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Localised sequence regions possessing high melting temperatures prevent the amplification of a DNA mimic in competitive PCR

David G. McDowell*, Nigel A. Burns and Helen C. Parkes

Department of Analytical Molecular Biology, Laboratory of the Government Chemist, Queens Road, Teddington, Middlesex TW11 0LY, UK

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ABSTRACT

The polymerase chain reaction is an immensely powerful technique for identification and detection purposes. Increasingly, competitive PCR is being used as the basis for quantification. However, sequence length, melting temperature and primary sequence have all been shown to influence the efficiency of amplification in PCR systems and may therefore compromise the required equivalent co-amplification of target and mimic in competitive PCR. The work discussed here not only illustrates the need to balance length and melting temperature when designing a competitive PCR assay, but also emphasises the importance of careful examination of sequences for GC-rich domains and other sequences giving rise to stable secondary structures which could reduce the efficiency of amplification by serving as pause or termination sites. We present data confirming that under particular circumstances such localised sequence, high melting temperature regions can act as permanent termination sites, and offer an explanation for the severity of this effect which results in prevention of amplification of a DNA mimic in competitive PCR. It is also demonstrated that when *Taq* DNA polymerase is used in the presence of betaine or a proof reading enzyme, the effect may be reduced or eliminated.

INTRODUCTION

Since competitive PCR was first described (1), numerous reports have detailed the use of this technique for the quantification of bacterial, viral and cellular nucleic acids (2–4). Such assays are based on the co-amplification of a target and an internal standard or mimic. The relative ratio of target to mimic yield at a range of mimic concentrations is used to calculate the concentration of target at the start of the reaction. A prerequisite for obtaining quantitative data by competitive PCR is that the internal standard and target amplify with the same efficiencies. Although it is generally accepted that differences in the length and sequence of the target and internal standard do not substantially affect amplification efficiency (5), a 10-fold difference in the amplification efficiencies of competitive standards of different lengths has been reported (6). Furthermore, target and mimic sequences, which differ by only a few base pairs, can form heteroduplexes (3) which can cause problems during the later cycles of PCR.

The investigation presented here was undertaken using a *Legionella pneumophila* based model system in order to determine the effects of sequence length and composition on quantification by competitive PCR. A number of mimics of different size, GC content and base distribution were developed and co-amplified with the intended target and with each other. The study demonstrates that mimics of increasing size and GC content result in reduced amplification efficiency and a consequent over estimation of target number by competitive PCR. The significance of base distribution within the sequence is determined and discussed. High melting temperature domains were shown to have profound effects on the reaction which could not be predicted by assessment of the overall GC content alone. Precautionary measures which may be taken in order to achieve maximal levels of accuracy for this type of assay are considered.

MATERIALS AND METHODS

PCR amplifications, unless otherwise stated, were carried out under the following conditions: 50 mM KCl, 10 mM Tris–HCl (pH 8.3 at 20°C), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin (Sigma), 0.2 mM of each dNTP (Pharmacia) and 0.6 U *Taq* polymerase (Perkin-Elmer). Other thermostable DNA polymerases used were *Pfu* (Stratagene), *Tth* (Perkin-Elmer) and Vent (New England Biolabs). Primers (Kings College, London, UK) were used at a final concentration of 1 pmol/µl. Glycerol, d(+)-trehalose, betaine (*N*,*N*,*N*-trimethylglycine monohydrate), tetramethylammonium chloride (TMAC) and NaCl were all obtained from Sigma. Betaine was prepared as a 5 M stock in sterile distilled water and stored at –20°C. Plasmid DNA was prepared using Wizard Miniprep Kits (Promega). Plasmid DNA was added to competitive PCRs in the range of 200 fg to 250 pg. Amplifications were performed using a GeneAmp® PCR system 2400 (Perkin-Elmer) in a final volume of 25 µl. The thermal profile consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. The 30 cycles were preceded by a denaturation step of 94°C for 5 min and immediately followed by a final extension of 72°C for 10 min. Analysis was by electrophoresis of 10 µl PCR product on 1.75% agarose gels containing 1× TBE buffer and ethidium bromide (0.5 g/ml). The gels were viewed by UV trans-illumination and documented by photography. Gels were analysed using a gel documentation and analysis work station (BioImage). PCR

*To whom correspondence should be addressed. Tel: +44 181 943 7346; Fax: +44 181 943 2767; Email: dgm@lgc.co.uk

sequence determination utilised an ABI 377 fluorescent sequencer using reagents and protocols recommended by the manufacturer (Perkin-Elmer).

Table 1. Mimics prepared for the model system using LEG primers

Name	GC content	T_m (°C)[a]	Mimic size (bp)	High T_m domains
SM1	unknown	unknown	900	unknown
SM2	45%	77.8	900	0
SM7	39%	75.3	588	0
SM8	45%	77.5	600	0
SM20	44%	79.1	304	0
SM21	40%	76.6	304	0
SM22	53% (T_{m+})	81.5	304	2
SM23	53% (T_{m-})	81.2	304	0
SM24	49%	80	304	3
SM31	47%	79.7	455	0
SM32	45%	77.3	450	0

Primer sequences: Leg 1, 5′-GTC ATG AGG AAT CTC GCT G-3′; Leg 2, 5′-CTG GCT TCT TCC AGC TTC A-3′.
[a]T_m calculated in 1× PCR buffer.

