ requires: M, 502. d($CDCl_3$, 200 MHz); 8.12, 7.82, 7.52, (7H,m,ArH), 5.68 (1H,s,H-1′), 4.38 (1H,dd,H-3′), 4.28 (1H,d,H-5′), 4.18 (1H,d,H-2′), 4.13 (2H,m,H-4′ and H-5′), 1.0 (28H,m, $4\times CH(CH_3)_2$).

Preparation of 1-[β-D-ribofuranosyl-2′-phenylthioformate-3′,5′-(1,1,3,3-tetraisopropyldisiloxyl)]naphthalene {2}

1-[β-D-Ribofuranosyl-3′,5′-(1,1,3,3-tetraisopropyldisiloxyl)]naphthalene (0.4g, 0.8mmole) in dry acetonitrile (25ml) was stirred for 24 hours at ambient temperature with 4-dimethylaminopyridine (0.6g, 4.8mmole) and phenylthiochloroformate (0.7g, 4.0mmole). Ethyl acetate (200ml) was added and the solution sequentially washed with equal volumes of a saturated solution of sodium hydrogen carbonate and with water. The crude product was obtained after drying the solution over magnesium sulphate and removing the solvent under reduced pressure. The product 1-[β-D-ribofuranosyl-2′-phenylthioformate-3′,5′-(1,1,3,3-tetraisopropyldisiloxyl)]naphthalene (0.45g, 88%) was isolated as a white solid by flash chromatography on silica gel (Merck Art 9385) with eluant of ethyl acetate: petrol (b.p.40/60) (1:12.5).

Found: $(M+NH_4)^+$656, $(M+H)^+$639. $C_{34}H_{46}O_6SSi_2$ requires: M, 638.d($CDCl_3$, 200 MHz); 7.86, 7.45, 7.27, (12H,m,ArH), 6.40 (1H,d,H-1′), 5.88 (1H,s,H-2′), 4.58 (1H,m,H-4′), 4.37 (1H,d,H-3′), 4.16 (2H,m,2×H-5′), 1.11 (28H,m,$4\times CH(CH_3)_2$).

Preparation of 1-[2′-deoxy-β-D-ribofuranosyl-3′,5′-(1,1,3,3-tetraisopropyldisiloxyl)]naphthalene {3}

1-[β-D-Ribofuranosyl-2′-phenylthioformate-3′,5′-(1,1,3,3-tetraisopropyldisiloxyl)]naphthalene (0.4g, 0.6mmole) and 2,2′-azobis(2-methylpropionitrile), (0.1g, 0.6mmole) and n-tributyltin hydride (2.8g, 2.7mmole) were refluxed in toluene (20ml) for 30 minutes. The title compound (0.12g, 51%) was isolated by removal of the solvent under reduced pressure and purification on silica gel (Merck Art 9385) with eluant of petrol (b.p.40/60): dichloromethane (1:1.5).

Found: $(M+NH_4)$ 504, (M+H) 487. $C_{27}H_{42}O_4Si_2$ requires: M, 486. d($CDCl_3$, 200 MHz); 7.89, 7.75, 7.48, (7H,m,ArH), 5.79 (1H,t,H-1′), 4.53 (1H,ddd,H-4′), 4.20 (1H,ddd,H-3′), 4.03 (2H,ddd,2×H-5′), 2.60 (1H,ddd,H-2′), 2.28 (1H,ddd,H-2′), 1.40 (28H,m,$4\times CH(CH_3)_2$).

Preparation of 1-(2′-deoxy-β-D-ribofuranosyl)naphthalene {4}

1-[2′-Deoxy-β-D-ribofuranosyl-3′,5′-(1,1,3,3 tetraisopropyldisiloxyl)] naphthalene (0.12g, 0.24mmole) in a mixture of pyridine (10%); water (10%) and tetrahydrofuran (80%), (2ml) was added to a solution of n-tetrabutylammonium fluoride (0.73mmole) in THF and allowed to stand at ambient temperature for 16 hours. The product, 1-(2′-Deoxy-β-D-ribofuranosyl) naphthalene (19mg, 35%) was isolated as a white solid after removal of the solvent *in vacuo* and purification by chromatography on silica gel (Merck Art 9385) with eluant of methanol:dichloromethane (1:9).

Found: $(M+NH_4)$, 262. $C_{15}H_{16}O_3$ requires: M, 244. d(CD_3OD, 200MHz); 8.05 (1H,m,ArH), 7.80 (^{3}H,m,ArH) 7.48 (^{3}H,m,ArH), 5.87 (1H,dd,H-1′), 4.35 (1H,ddd,H-3′), 4.05 (1H,ddd,H-4′), 3.75 (2H,dd,2H-5′), 2.48 (1H,ddd,H-2′), 1.99 (1H,ddd,H-2′)

Preparation of 1-(2′-deoxy-β-D-ribofuranosyl-5′-O-dimethoxytrityl)naphthalene {5}

1-(2′-Deoxy-β-D-ribofuranosyl)naphthalene (0.88g, 4 mmole) was dried by co-evaporation with anhydrous pyridine (2×10ml) and then stirred at ambient temperature for 2h with dimethoxytrityl chloride (1.3g, 4.3mmole) in anhydrous pyridine (10ml). Methanol (5ml) was added and the residual oil remaining after the solvent was removed *in vacuo* taken up in chloroform (50ml). The crude product was obtained by successively washing the organic solution with equal volumes of a saturated solution of sodium hydrogen carbonate (2×) and with water (2×) and then removal of the solvent under reduced pressure after drying over magnesium sulphate ($MgSO_4$). The title compound (0.6g; 30.5%) was isolated as a pale brown solid by chromatography on silica gel (Merck Art 9385) with eluant of pyridine: methanol: dichloromethane (1:3:196), followed by co-evaporation with toluene.

d($CDCl_3$, 200MHz); 8.03 to 6.82, (20H,m, ArH), 5.88 (1H, m, H-1′), 4.47 (1H, m, H-3′), 4.12 (1H, m, H-4′), 3.78 (7H, m, 2xOCH_3 and H-5′), 3.43 (1H, m, H-5′), 2.50 (1H, m, H-2′), 2.12 (1H, m, H-2′).

