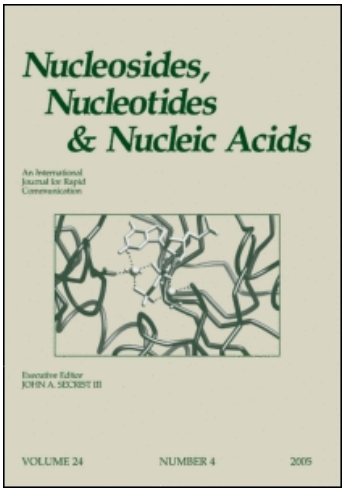

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5'-TAILED OCTANUCLEOTIDE PRIMERS FOR CYCLE SEQUENCING

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ABSTRACT: Recently we reported the use of octamer primers tailed with 5-nitroindole for use as primers in cycle sequencing reactions. Here we report the successful use of some other universal base analogues to improve the effectiveness of an octamer sequencing primer. These analogues are 5-nitroindazole, 3-nitropyrrole and benzimidazole.

Introduction

The synthesis of oligonucleotides for use as walking primers is a feature of almost all DNA sequencing projects, regardless of their scale. For cycle sequencing, oligonucleotide primers from 17 to 24 residues in length are generally used. To avoid both the expense and the delay inherent in the repeated synthesis of new primers, a number of attempts have been made to use presynthesised sets (libraries) of short oligonucleotides,[1,2] either as primers or for sequencing by hybridisation. An interesting approach uses combinations of contiguous short oligomers; their combination increases the stability of the duplexes formed and allows them to prime DNA synthesis.[3,4] This method has not been widely adopted and it seems to be unsuited to cycle sequencing because of the low annealing temperatures required.

We,[5] and others,[6,7] have considered the use of octamer primers for sequencing reactions. Octamers, however, are often ineffective in cycle sequencing. Because of the low dissociation temperatures (Td) of the 8-mer hybrids with the template, stable

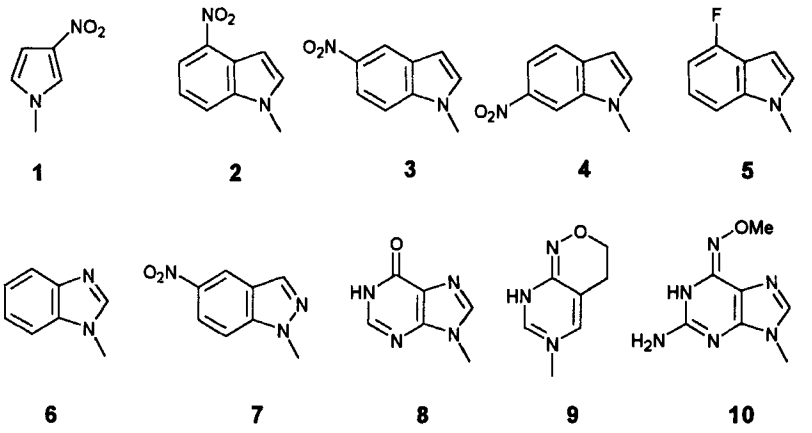

Figure 1. Base residues of 2'-deoxyribosyl nucleoside derivatives used to tail octamer primers for cycle sequencing.

primer/template/enzyme complexes are not formed. In this paper, we examine further the use of 8-mers with 5'-tails of universal base analogues, and show that some improve priming in sequencing reactions. An important feature of these tails is that such modifications do not increase the size of the primer library.

The 2'-deoxyribosyl derivatives of ten different base analogues have been examined: 3-nitropyrrole (**1**),[8] 4-, 5- and 6-nitroindole (**2-4**),[9] 5-fluoroindole (**5**), benzimidazole (**6**),[10] 5-nitroindazole (**7**), hypoxanthine (**8**) (which is often used as a universal base analogue[11,12]) and an equimolar mixture of the degenerate bases P (**9**) and K (**10**)[13] (Figure 1). These analogues have been used to 5'-tail the primers with up to twelve residues in an attempt to aid stabilisation of the 8-mer and the efficiency of each of these has been examined in cycle sequencing reactions.

