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Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS)

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ABSTRACT

We have improved the "polymerase chain reaction" (PCR) to permit rapid analysis of any known mutation in genomic DNA. We demonstrate a system, ARMS (Amplification Refractory Mutation System), that allows genotyping solely by inspection of reaction mixtures after agarose gel electrophoresis. The system is simple, reliable and non-isotopic. It will clearly distinguish heterozygotes at a locus from homozygotes for either allele. The system requires neither restriction enzyme digestion, allele-specific oligonucleotides as conventionally applied, nor the sequence analysis of PCR products. The basis of the invention is that unexpectedly, oligonucleotides with a mismatched 3'-residue will not function as primers in the PCR under appropriate conditions. We have analysed DNA from patients with α1-antitrypsin (AAT) deficiency, from carriers of the disease and from normal individuals. Our findings are in complete agreement with allele assignments derived by direct sequencing of PCR products.

INTRODUCTION

The analysis of nucleic acid sequence is central to biology. Determination of variation in DNA sequence between individuals underpins molecular genetics. Such analysis is routinely performed by examination of restriction fragment length polymorphism (RFLP) using the Southern blotting technique (1,2,3). This approach has proved enormously useful, generating a massive literature, despite the fact that it is relatively slow and only allows for the examination of the limited number of polymorphic base changes which either create or destroy a restriction endonuclease recognition site. Without doubt any method which enabled all polymorphic base changes in the genome to be examined in a facile manner would be invaluable to the molecular genetics community.

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PCR (4) has greatly facilitated the analysis of genomic DNA. It allows diagnosis of genetic diseases when combined with one of a variety of other techniques (5,6,7,8,9,10,11,12). We and others have reported the use of PCR and direct sequencing for diagnosis of inherited diseases (5,6,7,13). Allele-specific oligonucleotides (ASOs), (14,15) either radio-labelled (8) or non-isotopically tagged (9) have been applied to disease diagnosis in the conventional manner by probing dot blots of PCR products. Occasionally a point mutation giving rise to a specific phenotype may create or destroy a restriction enzyme recognition site (2). In such instances PCR products may (or may not) be cleaved when treated with the restriction enzyme. The presence or absence of the restriction site can be used to perform diagnoses as recently demonstrated for sickle cell anaemia (10). Similarly a polymorphic restriction site may be in linkage with an uncharacterised mutation allowing diagnoses to be performed in informative families by analysis of the amplified restriction site polymorphism (11,16,17).

We demonstrate here a general technique which allows the scrutiny of any point mutation polymorphism. The technique requires that the terminal 3'-nucleotide only of a PCR primer be allele specific. Thus the primer is synthesised in two forms. The 'normal' form is refractory to PCR on 'mutant' template DNA and the 'mutant' form is refractory to PCR on 'normal' DNA. In some instances a single 3'-mismatched base does allow amplification to proceed. We have shown that introducing additional deliberate mismatches near the 3' end of appropriate primers ameliorates this problem.

Molecular characterisation of the genes associated with the more common inherited disorders is constantly providing new information about the underlying, disease-associated mutations. Indeed recent sequencing of mutant ß-globin genes has only rarely resulted in the discovery of novel alleles (18). This implies that at the ß-globin locus characterisation of the molecular pathology is nearing completion (13). Diseases such as cystic fibrosis, as yet uncharacterised at the gene level, may have several RFLPs in linkage disequilibrium with the affected phenotype (19). Such RFLPs are useful for haplotype analysis and

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risk assessment of carrier status particularly where there is a family history of the disease (20). Furthermore, flanking sequences of some such RFLPs have been determined allowing PCR followed by restriction analysis for haplotype identification (16,17). Some concern has been expressed as to the reliability and reproducibility of such assays in the absence of rigorous and appropriate internal controls. In theory ARMS would allow rapid haplotype analysis in such situations, providing sufficient genomic sequence is known around the polymorphic restriction site.

The feasibility of our ARMS was demonstrated by the amplification of exon III and part of intron III in the human AAT gene (figure 1). Direct application of ARMS to the clinically significant S and Z alleles of AAT (21) was performed and the diagnoses were in agreement with the results of sequence analysis of the PCR products (5).

