Strategy for Controlling Preferential Amplification and Avoiding False Negatives in PCR Typing

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ABSTRACT

The use of the PCR method for routine testing has increased dramatically during recent years. Most assays involve coamplification either of an internal control, of several alleles at a given locus or of a variety of bands produced by low-stringency primer annealing. In such multiplex reactions, certain products will often amplify preferentially. Amplimers that are more sensitive can be outcompeted under suboptimal PCR conditions, leading to assignment of false negatives. Optimization of PCR parameters such as temperature steps, relative concentrations of primers and their annealing temperature do not alone ensure against false negatives when caused by stable double-stranded DNA (dsDNA) regions in the amplified sequence. A two-step strategy to solve this problem is presented in this paper: (i) *titration of the PCR with NaCl as a model inhibitor to establish the critical range within which false negatives occur;* (ii) *titration of the PCR with a dsDNA-destabilizing additive under false-negative-inducing conditions until the relative amplification efficiencies of coamplified fragments are adjusted. Betaine is introduced as a novel and efficient cosolute. These measures to achieve reliable PCR typing of a difficult target should be useful for many qualitative and quantitative multiplex PCR applications.*

INTRODUCTION

Preferential amplification of coamplified targets or internal controls may be a small but significant hazard in many multiplexed polymerase chain reaction (PCR) assays in routine use. It tends to occur in a random, all-or-nothing fashion affecting some DNA preparations more than others (17). False negatives created by preferential amplification have been described occasionally (3,9,14,16,17), but little systematic research on the causes and remedies of this problem exists. Although uncertainties can be reduced by repeating the experiments, this is not always practical. As an alternative, we describe a simple strategy to test and optimize the robustness of PCR typing by using a group of alleles of the human HLA-B locus as a model system. It can be adapted easily to improve the reliability of new and existing assays.

MATERIALS AND METHODS

Annealing temperatures of the primers were calculated with the Oligo program (12) distributed by MedProbe A.S. (Oslo, Norway). The melting profiles of double-stranded DNA (dsDNA) were calculated by an updated version of the POLAND program (15).

All PCR mixtures contained the following at a final concentration: 100 µg/mL bovine serum albumin, 0.2 mM of each deoxyribonucleoside triphosphate, 0.025 U/µL *Taq* DNA polymerase and one of the buffers below. Primers were used at a final concentration of 3 ng/µL each. Tris buffer was made according to the standard recipe including 50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris base, pH 8.3, at 20°C. Commercial 10× concentrated Tris buffer was also obtained (Boehringer Mannheim, Mannheim, Germany). Tricine buffers contained at a final concentration 10 mM tricine, pH 8.3 at 20°C, and variable concentrations of betaine, trehalose, glycerol and $MgCl_2$ (see legends to figures and Results section).

Betaine (*N*,*N*,*N*-trimethylglycine monohydrate), glycerol and sodium chloride were purchased from Sigma Chemical (Poole, England, UK). Betaine was stored as 5 M stock in dH_2O at -20°C. In contrast to the changes previously reported using a phosphate-buffered solution (11), no discoloration upon long-term storage was observed. Stocks as old as one year and from two different batches were used without a change in PCR performance. D(+)-trehalose was from Fluka (Buchs, Germany); its concentrations are given as % (wt/vol) of the unhydrated form. Glycerol concentrations are given as volume %. Tricine (*N*-tris(hydroxy methyl)-methylglycine) was bought from BDH Chemicals (St. Louis, MO, USA).