Preparation of the model system

A model system was established using randomly picked clones ranging in size from 300 to 900 bp derived directly from *Bacillus subtilis* or *Bacillus cereus* (non-specific products) and *L.pneumophila* following PCR using Leg 1 and Leg 2 primers (7) and further clones constructed from the pGEM *luc* Basic 2 plasmid (Promega) (Table 1) using primers designed to contain the first 20 nt of the *luc* target sequence, flanked by the LEG primer recognition sites (Table 2). These PCR fragments were ligated into the pCRII plasmid (Invitrogen) and transformed into the host, *Escherichia coli* DH5-α (Clontech). The transformants were screened by PCR using both Leg and M13 primers to determine the presence and size of the insert. Standard stocks of each mimic were prepared using Wizard Maxiprep kits (Promega) and the concentration of these targets determined by UV-absorption (260 nm).

Data analysis

Following analysis, the log value of the amount of target generated/amount of mimic product generated was plotted against the log of the initial number of target molecules added, to determine the number of starting molecules required to reach a final product ratio of 1:1. The experiments were repeated three times and the results expressed as a mean. Image intensities were corrected to allow for disparate product sizes and therefore the amount of ethidium bound. The amplification efficiencies of the DNA targets and mimics were determined in the presence of SYBR™ Green I™ using the LightCycler™ (Idaho Technology Inc.). PCR amplification was monitored in real time from cycles 0–40 (Fig. 1). Five to six cycles falling within the exponential phase of amplification, as determined by regression analysis, were used to determine the efficiency of amplification, as shown in Table 3. Melting-curve analysis was used to confirm amplification of the intended product by its melting temperature.

RESULTS AND DISCUSSION

In order to determine the effect of target length on competitive PCR, three targets were constructed with a constant %GC content of 45% and a range of sizes (450, 600 and 900 bp), and amplified in the presence of a number of 304 bp mimics with a range of %GC contents (40, 44 and 53%). The initial starting ratios of target and mimics required to give an equal yield of each product were determined as shown in Table 4. By studying how the required starting ratios varied with the %GC content (constant length) and with length (constant %GC), it can be seen that increased length or %GC content led to a reduced efficiency of amplification. Determination of the amplification efficiencies of both the targets and mimics by real-time analysis confirmed the reduction in the efficiency of amplification observed with length (Table 3). Furthermore, the amplification efficiency of the 304 bp mimic with the highest %GC content (53%) was greatly reduced compared with mimics of the same size. The reasons for this are not known. The highest %GC may result in either an overall stiffening effect on the helix or an increased chance of localised higher T_m regions with associated stiffness which may slow polymerase extension.

Table 2. Primer sequences used for the construction of mimics using the pGEM *luc* Basic 2 plasmid

Name	Primer sequence 5′–3′	Mimic generated
Leg1L 570N	**GTCATGAGGAATCTCGCTG**CTTTTGGCGAAGAATGAAAA	304 bp (40% GC)
Leg2L 816C	**CTGGCTTCTTCCAGCTTCA**CCCTTCCGCATAGAACTGCC	
Leg1L 60N	**GTCATGAGGAATCTCGCTGG**CGTCGAGTTTTCCGGTAAG	304 bp (53% GC)
Leg2L 306C	**CCTGGCTTCTTCCAGCTTCA**ATACAAAGGATATCAGGTGG	
Leg49%GC N	**GTCATGAGGAATCTCGCTG**ACAACGGCGCGGGAAGTTCA	304 bp (49% GC)
Leg49%GC C	**CTGGCTTCTTCCAGCTTCA**GATTATGTCCGGTTATGTAA	
Leg53%GC N	**GTCATGAGGAATCTCGCTG**CCTTTCGGTACTTCGTCCAC	304 bp (53% GC)
Leg53%GC C	**CTGGCTTCTTCCAGCTTCA**CCGCTTGAAGTCTTTAATTA	
Leg31L 480N	**GTCATGAGGAATCTCGCTG**CATAACCGGACATAATCATA	450 bp (45% GC)
Leg31L 861C	**CTGGCTTCTTCCAGCTTCA**ATGTATAGATTTGAAGAAGA	
Leg7L 500N	**GTCATGAGGAATCTCGCTG**TTAACGCCCAGCGTTTTCCC	600 bp (45% GC)
Leg7L 1061C	**CTGGCTTCTTCCAGCTTCA**CCTCTGGATCTACTGGGTTA	
Leg1 245N	**GTCATGAGGAATCTCGCTG**GCCACGCCCGCGTCGAAGAT	900 bp (45% GC)
Leg1 1106C	**CTGGCTTCTTCCAGCTTCA**CAGAGTCCTTTGATCGTGAC	

Sections of the oligonucleotides identical to the Leg primers sequences are underlined.