Results

An octamer primer which works reasonably well without any modification was used. Thus any deleterious effect of a modification was easy to determine and this formed a suitably challenging case for attempting improvement by tailing. 5'-tails (3, 6, 9 or 12 residues in length) for each of the modified bases (X) were added to the control 8-mer oligonucleotide primer comprising the eight terminal bases of the (-40) M13 universal

```
5' -                    GTCACGAC   (control)
5' -                XXX GTCACGAC
5' -            XXX XXX GTCACGAC
5' -        XXX XXX XXX GTCACGAC
5' - XXX XXX XXX XXX GTCACGAC
```

Figure 2. Octamer primers, based on universal primer, 5'-tailed with universal base analogues X. X is each of the analogues shown in Figure 1.

primer (5'- GTCACGAC) (Figure 2).[15] In each case, the reactions were performed using either 5 pmol or 25 pmol of primer and the annealing temperature was varied from 20°C to 50°C, in increments of 10°C, to determine the optimum annealing temperature for cycle sequencing.

At 25 pmol, the 8-mer tailed with three 2'-deoxyinosines produced some analysable data with an annealing temperature of 30°C, but it was of low intensity with a high background and short read length, containing many ambiguities. The data was significantly worse than that obtained for the control 8-mer, and all longer primers gave no analysable data. Tailing the 8-mer with an equal mixture of the degenerate pyrimidine (P) and the degenerate purine (K) residues at each position[16] was detrimental to priming. No analysable data was obtained with tails of any length, under any of the conditions used in contrast to the successful primers studied earlier,[16] in which the P/K mixture residues were dispersed non-consecutively amongst normal base residues.

We then turned our attention to other universal base analogues. These were expected to stabilise the primers but to behave indiscriminately in their interaction with the template. Bergstrom *et al* described 3-nitropyrrole as a universal base in primers for sequencing.[8] Four oligonucleotides with 3-nitropyrrole were tested as primers in cycle sequencing. The 8-mer with three 3-nitropyrrole residues gave approximately a 2-fold increase in signal intensity, but longer tails of 3-nitropyrrole gave no analysable data. Next, a set of 8-mers tailed with 5-nitroindole residues[9] was tested. As with 3-nitropyrrole, the longer tails were detrimental to the quality of the sequence data. With a tail of three 5-nitroindole residues, the intensity of the sequence data was 7-fold greater than for the control at an annealing temperature of 30°C;[5] at 40°C good quality sequence

data was still obtained, while the control failed to any give analysable data. The data obtained using the 5-nitroindole tailed primer is at least as good as that obtained using the full length 24-mer universal primer under these conditions.

Van Aerschot *et al* have reported the use of an acyclic 5-nitroindazole nucleoside analogue as a universal base analogue.[17] Despite the presence of an acyclic sugar moiety, oligonucleotide duplexes containing this compound retained good thermal stability, and oligonucleotides containing it were able to prime sequencing reactions.[18] The 2'-deoxyribonucleoside derivative of 5-nitroindazole (**15**) was therefore prepared for comparison with those of 4-, 5- and 6-nitroindole,[9] as well as that of 5-fluoroindole (**11**) and benzimidazole (**17**). Despite the fact that both 4- and 6-nitroindole have been shown to behave indiscriminately towards the natural bases, neither of these analogues, nor the 5-fluoroindole gave any analysable sequence information when their nucleoside derivatives were incorporated at the 5'-end of the 8-mer control primer. 5-Nitroindazole nucleoside (**15**) tailed oligomers in contrast behaved almost identically to those containing 5-nitroindole, with three residues giving good sequence data when the reactions were carried out at an annealing temperature of 30°C. With the nucleoside derivative of benzimidazole to tail oligomers, a tail of three residues was less effective, but still gave good sequence data. In all cases, longer tails were detrimental to the quality of the sequencing data obtained.

Previously we had demonstrated that the optimal tail length of 5-nitroindole residues was 3 or 4.[5] A similar set of primers was prepared containing one to six benzimidazole residues. All the primers performed better than the control 8-mer. The optimal length of the tail was found to be two, giving a 75% increase in signal intensity compared to the control primer. However, it was less effective than using the nucleoside derivative of 5-nitroindole tailed oligomers. A number of oligomers were prepared using tails consisting of both 5-nitroindole and benzimidazole (alternating 5-nitroindole and benzimidazole as well as three of one analogue followed by three of the other, Table 1). These were prepared in an attempt to minimise stacking between the universal bases; they gave signal intensities that, on the whole, were better than those obtained using benzimidazole alone, but were less effective than tails containing only 5-nitroindole. Also, longer tails (six residues) comprised of both of these universal bases were less effective than shorter tails containing just one analogue.