MATERIALS AND METHODS

DNA preparation

Genomic DNA was isolated from peripheral blood cells as described previously (5).

Oligonucleotide amplification, amplification refractory and sequencing primers

The common primers 1,2,5 and 6 (figure 1) were those described previously (5). Their respective sequences were d(CCCACCTTCCCCT CTCTCCAGGCAAATGGG), d(GGGCCTCAGTCCCAACATGGCTAAGAGGTG), d(TGTCCA CGTGAGCCTTGCTCGAGGCCTGGG) and d(GAGACTTGGTATTTTGTTCAATCATTAAG). Primer 2a and the 3,4,7 and 8 series of primers (figure 2) as well as the primers for the internal control, a 510 base pair fragment from the unusually long exon 26 of the human apolipoprotein B gene (22) were prepared as described (5) and were used without further purification. The sequencing primers for initial allele characterisation were those described earlier (5).

Allele characterisation by PCR and direct sequencing

Mutant and normal alleles of the AAT S and Z loci were confirmed by PCR amplification either as described (5), or as follows; target sequences were amplified in a 100µl reaction volume

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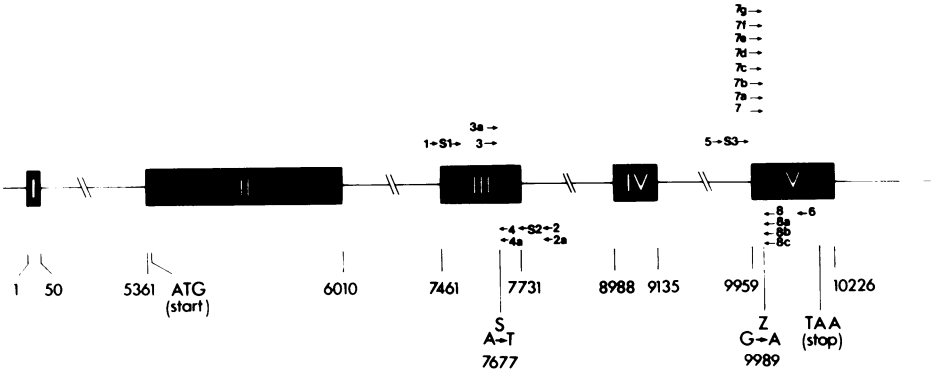

Figure 1
The human alpha-1-antitrypsin gene. Coordinates are as described by Long et al. (24). Position 1 is the proposed transcription start site. The solid boxes represent the five exons, the S and Z loci are shown, as are the respective mutations responsible for the S and Z phenotypes. The arrows below and above the gene represent the various primers used. Primers prefixed by S are those used in direct sequencing of PCR products to confirm genotypes prior to ARMS analyses. The remaining primers are those used to demonstrate the feasibility of the ARMS concept and those used in ARMS analyses, these primers are shown in detail in figure 2.

containing approximately 1µg genomic DNA, deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and thymidine triphosphate (TTP), each 1.5mM, 67mM Tris-HCl (pH8.8), 16.6mM ammonium sulphate, 6.7 mM magnesium chloride, 10mM 2-mercaptoethanol, 6.7µM EDTA and 1µM each appropriate amplification primer. Samples were heated at 100°C for 5 minutes to denature the DNA. Two units of Thermus aquaticus (Taq) DNA polymerase (23) (Anglian Biotechnology) were added to each sample. Samples were overlaid with light mineral oil (Sigma, 50µl) then heated at 60°C for 4 minutes for the first round of DNA synthesis. (See Discussion). Subsequent cycles consisted of a two minute denaturation step at 92°C and a combined primer annealing and DNA synthesis step at 60°C for 4 minutes. 33 cycles were performed and the DNA synthesis step of the final cycle was extended to 20 minutes. Direct sequencing of the PCR products was as described previously (5).

