# Alkaline-mediated differential interaction (AMDI): A simple automatable single-nucleotide polymorphism assay

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**The key requirements for high-throughput single-nucleotide polymorphism (SNP) typing of DNA samples in large-scale disease case-control studies are automatability, simplicity, and robustness, coupled with minimal cost. In this paper we describe a fluorescence technique for the detection of SNPs that have been amplified by using the amplification refractory mutation system (ARMS)-PCR procedure. Its performance was evaluated using 32 sequence-specific primer mixes to assign the HLA-DRB alleles to 80 lymphoblastoid cell line DNAs chosen from our database for their diversity. All had been typed previously by alternative methods, either direct sequencing or gel electrophoresis. We believe the detection system that we call AMDI (alkaline-mediated differential interaction) satisfies the above criteria and is suitable for general high-throughput SNP typing.**

Human population genetic studies were initiated following the discovery of the ABO blood groups by Landsteiner in 1900. For many years, they were limited to the serologically detectable red cell blood groups (1) but eventually included serological detection of the determinants of the highly polymorphic HLA system (2, 3). Starch gel electrophoresis and other related techniques for proteins provided an added dimension for studying population variability (4), but the scope of these various serological and protein-based assays for polymorphism detection was relatively limited. Restriction fragment length polymorphism (RFLP; refs. 5 and 6) provided the first basis for studying genetic polymorphism at the DNA level and revolutionized the scope of human genetic studies, as was soon realized by Solomon and Bodmer (7) and Botstein *et al.* (8). Since then a variety of assays for polymorphism detection at the DNA level has essentially completely replaced the former serological and protein-based assays even for routine ABO typing for blood transfusion. The impetus for large-scale human genetic studies comes both from an interest in what they can tell us about the interrelationships of human populations (9) and from their importance as a basis for studying inherited susceptibility to multifactorial diseases.

Studies on the association between alleles of the HLA system and a wide variety of diseases have provided the basis for the main approach that is now widely used for looking for marker associations with a disease as a clue to the definition of genetically based susceptibility (10). The simplest approach is the case-control study, in which a given marker, now usually a single nucleotide polymorphism (SNP), is tested on a disease cohort and an appropriate sample of controls with the basic aim of looking for a significant difference in the frequency of the marker in diseased and control individuals. This then, through linkage disequilibrium, provides the clue to the existence of a nearby susceptibility-determining variant. The widespread application of this approach requires high-throughput typing of DNA samples for SNPs (11).

**Development and Rationale for the Alkaline-Mediated Differential Interaction (AMDI) Assay.** The amplification refractory mutation system (ARMS)-PCR or allele-specific PCR assay (also known variously as SSP or ASP) has been widely used for HLA typing at the DNA level (12–16) as well as in other applications (17, 18). This assay can detect up to two cis-located motifs per reaction, using forward and reverse sequence-specific primers. The amplification of sequences that do not match at the 3′ end of the oligonucleotides is effectively blocked, so that the only DNAs that are amplified are those that carry the appropriate sequences at the two 3′ ends of the oligonucleotide primer. This assay can be used for detection of any SNP by choosing oligonucleotides specific for each of the sequences of the polymorphism, thus allowing detection of both types of homozygotes and the heterozygote. The conventional assessment of such an assay is based on the presence or absence of a visible amplification product on an electrophoretic gel. It is this step that limits throughput and the potential for automation of this otherwise simple and robust approach to the detection of SNPs, including in particular the wide variety of HLA allelic differences. Alternative assays, such as sequence-specific oligonucleotide hybridization, in which allele-specific probes anneal to a PCR product compatible with each of the alleles of a given locus, are at least as cumbersome in their implementation, and are also not readily amenable to full automation (19).

A key requirement for automating the ARMS-PCR and other related assays is to eliminate the electrophoresis step and have instead a means for assessing directly, preferably, in the well of a multiwell plate, the presence or absence of an allele-specific product. A recent approach to this is the use of doubly labeled fluorogenic probes (FRET or Taqman). We have used this technique to identify PCR products for high-throughput HLA class I genotyping (20), as have others (21–23). However, this assay depends on relatively expensive, specially designed and produced FRET probes. A newly designed probe is required for each SNP to be analyzed. A number of other assays have been described, such as invader (24), primer extension (25), ligation, and rolling circle (26), which achieve the same goal but all require specific fluorescent reagents, such as fluorescent nucleotides and/or additional enzymes that add significantly to both the cost and complexity of the assay.

As an alternative to labeled probe, we took an analogous approach to the light cycler technology, where PCR product is determined and quantified by continuous fluorescence monitoring using the dye SYBR green I. The use of such double-strand DNA-specific dyes is not a new concept and dyes such as ethidium bromide and acridine orange have been used previously for genotyping (27, 28).

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Abbreviations: AMDI, alkaline-mediated differential interaction; SNP, single-nucleotide polymorphism; ARMS, amplification refractory mutation system; CABS, 4-[cyclohexylamino]-1-butansulphonic acid.