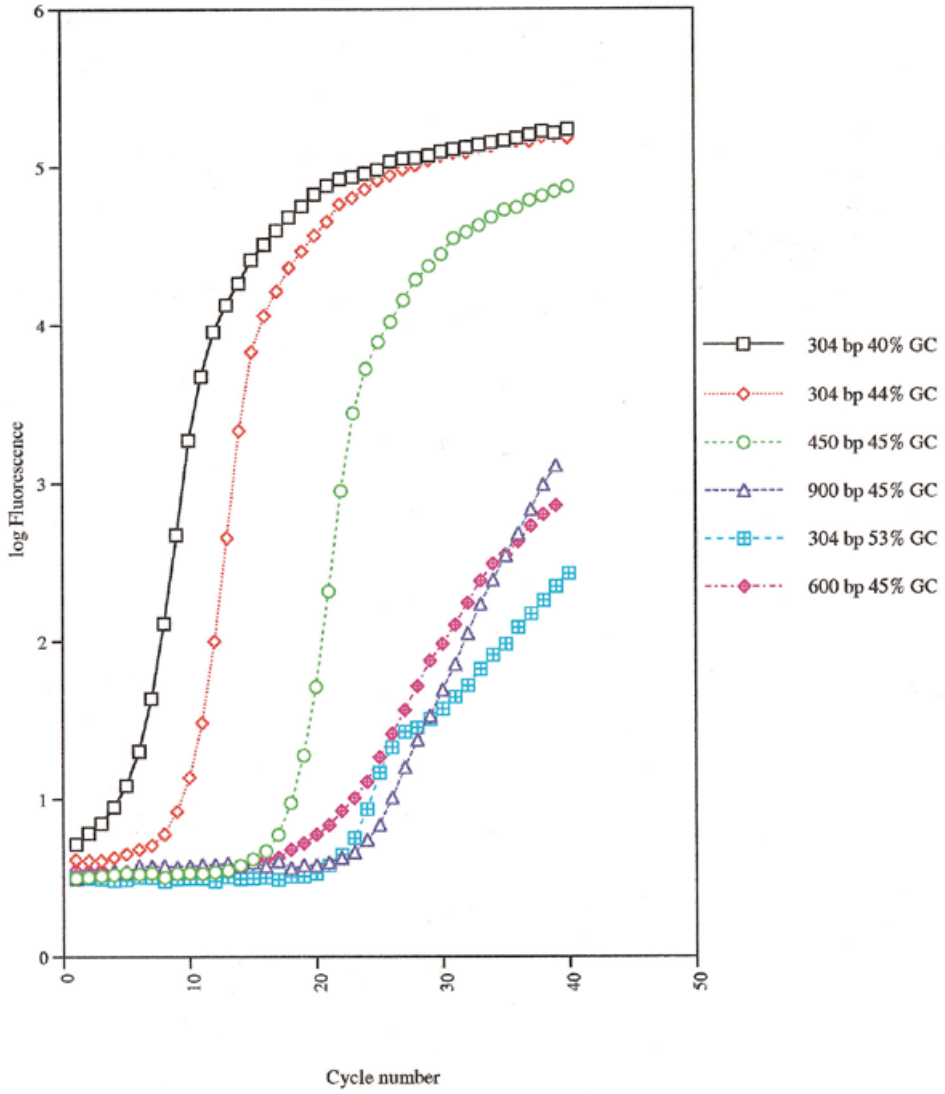

Figure 1. Fluoresence profiles generated for the DNA targets and mimics. Five picograms of each target and mimic was amplified for 40 cycles prior to melting curve analysis.

Table 3. The effect of sequence length on the amplification efficiencies of DNA targets and mimics

Target/mimic	Amplification efficiency (E)
900 bp (45%GC)	79%
600 bp (45%GC)	83%
450 bp (45%GC)	92%
304 bp (40%GC)	94%
304 bp (44%GC)	92%
304 bp [53%GC (T_{m+})]	51%

The mimic with the highest GC content (53%) failed to amplify in competitive reactions against a number of DNA targets, although it was amplifiable in isolation (Fig. 2). In this instance, the preferential amplification of the other DNA targets could not be overcome by altering cycling parameters or optimising primer and magnesium ion concentrations. It was presumed this resulted from a comparatively lower amplification efficiency resulting in the mimic being out competed by the other targets. Since both the target and mimic possess the same primer binding sites, preferential amplification of the target is suggested to result from differences in the internal sequences.

A number of secondary structures and GC-rich motifs have been implicated in causing problems with PCR (8,9). The presence of stable hairpin structures was not observed in the 53% GC mimic. Examination of the sequence composition of the mimic also revealed no G-tract quadruplexes or GC-rich motifs, both of which have been reported to inhibit primer extension by *Taq* DNA polymerase (9,10).

A range of co-solvents and co-solutes have been reported to improve the amplification from targets possessing high GC contents, including dimethyl sulfoxide (DMSO) (11), glycerol, trehalose, TMAC (12) and betaine (13). The effect of these compounds on competitive PCR was investigated. Figure 3 shows that while the majority of these had no visible effect on the preferential amplification of the target, the addition of betaine allowed the 53% GC mimic to be competitively amplified under these conditions. Betaine binds and stabilises AT base pairs whilst