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ARMS analysis of genomic DNA

The feasibility of the ARMS concept was demonstrated using duplicate samples of genomic DNA from one normal individual. AAT exon III primers 1 and 2 were used with one sample; primers 1 and 2a (3'C/T mismatch) were used with the other sample. AAT exon V primers 5 and 6 were present in both samples serving as an internal control. All primers are shown in figure 1. PCR reactions were performed and examined by agarose gel electrophoresis (3% Nu-sieve) as previously described (5).

In applying ARMS to subsequent mutation analysis, primers 'Control 1' d(CTCTGGGAGCACAGTACGAAAAACCACTT) and 'Control 2' d(AA TGAATTTATCAGCCAAAACTTTTACAGG) were included in all reactions and served to provide an internal control PCR product. The control primers amplify a 510 base pair product within exon 26 of the human apolipoprotein B gene (22).

Genomic DNAs of characterised AAT genotypes MM,MS,MZ and ZZ were subjected to PCR so as to amplify the internal control fragment. In separate pairs of reactions each DNA was either coamplified with the appropriate 'normal' or 'mutant' primer paired with a common primer for the respective AAT locus. These primers are shown in figure 2.

The reactions for the ARMS analyses were performed in a volume of 100µl containing approximately 1µg genomic DNA. dATP, dCTP, dGTP and TTP were each 1.5mM in 67mM Tris-HCl (pH8.8), 16.6mM ammonium sulphate, 6.7mM magnesium chloride, 10mM 2-mercaptoethanol, 6.7µM EDTA and 1µM each appropriate amplification primer. Samples were heated at 100°C for 5 minutes to denature the DNA. Two units Taq DNA polymerase (Anglian Biotechnology) was added to each sample. Samples were overlaid with light mineral oil (Sigma, 50µl) then heated at 60°C for 4 minutes for the first round of DNA synthesis. Subsequent rounds of amplification comprised two minutes denaturation at 92°C followed by combined primer annealing and DNA synthesis at 60°C for four minutes. 33 cycles were performed in this way with the final synthesis step extended to 20 minutes. 18µl from each reaction was combined with 2µl of 50% glycerol 0.2% bromophenol blue in 1X TBE then electrophoresed on 1.4% agarose gels in 1X TBE containing 0.5µg/ml ethidium bromide.

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5' ATTCCCCAACCTGAGGGTGACCAAGAAGCTGCCCACACCTCTTAGCCATGTTGGGACTGAGGCCCATCAGGACTGGC 3'
3' TAAGGGGTTGGACTCCCACTGGTTCTTCGACGGGTGTGGAGAATCGGTACAACCCTGACTCCGGGTAGTCCTGACCG 5'
   |                        Primer 2   GTGGAGAATCGGTACAACCCTGACTCCGGG 5'        |
 7735                       Primer 2a  TTGGAGAATCGGTACAACCCTGACTCCGGG 5'      7811


      5' GCCTGATGAGGGGAAACTACAGCACCTGGT  Primer 3a
      5' GCCTGATGAGGGGAAACTACAGCACCTGGA  Primer 3
5' CTTCCTGCCTGATGAGGGGAAACTACAGCACCTGGaAAATGAACTCACCCACGATATCATCACCAAGTTCCTGGAAA 3'
3' GAAGGACGGACTACTCCCCTTTGATGTCGTGGACCtTTTACTTGAGTGGGTGCTATAGTAGTGGTTCAAGGACCTTT 5'
   |                        Primer 4   TTTTACTTGAGTGGGTGCTATAGTAGTGGT 5'        |
 7642                       Primer 4a  ATTTACTTGAGTGGGTGCTATAGTAGTGGT 5'      7718


