

The elimination of primer-dimer accumulation in PCR

Jannine Brownie, Susan Shawcross¹, Jane Theaker, David Whitcombe*, Richard Ferrie, Clive Newton and Stephen Little

Zeneca Diagnostics, Gadbrook Park, Northwich, Cheshire CW9 7RA, UK and ¹Department of Biological Sciences, Manchester Metropolitan University, Chester Street, Manchester M1 5GD, UK

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ABSTRACT

We attempted to produce primer-dimers (PDs) from a variety of primers with differing types and extents of complementarity. Where PDs were produced they were cloned and sequenced. We were unable to produce detectable PDs either with individual primers alone or with similar sequence primers even if they had 3' complementarity. These observations led to the hypothesis that a system could be developed whereby the accumulation of PDs in a PCR may be eliminated. We demonstrate a method for the general suppression of PD formation that uses a sequence of additional nucleotides (a Tail) at the 5' ends of amplimers. Tailed amplimers are present at low concentration and only participate during early cycles of PCR. In subsequent PCR cycles, amplification is achieved using a single primer that has the same sequence as that of the Tail portion of the early cycle primers, here we refer to this as a Tag. When products are small, as with PDs, there is a high local concentration of complementary sequences derived from the Tail. This favours the annealing of the complementary ends of a single strand produced by tailed primer interactions and gives rise to 'pan-handle' structures. The formation of these outcompetes the annealing of further Tag primers thereby preventing the accumulation of non-specific PD products. This aids the design of large multiplex reactions and provides a means of detecting specific amplicons directly in the reaction vessel by using an intercalating dye.

INTRODUCTION

In the PCR, template-independent primer interactions can take place that give rise to non-specific products, notably primer-dimers (PDs) (1). Because primers are present at high concentrations, weak interactions can occur between them. Complementarity of just one nucleotide between amplimer 3'-ends can give rise to PD artefacts after 30 cycles (2). The same study showed that in an analogous PCR, where there was no 3' complementarity, PDs were still produced, albeit after 40 cycles. Another study by Ferrie *et al.* (3) showed that under cold-start conditions, every possible combination of two different primers of a multiplex ARMS reaction would give rise to PDs, irrespective of any primer complementarity. Furthermore, each of the primers individually

failed to produce detectable PDs (Ferrie *et al.*, unpublished observations). From these findings we reasoned that PDs derived from inter-primer extension are good substrates for amplification in subsequent cycles of PCR and that intra-primer interactions would produce inefficiently amplified products and so enhance amplification of the 'true' targets.

PD formation can be reduced by careful primer design, the application of stringent conditions, the use of 'hot-start' (4,5), touch-down PCR (6) and/or enzyme formulations such as AmpliTaq Gold™ (7). There are many instances when it has been desirable to combine several amplimer pairs in a multiplex reaction (e.g 3,8–15). One problem encountered when developing multiplex reactions is the increasing difficulty of eliminating all interactions that promote the formation of PDs. In such situations where the reactions may contain many primers at high concentration, it becomes impossible to eliminate PDs using the above methods. We have solved these problems using primers which are genome specific at their 3'-ends and carry common extensions (Tails) at their 5'-ends. In addition, the reactions include Tail-specific primers (Tags) which can prime from the newly synthesised sequence that is complementary to the Tails. Low concentrations of specific primers can therefore be used in combination with higher concentrations of Tag to produce a wide range of specificities without introducing large quantities of primer.

A switch from genomic priming, by the genome-specific portions of the primers to Tail priming by the Tag, is brought about by elevating the PCR annealing temperature. Switching is accomplished by designing the primers such that the T_m of the Tag annealed to its complementary sequence is higher than that of the genome-specific primed duplex. After at least two cycles of genomic priming, the complement of any Tag sequence is incorporated into the amplicon ends. After this has occurred the annealing temperature is raised and the temperature switch effected. The subsequent amplification is then driven entirely by the Tag primers. Tag-driven PCR is also favoured by incorporating the Tag primer at a higher concentration relative to the tailed genomic primer analogous to Tags as used in MVR-PCR (16).