Similar results were observed with *Taq* (*Thermus aquaticus*) DNA polymerase from Life Technologies (Paisley, England, UK), Boehringer Mannheim and Promega (Southampton, England, UK) and *Tbr* (*Thermophilos brockianus*) DNA polymerase from Biometra (Göttingen, Germany). Primers were synthesized on an Applied Biosystems Model 392 DNA/RNA Synthesizer (Perkin-Elmer/Applied Biosystems

Table 1. Primers and Amplified Sequences

Primer Pair	Primer Sequence 5′ → 3′	Gene	PCR Product Size
B7CREG1 B27R	CGCCGCGAGTCCGAGAG CCACGTCGCAGCCATACATA	HLA-B27 (target)	434 bp
B7CREG3 B0760R	CTCCCACTCCATGAGGTATTTCC CCGCGCGCTCCAGCTTG	HLA-B60/B41 (targets)	786 bp
tnfbl tnfbr	CGTGCTTCGTGCTTTGGACTA AGCTGGTGGGGACATGTCTG	TNF-beta (internal control)	737 bp
xabl xabr	AACTGCAGAGCGACTTCCATTC AGGTCATGCAGGGGGTAGTCCA	XA/XB (internal controls)	893 bp/10 014 bp

Division, Foster City, CA, USA), deprotected, precipitated with ethanol and used without further purification.

Genomic DNA was prepared by phenol/chloroform extraction and dissolved in Tris base/EDTA buffer, pH 8.0. Between 50 and 500 ng per 20 µL reaction were used in HLA typing. For NaCl titration experiments, DNA was desalted with 3 × 10 vol of double-distilled water in Microcentricon®-30 ultra-filtration tubes (Amicon, Beverly, MA, USA), and 100 ng in 2 µL were added per reaction tube. The TNF-B27 hybrid was constructed as described in the Results section.

The PCR was carried out in a total volume of 20 µL on a Perkin-Elmer DNA Thermal Cycler 480 (Norwalk, CT, USA) with a 4-min 94°C predenaturation; 5 cycles of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C; and 9 min at 72°C