Table 1. 5 and 6 denote 5-nitroindole and benzimidazole nucleosides respectively. Signal intensities are a fluorescent photomultiplier tube output measurement recorded by the automated sequencer for each set of reactions.

Primer	Range of intensities
GTCATGAC	138-88
555GTCATGAC	470-347
6655GTCATGAC	296-220
5566GTCATGAC	173-151
6565GTCATGAC	317-241
5656GTCATGAC	288-226
555666GTCATGAC	149-123
666555GTCATGAC	126-99

Discussion

Standard approaches to DNA sequencing either place fragments of the DNA to be sequenced adjacent to a single priming site or employ walking with custom primers. Thus, subcloning the fragments into, for example, pUC plasmids allows a pair of vector primers to be used repeatedly. The synthesis of custom primers is, however, expensive and wasteful if each primer is used only once. In an alternative approach, a library of reusable short primers might be used for primer walking. A complete library of octamers comprises 65,536 (4^8) oligomers, an unwieldy number to synthesise and store for most facilities. Nevertheless, a subset of the complete library, containing only 1500 primers, has the following probabilities of yielding a suitable primer: a 90% probability within 99 bases of the end of the previous sequence read; a 99% probability within 198 bases and 99.9% probability within 297 bases.[5]

Since carrying out our initial experiments, Mirzabekov's group has reported using oligonucleotides tailed with 5-nitroindole residues for hybridisation on microchips.[19,20] In these papers, one or two 5-nitroindole residues were incorporated at either or both ends of short oligonucleotides (pentamers, or hexamers) to aid duplex stabilisation. We have now assessed ten base analogues for their effectiveness as 5'-tails on octamer oligonucleotides used as primers in cycle sequencing reactions. More than 80 modified

primers have so far been tested. Polymerase priming is more demanding of a modified oligonucleotide than hybridisation alone. Nevertheless, we have been able to show that some tailed 8-mers produced improved sequence data compared to the corresponding control 8-mers. Thus, the use of modified 8-mer libraries is a realistic possibility.

In an extension of this work, we have determined the complete sequence of one strand of the vector M13mp18, which had previously only been collated from other sources.[21] Thirty-five pairs of primers, either octamers or the same oligonucleotide sequence tailed at the 5' end with four residues of 5-nitroindole were used. The presence of a 5'-tail consistently allowed the use of higher annealing temperatures, and, as before,[5] some sequences produced useful sequence data only when tailed. Details will be reported elsewhere.

In conclusion, the addition of a 5'-tail of a number of universal bases stabilises short (octamer) primers so that they may be used in cycle sequencing. Of the ten base analogues which we have investigated, the best for this purpose were 5-nitroindole and 5-nitroindazole. Taking into account the relative ease of synthesis of the former, this is the preferred tail. Of the analogues tested, four were ineffective; 3-nitropyrrole and benzimidazole tails also improved priming efficiency. These results are summarised in Table 2. Further development of these findings could both expedite and decrease the cost, and facilitate automation of primer walking.

Experimental

General methods ^{1}H-NMR and ^{31}P-NMR spectra were obtained on a Bruker DRX 300 spectrometer, and spectra were obtained in d^{6}-DMSO. ^{1}H chemical shifts are given in ppm relative to Me_4Si and J values in. ^{31}P-NMR spectra are externally referenced to phosphoric acid. Ultraviolet spectra were recorded on a Perkin Elmer Lambda 2 spectrophotometer. Samples were dissolved in 1% aqueous methanol unless otherwise stated. Tlc was carried out on pre-coated F_{254} silica plates and column chromatography with Merck kieselgel 60. Unless otherwise stated reactions were worked up as follows: after removal of the solvent, the product was dissolved in chloroform and washed with aqueous sodium bicarbonate solution. The combined organic fractions were dried over sodium sulphate and evaporated.

Table 2. Relative efficiencies of each of the base residues to promote priming in cycle sequencing reactions when added as a 5'-tail to the control sequence. The control sequence is given in Figure 2, the bases **1-10** are shown in Figure 1. Data is taken for cycle sequencing reactions using an annealing temperature of 30°C, and using a tail of three residues.