Preparation of 1-[2′-Deoxy-β-D-ribofuranosyl-5′-O-dimethoxytrityl-3′-(cyanoethoxy-N,N-diisopropylamino phosphine)]naphthalene {6}

1-(2′-Deoxy-β-D-ribofuranosyl-5′-dimethoxytrityl)naphthalene (1.5g, 2.75mmole) was dissolved in anhydrous dichloromethane (20ml) containing N,N′diisopropylethylamine (1.9ml, 11mmole). The solution was stirred at room temperature under a slow stream of dry argon, while 2-cyanoethoxy-N,N-diisopropylchlorophosphine (0.8ml, 3.3mmol) was added dropwise. Stirring was maintained at room temperature for 30 minutes and the reaction was quenched by the addition of anhydrous methanol (2ml). The solution was diluted with ethyl acetate (200ml) and was washed with brine (2×100ml) and water (100ml), dried ($MgSO_4$,

filtered and evaporated to an oil which was redisolved in the minimum volume of dichloromethane:ethyl acetate: triethylamine (42:55:3) and applied to a silica column. Elution with the same solvent gave the title compound as a colourless viscous oil (1.1g, 54%).

d($CDCl_3$, 200MHz); 7.83 (^{3}H,m,ArH), 7.33 (1^3H,m,ArH), 6.82 (4H,m,ArH), 5.88 (1H,dd,H-1′), 4.65 and 4.48 (1H,m,H-3′), 4.3 and 4.15 (1H,m,H-4′), 3.8 (8H,m,2×OCH_3 and H-5′), 3.6 (2H,m,CH_2OP),2.6 (2H,m,CH_2CN), 2.57 (1H,m,H-2′), 2.18 (1H,m,H-2′), 1.31 and 1.17 (14H,m,2×$(CH_3)_2CH$).

Preparation of 1-(2′,3′-anhydro-β-D-ribofuranosyl)naphthalene {7}

A solution of 1-(β-D-ribofuranosyl)naphthalene (5.0g, 19.2mmole) in acetonitrile (50ml) was treated with acetonitrile: water (99:1) (5ml) and 2-acetoxyisobutyryl chloride (13.0g, 80mmole) and heated at 80°C for 1 hour. The reaction mixture was concentrated under reduced pressure and EtOAc (200ml) added. The solution was sequentially washed with saturated aqueous $NaHCO_3$ (3×200ml), and H_2O (3×200ml) and concentrated under reduced pressure after drying over $MgSO_4$. The resulting brown oil was taken up in MeOH (30ml) and treated with sodium methoxide (2M, 30ml) at ambient temperature for 16 hours. The crude product was obtained by stirring with Amberlite IRC 50 (5g, 50meq) for 16 hours and by removing the solvent *in vacuo*. The product 1-(2′,3′-anhydro-β-D-ribofuranosyl)naphthalene (2.5g, 54%), m.p. 88−90°C, was isolated as a white solid by chromatography on silica gel (Merck Art No 9385) with eluant of Et_3N:MeOH:CH_2Cl_2 (1:10:189).

Found: (M+H) 243, (M+NH_4) 260. $C_{15}H_{14}O_3$ requires: M, 242. d($(CD_3)_2SO$, 200MHz); 8.12, 7.93 and 7.58 (7H,m,ArH), 5.77 (1H,s,H-1′), 4.94 (1H,t,OH), 4.16 (1H,dd,H-4′), 4.13 (1H,d,H-2′ or H-3′), 3.99 (1H,d,H-3′ or H-2′), 3.5 (2H,m,2H-5′).

Preparation of 1-(3′-deoxy-β-D-ribofuranosyl)naphthalene {8}

1-(2′,3′-Anhydro-β-D-ribofuranosyl)naphthalene (2.5g, 10.3mmole) in dry THF (50ml) was added gradually to a stirred suspension of $LiAlH_4$ (1.52g, 40mmole) in THF (50ml) maintained in an atmosphere of N_2 for 16 hours at ambient temperature. Excess $LiAlH_4$ was destroyed by the gradual addition of THF/H_2O (95:5, 50ml) and the Li/Al complex decomposed with dilute HCl (1M, 50ml). The crude product was obtained by the addition of butan-1-ol (300ml) and by successively washing the organic solution with $NaHCO_3$ (aq) (2×150ml) and water (2×150ml) followed by evaporation under reduced pressure. The product, 1-(3′-deoxy-β-D-ribofuranosyl)naphthalene (0.44g, 17%), m.p. 145−147°C, was isolated as a white crystalline solid after chromatography on silica gel (Merck, Art No.9385) with eluant of MeOH:CH_2Cl_2 (1:9) and evaporation of the solvent *in vacuo* followed by recrystallisation from MeOH:H_2O (1:1).

Found: (M+H) 245, (M+NH_4) 262. $C_{15}H_{16}O_3$ requires: M, 244. d(CD_3OD,200MHz); 8.18 (1H,m,ArH), 7.82 (^{3}H,m,ArH), 7.5 (^{3}H,m,ArH), 5.54 (1H,d,H-1′),4.48 (1H,m,H-4′), 4.52 (1H,m,H-2′), 3.88 (2H,dd,2H-5′) 1.93 (2H,m,2H-3′).