Any product, including PDs, that may have formed during the early PCR cycles will have the complement of any Tag sequence incorporated into its ends. The single strands from these products therefore have complementary ends if the same Tail sequence is used with each primer. The annealing of the complementary ends of small products is preferred because of their high local concentration and this promotes the formation of 'pan-handle' structures that outcompetes the annealing of further Tag primers.

*To whom correspondence should be addressed. Tel: +44 1606 44491; Fax: +44 1606 49366; Email: david.whitcombe@ukbla71.zeneca.com

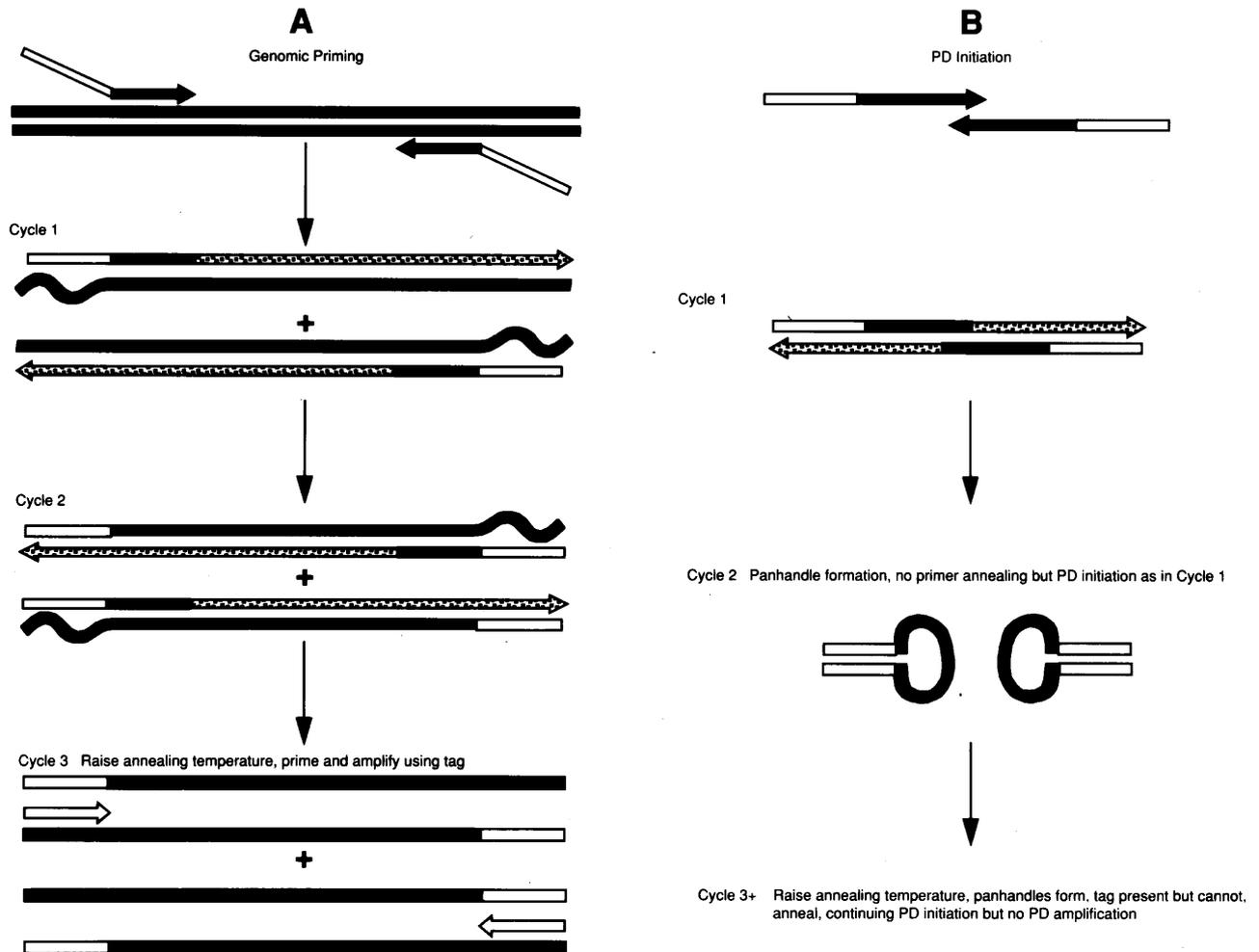


Figure 1. HANDS scheme. (A) Amplification of desired amplicon; (B) the prevention of accumulation of PDs through Panhandle formation. Open boxes are Tail and Tag sequences; stippled boxes are newly synthesised sequences; filled boxes are genomic sequences. Arrow heads indicate the direction of DNA synthesis.