      5' CCGTGCATAAGGCTGTGCTGACCCTCGACA  Primer 7g
      5' CCGTGCATAAGGCTGTGCTGACCCTCGACG  Primer 7f
      5' CCGTGCATAAGGCTGTGCTGACCATAGACA  Primer 7e
      5' CCGTGCATAAGGCTGTGCTGACCATAGACG  Primer 7d
      5' CCGTGCATAAGGCTGTGCTGACCATCGCCA  Primer 7c
      5' CCGTGCATAAGGCTGTGCTGACCATCGCCG  Primer 7b
      5' CCGTGCATAAGGCTGTGCTGACCATCGACA  Primer 7a
      5' CCGTGCATAAGGCTGTGCTGACCATCGACG  primer 7
5' TCCAGGCCGTGCATAAGGCTGTGCTGACCATCGACgAGAAAGGGACTGAAGCTGCTGGGGCCATGTTTTTAGAGGCC 3'
3' AGGTCCGGCACGTATTCCGACACGACTGGTAGCTGcTCTTTCCCTGACTTCGACGACCCCGGTACAAAAATCTCCGG 5'
   |                        Primer 8   CTCTTTCCCTGACTTCGACGACCCCGGTAC 5'        |
 9954                       Primer 8a  TTCTTTCCCTGACTTCGACGACCCCGGTAC 5'     10030
                            Primer 8b  CTCATTCCCTGACTTCGACGACCCCGGTAC 5'
                            Primer 8c  TTCATTCCCTGACTTCGACGACCCCGGTAC 5'
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Figure 2
ARMS primers. The top panel shows the primers used to test the ARMS concept. Primer 2 is complementary to the coding strand of the AAT gene. Primer 2a shows the 3'-OH mismatched T residue. Primers 2 and 2a are used in conjunction with primer 1 (figure 1). The centre panel shows the ARMS primers employed at the AAT S locus. The lower case A/T base pair is the AAT S locus and the depicted sequence is the normal sequence. The AAT S variant DNA has a T/A base pair at this position. Primers 3 and 4 correspond to 'normal' sequence, primers 3a and 4a correspond to 'mutant' sequence. The lower panel shows the the ARMS primers employed at the AAT Z locus. The lower case G/C base pair is the Z locus and the normal sequence is shown. The AAT Z variant DNA has an A/T base pair at this position. Primers 7 and 8 correspond to the 'normal' sequence, primers 7a and 8a correspond to the 'mutant' sequence. Primers 7, 8, 7a and 8a have not been destabilised. The remaining primers in the 7 and 8 series are destabilised and the deliberately introduced mismatches are underlined. Primers 7b, 7d, 7f and 8b correspond to 'normal' sequence (discounting the deliberate mismatches) likewise primers 7c, 7e, 7g and 8c correspond to 'mutant' sequence, again discounting the introduced mismatches. The position numbers are as described by Long et al. (24).

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RESULTS

ARMS primers

Figure 1 shows the ARMS primers in relation to the human AAT gene. Figure 2 shows each ARMS primer sequence in detail with respect to the gene. The position numbers are measured from the proposed transcription start site of the AAT gene (24). Discounting the variable 3' nucleotides and deliberately introduced mismatches, the 2,3,7 and 8 series primers are 59% GC 30mers and the 4 series primers are 41% GC 30mers. The common primers 1,2,5 and 6 are a 63% GC 30 mer, 60% GC 30mer, 67% GC 30mer and a 31% GC 29mer respectively.

Feasibility of the ARMS concept

We have shown previously that the AAT gene regions bounded by primers 1 and 2 and by primers 5 and 6 (figures 1 and 2) can be coamplified without affecting the efficiency of amplification of either target performed in isolation (5). We chose to introduce a 3' terminal base change into primer 2. Specifically the 3' dG residue was replaced by T to provide primer 2a. This substitution generates a template/primer C/T mismatch. When primers 1,2,5 and 6 are combined in a PCR, both the 360 bp product bounded by primers 1 and 2, and the 220 bp product bounded by primers 5 and 6 are observed (figure 3, lane 1). Substitution of primer 2 by primer 2a however blocks amplification of the 360 bp product while the internal control 220 bp product is still generated (figure 3, lane 2). This result is attributable to the lack of a 3' exonucleolytic proofreading activity of Taq DNA polymerase (23) in agreement with the observations of Tindall and Kunkel (25).