Preparation of 1-(3′-deoxy-5′-O-dimethoxytrityl-β-D-ribofuranosyl)naphthalene {9}

1-(3′-Deoxy-β-D-ribofuranosyl)naphthalene (0.44g, 1.8mmole) in dry pyridine (10ml) was treated with dimethoxytrityl chloride (0.97g, 2.9mmole) for 2 hours at ambient temperature. Excess dimethoxytrityl chloride was destroyed by the addition of MeOH (10ml) and CH_2Cl_2 (50ml) was added. The crude product was obtained by successively washing the organic solution with $NaHCO_3$ aq (2×50ml) and with water (2×50ml) and by evaporation of the solvent *in vacuo* after drying over $MgSO_4$. The title compound (0.7g, 71%) was isolated as a pale brown foam after flash chromatography on silica gel (Merck, Art 9385) with eluant of MeOH:CH_2Cl_2 (1:99) and by removal of the solvent *in vacuo* followed by co-evaporation with toluene.

d(CD_3OD, 200MHz); 8.18 (1H,m,naphthalene protons), 7.83 (^{3}H,m,naphthalene protons), 7.5 (^{3}H,m,naphthalene protons), 7.24 and 6.86 (1^3H,m,ArH), 5.6 (1H,br.s.H-1′), 4.62 (1H,m,H-4′), 4.37 (1H,m,H-2′) 3.78(6H,d,2xOCH_3), 3.43 (2H,dd,2H-5′), 2.07 (1H,ddd,H-3′), 1.86 (1H,ddd,H-3′).

Preparation of 1-[3′-deoxy-5′-O-dimethoxytrityl-2′-(methoxy-N,N-diisopropylaminophosphine)-β-D-ribofuranosyl]naphthalene {10}

1-[3′-Deoxy-5′-O-dimethoxytrityl-β-D-ribofuranosyl]naphthalene (0.78, 1.3mmole) and N,N-diisopropylethylamine (0.67g, 5.2mmole) in anhydrous CH_2Cl_2 (30ml) at −40°C was stirred with chloro-N-diisopropylmethoxyphosphine (0.26g, 1.3mmole). The reaction mixture was maintained under an atmosphere of dry N_2 and attained ambient temperature after 1 hour. The title compound, along with trace amounts (<5%) of an unknown compound, (0.8g, 87%) was isolated as a yellow solid (mixture of diasteroisomers) by removing the solvent in vacuo followed by flash chromatography on silica gel (Merck Art No. 9385) with eluant of Et_3N:EtOAc (1:66) and by evaporation of the solvent.

d($CDCl_3$, 200 MHz); 8.3 and 8.17 (1H,m,naphthalene protons), 7.80 (^{3}H,m,naphthalene protons), 7.53 (^{3}H,m,naphthalene protons), 7.28 (9H,m,ArH), 6.83 (4H,m,ArH), 5.8 and 5.75 (1H,s,H-1′), 4.65 (1H,m,H-4′), 4.5(1H,m,H-2′), 3.78 (6H,s,2×OCH_3), 3.65 (2H,m,2H-5′), 3.45 and 3.36 (^{3}H,d,$POCH_3$), 2.09 and 2.02 (2H,m,2H-3′), 1.21 (14H,m,2$CH(CH_3)_2$).

Preparation of 16-O-dimethoxytritylhexadecan-1-ol {11}

1,16-Hexadecanediol (1.0g, 3.8mmole) was treated with dimethoxytrityl chloride (1.28g, 3.8mmole) in pyridine (30ml) at ambient temperature for 16 hours. The solvent was removed *in vacuo* and the product, 16−0-dimethoxytritylhexadecan-1-ol, (0.35g, 16%) was isolated as a brown oil by flash chromatography on silica gel (Merck Art 9385) with eluant of MeOH:CH_2Cl_2 (1:99).

Found: (M+H) 561. $C_{37}H_{52}O_4$ requires: M, 560. d(CD_3OD, 250MHz); 7.40, 7.76 and 6.81 (1^3H,m,ArH), 3.75 (6H,s,2×OCH_3), 3.52 (2H,t,C(16)H_2), 3.02 (2H,t,C(1)H_2), 1.33 (28H,m,14×CH_2).

Preparation of N,N-diisopropylamino(16−0-dimethoxytrityl-hexadecan-1-O)methoxyphosphine {12}

16-O-dimethoxytritylhexadecan-1-ol in CH_2Cl_2 (15ml) was stirred with chloro(methoxy)-N,N-diisopropylaminophosphine (0.36g, 1.79mmole) in the presence of N,N-diisopropylethylamine (0.92g, 7.2mmole) at ambient temperature for 2 hours. The title compound (0.62g, 48%) was isolated as a yellow oil by removing the solvent *in vacuo* and by chromatography on silica gel (Merck Art 9385) with eluant of Et_3N:hexane (1:9).

Found: (M+H) 722. $C_{44}H_{68}NO_5P$ requires: M, 721.