The combination of low tailed genomic primer concentrations and the formation of 'pan-handle' structures suppresses PD formation. We have incorporated each of these aspects into a system for the routine elimination of PDs; Homo-Tag Assisted Non-Dimer System (HANDS) (Fig. 1).

MATERIALS AND METHODS

Oligonucleotides

The nucleotide sequences of the synthetic oligonucleotides are shown in Table 1. Primers for the PD synthesis reactions comprise two groups and are aligned in Figure 2. An analysis of their homology was carried out using MegAlign, of DNASTar (Table 2).

Oligonucleotides S1 and S2 amplify across the multiple cloning site of pCRScript SK⁺ (Stratagene); oligonucleotides S3 and S4 are sequencing primers for pCRScript SK⁺ and are nested relative to S1 and S2.

Oligonucleotides G1 and G2 are designed to amplify a fragment of the human apolipoprotein B (Apo B) gene (17) oligonucleotides G1T1 and G2T1 are tailed equivalents of G1 and

G2. Oligonucleotides H1T1, H2T1, I1T1, I2T1, J1T1 and J2T1 are three pairs of tailed ARMS (18) primers for the respective detection of the W1282X (19), G542X (20) and Δ F508 (21) cystic fibrosis (CF) mutations.

PD synthesis and cloning

PCR reaction mixes comprised 10 mM Tris-HCl, (pH 8.3) 1.2 mM MgCl₂, 50 mM KCl, 0.01% gelatin, dNTPs (100 μ M each) and 1 U DNA AmpliTaq polymerase (P. E. Applied Biosystems). Primers were 1 μ M and reaction volumes were 50 μ l. PCRs were performed using a Hybaid Omnigene cycler. Thermal cycling comprised 94°C, 2 min; 60°C, 2 min; 72°C, 2 min for 35 cycles followed by a final extension of 72°C for 10 min.

Separate PD aliquots (~1 pmol) were combined with pCRScript SK⁺ (10 pg, Stratagene). Each vector/PD combination was purified using GeneClean III as directed by the manufacturer (BIO 101 Inc.) then eluted in water and ligated. Ligation aliquots were used to transform *Escherichia coli* NM522 and apparent insert positive clones were identified by blue/white screening using X-gal and IPTG.

Table 1. Oligonucleotides used in this study

Primer	Sequence (5'-3')
A	ACGCGTAGACGTCCAGTCAGACGTGTGCAG
B	CTACGCGTGACACATGCGACTGACCATGAG
C	ACGCGTAGACGTCCAGTCAGACGTGTGCAT
D	CTACGCGTGACACATGCGACTGACCATATG
E	GACGTACGACGTCCAGTCAGACGTGTGCAT
F	GATAGCGAGACACATGCGACTGACCATATG
S1	AGCGGATAACAATTTACACAGG
S2	ATTAAGTTGGGTAACGCCAGGG
S3	AAACAGCTATGACCATGATTACG
S4	GTTTTCCCAGTCACGACGTTG
G1	CAGCCAAAACCTTTTACAGGGATGGAGAACG
G1T1	GCGTACTAGCGTACCACGTGTCGACTCAGCCAAAACCTTTTACAGGGATGGAGAACG
G2	CTCTGGGAGCACAGTACGAAAAACCACCT
G2T1	GCGTACTAGCGTACCACGTGTCGACTCTCTGGGAGCACAGTACGAAAAACCACCT
H1T1	GCGTACTAGCGTACCACGTGTCGACTAAGGTGAGTATAAAGGAAGTCTGCATCAG
H2T1	GCGTACTAGCGTACCACGTGTCGACTCCTGTGGTATCACTCCAAGGCTTTCCAT
I1T1	GCGTACTAGCGTACCACGTGTCGACTAAAATTTTACGCAATGTTGTTTTGACCAAC
I2T2	GCGTACTAGCGTACCACGTGTCGACTCACTCAGTGTGATTCCACCTTCTGA
J1T1	GCGTACTAGCGTACCACGTGTCGACTCACTAATGAGTGAACAAAATTCTCACCATT
J2T2	GCGTACTAGCGTACCACGTGTCGACTGCCTGGCACCATTAAGAAAATATCATTGG
K1T1	GCGTACTAGCGTACCACGTGTCGACTCGTGATTTGATAAATGACCTAATAATGATGG
K2T1	GCGTACTAGCGTACCACGTGTCGACTCACTAATGAGTGAACAAAATTCTCACCATT
L1T3	GACGATACGACGGGCGTACTAGCGTAAAATTTTACGCAATGTTGTTTTGACCAAC
L2T1	GCGTACTAGCGTACCACGTGTCGACTTACCTTGCTAAAGAAATTTCTGTCTCGTTG
T1	GCGTACTAGCGTACCACGTGTCGACT
T2	ACTAGCGTACCACGTGTCGACT
T3	GACGATACGACGGGCGTACTAGCGTA