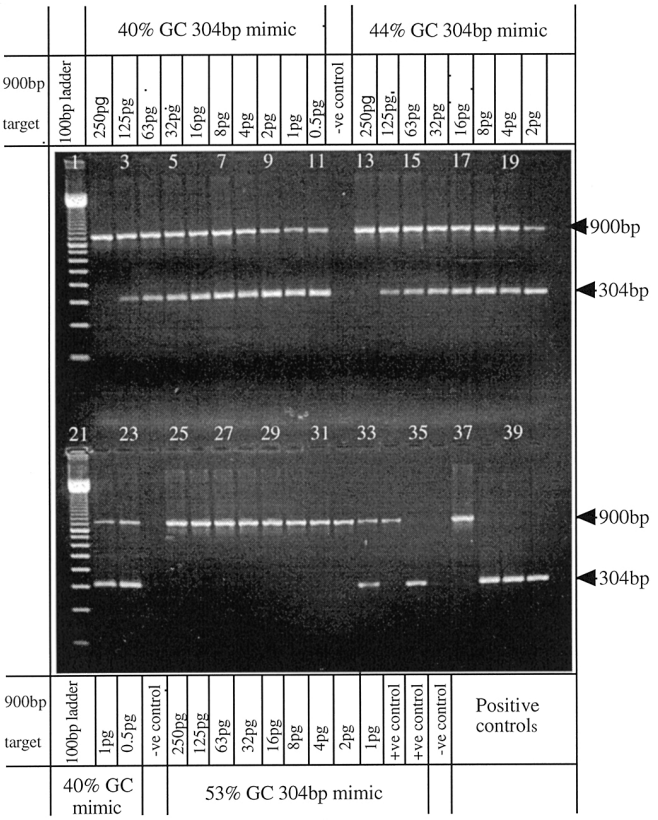

Figure 2. Competitive PCR products generated using a 900 bp DNA target and 304 bp mimics of varying %GC contents. Lane 1, 100 bp ladder; lanes 2–11, dilution series of a 900 bp target amplified in the presence of 5 pg 40% GC 304 bp mimic; lane 12, negative control; lanes 13–23, dilution series of a 900 bp target amplified in the presence of 5 pg 44% GC 304 bp mimic; lane 21, 100 bp ladder; lane 24, negative control; lanes 25–33, dilution series of a 900 bp target amplified in the presence of 5 pg 53% GC 304 bp mimic (T_{m+}); lane 34, 900 bp target (5 pg); lane 35, 53% GC 304 bp mimic (5 pg) (T_{m+}); lane 36, negative control; lane 37, 900 bp target (5 pg); lane 38, 40% GC 304 bp mimic (5 pg); lane 39, 44% GC 304 bp mimic (5 pg); lane 40, 53% GC 304 bp mimic (T_{m+}) (5 pg).

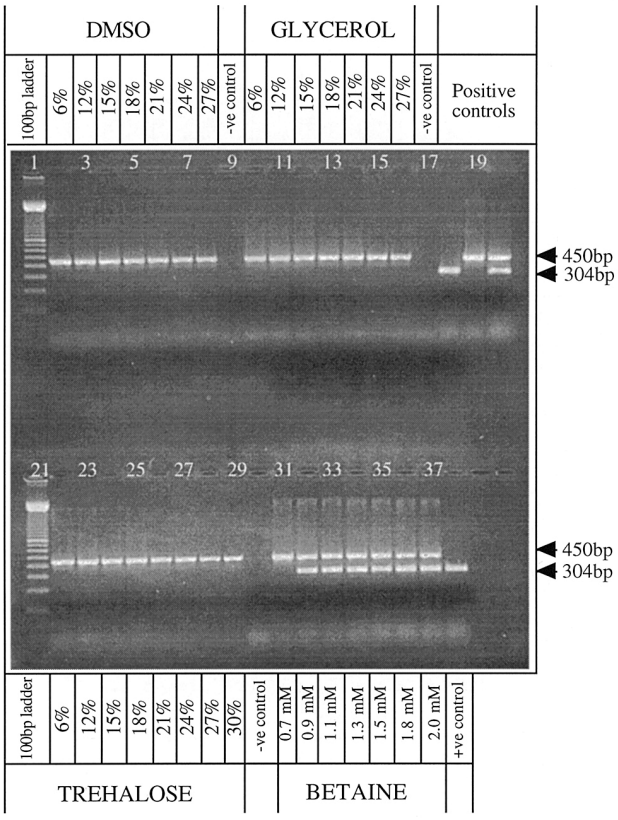

Figure 3. The effect of selected co-solvents on the competitive amplification of a 450 bp DNA target and a 53% GC (T_{m+}) 300 bp mimic. Lane 1, 100 bp marker (Gibco); lane 2, 6% DMSO; lane 3, 12% DMSO; lane 4, 15% DMSO; lane 5, 18% DMSO; lane 6, 21% DMSO; lane 7, 24% DMSO; lane 8, 27% DMSO; lane 9, negative control; lanes 10–16, samples amplified in the presence of glycerol (the concentrations used were the same as those for DMSO); lane 17, negative control; lane 18, 53% GC 304 bp mimic (100 pg); lane 19, 450 bp target (100 pg); lane 20, 450 bp target (100 pg) + 44% GC 304 bp mimic (100 pg); Lane 21, 100 bp marker; lanes 22–28; samples amplified in the presence of trehalose (the concentrations used were the same as those for DMSO); lane 29, 30% trehalose; lane 30, negative control; lane 31, 0.7 M betaine; lane 32, 0.9 mM; lane 33, 1.0 M betaine; lane 34, 1.1 M betaine, lane 35, 1.2 mM; lane 36, 1.3 M betaine; lane 37, 1.4 M betaine; lane 38, 44% GC 304 bp mimic (100 pg).

destabilising GC base pairings resulting in a net specific destabilisation of GC-rich regions (13). A requirement for DNA destabilising co-solutes has also been reported when the target contains regions whose melting temperatures exceeds 81°C (14,15). When the thermal stability profile of the 53% GC mimic was calculated using a modified version of the programme developed by Poland (16,17), two regions with localised T_ms >81°C were observed (Fig. 4). In order to assess whether these regions contributed to the loss of amplification observed with this mimic, two additional mimics were constructed. One mimic was designed with an almost identical (<0.2% higher) overall GC content (53% T_{m-}) but lacking any high T_m domains, and a second with a lower overall GC content (49% GC) but containing three high T_m domains (Fig. 5). Competitive PCRs using these mimics showed the 53% GC (T_{m-}) mimic amplified in the competitive reaction despite the overall higher GC content, whilst the 49% GC mimic failed to amplify (Fig. 6).

Table 4. The effect of sequence length on the initial starting ratio of molecules required to produce an equal amount of target and mimic product

Mimic 304 bp (%GC)	Target 45% GC		
	900 bp	600 bp	450 bp
40%	4:1	0.7:1	0.2:1
44%	0.4:1	0.2:1	0.06:1
53% (T_{m+})	not obtained	not obtained	not obtained

All of the DNA targets used in the competitive PCRs had a GC content of 45%. Required starting ratios are given as mimic:target.