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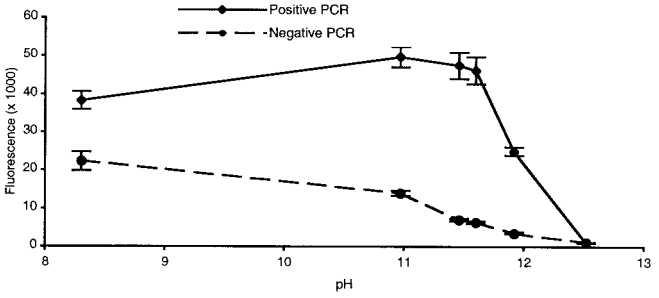


**Fig. 1.** Effect of pH on negative and positive PCR products. The conditions are as described in *Methods*, except that the pH of the CABS buffer has been adjusted as indicated. The oligonucleotides used were the sense 283 (5′-GATCGTTCGTGTCCCCACAG-3′) and antisense 314 (5′-CTGGTACTCCCCCAG-GTCA-3′) (15). The first point at pH 8.4 is the result of a reaction at the normal pH of the PCR reaction and in the absence of CABS buffer. Each point is the mean of six reactions with bars indicating the standard deviation.

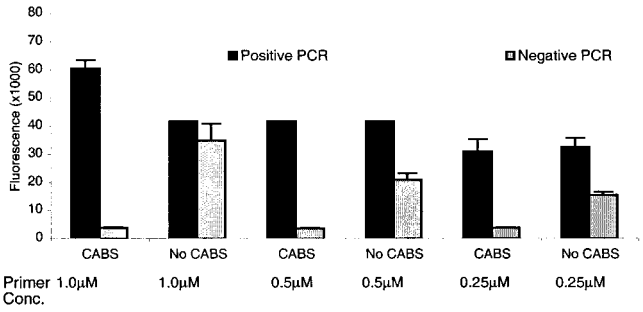


**Fig. 2.** Effect of 0.2 M CABS (pH 12.7) on the background fluorescence. The black columns represent specific PCR product and the shaded columns represent no specific product. Conditions are as described in *Methods*. The oligonucleotides used were sense 68 (5′-GTTTCTTGGAGTACTCTACGT-3′ and antisense 104 (5′-CCCGCCTGTCTTCCAGGAA-3′) (15). The height of the column is the average of five readings, and the bar extension is the magnitude of the standard deviation.

However, the high and variable background fluorescence caused by the binding of these dyes to oligonucleotide-based products of the PCR makes direct fluorometric analysis difficult. Various methods have been used to alleviate this problem, including the use of single-strand-specific exonucleases to digest away the imperfectly formed secondary structures. This approach, however, imposes constraints of additional reactions with timed endpoint analysis. The light cycler, on the other hand, which measures real time PCR, takes a single fluorescent reading at each cycle. This reading is usually at the end of each PCR extension step and at a temperature above the $T_m$ of the primers. Melting curve analysis can then be used to detect the specific product (29).

Careful assessment of results obtained with the light cycler (30) and the use of exonuclease gave indications of how to address the problems of high background fluorescence. A realization that primer/dimer formation must contribute led to the substitution of AmpliTaq Gold polymerase (Perkin–Elmer) or other similar enzymes for the usual *Taq* polymerase. Also, the inclusion of a partial denaturation step before the fluorescence reading was used in an attempt to eliminate the shorter primer/dimers in preference to the longer specific products. This possibility was examined by addition of a constant volume of SYBR green I (Molecular Probes) diluted in increasing concentrations of sodium hydroxide, enabling fluorescence to be measured and recorded over a wide range of pH, from 8–12. Results, although promising, were not reproducible because lack of buffering capacity made tight control of pH adjustment inadequate. The experiment was repeated, this time substituting an appropriate buffering system using 4-[cyclohexylamino]-1-butansulphonic acid (CABS; Sigma), which has a useful pH range of 10.0 to 11.4. SYBR green I diluted in a constant volume of CABS buffer of varying pH allowed for fluorescence monitoring over a suitable pH range comparable to that achieved with the sodium hydroxide alone. Fluorescence measurements showed that as the pH increased, the background fluorescence decreased. The specific product, however, retained measurable fluorescence up to a pH, after which all fluorescence was lost (Fig. 1).

A pH of 11.7 was chosen as optimal. At this pH, fluorescence of specific PCR product is maintained whereas background fluorescence is reduced. As expected, when the pH is too high, the product is denatured and fluorescence is lost.

To achieve a final pH of 11.7 for fluorescence detection, consideration must be given to both the pH of the PCR buffer and the pH of the fluorescence-detection buffer CABS. An equal volume of 200 mM CABS (pH 12.7) when added to a final PCR performed in Tris buffer (pH 8.4) meets this requirement.

Representative results showing a comparison between fluorescence measured in the presence or absence of CABS fluorescence-detection buffer are given in Fig. 2.