A	Group 1	
Primer A	ACG-CGTA-GACGTCCAGTCAGACGTGTGCAG	
Primer C	ACG-CGTA-GACGTCCAGTCAGACGTGTGCAT	
Primer E	GACGTACGACGTCCAGTCAGACGTGTGCAT	
B	Group 2	
Primer B	CTACGCGTGACACATGCGACTGACCATGAG	
Primer D	CTACGCGTGACACATGCGACTGACCATATG	
Primer F	GATA-GCGTGACACATGCGACTGACCATATG	

Figure 2. DNASTar multiple sequence alignment of primers used to attempt PD synthesis. (A) Alignment of the primers comprising group 1. (B) Alignment of the primers comprising group 2.

DNA sequencing of PD clones

Plasmid aliquots (~1 ng) were amplified in 100 µl reactions using 2 U AmpliTaq Gold (P. E. Applied Biosystems) and 500 nM primers S1 and S2 in the buffer described for PD synthesis. Thermal cycling using a P. E. Applied Biosystems 9600 cycler comprised 15 min at 94°C followed by 35 cycles of 94°C, 40 s; 65°C, 40 s and a final extension at 72°C for 7 min. PCR products were pre-treated to remove excess primers and dNTPS and purified using the T7 Sequenase PCR product sequencing kit (Amersham). They were then precipitated with isopropanol, sequentially washed with 70% ethanol and 95% ethanol, air-dried and resuspended in water (25 µl).

Table 2. Reagent and primer variables for amplicon detection by intercalation

Replicate group type	Primer(s)	Taq (units)	DNA (ng)
+ Tails	G1T1 + G2T1	2.5	0
+ Tails + Tag	G1T1 + G2T1 + T2	2.5	0
Genomic priming	G1 + G2	2.5	0
+ Tails	G1T1 + G2T1	2.5	10
+ Tails + Tag	G1T1 + G2T1 + T2	2.5	10
Genomic priming	G1 + G2	2.5	10
+ Tails	G1T1 + G2T1	0	10
+ Tails + Tag	G1T1 + G2T1 + T2	0	10
Genomic priming	G1 + G2	0	10
+ Tails	G1T1 + G2T1	0	0
+ Tails + Tag	G1T1 + G2T1 + T2	0	0
Genomic priming	G1 + G2	0	0

Forward and reverse sequencing reactions were carried out using 0.5 µg PCR product and primers S3 and S4 (6.4 pmol primer per reaction). Dye terminator cycle sequencing was performed using a Taq dye terminator kit (P. E. Applied Biosystems) by a P. E. Applied Biosystems Catalyst 800 Turbo Robot and analysed using a P. E. Applied Biosystems 377 automated DNA sequencer. A contig from the unedited forward and reverse sequences for each clone was generated using the

SeqMan software of DNASTar. Finally, the respective contigs for the cloned PDs from each primer pair were aligned using the MegAlign software of DNASTar. DNASTar software was licensed from Lasergene Inc.