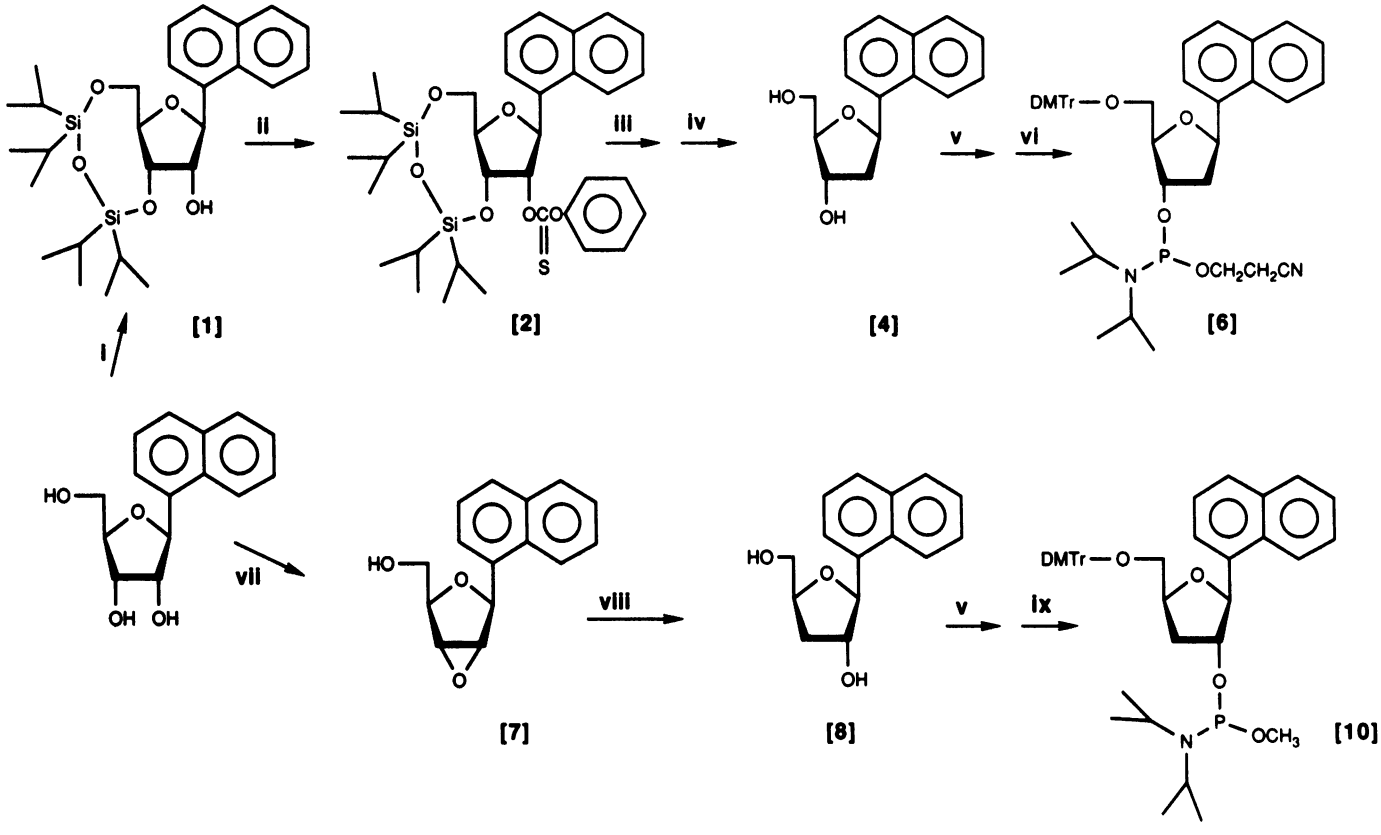

Figure 1. Synthetic route for the preparation of compounds 6 and 10. Abbreviations used; Pyr, pyridine; DMAP, 4-dimethylaminopyridine; AIBN, 2,2'-azobis(2-methylpropionitrile); DMTrCl, 4,4'-dimethoxytrityl chloride; THF, tetrahydrofuran.

d($CDCl_3$,200MHz); 7.3 and 6.8 (1^3H,m,Ar), 3.80 (6H,s,2xOCH_3), 3.59 (2H,m CH_2OP), 3.4 (^{3}H,d,$POCH_3$), 3.03 (2H,t,CH_2ODMTr), 1.25 (42H,m,14CH_2, 2$CH(CH_3)_2$).

The scheme for the synthesis of compounds 6 and 10 is shown in Figure 1. The structure of compound 12 is shown in Figure 2.

Oligonucleotide design, synthesis and alkaline phosphatase conjugation

The primary structures of the non-amplifiable tails were generated using a computer programme to generate random nucleotide sequences (D.E.Jenner, unpublished). Particularly G-rich sequences or sequences with more than three consecutive G residues were not used. All oligonucleotides were synthesised using an Applied Biosystems 380A or 380B DNA synthesiser and are presented in Table 1. Oligomers 1 to 6 were those used as templates in the *Taq* DNA polymerase inhibition assay using oligonucleotide 7 as the primer. Oligomers 8, 9 and 10 were used as ARMS (14) primers. Oligomers 11 to 13 were immobilised to microtitre dish wells to test the specificity of capturing the test oligomers 14 to 16 (Table 1). Oligomers 11 and 12 were also used as the immobilised capture primers for the ARMS products of oligomers 9 and 10 with the common ARMS primer 11 which generates a single stranded tail complementary to the signal oligonucleotide 17. The 5'$(dT)_{10}$ residues of the capture and signal oligomers were included as spacer elements between the hybridisation regions and their respective conjugants.

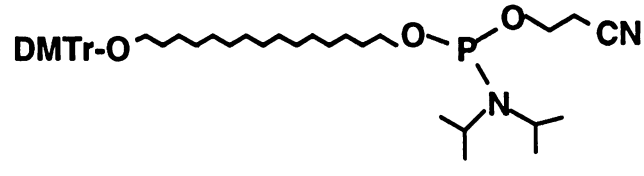

Figure 2. Structure of C_{16} phosphoramidite (compound 12).

Oligonucleotides 1–4, 6 and 7 were purified by acrylamide gel electrophoresis, u.v. shadowing, butan-1-ol concentration, phenol/chloroform extraction and ethanol precipitation. Oligonucleotide 5 was purified by HPLC using an anion exchange column (Mono Q, Pharmacia) equilibrated with Buffer A (10mM NaOH, 0.5M NaCl). The oligonucleotide was eluted with a gradient of Buffer B (10mM NaOH, 0.9M NaCl) 0–100% in 60 minutes. Fractions corresponding to the product peak were pooled and desalted using a PD10 column (Pharmacia) then concentrated by drying under vacuum. Particularly rigorous purification of oligonucleotides 1–6 was essential to avoid the presence of coupling failure sequences giving misleading results in the *Taq* DNA polymerase inhibition assay. Oligonucleotides 8–10 were purified by reverse phase HPLC.

The purity of the oligonucleotides was checked by 5'-^{32}P labelling using 5'-g^{32}P ATP (Amersham) and T4 polynucleotide

Table 1. Oligonucleotides used in this study.