ARMS analysis of the AAT S locus

Genomic DNAs, either homozygous normal with respect to the AAT gene S allele or heterozygous S were each amplified as described in Materials and Methods. Each DNA was separately amplified using primers 2 and 3 and primers 2 and 3a. Primer 3 corresponds to the normal sequence at the S locus and primer 3a corresponds to the S variant sequence. In all reactions the internal control primers were also included.

On the normal DNA substrate, product was derived only from the internal control primers and primers 2 and 3. No product was

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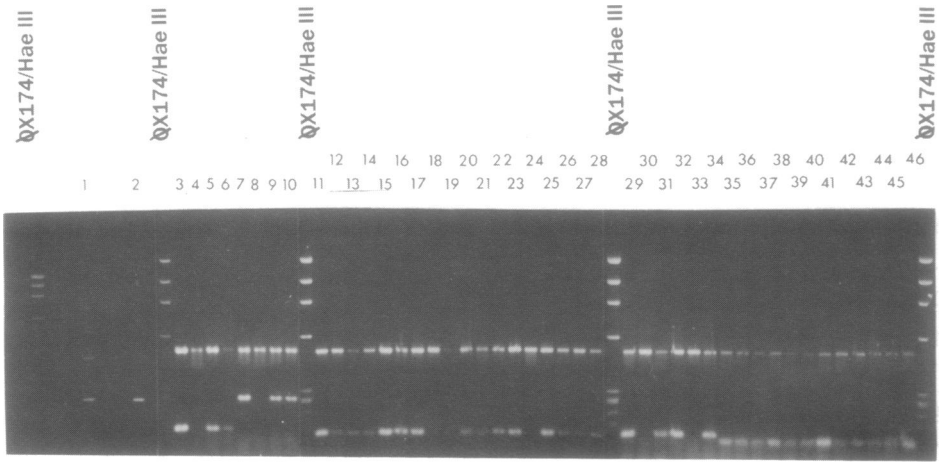

Figure 3
Agarose gels showing the feasibility of the ARMS concept (lanes 1 and 2) and ARMS analyses at the AAT S locus (lanes 3 to 10) and AAT Z locus (lanes 11 to 46) Specific reactions are as described in the text (results section).

observed when primer 3a replaced primer 3. When heterozygous S DNA replaced homozygous normal DNA the expected 152 bp product was generated when either primer 3 or 3a was included in the reaction (figure 3, lanes 3 to 6). Primer 3 generates an A/A mismatch with S variant DNA and primer 3a generates a T/T mismatch with normal DNA. When the ARMS detection primers were designed for the opposing strand at the S locus (primers 4, normal and 4a, S variant) and used for amplification with a common primer 1, similar results were obtained. The 510 bp internal control was generated but the 267 bp product was observed only when the normal primer was applied to normal DNA or the normal or S variant primer was applied to heterozygous S DNA. The 267 bp product was not generated when the S variant primer was applied to normal DNA (figure 3, lanes 7 to 10). Primer 4 generates a T/T mismatch with S variant DNA and primer 4a generates an A/A mismatch with normal DNA.

ARMS analysis of the AAT Z locus

In analogous experiments to the ARMS analyses of the AAT S locus we amplified genomic DNAs characterised as normal, heterozygous and homozygous at the AAT Z locus. All reactions contained the

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internal control primers 1 and 2. Initial experiments contained primer 7 (normal) or 7a (mutant) for amplification with common primer 6 to yield a 150 bp product (figures 1 and 2). Alternative experiments targetting the opposing strand and employing primers 8 (normal) or 8a (mutant) for amplification with common primer 5 (figures 1 and 2) would give products of 129 bp. Figure 3, lanes 11-16 and lanes 35-40 shows the products generated by the respective use of primer 7 with normal DNA, primer 7a with normal DNA, primer 7 with heterozygous DNA, primer 7a with heterozygous DNA, primer 7 with homozygous Z (ZZ) DNA, primer 7a with ZZ DNA, primer 8 with normal DNA, primer 8a with normal DNA, primer 8 with heterozygous DNA, primer 8a with heterozygous DNA, primer 8 with ZZ DNA and primer 8a with ZZ DNA. In contrast to the ARMS data for the AAT S locus, the corresponding results for the AAT Z locus show reduced specificity in that products were evident using either normal primer with ZZ DNA and either mutant primer with normal DNA. Primer 7 with ZZ DNA generates a primer/template G/T mismatch. Conversely primer 7a with normal DNA generates an A/C mismatch. Primer 8 with ZZ DNA generates a C/A mismatch and primer 8a with normal DNA generates a T/G mismatch.