Control	1	2	3	4	5	6	7	8	9/10
+	++	-	+++++	-	-	+++(+)	+++++	+/-	-

1-(2-Deoxy-β-D-ribofuranosyl)-5-fluoroindole 11. To a solution of 5-fluoroindole (1g, 7.4mmol) in acetonitrile (25ml) was added sodium hydride (60%, 0.33g, 8.25mmol) and the solution stirred at room temperature for 30 minutes. To this was added 2-deoxy-3,5-di-O-*p*-toluoyl-α-D-ribosyl chloride (3.45g, 8.8mmol) and stirring continued for 2 hours. The solution was evaporated, worked up and chromatographed ($CHCl_3$) to give a clear gum; ^{1}H-NMR δ 2.37 (3H, s, toluoyl CH_3), 2.40 (3H, s, toluoyl CH_3), 2.64-2.69 (1H, m, H2'), 2.90-3.00 (1H, m, H2"), 4.48-4.61 (3H, m, H5', H5", H4'), 5.67-5.70 (1H, m, H3'), 6.50-6.52 (1H, m, H3), 6.56-6.60 (1H, m, H1'), 6.87-6.94 (1H, m, H7), 7.30-7.38 (5H, m, toluoyl CH, H2), 7.65-7.70 (3H, m, H4, H6), 7.85-8.31 (4H, m, toluoyl CH). This was dissolved in methanolic ammonia (25ml) and stirred at room temperature for 3 days. The solvent was removed and the product chromatographed ($CHCl_3$/5% MeOH) to give a white foam, (1.12g, 60%); ^{1}H-NMR δ 2.16-2.23, 2.43-2.52 (2H, m, H2'), 3.43-3.56 (2H, m, H5', H5"), 3.78-3.82 (1H, m, H4'), 4.30-4.36 (1H, m, H3'), 4.88 (1H, t, 5'-OH), 5.27 (1H, d, 3'-OH), 6.34 (1H, t, J = 6.5, H1'), 6.47 (1H, m, H3), 6.93-7.0 (1H, m, H7), 7.28-7.32 (1H, m, H2), 7.56-7.61 (1H, m, H6), 7.64-7.65 (1H, m, H4). (Indole protons are coupled to fluorine). uv λmax/nm 270 (ε=6000), 318 (ε=250), λmin 237, 309, (ε_{260} (μM) = 5).

1-[5-(4,4'-Dimethoxytrityl)-2-deoxy-β-D-ribofuranosyl]-5-fluoroindole 12. To a solution of the 5-fluoroindole nucleoside (1g, 4mmol) in pyridine (25ml) was added dimethoxytrityl chloride (1.5g, 4.4mmol) and the solution stirred at room temperature overnight. The solvent was removed and the product chromatographed ($CHCl_3$/1% MeOH) to give a yellow foam, (1.86g, 84%); ^{1}H-NMR δ 2.26-2.34, 2.54-2.63 (2H, m,

H2', H2"), 3.09-3.33 (2H, m, H5', H5"), 3.70 (6H, 6, 2 x OCH_3), 3.93-3.97 (1H, m, H4'), 4.38-4.43 (1H, m, H3'), 5.37 (1H, d, 3'-OH), 6.40 (1H, t, J = 6.4, H1'), 6.46-8.58 (18H, m, aromatic CH).

1-[3-O-(P-β-Cyanoethyl-*N,N*-diisopropylaminophosphinyl)-5-(4,4'-dimethoxytrityl)-2-deoxy-β-D-ribofuranosyl]-5-fluoroindole 13. To a solution of the above nucleoside (1.1g, 2mmol) in dichloromethane (10ml) under argon was added diisopropylethylamine (1.1ml, 6mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.68ml, 1.5mmol) and the solution stirred at room temperature for 1 hour. The reaction was quenched with methanol (100µl) and then the solution was poured into ethyl acetate (100ml) and washed with 5% Na_2CO_3 solution (2 x 25ml) followed by saturated brine (2 x 25ml) and then evaporated to a gum. This was chromatographed (ethyl acetate/hexane/triethylamine, 10:10:1) to give, after precipitation from ice-cold hexane, a white foam, (1.17g, 79%); ^{31}P-NMR δ 148.50, 147.83.