Oligomer	Nucleotide sequence (5'→3')
1	AATTCCGTGCATAAGGCTGTGCTGACCATCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG
2	AATTCCGTGCATAAGGCTGTGCTGNNNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG
3	AATTCCGTGCATAAGGCTGTGCTGNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG
4	AATTCCGTGCATAAGGCTGTGCTGNNNNTCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG
5	AATTCCGTGCATAAGGCTGTGCTG(C16)TCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG
6	AATTCCGTGCATAAGGCTGTGCTG(T(Me))4TCGACGAGAAAGGGACTGAAGCTGCTGGGGCCATG
7	CATGGCCCCAGCAGCTTCAGTCCCTTTCTC
8	GTGTCGTCCCGCAGTCAATGNNGGGTAGTGTGAAGGGTTCATATGCATAATC
9	TATAGGCAAGTAAGTTGATANNGCCTGGCACCATTAAAGAAAATATCATCTT
10	ACGATTGTTAACCAAGATCTNNGCCTGGCACCATTAAAGAAAATATCATTGG
11	TTTTTTTTTTTATCAACTTACTTGCCTATA
12	TTTTTTTTTTAGATCTTGGTTAACAATCGT
13	TTTTTTTTTTCGGTCCTCATAATACATACT
14	TTTTTTTTTTATAGGCAAGTAAGTTGATA
15	TTTTTTTTTTACGATTGTTAACCAAGATCT
16	TTTTTTTTTTAGTATGTATTATGAGGACCG
17	TTTTTTTTTTCATTGACTGCGGGACGACAC

Nucleotide sequence of oligonucleotides used in this study. Oligomer 1 is the control template for the *Taq* DNA polymerase inhibition assay. Oligomer 2, N represents a 2'-deoxyribofuranosyl naphthalene moiety, 3',5' phosphodiester linked (prepared using compound 6). Oligomers 3 and 4, N represents a 3'-deoxyribofuranosyl naphthalene moiety, 2',5' phosphodiester linked (prepared using compound 10). Oligomer 5, where C_{16} represents addition of the C_{16} PA (compound 12). Oligomer 6, in which T(Me) represents a methyl phosphonate substituted thymidine residue. Oligomer 7 is complementary to the thirty 3'-residues of oligonucleotides 1–6. Oligomers 8–10, (where N represents a 3'-deoxyribofuranosyl naphthalene moiety, 2',5' phosphodiester linked), are the primers that generate amplification refractory mutation system (ARMS, 14) products with single-stranded tails for the cystic fibrosis ΔPhe_{508} mutation (15). Oligomer 8 generates a single-stranded tail complementary to the signal generating oligonucleotide 17. Oligomer 9 generates a single-stranded tail complementary to the capture oligonucleotide 11. Oligomer 10 generates a single-stranded tail complementary to the capture oligonucleotide 12.

kinase followed by polyacrylamide gel electrophoresis under denaturing (7M urea) conditions.

Oligonucleotides 14–17 were synthesised as 5' aminoalkyl derivatives using aminoalkyl phosphoramidite (Amino-link 2, Applied Biosystems) in the final synthesis cycle on the DNA synthesiser. These oligonucleotides were conjugated to AP using the E-LINK™ oligonucleotide labelling kit as directed by the supplier (Cambridge Research Biochemicals).

Taq DNA polymerase inhibition assay

Oligonucleotide 7 (30 pmol) was 5' radiolabelled using 5'-g^{32}P ATP 75μCi (15 pmole), (Amersham) and T4 polynucleotide Kinase (4 units), (Boehringer). The reaction was terminated by heating at 100°C for 5 minutes, followed by a phenol: chloroform (1:1) extraction and ethanol precipitation.

Reactions were performed in 100μl containing 1.2mM $MgCl_2$, 50mM KCl, 10mM Tris HCl (pH 8.3) and 0.01% gelatin, 1 pmole of radiolabelled oligonucleotide 7, 3 pmoles of the appropriate long oligonucleotide 1–6 and two units of *Taq* DNA polymerase (Cetus—Amplitaq). The dNTPs, where included, were added to a final concentration of 100μM each. The reactions were incubated at 91°C for 2 minutes then 60°C for 58 minutes. 5μl aliquots were removed from each reaction, mixed with 5μl formamide dye (80% formamide, 10mM NaOH 1mM EDTA and 0.1% Bromophenol Blue) and denatured by heating at 100°C for 5 minutes. The aliquots were electrophoresed on a 15% denaturing polyacrylamide gel, 8M urea in TBE buffer (0.089M Tris borate, 0.089 boric acid, 0.002M EDTA) and autoradiographed. The general scheme of the assay is shown in Figure 3.

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5'CATGGCCCCAGCAGCTTCAGTCCCTTTCTC.....3'
3'GTACCGGGGTCGTCGAAGTCAGGGAAAGAGCAGCT(X)nGTCGTGTCGGAATACGTGCCTTAA5'
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Figure 3. Design of extension blocking experiment. The top oligonucleotide is the primer with a dotted line indicating the expected extension under normal conditions. The bottom oligonucleotide shows the general design of the template where X indicates the site of the analogue incorporation. In the positive control template X is replaced by the dinucleotide AG.

Thiolation of capture oligonucleotides, oligonucleotides 11, 12 & 13

The capture oligonucleotides (200μl, 0.2μMol synthesis) were mixed separately with 4mg/ml iminothiolane in 0.2M Na_2CO_3/$NaHCO_3$ pH 9.6 (300μl) and incubated at ambient temperature for 1 hour. The reactions were diluted to 1ml with phosphate buffered saline (PBS) and desalted on a Nap-25 column (Pharmacia). The columns were washed with PBS (2.2ml). The first 1.6ml eluted was retained and the oligonucleotide concentrations were measured by u.v. spectrometry.

Immobilisation of thiolated oligonucleotides to microtitre dishes

Oligonucleotide immobilisation to microtitre wells was performed using a modification of the method described by Running and Urdea (16). Poly (Phe-Lys) (Sigma) was dissolved to 100μg/ml in 50mM Na_2CO_3/$NaHCO_3$, pH 9.6 and 100μl of this solution was added to each well of five clear polystyrene microtitre dishes (Nunc). The dishes were incubated overnight at 4°C then washed 3 times with PBS/0.05% Tween. Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) was dissolved at 8.7mg/ml in dry dimethylformamide and this solution was diluted 10 times with conjugation buffer (100mM triethanolamine HCl, 1mM $MgCl_2$, 1mM $ZnSO_4$, pH 7.4). 100μl of this solution was added to each microtitre well and the plates were incubated at ambient temperature for 1 hour. The wells were washed 5 times with water.