Tag design

Tag sequences were designed to exhibit the following characteristics: (i) they should have no known genomic target themselves; (ii) they should have high T_m s; (iii) they should not be prone to PD formation by themselves or with other primers in the reactions; and (iv) they should have no stable secondary structure. Oligonucleotides (13mers) were designed incorporating the least frequent neighbouring pairs of bases (22) and then examined for their absence in known human genomic sequences in the GenBank database. They were then combined to generate candidate 26mers which were examined for self- and human genomic DNA complementarity. Tag sequences are shown in Table 1.

DNA extraction

The panel of samples was selected to include normal DNAs, DNA from a CF $\Delta F508$ homozygote, examples of W1282X/+, G542X/+ heterozygotes and W1282X/ $\Delta F508$, G542X/ $\Delta F508$ compound heterozygotes. All DNA samples were prepared as described previously (3).

PCR reaction conditions

PCR reactions were carried out in the buffer described for PD PCR. These were with or without 5 μ l (~20 ng) of human genomic DNA as specified case-by-case. All tubes were soaked at 94°C for 5 min, unless otherwise stated, before adding 2 U of *Taq* DNA polymerase (Kodak) in 5 μ l buffer. Thermal cycling was performed in 0.65 ml tubes using a P. E. Applied Biosystems 480 Thermal Cycler. All procedures generally accepted for avoiding PCR carry-over contamination were employed (23). Aliquots from the PCRs were analysed by gel electrophoresis through 3% Nusieve agarose gels.

Preparation of Homo- and Hetero-tailed PDs

Twelve PCRs (100 μ l each) were performed without genomic DNA using primers K1T1 and K2T1 (5 μ M each) to give Homo-tailed PD 1. Thermal cycling comprised 39 cycles of 94°C, 1 min; 60°C, 5 min; 72°C, 1 min. Hetero-tailed PD 2 was prepared similarly using primers L1T3 and L2T1, providing the T3 and T1 5' Tails. After PCR, reaction mixes for each PD were pooled and the whole of each sample was electrophoresed on a 1.5% Nusieve agarose preparative gel. PDs were extracted and purified using a Qiaex II Agarose Gel Extraction Kit (Qiagen), ethanol precipitated and resuspended in 10 mM Tris-HCl pH 8.5 (30 μ l). PD dilutions were quantified by gel electrophoresis against ϕ X174 *Hae*III DNA (1 μ g).

PCR of tailed PDs using Tag primers

Both PDs were included in separate PCRs over the range of 10–10¹⁰ molecules/50 μ l reaction. For each PD there was an equivalent no PD negative control. PCRs for PD1 contained Tag T1 (1 μ M); PCRs for PD2 contained Tag T1 and Tag T3 (0.5 μ M each). Thermal cycling comprised 35 cycles of 94°C, 1 min;

60°C, 1 min; 72°C, 1 min, with a final extension at 72°C for 10 min. Amplicon detection used 3% Nusieve agarose gels.

The T1 Tag/Tail sequence had a predicted T_m of 72.5°C at 0.5 μ M concentration in the PCR buffer as calculated using the Oligo 5.0 software. The PCR for PD1 above was duplicated except that a combined annealing and extension step of 74°C for 2 min was used.

Direct detection of amplicons by intercalation

Four cold-start PCRs (100 μ l each) were carried out in triplicate containing YO-PRO-1 {3-Methyl-2-[[1-[3-(trimethylammonio)propyl]-1,4-dihydroquinolin-4-ylidene]methyl]benzo-1,3-oxazolium diiodide} (1 μ M) from Molecular Probes Inc. Primers, template and polymerase additions were as shown in Table 2. Thermal cycling conditions for Tag-driven reactions were 94°C for 5 min followed by four cycles of 94°C, 1 min; 64°C, 1 min; 72°C, 1 min; then 46 cycles of 94°C, 1 min; 68°C, 1 min then a final soak at 72°C for 10 min. Thermal cycling conditions for non-tailed primer driven reactions were 94°C for 5 min followed by 40 cycles of 94°C, 1 min; 64°C, 1 min then a final soak at 72°C for 10 min. A portion (85 μ l) from each reaction was transferred to separate wells of flat bottomed 96-well plates (Dynatech) and analysed on a Fluoroskan fluorimeter (Denley). The excitation wavelength was 485 nm and the emission filter was 538 nm. Aliquots (25 μ l) from the PCRs were then analysed by gel electrophoresis.