1-(3,5-Di-O-p-toluoyl-2-deoxy-β-D-ribofuranosyl)-5-nitroindazole 14. To a solution of 5-nitroindazole (1g, 6.1mmol) in acetonitrile (25ml) was added sodium hydride (60%, 0.27g, 6.8mmol) and the solution stirred at room temperature for 30 minutes. To this was then added 2-deoxy-3,5-di-O-*p*-toluoyl-α-D-ribosyl chloride (2.9g, 7.5mmol) and stirring continued for a further 2 hours. The solution was evaporated and worked up as usual and chromatographed (ether/hexane, 1:1) to give two products. The first product was identified as the desired isomer, (2.0g, 63%), the second product was the N^2-regio isomer. Found: C, 65.14; H, 4.76; N, 8.14. $C_{28}H_{25}N_3O_7$ requires C, 65.2; H4.9; N, 8.2. ^{1}H-NMR δ 2.36, 2.40 (6H, 2 x s, 2 x CH_3), 2.74-2.82, 3.31-3.42 (2H, m, H2', H2"), 4.31-4.36, 4.45-4.51 (2H, m, H5', H5"), 4.55-4.59 (1H, m, H4'), 5.82-5.86 (1H, m, H3'), 6.96 (1H, t, J = 6, H1'), 7.27-7.38, 7.79-7.97 (8H, m, phenyl CH), 8.06 (1H, d, J = 9.3, H7), 8.24 (1H, dd, J_1 = 9.2, J_2 = 1.9, H6), 8.51 (1H, s, H3), 8.81 (1H, d, J = 1.8, H4). ^{13}C-NMR δ 21.31, 21.37 (2 x CH_3), 35.77 (C2'), 64.06 (C5'), 75.08 (C4'), 81.82 (C3'), 86.31 (C1'), 111.07 (C7), 119.25 (C6), 121.69 (C3), 123.67, 126.72 (C8, C9), 129.32-129.69 (phenyl CH), 137.80 (C4), 141.95, 142.44, 143.87, 144.21 (phenyl C), 165.53 (C5). u.v. λmax (nm) ($CHCl_3$) 246 (ε=40000), 308 (ε=7200).

1-(2-Deoxy-β-D-ribofuranosyl)-5-nitroindazole 15. A solution of the 5-nitroindazole nucleoside, **6**, (1.9g, 3.7mmol) in ammonia saturated methanol (25ml) was

stirred at room temperature for 2 days. The solvent was removed and the product chromatographed ($CHCl_3$/5% MeOH) to give a yellow gum, (1.04g, 100%); ^{1}H-NMR δ 2.28-2.38 (1H, m, H2'), 2.85-2.95 (1H, m, H2"), 3.29-3.53 (2H, m, H5', H5"), 3.82-3.90 (1H, m, H4'), 4.43-4.51 (1H, m, H3'), 4.64 (1H, t, 5'-OH), 5.28 (1H, d, 3'-OH), 6.65 (1H, t, J = 6, H1'), 7.98 (1H, d, J = 9, H7), 8.22 (1H, dd, J_1 = 9, J_2 = 2, H6), 8.44 (1H, s, H3), 8.77 (1H, d, J = 2, H4).

1-[3-O-(P-β-Cyanoethyl-*N,N*-diisopropylaminophosphinyl)-5-(4,4'-dimethoxytrityl)-2-deoxy-β-D-ribofuranosyl]-5-nitroindazole 16. To a solution of **3** (1g, 3.6mmol) in pyridine (25ml) was added dimethoxytrityl chloride (1.3g, 3.8mmol) and the solution stirred at room temperature overnight. The solvent was removed and the product worked up as usual and chromatographed ($CHCl_3$/1% MeOH) to give the *5'-dimethoxytrityl derivative* as a yellow foam, (1.06g, 51%); ^{1}H-NMR δ 2.42-2.54 (1H, m, H2'), 2.92-3.19 (3H, m, H2", H5', H5"), 3.78 (4H, s, 2 x OCH_3), 4.02-4.22 (1H, m, H4'), 4.65-4.74 (1H, m, H3'), 5.47 (1H, d, 3-OH), 6.70-8.71 (14H, m, H1', Ar-H), 8.17 (1H, d, H7), 8.38 (1H, dd, H6), 8.50 (1H, s, H3), 8.93 (1H, s, H4). To a solution of the 5'-dimethoxytrityl derivative (0.5g, 0.86mmol) in dichloromethane (10ml) under argon was added diisopropylethylamine (0.45ml, 2.6mmol) followed by 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.29ml, 1.15mmol) and the solution stirred at room temperature for 1 hour. The reaction was worked up as described above and the product chromatographed (EtOAc/CH_2Cl_2/Et_3N, 10:10:1) to give the product as a yellow foam. (0.46g, 68%); ^{31}P-NMR δ 147.57, 146.87.