Thiolated oligonucleotides, 10μM in PBS, were added to appropriate microtitre wells (100μl per well). These were incubated overnight at 4°C. The wells were washed twice with PBS/0.05% Tween, twice with 5% lactose, 0.5% gelatin, 0.1% NaN_3, 6mM PBS, pH 7.5. Finally the coated dishes were dried overnight in a laminar flow cabinet. The dishes were stored at 4°C *in vacuo*.

RESULTS

Novel phosphoramidites can block extension by *Taq* DNA polymerase

A number of novel phosphoramidites were synthesised allowing non-standard nucleoside moieties to be incorporated into oligonucleotides by automated synthesis. 1-(2'-Deoxy-β-D-ribofuranosyl)naphthalene {4} was prepared by the general procedure described by Robins and Wilson (17). Condensation of naphthalene with a fully protected ribose by the literature method (13) allowed us to obtain 1-(β-D-ribofuranosyl)naphthalene in a reasonable yield. (For the sake of simplicity, we refer to this analogue as 'naphthosine'.) Naphthosine was converted to 2'-deoxynaphthosine {4} by reduction of the 2'-phenylthioformate derivative {2}, prepared after selective protection of the 3'- and 5'-hydroxyls. After desilylation, the 5'-hydroxyl was protected by formation of the dimethoxytrityl ether and the 3'-hydroxyl was phosphitylated by standard methods to give {6},(18, 19). A shorter route to this compound, involving

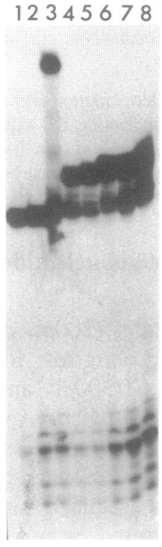

Figure 4. Autoradiograph of polyacrylamide gel after electrophoresis of the *Taq* DNA polymerase inhibition assay reactions. Lane 1; 5′^{32}P primer (oligomer 7): lane 2; 5′^{32}P primer under assay conditions without template: lane 3; 5′^{32}P primer under assay conditions with control oligomer 1 template; lanes 4 to 8; 5′^{32}P primer under assay conditions with substituted oligomers 2 to 6 as templates. If DNA polymerisation is not prevented by the polymerisation blocking moiety the full length oligonucleotide will be copied to give a 63 mer product as expected in control reaction (lane 3). However, if the blocking moiety stops any further *Taq* DNA polymerase incorporation then a 35 mer product is expected (lanes 4 to 8), representing chain extension up to the substitution.

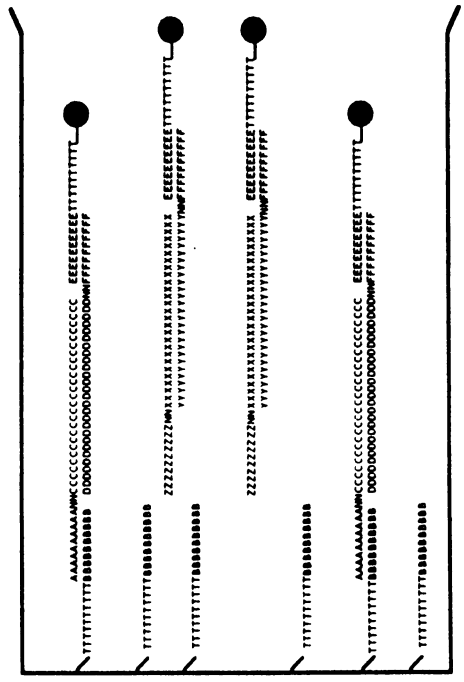

Figure 5. Diagram outlining the scheme of the microtitre dish assay: microtitre well with sequence 'oligo B' immobilised via the oligo dT tail. PCR products from 'oligo C' and 'oligo D' with respective 'oligo A' and oligo F' tails; 'oligo A' is complementary to 'oligo B' and is captured The PCR products from 'oligo X' and 'oligo Y' with respective 'oligo Z' and oligo F' tails; 'oligo Z' is not complementary to 'oligo B' and is not captured. A common detection primer, 'oligo E' conjugated to a signal generation system (filled circle) via an oligo dT tail can be used to detect any immobilised PCR product. In an adjacent microtitre well an immobilised oligonucleotide complementary to 'oligo Z' would capture a PCR product incorporating the 'oligo Z' tail and not one incorporating the 'oligo A' tail.

the condensation of naphthalene with 1,3,5-tri-*O*-acetyl-2-deoxyribose has recently been developed (D. Picken and M. Gray, Personal communication). The 3′-deoxy analogue {8} was obtained by conversion of naphthosine to the 2′, 3′ anhydro compound {7} by treatment with 2-acetoxyisobutyryl chloride followed by reaction with sodium methoxide (20). The furanose sugar epoxide proved to be resistant to reductive ring opening with $LiET_3BH$ (21), but the highly regioselective conversion to {8} was achieved with $LiAlH_4$ at 60°C for 1 hr. The 5′-protected phosphoramidite {10} was obtained by standard procedures (18, 19). The synthetic routes to these two phosphoramidites are shown schematically in Figure 1.

The C_{16} spacer arm phosphoramidite (Figure 2) was prepared by sequential dimethoxytritylation and phosphitylation of 1,16-hexadecanediol. (While this manuscript was in preparation an alternative use for this reagent has been reported by Brown and co-workers (22) who suggest that the presence of the hexadecane moiety at the 3′-end of oligonucleotides may increase the lipophilicity of antisense probes.)

Each analogue was redissolved to a concentration of 0.1M in anhydrous acetonitrile and attached to one of the spare ports on the DNA synthesiser. By extending the wait time during coupling to five minutes, coupling yields of 95% or greater were achieved. In addition to oligonucleotides containing these non-base-pairing analogues, we also prepared oligonucleotide templates with potential blockade caused by runs of ionically neutral methyl phosphonates in place of phosphate groups.