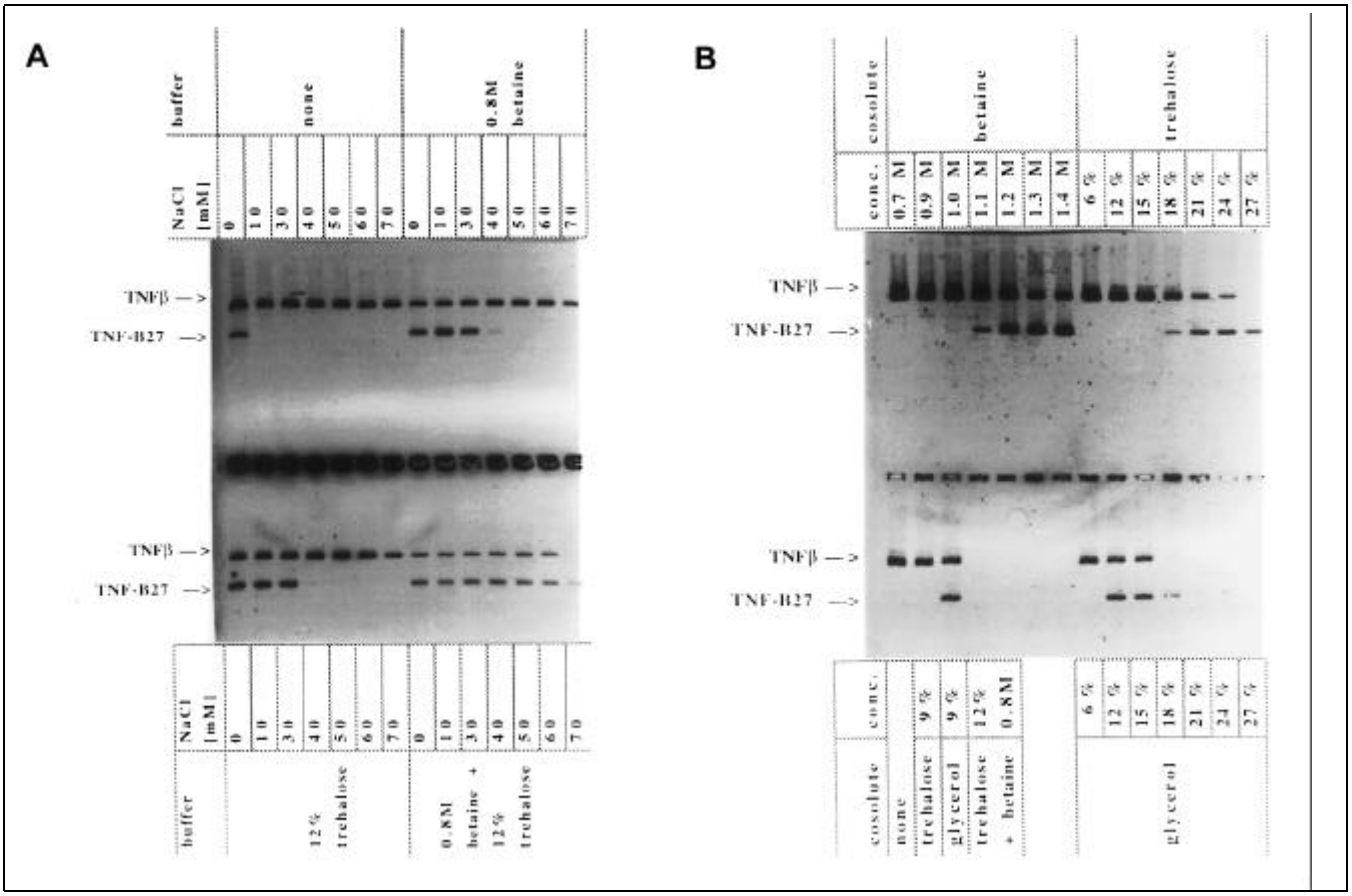

Figure 1. Preferential amplification due to internal sequence differences was reversed by sufficient concentrations of dsDNA destabilizing cosolute. Panel A: Effect of NaCl on TNF vs. TNF-B27 yields in the presence of dsDNA destabilizing additives. TNF-B27 (the smaller and more GC-rich amplimer) and TNF (longer product) were reamplified together across a range of NaCl concentration. Amplifications were carried out, without the addition of cosolutes, with 0.8 M betaine and with 12% trehalose. Panel B: Effect of different dsDNA destabilizing additives on the PCR in the presence of constant 60 mM NaCl. Amplified fragments are the same as in Panel A. Titrations of 0.7–1.4 M betaine, 6%–27% trehalose and 6%–27% glycerol are shown.

final extension.

Analysis of the PCR products was done by electrophoresis of 7 µL in 1.5% agarose gels containing 4% glycerol and 0.5× TBE buffer. Staining was with ethidium bromide. Pictures of the stained gels were taken with the Eagle Eye® Video System (Stratagene, Cambridge, England, UK) and are shown as negatives of the original image.

Table 2. Primer Sequences Used in the Generation of the TNF-B27 Hybrid Amplimer

tnfbl-B27	5′ CGTGCTTCGTGCTTTGGACTA*GtAc*CCGCGGGCGCCGT 3′
tnfbr-B27	5′ AGCTGGTGGGGACATGTCTG*Ta*CTGGAGGGTGTGAGACCCTG 3′

Bases identical to bases in the TNF-beta primer are underlined. *Rsa*I restriction sites are shown in italics; mismatches, in lower-case letters. The remaining 3′ ends correspond to the B27 sequence immediately adjacent to the primers B7CREG1 and B27R.

RESULTS

Primer Design

Primers were chosen to bridge exons 2 and 3 of the HLA-B gene where most of its allelic polymorphisms are located (Table 1). Sequences amplified as control were from TNF-beta and X genes. The X gene locus contains a duplication that resulted in one or two control PCR products, depending on the haplotype. Control primers were designed to amplify DNA fragments longer than the HLA-B targets. Several precautions were taken at this stage to maximize PCR product yield and specificity. The primers were checked for the absence of stable hairpins and 3′ primer dimers. Care was taken to avoid the base thymidine in the allele-specific 3′-mispairings (8). The frequency and stability of potential mispriming sites within the human genome (5) was checked by a data bank search with a 12-bp-long 3′ fragment of each primer sequence. Similar annealing temperatures were attempted for all primers.