In an attempt to increase the specificity of the ARMS primers we chose to deliberately introduce an additional mismatch near their 3'-ends. When primers 7f and 7g which have a deliberate C/T mismatch seven bases from the 3'-end were introduced to replace primers 7 and 7a (figure 2) specificity was improved. Figure 3, lanes 17-22 shows the products of these reactions. In particular, lane 18 shows the virtual absence of the 150 bp product when the mutant primer (7g) is applied to normal DNA. Unfortunately the normal primer (7f) still generates a small amount of product with ZZ DNA (figure 3, lane 21), but much reduced with respect to the yield with the mutant primer on ZZ DNA with equivalent internal control products. When primers 7d and 7e which have a deliberate A/G mismatch five bases from their 3'-ends were introduced to replace primers 7 and 7a similar results were obtained (figure 3 lanes 23 to 28) to those with primers 7f and 7g. When primers 7b and 7c replaced primers 7 and 7a in the system the desired specificity was observed. Primers 7b and 7c have a deliberate C/T mismatch three bases from their 3'-ends. Specifically only primer

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7b generated a 150 bp product with normal DNA (fig. 3 lane 29). The 'mutant' primer 7c failed to do so (fig. 3 lane 30). Both primers 7b and 7c generated product from heterozygous DNA (fig. 3 lanes 31 and 32). Primer 7b failed to generate the 150 bp product with ZZ DNA whereas the 'mutant' primer 7c did generate the 150 bp product (fig. 3 lanes 33 and 34). Similar exchange of primers 8 and 8a for primers 8b and 8c (which have an A/A mismatch four bases from their 3'-ends) showed marginal increased specificity (fig. 3 lanes 41 to 46).

DISCUSSION

Interest is increasingly being focused on the mutations in the human genome which produce disease states. The number of such mutations characterised at the DNA sequence level is increasing rapidly and this has been substantially aided by the PCR/direct sequencing approach for the analysis of genomic DNA. We previously reported that PCR followed by direct sequencing was absolutely specific for the diagnosis of AAT deficiency (5). In this communication we present ARMS, a system allowing the direct analysis of any locus of interest and thus generally applicable to any inherited disease provided sufficient sequence data is available. ARMS is simple, rapid and reliable providing the capability for accurate pre- and postnatal diagnosis and a means for heterozygote detection. ARMS is still of benefit even if disease-associated mutations, as yet uncharacterised, are linked to characterised polymorphisms. In such instances the technique will allow detailed haplotype analyses to be performed with a minimal quantity of DNA. Accurate prenatal diagnoses are achievable in a few hours if maternal contamination of the foetal material is avoided. An important practical consideration with this approach (as with other PCR-based strategies) is that it is unecessary to prepare high quality DNA suitable for restriction enzyme digestion.

A prerequisite of ARMS is the absence of a 3'-exonucleolytic proofreading activity associated with the DNA polymerase employed. The lack of such an exonuclease associated with Taq DNA polymerase has been confirmed here by the successful application of ARMS and independently (23,25). Another requirement in the

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application of ARMS is that 3'-OH terminal mismatched primers are refractory to extension by the chosen DNA polymerase. This was not apparent from the work of Tindall and Kunkel (25), since their exonuclease assay required, and did generate, polymerase products from C/A mismatched primer/template complexes. Taq polymerase refractory mismatches have been demonstrated in this work for some mismatched primers. In instances where the mismatch is not refractory to extension (as demonstrated with primer/template G/T, A/C, C/A and T/G mismatches at the AAT Z locus) further deliberate mismatches to destabilise the primer/template complexes render the primers increasingly refractory as the additional mismatch is moved progressively closer to the 3' end.