The data obtained suggested that the high T_m domains were responsible for the repression of amplification. During denaturation the template strands will begin to separate at the lowest melting temperature domain. If the localised sequence regions possessing high T_m values were not denatured completely then the template

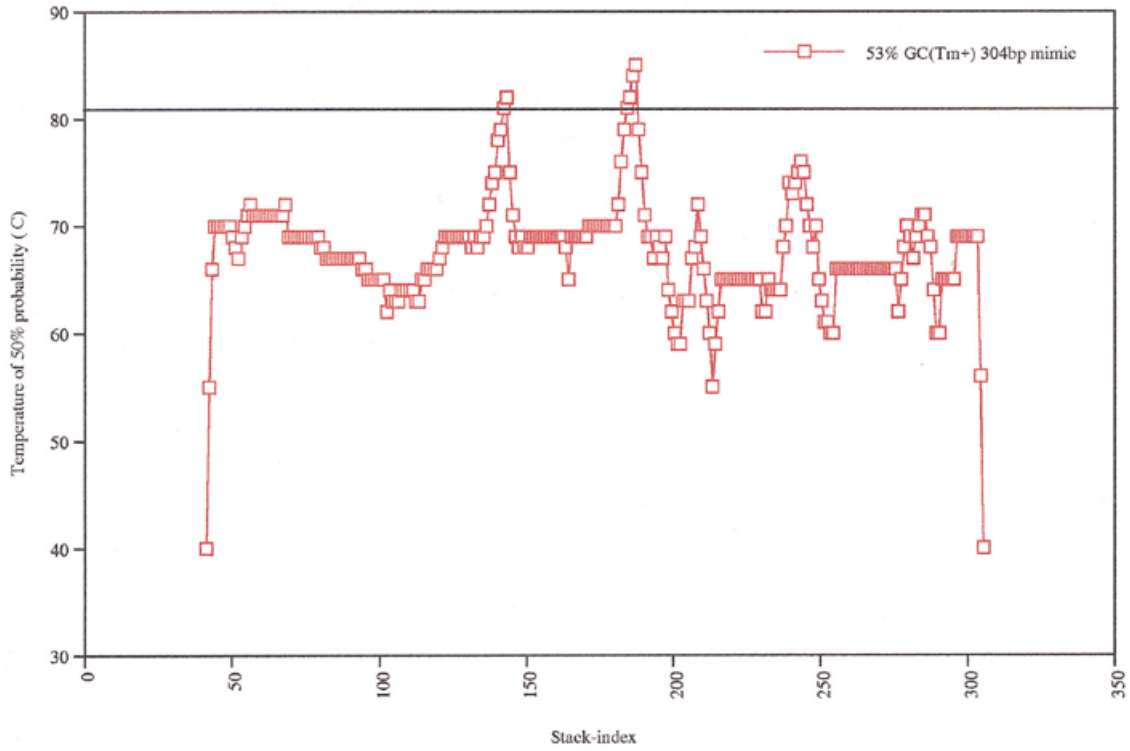

Figure 4. Thermal stability profile of the 53% GC (T_{m+}) 304 bp mimic.

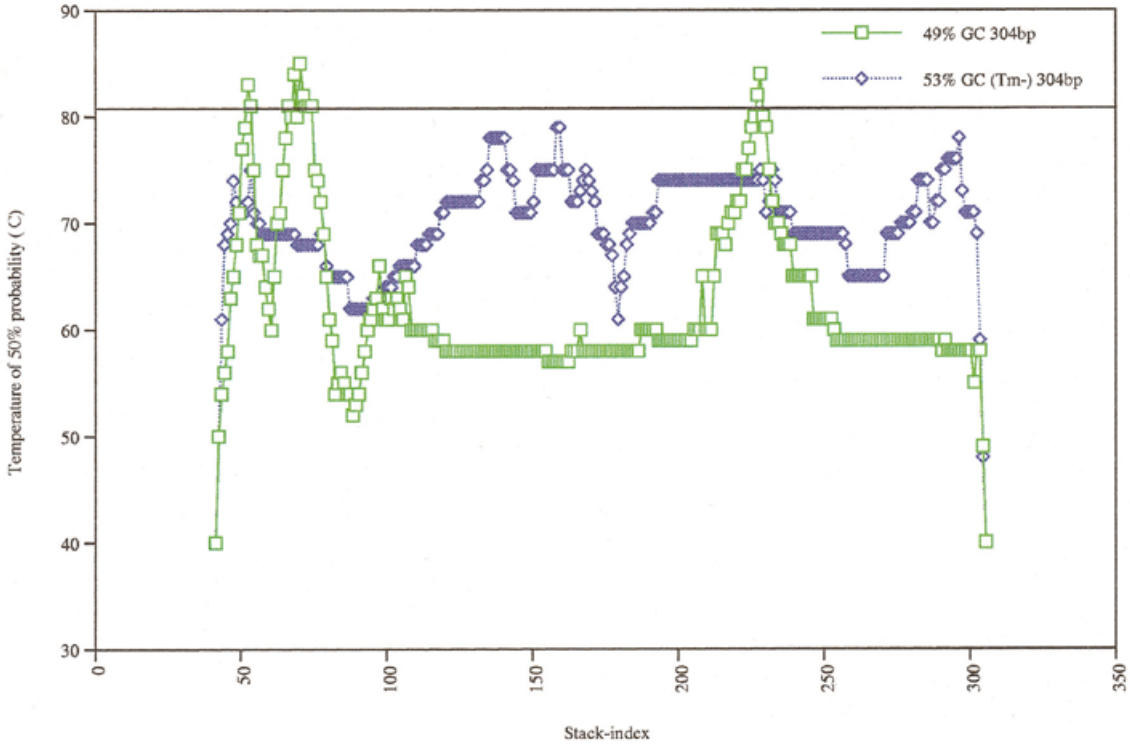

Figure 5. Thermal stability profiles of the 49 and 53% GC (T_{m-}) 304 bp mimics.

would only partially unwind, inhibiting the ability of *Taq* DNA polymerase to read through these regions.