Table 3. Analysis of primer homologies and PD formation according to primer pair

Primers	Primer groups	Homology (%)	5' Overlap	3' Overlap	PD formed	Clones
A+A	1+1	100	6	0	No	0
A+B	1+2	35	8	0	Yes	3
A+C	1+1	96	6	0	No	0
A+D	1+2	35	8	0	Yes	7
A+E	1+1	74	0	0	No	0
A+F	1+2	35	0	0	Yes	6
B+B	2+2	100	0	0	No	0
B+C	2+1	35	8	0	Yes	8
B+D	2+2	93	0	0	No	0
B+E	2+1	42	1	0	Yes	6
B+F	2+2	77	0	0	No	0
C+C	1+1	100	6	2	No	0
C+D	1+2	35	8	3	Yes	6
C+E	1+1	77	0	2	No	0
C+F	1+2	35	0	3	Yes	nd
D+D	2+2	100	0	6	No	0
D+E	2+1	42	1	3	Yes	nd
D+F	2+2	83	1	6	No	0
E+E	1+1	100	0	2	No	0
E+F	1+2	46	0	3	Yes	nd
F+F	2+2	100	0	6	No	0



Figure 3. DNA sequence alignments of cloned PDs. Contigs derived from the forward and reverse sequencing of individual cloned PDs aligned against the primers from which they were produced. Rev comp. indicates that it is the reverse complement of the primer sequence that is shown. Nucleotides shown in lower case appear to be deleted from the primers during PD formation

Multiplex HANDS reactions

Ten multiplex reactions were performed on a variety of genomic DNAs and a 'no DNA' control using tailed primers G1T1, G2T1, H1T1, H2T1, I1T1, I2T1, J1T1 and J2T1 at either 10 or 20 nM and the T1 Tag at 1 µM. The CF genotypes of the DNAs were ΔF508/ΔF508, ΔF508/W1282X, ΔF508/G542X, W1282X/+, G542X/+ and +/+. Thermal cycling conditions were 94°C for 5 min followed by four cycles of 94°C, 1 min; 60°C, 1 min; 72°C, 1 min; then 35 cycles of 94°C, 1 min; 74°C, 2 min; then a final soak at 72°C for 10 min. Aliquots (25 µl) from the PCRs were analysed by gel electrophoresis.

RESULTS

PD synthesis

The accumulation of PDs from a primer from either group 1 or group 2 was combined with another from the same group (Fig. 2) was not detectable. PD synthesis was pronounced when primers from each group were combined. Any one primer in isolation would not give rise to detectable PDs as shown previously (R.Ferrie, unpublished). These observations were upheld irrespective of any

complementarity between primers (Table 3). An analysis of alignments of cloned PD sequences and the primers from which they were formed is shown in Figure 3.

The Homo-Tag inhibitory effect

PD1 carried Homo-Tails for use with a single Homo-Tag. PD 2 was for use with separate Tag sequences. Secondary amplification of these PDs with the respective Homo-Tag primer or Tag primer pair confirmed inhibition of PD formation in the Homo-Tag system but not the Hetero-Tag system (Fig. 4). Homo-Tailed PD1 was only visible on gel analysis when 10¹⁰ target molecules were present in the reaction. In contrast, the Hetero-Tailed PD 2 was efficiently amplified to produce a visible band on a gel when as few as 10 molecules of target were present in the reaction. The Homo-Tag format therefore suppressed PD formation by a factor of 10⁹.