A Preferential Amplification Model

Differences in PCR efficiency between coamplified amplimers are caused by primer-specific as well as template-specific effects. To study the influence of PCR conditions on each cause separately, a hybrid template was constructed that had an identical internal sequence of the HLA-B27 allele, but sites for the TNF instead of the B27 primers. This was achieved by two rounds of reamplification. In the first, oligonucleotides were used that carried sequences of the B27 fragment 3′ to that of the original TNF-beta control primers (Table 2). By reamplifying B27 PCR products with these primers, a hybrid product was obtained. This product was diluted $1/10^8$ and reamplified a second time using TNF-beta primers only, thereby diluting out residual B27 and hybrid primers. A single band of the expected size was obtained. The successful generation of the hybrid template was further confirmed by digestion with *Rsa*I, which cut within the internal sequence and at two new sites generated by the TNF-B27 primers (not shown). Products of this second round of reamplification were quantified by UV absorption (260 nm) and mixed with equal copy numbers of TNF or B27 PCR amplimers. These mixtures were diluted to give similar PCR product yields as the genomic template when amplified under the same conditions (about 10 000 copies of each amplimer).

Effects of NaCl and dsDNA Destabilizing Additives

Figure 1A shows the influence of NaCl on the coamplification of the two model amplimers. TNF-B27, the smaller and more GC rich of the two products, was specifically inhibited by 10 mM NaCl (upper right of the gel). The use of dsDNA destabilizing reagents has been widely recommended to improve the PCR of GC-rich templates. Amplification of TNF-B27 could be improved to tolerate up to 30 mM NaCl when 0.8 M betaine or 12% trehalose was added to the PCR (upper left and lower right). However, false negatives (i.e., no detectable yield of TNF-B27 in the presence of a strong TNF product band) still occurred in the range from 40 to 70 mM NaCl. Similar results were obtained with glycerol and formamide as PCR additives or by using PCR buffers containing KCl or NH_4Cl (not shown). Interestingly, the results indicated that preferential amplification of TNF "control" in presence of salt could be changed to preferential amplification of the TNF-B27 "target" when both additives were used together (lower left, 70 mM NaCl).

The following experiment (Figure 1B) was designed to test whether this effect was specific for a combination of trehalose and betaine or was a more general characteristic of dsDNA-destabilizing conditions. PCR was carried out in the presence of 60 mM NaCl, the concentration at which the maximal difference in TNF and TNF-B27 yields had been found. Figure 1B demonstrates that this salt concentration imposes no limits on the PCR of TNF-B27, provided that the concentration of betaine, trehalose or glycerol is also raised. Moreover, high enough concen-

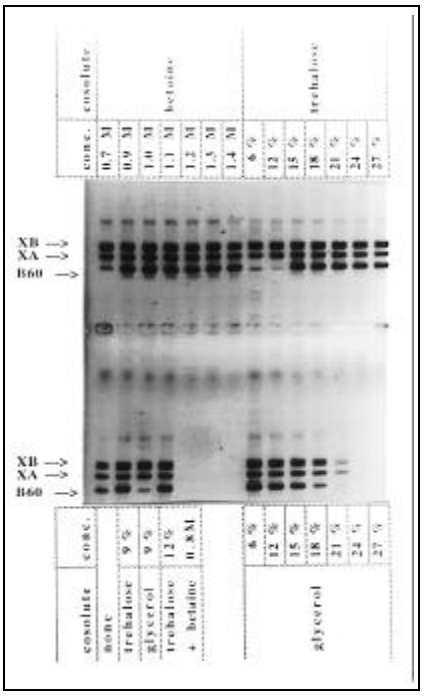

Figure 2. Betaine can prevent false negatives in a complex PCR typing system. Target HLA-B60 was amplified together with the XA and XB controls using genomic DNA from the human cell line PE117. Titrations of cosolutes were performed as for the model system in Figure 1B.

trations reversed preferential amplification, making the TNF "control" but not the TNF-B27 "target" more sensitive to inhibition. Although all three additives had this effect, the best overall yields in the model system were achieved with 1.3 M betaine.