To investigate the role of these domains further, a competitive reaction (using the 53% T_{m+} GC mimic) was performed in the presence of betaine for 24 cycles, allowing a significant amount of both target and mimic product to be generated. The buffer system was then changed and amplification continued in the absence of betaine for a further 16 cycles. Gel analysis revealed the accumulation of truncated products in the absence of betaine (Fig. 7). The truncated products were isolated and the sequence

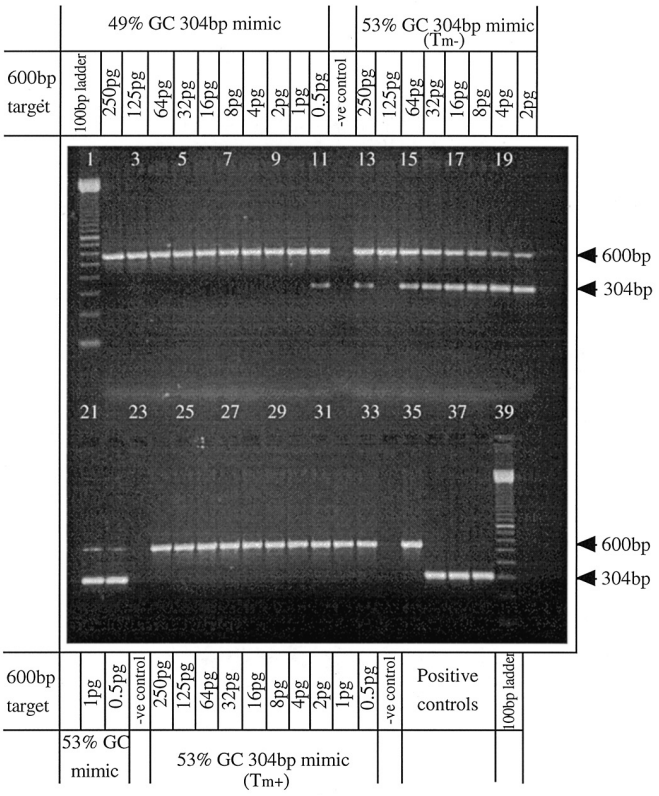

Figure 6. Competitive amplification of a 600 bp DNA target in the presence of 304 bp mimics with modified internal sequences. Lane 1, 100 bp marker (Gibco); lanes 2–11, dilution series of the 600 bp target amplified in the presence of 5 pg 49% GC 304 bp mimic; lane 12, negative control; lanes 13–22, dilution series of the 600 bp target amplified in the presence of 5 pg 53% GC (T_{m-}) 304 bp mimic; lane 23, negative control; lanes 24–33, dilution series of the 600 bp target amplified in the presence of 5 pg 53% GC 304 bp mimic (T_{m+}); lane 34, negative control; lane 35, 600 bp target (5 pg); lane 36, 49% GC 304 bp mimic (5 pg); lane 37, 53% GC 304 bp mimic (T_{m+}) (5 pg); lane 38, 53% GC 304 bp mimic (T_{m-}) (5 pg); lane 39, 100 bp marker (Gibco).

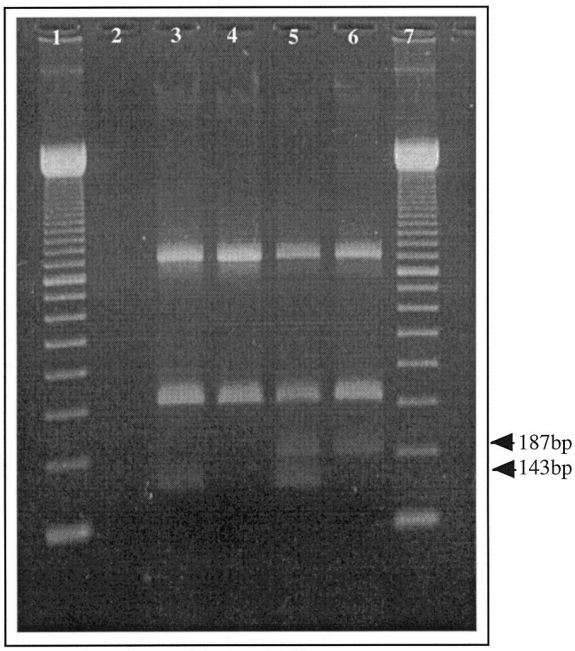

Figure 7. Amplification of truncated PCR products following the removal of betaine. Competitive reactions were initially amplified for 24 cycles in the presence of betaine (1.4 M). Following the removal of betaine (lanes 3, 5 and 6), PCR products derived from the first round of amplification were subjected to a further 16 cycles of amplification. Lane 1, 100 bp ladder; lane 2, negative control; lane 4, positive control [900 bp target (100 pg) + 53% GC 304 bp mimic (T_{m+}) (100 pg)], betaine not removed after initial round of amplification; lanes 3, 5 and 6, 900 bp target (100 pg) + 53% GC 304 bp mimic (T_{m+}) (100 pg); lane 7, 100 bp ladder.

determined by cycle sequencing to confirm the site of termination. The sequence information revealed two features regarding the premature termination event. The PCR products terminated at the sites of high T_m domains predicted from the thermal stability profile of the mimic. Also, in the majority of cases, an extra deoxyadenosine residue was added to the 3′ terminus of the truncated product, presumably as a function of the template-independent terminal transferase activity associated with *Taq* DNA polymerase (18,19).