Empirically, the degree of specificity observed with mismatched primers (and thus the requirement for additional destabilisation), correlates with the mismatch type. C/T, A/A and T/T mismatches (which are all either purine/purine or pyrimidine/pyrimidine mismatches) are considerably more refractory to extension by Taq polymerase than G/T, T/G, A/C or C/A mispairs (all purine/pyrimidine mismatches). We have not yet optimised the position for introduction of the deliberate mismatches, nor the type of mismatch, neither have we examined the effect of deliberate base-pair insertions, deletions or modifications which may also be expected to appropriately destabilise non-refractory primers.

It is likely that any of these approaches to deliberately destabilise the ARMS primers and hence improve specificity may be enhanced by reducing dNTP, magnesium and primer concentrations or simply increasing the amplification annealing/extension temperature. Conversely an increase in concentration of these reagents or a decreased amplification annealing temperature might be expected to have an adverse effect on specificities of primers which previously generated no unwanted product.

We have deliberately chosen relatively unforgiving amplification conditions in this series of experiments so as to challenge the basic concept fully. Removal of tubes from the heating block to facilitate the addition of enzyme after initial DNA denaturation at 100°C was performed in the experiments described herein.

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Undoubtedly this allows cooling of reaction mixtures and is difficult to control precisely. Products were generated at the AAT Z locus with mismatched, non-destabilised primers and primers with additional mismatches 7, 5 and 4 bases from their 3'-termini. It is conceivable that the generation of these products will be avoided, if, after heat denaturation of the genomic DNA in the presence of primers the reactions are not allowed to briefly cool during enzyme addition. These products may result from a proportion of template molecules being primed at a less stringent lower temperature than the routine extension temperature. Any extension products so derived would then be correctly paired with the ARMS primer in subsequent cycles, and so generate the observed unwanted products.

Destabilisation of the ARMS primers where necessary such that anomalous products are not generated has been one of our approaches to the development of this technique at this early semi-manual stage. Obviously, further refinement is possible by optimising such variables as magnesium, dNTP or Taq polymerase concentrations and the precise temperature throughout the ARMS cycles. Careful control of the later variable in particular should be achievable with fully automated ARMS instrumentation. Indeed, addition of Taq polymerase to ARMS reaction mixtures at 60°C and ensuring that the reaction temperature never falls below this, may significantly increase reaction specificity and avoid generation of products on mismatched templates.

The AAT Z mutation is caused by a G to A transition immediately preceded by a C residue. The AAT S phenotype results from an A to T mutation. Analysis of single base mutations within coding regions causing human genetic disease shows that 35% of such mutations have occured within CpG dinucleotides and that over 90% of these were either C to T or G to A transitions (26). Since such mutations would generate the same primer/template mismatches as at the AAT Z locus, it is expected that destabilisation of ARMS primers will be required for at least 30% of ARMS potential applications.

We have chosen to use large (30mer) primers in this assay since this allows the use of high annealing temperatures to improve specificity and reduces the chance of mispriming elsewhere on

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genomic DNA. An alternative approach might be to use shorter primers which span a point mutation such that discrimination between 'normal' and 'mutant' loci is achieved by hybridisation of the primers in an allele-specific manner under appropriately stringent conditions. This type of analysis, also of the AAT Z locus, has been performed by Dermer and Johnson (27). It is important to note however that in this analysis the specificity of the primers was not absolute, the absence of internal controls could conceivably give rise to incorrect diagnoses and hybridisation to blots of the reaction mixtures was required. Other disadvantages would be that different conditions would have to be determined for each locus of interest which would complicate the simultaneous examination of multiple loci. There would also be the danger of the primers priming at loci other than those desired.

It has not escaped our notice that ARMS may have many other applications in medicine and molecular biology. The technique will be useful for the precise typing of infectious pathogens where characteristic strain-specific base changes can be identified. The analysis of oncogene activation is rendered straightforward as is the detection of deletions in DNA. Many further applications can also be envisaged in the research context.

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Patent applications relating to the methods described here are pending.

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