Application to PCR Typing

Next, the optimization protocol developed in the model system was tested with other primer combinations and genomic DNA (Figure 2). The X-gene control was chosen to develop PCR genotyping for B27, B60 and several other HLA-B alleles. Betaine buffer did not affect the ability of *Taq* DNA polymerase to discriminate allele-specific 3′ mismatches, and no false negatives or positives were observed in the typing of over 150 control samples (Th. Weissensteiner and J.S. Lanchbury, unpublished). These systems were more complex: more potential mispriming sites existed in the genomic template (a target and two controls were coamplified and two different primer pairs were used). Nevertheless, the original tendency for preferential amplification of the control fragments (XA and XB) could be reversed with every target primer pair that was tested. The B60 amplimer shown in Figure 2 was the longest target and also the most sensitive to NaCl inhibition. Preferential amplification of X-gene control fragments can be seen at and below 0.7 M betaine and 12% trehalose (upper part of the gel). This could be misinterpreted as "B60-negative" typing results. Betaine and trehalose improved the relative, as well as absolute, yield of the B60 product. A concentration of 1.2 M betaine was sufficient to reverse preferential amplification of the X-gene controls vs. all HLA-B targets. Glycerol, however, did not give the HLA-B amplimer a competitive advantage in this system (Figure 2, lower right).

To see whether the above findings could be of general importance for other primer and PCR buffer systems, a recently published PCR assay (1) was tested in the same way (Figure 3). Substituting $(NH_4)_2SO_4$ for KCl has been claimed to improve some amplifications. The authors used control primers with a 10°C lower T_m and at 1/10 of the concentration of the target primers. As expected, this measure gave the target a competitive advantage at low salt concentrations. On the other hand, the control sequence was both longer and its dsDNA form less thermally stable. Figure 3 shows that the control is preferentially amplified at high salt concentrations. No false negatives were induced by NaCl when 17 mM ammonium

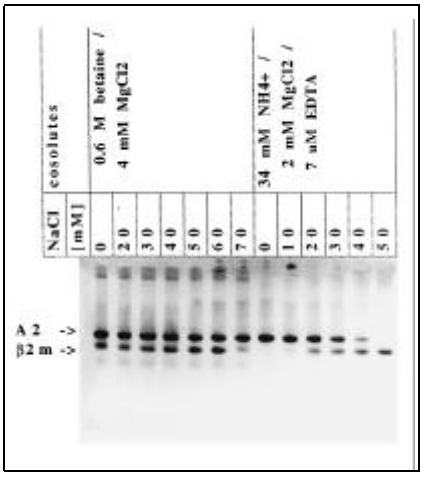

Figure 3. Detection and reversal of preferential amplification in an established PCR assay. The larger of the two coamplified fragments was the HLA-A target (A2); the smaller, the beta-2-microglobulin control (β2m). NaCl titrations from 0–70 mM and 0–50 mM are shown with and without the addition of betaine. Composition of betaine-free buffer was as in the original paper (1), except that tricine was used instead of Tris.

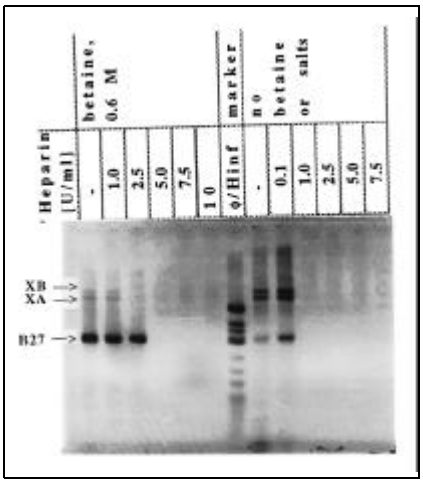

Figure 4. Betaine can improve the tolerance of a PCR assay to heparin inhibition. Genomic DNA from a HLA-B27-positive individual was typed, using the same controls as in Figure 2 but with B27 as the target allele. Heparin was added to the PCRs with and without betaine in concentrations from 0–10 U/mL.

sulfate was omitted from the PCR buffer and when betaine was added under otherwise identical PCR conditions.