The inclusion of enzyme blends in long-range PCR has been shown to increase the efficiency of the amplification, previously limited by the incorporation of mismatched basepairs by *Taq* DNA polymerase (20). Whilst this is more likely to be significant with longer targets, we decided to investigate whether a similar effect could be demonstrated with the high T_m domain containing target. The addition of enzyme blends containing either *Pfu* or *Vent* DNA polymerases to the competitive PCR reversed the preferential amplification of the target over that of the 53% (T_{m+}) GC mimic (Fig. 8). Since templates with higher %GC contents than that of the mimic are routinely amplified in competitive PCR, the reduction in the amplification efficiency of the mimic must be caused by the cumulative effect of the high T_m domains. It appears that primer extension by *Taq* polymerase is inhibited by these domains.

The preferential amplification of targets can be caused by differences in salt concentration (21). High salt concentrations were found to suppress the amplification of a 53% (T_{m-}) GC 304 bp mimic (Fig. 9). This mimic contains a number of domains with denaturation temperatures close to 81°C. Increases in salt concentration have a stabilising effect on these template regions, increasing the melting temperature of these domains to the point at which an inhibitory effect on amplification is observed. Indeed, amplification in the presence of a range of salt concentrations may be a useful method for detecting the presence of high T_m regions which may prove inhibitory to extension and serve as pause sites, particularly where sequence information is not available or as a confirmatory test following suitable sequence analysis. The addition of betaine countered the effects of the increased salt levels allowing the generation of mimic PCR products.

Most chain extension studies on DNA polymerases have focused on the process of elongation. However, despite the fact that polymerase activity needs to be rapid and accurate for effective function, the problem of pause sites has yet to be satisfactorily addressed (22). *In vitro* replication studies of øX174 demonstrated the formation of truncated products as the result of barriers in the sequence (23) although these were only observed with T4 DNA polymerase but not with *E.coli* DNA polymerase I. Interestingly in this instance, the precise stopping point indicated

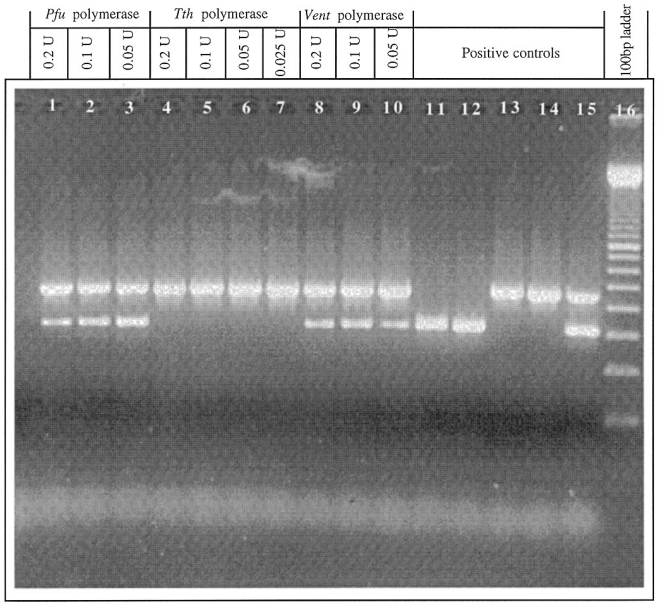

Figure 8. The effect of high fidelity DNA mixtures on the competitive amplification of a 450 bp DNA target and a 53% GC 300 bp mimic. Lane 1, *Taq* DNA Polymerase (0.6 U) + *Pfu* Polymerase (0.2 U); lane 2, *Taq* DNA Polymerase (0.6 U) + *Pfu* Polymerase (0.1 U); lane 3, *Taq* DNA Polymerase (0.6 U) + *Pfu* Polymerase (0.05 U); lane 4, *Taq* DNA Polymerase (0.6 U) + *Tth* Polymerase (0.02 U); lane 5, *Taq* DNA Polymerase (0.6 U) + *Tth* Polymerase (0.2 U); lane 6, *Taq* DNA Polymerase (0.6 U) + *Tth* Polymerase (0.1 U); lane 7, *Taq* DNA Polymerase (0.6 U) + *Tth* Polymerase (0.05 U); lane 8, *Taq* DNA Polymerase (0.6 U) + Vent Polymerase (0.2 U); lane 9, *Taq* DNA Polymerase (0.6 U) + Vent Polymerase (0.1 U); lane 10, *Taq* DNA Polymerase (0.6 U) + Vent Polymerase (0.05 U); lane 11, *Taq* DNA Polymerase (0.6 U) + 53% GC (T_{m+}) 304 bp mimic (100 pg); lane 12, *Taq* DNA Polymerase (0.6 U) + 44% GC 304 bp mimic (100 pg); lane 13, *Taq* DNA Polymerase (0.6 U) + 450 bp target DNA (100 pg); Lane 14, *Taq* DNA Polymerase (0.6 U) + 450 bp target DNA (100 pg) + 53% GC (T_{m+}) 304 bp mimic (100 pg); lane 15, *Taq* DNA Polymerase (0.6 U) + 450 bp target DNA (100 pg) + 44% GC 304 bp mimic (100 pg); lane 16, 100 bp ladder.