The T1 Tag/Tail sequence had a predicted T_m of 72.5°C in the PCR buffer. The annealing temperature of the T1 Tag primed PCR of PD1 was raised to 74°C. The inhibition of PD formation of the Homo-Tag system was consequently reduced due to destabilisation of the 'pan-handle' structure. By increasing the annealing temperature of the PCR cycles above the T_m of the Tail

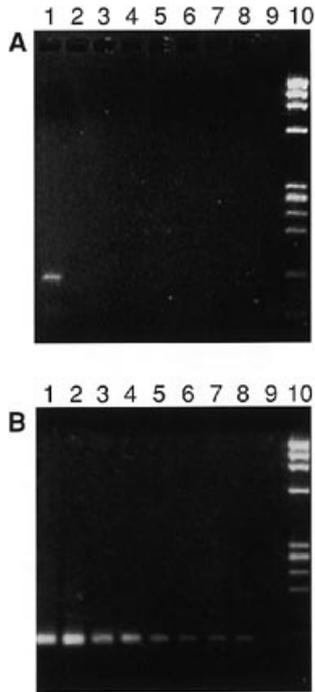


Figure 4. PD reamplification. (A) Homo-Tailed PD1 reamplified using Tag primer T1. (B) Hetero-Tailed PD2 reamplified using Tag primers T1 and T3. (A and B) Lane 1, 10^{10} PD molecules; lane 2, 10^8 PD molecules; lane 3, 10^7 PD molecules; lane 4, 10^6 PD molecules; lane 5, 10^5 PD molecules; lane 6, 10^4 PD molecules; lane 7, 10^3 PD molecules; lane 8, 100 PD molecules; lane 9, 10 PD molecules; lane 10, ϕ X174/*Hae*III size marker.

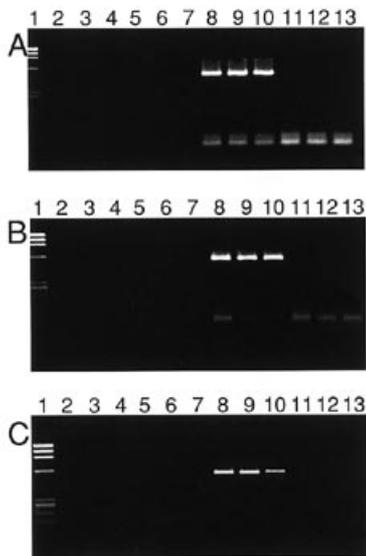


Figure 5. (A) ApoB PCR with conventional primers; (B) ApoB PCR with tailed primers; (C) ApoB HANDS PCR. (A–C) Lane 1, ϕ X174/*Hae*III size marker; lanes 2–4, with template DNA, without *Taq* DNA polymerase; lanes 5–7, without template DNA, without *Taq* DNA polymerase; lanes 8–10, with template DNA, with *Taq* DNA polymerase; lanes 11–13, without template DNA, with *Taq* DNA polymerase.

sequence, PD1 was re-amplified when fewer (10^6) template molecules were present in the reaction (data not shown).

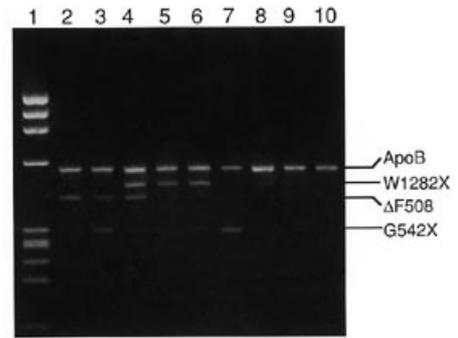


Figure 6. Multiplex ARMS using HANDS. Lane 1, ϕ X174/*Hae*III size marker; lanes 2–10, ARMS analyses of CF genotypes; lane 2, Δ F508/ Δ F508; lane 3, Δ F508/G542X; lane 4, Δ F508/W1282X; lanes 5 and 6, W1282X/+; lane 7, G542X/+; lanes 8–10, +/+. Control and ARMS allele-specific amplicons are shown.

Table 4. Fluorescence readings after YO-PRO-1 intercalation

Substrate DNA	Genomic (untailed)	Tailed	Tailed + Tag (HANDS)
Yes	35.18	34.13	25.56
No	33.27	30.79	17.34

Direct detection of amplicons by intercalation

Gel analysis of PCRs containing YO-PRO-1 showed no visible amplicon when *Taq* DNA polymerase was omitted. Genomic (untailed) primers gave rise to specific amplicon in the presence of genomic DNA only and to PDs either in the presence or absence of genomic DNA. Similar results were obtained using tailed equivalents to the genomic primers. When the Tag primer was included to drive PCR, only the specific amplicon was produced (Fig. 5). Table 4 shows mean fluorescence readings from the reactions that contained *Taq* DNA polymerase.