Betaine Increases the Tolerance to Heparin Contamination

Heparin is a common contaminant in DNA preparations from clinical blood samples and certain cell types like peritoneal mast cells. It inhibits many DNA-modifying enzymes and, at least in one case, inhibition depends on motifs in the DNA sequence (4). Sequence-specific inhibition by heparin in the PCR would lead to preferential amplification. However, unlike NaCl, heparin caused only a general reduction of the PCR yield, with no primer or amplimer specific effects (Figure 4). Amplification was highly sensitive to heparin, but addition of betaine improved tolerance by an estimated 2.5-fold to 25-fold. More importantly, when betaine was added, the HLA-B27 target gained a competitive advantage over the X-gene controls, thus avoiding the danger of false-negative results.

Betaine Increases the Optimal Range for $MgCl_2$ Concentrations

A minimal concentration of 1.5 mM magnesium was needed for the amplification of the TNF fragment in KCl-free buffer in the presence of 0.6 M betaine (Figure 5, left). Yields of TNF were virtually unaffected up to 5.25 mM $MgCl_2$. Production of spurious PCR products (bands above and below the TNF product) was minimal at 6 mM magnesium concentrations. In the absence of betaine (Figure 5, right), the yields were slightly improved at 0.75 mM $MgCl_2$, but the optimum concentration produced yields that peaked at 1.5 mM. Above this, the yield of the TNF fragments decreased and the relative amount of spurious products increased.

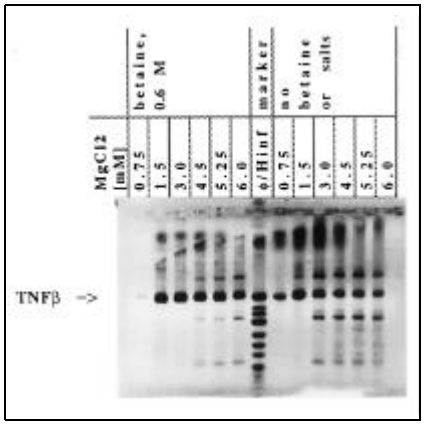

Figure 5. PCR in betaine buffer requires higher concentrations of Mg^{2+} and shows a broader optimal range. PCRs performed with TNF-beta primers using human genomic DNA as a template. $MgCl_2$ concentrations 0.75–6.0 mM were titrated in buffer with 0.6 M betaine or without.

Table 3. Guidelines for Minimizing the Occurrence of False Negatives Due to Preferential Amplification in PCR

1. **Design *primers* to give the target amplimer a competitive advantage over the internal control.**
   Choose target and control fragment to have a similar GC content.
   Make the control longer than the target amplimer.
   Design control primers to have a lower T_m than the target primers (ca. -10°C).
2. **Test for preferential amplification of the internal control due to *internal sequence differences*.**
   Titrate multiplex PCR with NaCl (0–80 mM in 10-mM increments). Is the internal control less sensitive to inhibition than the target?
   If so, proceed with step 3.
3. **Optimize buffer**.
   Omit monovalent cations like potassium and ammonium in the PCR buffer. Choose the NaCl concentration in step 2 at which the maximal difference in the yields of target vs. control occurred. Titrate optimal concentration of a dsDNA-destabilizing cosolute under critical NaCl inhibition (i.e., 0–1.5 M betaine in 0.3-M increments). Target yields should be somewhat greater than that of the control. If optimized buffer gives low yields in the absence of NaCl, adjust the magnesium concentration (2× of the optimum concentration used in standard PCR buffer may be necessary for some primers).