The rationale behind the design of the novel phosphoramidites was that by providing no hydrogen bond potential they would not form Watson–Crick base pairs with normal nucleotides, and therefore chain extension by DNA polymerase should be blocked when these residues were incorporated within a single-stranded DNA template. In order to test this hypothesis we prepared a number of oligonucleotides using these compounds. We attempted to extend a ^{32}P-labelled complementary oligonucleotide primer using *Taq* DNA polymerase with these longer, analogue-substituted oligonucleotides as templates.

The templates were designed with a gap of five bases between the primer complementarity region and the putative blocking sites.

Table 2. Specificity of capture and detection oligomers bound to microtitre plates.

Signal oligomer	Plate oligomer		
	11	12	13
14	1.2	0.04	0.08
15	0.03	0.16	0.03
16	0.04	0.03	0.79

Values are absorbance units at 405 nm. Oligonucleotides 11, 12 and 13 are complementary to oligonucleotides 14, 15 and 16 respectively. (n=2).

Table 3. Results of binding PCR products to microtitre plates using single-stranded tails.

Sample (n=8)	Negative control (n=8)
0.71	0.26

Values are absorbance units at 405 nm.

This allowed us to ascertain that chain extension had indeed started and that polymerase activity was not generally inhibited. The results of these experiments are shown in Figure 4. When a normal (non-substituted) control oligonucleotide template was used, the primer was extended the full length (Lane 3). However when the template contained 2 or more of our nucleotide analogues (lanes 4−7), extension was terminated after 4 or 5 bases had been incorporated, indicating that extension was inhibited at the point of insertion of the first of the nucleotide analogues.

Production of single-stranded tails in PCR

Having demonstrated that the non- nucleotidic analogues could block extension by *Taq* DNA polymerase, we wished to demonstrate that single-stranded tails on PCR products could be generated. To accomplish this we designed an assay in which a single-stranded tail on a PCR product could be captured by an oligonucleotide covalently bound to a microtitre plate. The PCR product was detected by hybridising another oligonucleotide (the detection oligonucleotide), to the tail at the other end of the PCR product (see Fig 5 and Table 3). The detection oligonucleotide was covalently linked to alkaline phosphatase. The oligonucleotide coated microtitre plates were tested for specificity using complementary oligonucleotides which were also covalently linked to alkaline phosphatase for eventual signal generation. Hybridisation was detected using p-nitrophenyl phosphate (PNPP) a colourimetric substrate for alkaline phosphatase (23). The results, (Table 2) show that there is high degree of specificity of capture for three different pairs of binding and capture oligonucleotides. Microtitre plates functionalised as described and tested in this way were stable over one year when stored at 4°C.

PCR primers to amplify exon X of the human CFTR (cystic fibrosis transmembrane regulator) gene (13) containing 2 naphthosine residues and a 20 base tail were synthesised (oligonucleotides 8−10, see table 1). These primers were used in PCRs of human genomic DNA and produced bands of the expected size (data not shown).

The microtitre plate system was used to detect the CFTR PCR products generated. A control PCR reaction was performed whereby the tail at the capture end of the PCR product was designed to be non-complementary to the immobilised capture oligonucleotide. The 20 base tail at the signal end was common to both PCR reactions. Table 3 shows the discrimination between these two PCR products in the assay indicating that the expected single-stranded tails were present on the PCR products.

DISCUSSION

A number of novel phosphoramidites were synthesised in the expectation that they could be used to synthesise primers that would allow the production of single-stranded tails on PCR products. We have shown that templates incorporating these compounds block chain extension by *Taq* DNA polymerase. We have also demonstrated the use of these compounds in a simple PCR assay that does not require denaturation or partial degradation of PCR products.

There are a number of advantages of using oligonucleotides as capture and detection affinity partners compared to other assay systems.

1) The theoretical availability of an almost infinite number of binding pairs. This would be particularly convenient for multi-analyte screening; for example, the screening for a number of different mutations or polymorphisms in the same genome using ARMS (14) and the identification of specific pathogens in biological specimens. Such utility cannot be attributed to other systems such as Dynabeads (24) where a single binding pair (biotin and streptavidin) is employed. Clearly, the advantage of the non-amplifiable tails allows the isolation of a specific PCR product from a cocktail of amplified DNA species.

2) It is not necessary to denature PCR products for either capture or detection. This does not therefore allow hybridisation of the captured DNA with an internal oligonucleotide. This does not present a problem however, since internal sequences of any given PCR product can be analysed simultaneously by ARMS (14).

3) All reactions can be performed in standard hybridisation buffers. When using antigen/antibody binding pairs it may be necessary to sacrifice sensitivity in order to optimise antigen antibody interactions.

4) The system is potentially more versatile than other systems. In theory, once assay conditions are optimised for one pair of oligonucleotides these conditions should apply for all oligomers of similar length and base composition.

It should be noted that we have used single-stranded tails as both detection and capture moieties. This gives two levels of specificity. It may be more convenient to use a single-stranded tail at only one end of the PCR product for capture. Another signal generation system e.g. direct fluorescence of the other fluoresceinylated PCR primer could then be used.

Mispriming is a possible complication of any PCR based assay. The likelihood of this phenomenon occurring in the non-amplifiable tail assay can be reduced by initially testing target sequences with conventional amplimers. This potential problem can however, be completely avoided if the non-amplifiable tail oligonucleotides are used as nested primers in a secondary PCR reaction. This should pose only minor inconvenience if the PCR reactions are carried out in microtitre dishes to compliment the microtitre capture and detection format.

It was found in our *Taq* DNA polymerase blocking assay for these compounds that there was some 'readthrough' of the first base analogue. Indeed when we prepared templates with a single base analogue residue there was often poor termination (data not shown). This suggests that *Taq* DNA polymerase is capable of inserting one base opposite the site of the base analogue. However with all the compounds described here, we found that two or more residues were sufficient to terminate extension. Presumably if one nucleotide is added opposite a non-base-pairing analogue then the capacity to add another is particularly compromised, unless the potential for correct hydrogen bonding is restored.