Multiplex HANDS reactions

Multiplex HANDS reactions with DNAs of different CF genotypes were undertaken using tailed ARMS (18) primers. Only when the appropriate allele was present was the respective amplicon produced; furthermore, there was no visible PD accumulation (Fig. 6).

DISCUSSION

PDs are routinely observed in PCRs. They are not derived from template DNA and they can complicate experimental analysis. For example, PDs might be expected to obscure the true result in multiplex PCRs, RAPD analyses, differential display, amplicon cloning and in quantitative PCR methods. We have gone some way to understanding how PDs are produced by synthesising, cloning and sequencing them. We show that there is no one single mechanism for their formation since there are examples where (i) nucleotides are deleted from the 3' end of one or both amplimers, (ii) a seemingly random sequence of nucleotides is inserted between the 3' ends of amplimers, (iii) a sequence of nucleotides derived from one of the amplimers is inserted between the amplimers, and (iv) where there is 3' complementarity, overlap extension of the

amplimers is observed. We cannot propose a favoured mechanism for PD formation because the PDs here were cloned before characterisation. This could affect the frequency of one type relative to another, skewed through replication *in vivo*.

Our results lead us to conclude that PDs formed from primers with Homo-Tails form a 'pan-handle' structure and that this inhibits PD accumulation during PCR. When PDs are formed from primers with Hetero-Tails PD formation was not inhibited because of the absence of complementarity of the ends of the single strands of a PD. Without complementary ends a 'pan-handle' structure cannot form. Further evidence substantiating these conclusions comes from the results of the preliminary PD synthesis experiments and from the reduced inhibition when Homo-Tailed PDs are amplified using the Homo-Tag with the PCR annealing temperature slightly above the T_m of the Tag sequence. This is likely due to the 'breathing' or destabilisation of the 'pan-handle' structure which allows some annealing of the Tag primer. Even transient Tag annealing and extension by one nucleotide would render this duplex more stable than the duplex of the 'pan-handle' because of the small temperature increment between T_m and annealing temperature.

The elimination of PD accumulation in PCR should help to overcome the common problems alluded to above. In addition we envisage other benefits will be derived from HANDS. These could include a massive simplification of the design of multiplex PCR where genome-specific Homo-Tailed primers are present at low concentrations. All amplicons of the multiplex would then be subject to the bulk of their amplification by just one Tag primer. Not only would this reduce the total primer present needed for the simultaneous amplification of many amplicons but it would standardise the annealing temperature for all amplicons in the multiplex. We have demonstrated this using tailed ARMS primers and demonstrated that full allele specificity was retained after combining ARMS and HANDS.

We have also shown the ability to detect amplicons by intercalation of a fluorescent dye in the knowledge that increased fluorescence due to intercalation is derived only from the intercalation into amplicon and not into artefactual PDs. The fluorescence analysis (Table 4) indicates a significant background reading due to the inherent fluorescence of the intercalator used here. Nevertheless, it is clear that the signal is greater when genomic DNA is present in the PCR when HANDS is applied. There is little difference in fluorescence readings from genomic priming with tailed or untailed primers alone due to the generation of PDs. It is also clear that PD accumulation is genuinely suppressed when PCR is driven by a single Tag primer (Fig. 5). It therefore follows that homogeneous amplicon detection is possible using direct intercalation and so the reaction vessel need not be opened. HANDS therefore provides the means to eliminate another problem associated with PCR, that of carry-over contamination.

ACKNOWLEDGEMENTS

The PCR process is covered by patents held by Hoffmann-La Roche. HANDS is the subject of European Patent Application, Publication No. 0 731 177 (Zeneca Limited). ARMS™ is the subject of European Patent No. 0 332 435 (Zeneca Limited) and corresponding patents worldwide.

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