DISCUSSION

The critical finding of this study is that the preferential amplification of a longer, but less GC-rich, fragment can be altered in favor of a shorter but more GC-rich amplimer. As shown by the TNF-beta/TNF-B27 model system, these effects are not due to the primers. Because of differences in the amplified sequences, preferential amplification affects all multiplex PCR systems. To understand it, however, both the thermodynamics and the kinetics of the DNA association/dissociation reactions must be considered. The situation is further complicated by the fact that the influence of a solute mixture on dsDNA stability is not always predictable from that of solutions of its isolated components (10). Extrapolations from these data to conditions in the PCR, as in the following discussion, must therefore be tentative.

Betaine, a Versatile Novel Cosolute for PCR

Optimal yield and reliability of PCR typing was achieved by introducing betaine, a cosolute whose use in the PCR has not been described before. Betaine is nontoxic, inexpensive and has a lower viscosity than glycerol. In addition, this cosolute proved suitable for the preparation of premixed and dried PCR reagents, and it was compatible with various restriction enzymes, dot blotting and chemiluminescent detection of PCR products (Th. Weissensteiner et al., unpublished). Betaine increases the thermal stability of proteins (13) and prevents nonspecific interaction of topoisomerase with single-stranded DNA (ssDNA) at high temperatures (7). Nonspecific binding is also the mechanism by which the polyanion heparin inhibits DNA-processing enzymes. An increased thermal stability (and therefore dsDNA specificity) of *Taq* DNA polymerase may be one reason for the increased tolerance of the PCR to heparin when betaine buffer is used.

Another attractive property of

betaine is its ability to bind and stabilize AT base pairs. Together with its general DNA-denaturing effect, the net result is a specific destabilization of GC base pairings under isothermal equilibrium conditions (11). Such a property could be very useful if it is applied also to the non-equilibrium conditions of rapid thermal cycling. First, reduced primer affinity is likely to set the upper limits for the use of dsDNA-destabilizing PCR conditions. In theory, much higher concentrations of cosolute should be tolerated if betaine is used together with an AT-rich primer.

Second, differences in the amplification efficiencies of two different amplimers, due to the thermal stability of their internal sequences, could be reduced. A related substance, tetramethyl-ammonium chloride (TMACl), has been reported to enhance PCR specificity when as little as 10–100 μM concentrations are added to the standard KCl buffer (6). It was found during this study that even 50 mM TMACl is tolerated when KCl is reduced accordingly, and TMACl is able to reverse preferential amplification (not shown). Betaine as a nonionic substance could be used in concentrations over 1 M. However, much higher concentrations than these would be needed to isostabilize TNF and TNF-B27 amplimers, even when their difference in length is taken into account [isostabilization of AT and GC base pairs requires 3.3 M TMACl or 5.2 M betaine (11)]. The reversal of preferential amplification seen in the model system can therefore not simply be explained by a reversal in their relative T_m. Moreover, preferential amplification could also be reversed by trehalose and glycerol, which share little chemical homology with TMACl or betaine (Figure 1B). Whether this is due to a GC-specific destabilization or perhaps kinetic effects remains to be investigated, but reagents with sequence-specific dsDNA stabilizing or destabilizing properties could be of great value for PCR and other DNA and RNA polymerase reactions.

Multiplex PCR Under Suboptimal Conditions

Conventional strategies have relied on amplimer length and primer efficiency to adjust the relative sensitivities of coamplified targets and controls. While this may be sufficient for such variables as the amount of DNA used and its integrity, a decreased yield of control product also reduces the sensitivity of the assay. More importantly, it does not always guarantee against a selective dropout of the target amplimer under other suboptimal PCR conditions. It appears that a combination of dsDNA destabilization, with either higher primer efficiency or shorter length, is

necessary to give an amplimer with a high T_m a competitive advantage.

The danger of false negatives due to preferential amplification should also be considered when setting up other multiplex PCR systems. This paper shows how preferential amplification can be tested and controlled by a number of simple steps (see Table 3). The same strategy should help to increase the reproducibility of low-stringency PCR banding patterns, such as random amplified polymorphic DNA (RAPD) (2) and differential display PCR, and improve the reliability of long-range amplifications.

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