1-(2-Deoxy-β-D-ribofuranosyl)benzimidazole 17. 1-(2-Deoxy-β-D-ribofuranosyl)benzimidazole was a gift from Dr. Arthur Van Aerschot, or was prepared according to the method of Revankar.[14] ^{1}H-NMR δ 2.25-2.32 (1H, m, H2'), 2.55-2.64 (1H, m, H2"), 3.50-3.61 (2H, m, H5', H5"), 3.84-3.88 (1H, m, H4'), 4.36-4.41 (1H, m, H3'), 4.97 (1H, t, 5'-OH), 5.35 (1H, d, 3'-OH), 6.35 (1H, t, J = 6.2, H1'), 7.18-7.27 (2H, m, Ar), 7.64-7.71 (2H, m, Ar), 8.49 (1H, s, H-2). u.v. λmax (nm) 279 (ε=3200), 272 (ε=3800), 245 (ε=6700), (ε260 (μM) 3.4).

1-[5-(4,4'-Dimethoxytrityl)-2-deoxy-β-D-ribofuranosyl]benzimidazole 18. This was prepared as described above. From 0.5g of the nucleoside **2** was obtained after chromatography ($CHCl_3$/2% MeOH) 0.93g (81%) of the dimethoxytritylated

benzimidazole; ^{1}H-NMR δ 2.33-2.41 (1H, m, H2'), 2.67-2.76 (1H, m, H2''), 3.13-3.25 (2H, m, H5', H5''), 3.27 (6H, s, 2 x OCH_3), 3.97-4.01 (1H, m, H4'), 4.39-4.56 (1H, m, H3'), 5.36 (1H, d, 3'-OH), 6.39 (1H, t, J = 6.4, H1'), 6.74-6.91, 7.16-7.33, 7.65-7.68 (17H, m, Ar), 8.34 (1H, s, H2).

1-[3-O-(P-β-Cyanoethyl-*N,N*-diisopropylaminophosphinyl)-5-(4,4'-dimethoxytrityl)-2-deoxy-β-D-ribofuranosyl]benzimidazole 19. This was prepared as described above. 0.9g of the dimethoxytritylated benzimidazole gave 0.86g (70%) of the phosphoramidite. ^{31}P-NMR δ 147.73, 147.10.

Preparation of oligonucleotides. Phosphoramidite monomers of 3-nitropyrrole, 5-nitroindole, dP and dK were purchased from Glen Research, and 2'-deoxyinosine monomer was supplied by Cruachem. 4- and 6-nitroindole monomers were synthesised as previously described.[9] Oligonucleotides were synthesised on an Applied Biosystem 380B synthesiser with the normal synthesis cycle. Purification was carried out by electrophoresis using 20% polyacrylamide gels followed by extraction (0.5M ammonium acetate, 1mM EDTA buffer), concentration and desalting using a Sephadex G-25 column (NAP-10, Pharmacia). Oligonucleotides containing dP and dK were purified trityl-on by reverse phase HPLC (C-18, 0.1M triethylammonium acetate buffer, pH7) and then detritylated (80% acetic acid) after concentration, and desalted as above. Some of the primers were characterised by mass spectroscopy using a Hewlett-Packard G205A MALDI-TOF spectrometer with negative polarity in a diammonium hydrogen citrate/2,6-dihydroxyacetophenone (1:1) matrix; they gave the expected masses.

Sequencing reactions. Sequencing was performed on a Vistra DNA Labstation 625 (Amersham Pharmacia Biotech) using ThermoSequenase™ dye-terminators with automated work-ups and samples were run on an ABI 373 fluorescent sequencer. Template used was 1μg of caesium chloride purified single-stranded M13mp8 DNA. Thermal cycling conditions used were: 95°C for 5 minutes followed by 25 cycles of: 95°C for 30 seconds, annealing for 15 seconds, 60°C for 4 minutes. Annealing temperatures range from 20-50°C. Signal intensity is a fluorescence photomultiplier tube output measurement.

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