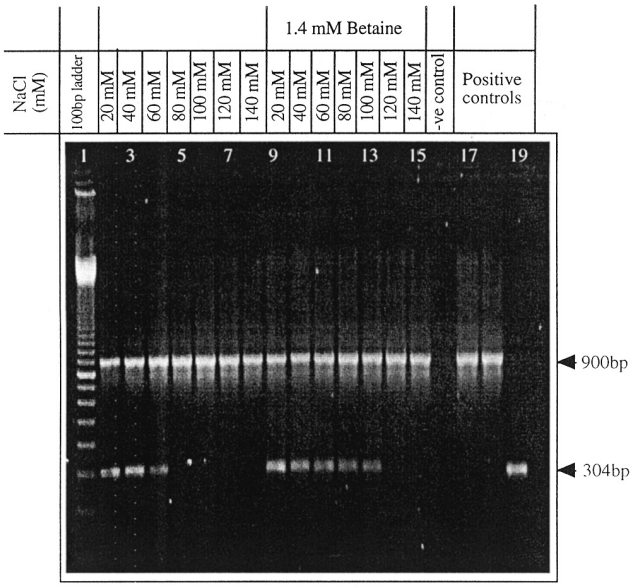

Figure 9. The effect of NaCl concentration on competitive PCR [450 bp/45% GC + 304 bp/53% GC (T_{m-})] in the absence and presence of betaine. Lane 1, 100 bp ladder; lane 2, 10 mM NaCl; lane 3, 20 mM NaCl; lane 4, 30 mM NaCl; lane 5, 40 mM NaCl; lane 6, 50 mM NaCl; lane 7, 60 mM NaCl; lane 8, 70 mM NaCl; lane 9, 80 mM NaCl; lane 10, negative control; lane 11, 10 mM NaCl + 1.4 M betaine; lane 12, 20 mM NaCl + 1.4 M betaine; lane 13, 30 mM NaCl + 1.4 M betaine; lane 14, 40 mM NaCl + 1.4 M betaine; lane 15, 50 mM NaCl + 1.4 M betaine; lane 16, 60 mM NaCl + 1.4 M betaine; lane 17, 70 mM NaCl + 1.4 M betaine; lane 18, 10 mM NaCl + 1.4 M betaine lane 19, 450 bp target + 1.4 M betaine; lane 20, 53% GC (T_{m+}) 304 bp mimic + 1.4 M betaine.

the possible addition of an extra base which did not fit the termination hypothesis proposed. The more recent use of the term pause sites indicates a temporary stop in processivity resulting from localised sequence. Given sufficient time, this implies the polymerase incorporation would continue to full length. However, an erroneous incorporation of an extra base caused by the local sequence at the pause site, would be highly resistant to further elongation as with ARMs style assays (24). The øX174 pause sites were postulated to result from hairpin structures in the sequence. Similar results were presented using four forms of the *E.coli* DNA polymerase III and showed differences in pause sites for the different forms (25), which could potentially result from differences in the ability of the various forms of the enzyme to read through particular regions or their potential for erroneous incorporation at these sites.

Whilst a number of secondary palindromic sequence and others as given earlier have been reported to cause pausing of DNA elongation, other pause sites lacking such regions and proven not to be stimulated by spatially separated sequences led to the postulation that specific primary sequences could also cause this effect (26). Some of the primary sequences responsible for extension arrest may result from stretches of alternating purine and pyrimidine residues (27). Such sequences have been shown to favour a left-handed helix (28), forcing the polymerase to pause until equilibrium results in the formation of a right-handed helix. Ten such residues have been shown to be a strong arrest site (27).

In the system described here, the sequence does not suggest such a pause site resulting from the localised formation of a left-handed or Z helix. The effect of high T_m and GC-rich regions may be to give both localised regions of stiffness and resistance to unwinding, inhibiting the polymerase. The reversal of polymerase pausing by betaine as presented here may have two explanations. A weak and non-cooperative binding of betaine with AT pairs in the major groove of the B form of the double helix serves to stabilise this form and move equilibrium in this direction (13), whilst increasing the hydration of the molecule leads to an overall improvement in flexibility (22). This is thought to facilitate a conformational change in the enzyme–DNA–dNTP complex which is a limiting step in polymerisation and serves as an additional check for correct base pairing, since only correct base pairs can induce a rapid isomerisation of the ternary complex leading to catalysis of the nucleotide phosphoryl transfer reaction (29). Betaine also exerts an isostabilising effect on the differential thermal stability of A:T and G:C base pairs. This isostabilising effect results from the large destabilising effect of betaine on G:C pairs whilst a lesser destabilising effect is exerted on A:T pairs (13), presumably resulting from the binding of betaine to these residues.

There appear to be a variety of DNA sequences and structures which may render regions of DNA inaccessible to the polymerase. The subsequent pausing may result in termination or delayed extension depending on the system used. The significance of the pausing may also vary according to the system. The aim of the work

on competitive PCR presented here was to improve the accuracy of quantification by investigating critical parameters. In such a system, any perturbation in amplification efficiency resulting from pause sites could have a profound effect on the relative amplification efficiency of target and mimic as shown here. In PCR amplification systems, the potential for variation is shown to be large in certain circumstances. The use of the *Taq* polymerase, in particular, exacerbates the problem since pausing appears to be followed by a template-independent polymerase activity (18,19). The resultant mismatched 3′ base is not extendible by the polymerase since there is no 3′–5′ exonuclease activity. In such circumstances, pause sites may become termination sites with the partial extension products being effectively permanently removed from the amplification process. This leads to a reduction in amplification efficiency and, in a competitive PCR system, may give a marked difference in relative yield from a relatively small difference in amplification efficiency. In designing a competitive PCR, maintaining the minimal feasible difference in size and GC content may not be sufficient to ensure comparable amplification efficiencies. Equally, careful design of a mimic to avoid pause sites may not be beneficial unless the target can also be assured to lack similar pause sites. The use of betaine and/or the addition of a small amount of proof-reading enzyme may prove to be of great value in competitive systems where quantification requires equal amplification efficiencies, in other systems where balanced product yields are required such as multiplex PCR or where high amplification efficiencies are required such as in long PCR (N.A. Burns, unpublished data). The prophylactic use of betaine and/or proof-reading enzymes has demonstrated general benefits for a range of PCR applications and should perhaps be considered by laboratories seeking to ensure the highest levels of quality and confidence are achieved.

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