This conclusion may only be applicable to *Taq* DNA polymerase however. Other groups have examined incorporation of nucleotides opposite abasic sites in a template molecule by other DNA polymerases (25−27). Specifically, it has been demonstrated that *Drosophila* DNA polymerase a does extend past an abasic site but extension is compromised (25). Avian myeloblastosis virus reverse transcriptase, the Klenow fragment of *E.coli* DNA polymerase I and calf thymus DNA polymerase a all appear to have similar properties (26). Another study suggests that *Drosophila* DNA polymerase a does not extend past a similar abasic site (27) in contradiction to Ref 24. It is difficult to reconcile these differences in findings for incorporation opposite a abasic sites with the same enzyme (25 and 27). These effects may be both primer and template sequence dependent since

base stacking does effect the efficiency of incorporation opposite these abasic sites (25). Such findings could possibly reflect our observations that two or more base analogues may be required to completely block *Taq* DNA polymerase primer extension. However, different DNA polymerases are likely to exhibit different properties and we have not extended our studies to the blockade characteristics of the nucleotide analogues described herein to DNA polymerases other than *Taq* DNA polymerase. It is therefore conceivable that these nucleotide analogues may have utilities other than those outlined in this work. For instance, if the nucleotide analogues do not block primer extension *in vivo* through being excised by DNA repair processes, these analogues may allow the precise directional cloning of PCR products or the ordered ligation of many PCR products for subsequent cloning. Such benefits would be additional to the diagnostic application arising as a result of the ability to produce PCR products containing single-stranded tails by the use of primers synthesised with these novel phosporamidites. Experiments to address these questions are in progress and will be reported separately. Non-amplifiable tails should prove to be particularly useful in the design of automatable PCR assay formats for both inherited and infectious diseases.

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REFERENCES

1. Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I. and Khorana, H. G. (1971) *J. Mol. Biol.*, **56,** 341–361.
2. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985) *Science*, **230,** 1350–1354.
3. Yamane, A., Nakagami, S., Kawasoe, T. and Miyoshi, K. (1988) *Nucl.eic Acids Res. Symp. Ser.*, 20, 91–92.
4. Gregersen, N., Winter, V., Bruun Petersen, K., Koch, J., Klvraa, S., Rüdiger, N., Heinsvig, E-M. and Bolund, L. (1989) *Clin. Chim. Acta.*, **182,** 151–164.
5. Houlston, R. S., Snowden, C., Green, F., Alberti, K. G. M. M. and Humphries, S. E. (1989) *Hum. Genet.*, **83,** 364–368.
6. Saiki, R. K., Walsh, P. S., Levenson, C. H. and Erlich, H. A. (1989) *Proc. Natl. Acad. Sci. U.S.A.*, 86, 6230–6234.
7. Kemp, D. J., Smith, D. B., Foote, S. J., Samaras, N. and Peterson, M. G. (1989)*Proc. Natl. Acad. Sci. U.S.A.*, **86,** 2423–2427.
8. Keller, G. H., Huang, D-P., Shih, J. W-K. and Manak, M. M. (1990) *J. Clin. Microbiol.*, 28, 1411–1416.
9. Saiki, R. K., Chang, C-A., Levenson, C. H., Warren, T. C., Boehm, C. D., Kazazian, H. H. and Erlich, H. A. (1988) *N. Engl. J. Med.*, 391, 537–541.
10. Sauvaigo, S., Fouqué, B., Livache, T., Bazin, H., Chypre, C. and Téoule, R. (1990) *Nucleic Acids Res.*, 18, 3175–3183.
11. Gyllensten, U. B. and Erlich, H. A. (1988) *Proc. Natl. Acad. Sci. U.S.A.*, **85,** 7562–7657.
12. Higuchi, R. G. and Ochman, H. (1989) *Nucl. Acids Res.*, **17,** 5865.
13. Ohrui, H., Kuzuhara, H. and Emoto, S. (1972) *Agr. Biol. Chem.*, **36,** 1651–1653.
14. Newton, C. R., Graham, A., Heptinstall, L. E., Powell, S. J., Summers, C., Kalsheker, N., Smith, J. C. and Markham, A. F. (1989) *Nucleic Acids Res.*, **17,** 2503–2516.
15. Riordan, J. R., Rommens, J. M., Kerem, B-T., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J-L., Drumm, M. L., Jannuzzi, M. C., Collins, F. S. and Tsui, L-C. (1989) *Science*, **245,** 1066–1072.
16. Running, J.A. and Urdea, M.S. (1990) *BioTechniques* **8** 276–277.
17. Robins, M. J. and Wilson, J. S. (1981) *J. Am. Chem. Soc.*, **103,** 932–933.
18. Jones, R. A. (1984) In Gait, M. J. (ed.) Oligonucleotide synthesis-A practical approach. IRL Press, Oxford pp. 27–28.
19. Atkinson, T. C. and Smith, M. (1984) In Gait, M. J. (ed.) Oligonucleotide synthesis-A practical approach. IRL Press, Oxford pp. 41–45.
20. Russell, A. F., Greenberg, S. and Moffat, J. G. (1973) *J. Am. Chem. Soc.*, **95,** 4025–4030.
21. Hansske, F. and Robins, M. J. (1985) *Tetrahedron Lett.*, **26,** 4295–4298.
22. Mackellar, C., Graham, D., Will, D. W., Burgess, S. and Brown, T. (1992) *Nucl. Acids Res.*, 20, 3411–3417.
23. Garen, A. and Levinthal, C. (1960) *Biochim. Biophys. Acta* **38** 470–483.
24. Wahlberg, J., Lundeberg, J., Hultman, T. and Uhlén, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 6569–6573.
25. Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J. and Goodman, M. F. (1987) *J. Biol. Chem.*, **262,** 6864–6870.
26. Takeshita, M., Chang, C-N., Johnson, F., Will, S. and Grollman, A. P. (1987) *J. Biol. Chem.*, **262,** 10171–10179.
27. Ng, L., Weiss, S. J. and Fisher, P. A. (1989) *J. Biol. Chem.*, **264,** 13018–13023.