Further experiments were designed to show the individual contribution of the oligonucleotides, the primer/dimers, and the double-stranded template to the final fluorescence. The oligo contribution can be easily assessed by the exclusion of both AmpliTaq Gold polymerase and template from the PCR reaction (Fig. 3). In a similar way, the primer/dimer contribution can be evaluated by a PCR in which the DNA template is absent. A comparison between the latter and the former clearly shows a primer/dimer effect on fluorescence. Appropriate comparisons also can be used to evaluate the contribution of the DNA template (Fig. 3).

## Methods

**DNA.** DNA is prepared by using standard salting-out procedures. The sources for the HLA typing were lymphoblastoid cell lines established from individuals of known HLA type.

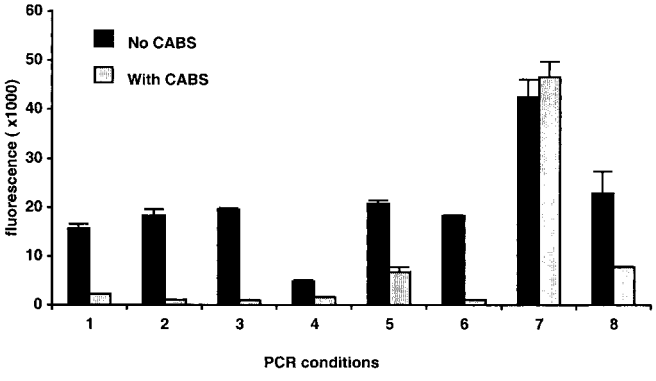


**Fig. 3.** Effect of individual constituents of the PCR on background fluorescence in the presence or absence of CABS. Each column represents the result of three PCRs performed: 1, in the absence of *Taq* Gold polymerase, but in the presence of oligonucleotides and template; 2, in the absence of *Taq* Gold polymerase and template, but in the presence of oligonucleotides; 3, in the absence of *Taq* Gold polymerase, template, and one oligonucleotide but in the presence of one oligonucleotide; 4, in the absence of primers and template, but in the presence of *Taq* Gold polymerase; 5, in the absence of template, but in the presence of both primers and polymerase; 6, as in case 3, except in the presence of the alternative primer of the primer combination; 7, in which specific amplicon is produced; and 8, in which no amplicon is expected. Conditions 1, 2, and 6 show the effect of CABS in reducing the background fluorescence caused by the single-strand oligonucleotides. The reaction with CABS in conditions 5 and 8 shows the residual effect caused by primer/dimers, in the case of 8 with additional template DNA.

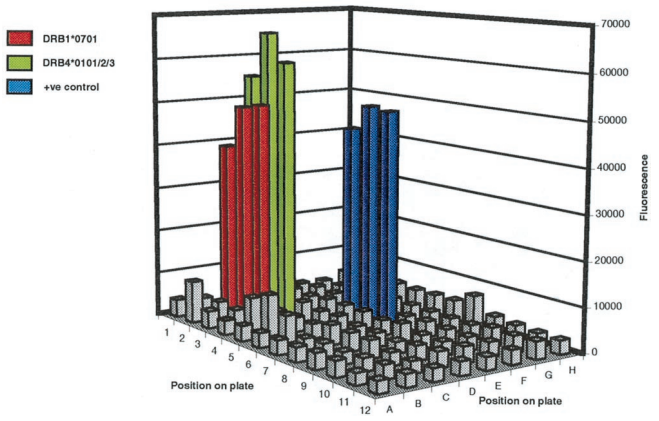


**Fig. 4.** HLA-DR typing of a lymphoblastoid cell line by using the AMDI assay. Thirty-two primer pairs, including one positive control, are arranged in triplicate on a 96-well plate. The heights of the columns indicate the actual reading at a given position on the plate. The only positives are the positive control and those for the DRB1*0701 and DRB*40101/2/3 oligonucleotide pairs. These are the only combinations expected to react from the known HLA type of the lymphoblastoid cell line.

**PCRs.** All PCR reactions were performed with 100–120 ng of genomic DNA template in a final reaction volume of 15 $\mu$l. The reaction mixture consisted of 67 mM Tris·HCl (pH 8.4) at 25°C, 16.6 mM ammonium sulfate, 2 mM magnesium chloride, 0.01% (vol/vol) Tween 20, 200 $\mu$M of each dNTP, 1 $\mu$M of each primer, and 0.5 units Hotstart *Taq* DNA polymerase (Qiagen) or AmpliTaq Gold (Perkin–Elmer). Thermal cycling was performed in a MJ Research (Cambridge, MA) machine PTC-225 by using 96-well thermo-Fast plates (Advanced Biotechnologies, Epsom, U.K.). Each well was sealed with a layer of mineral oil, and the plate was then sealed with an adhesive sealing sheet to prevent evaporation and cross contamination. The cycling parameters were as follows. One cycle of 95°C for 15 min followed by 5 cycles of 95°C for 25 sec, 70°C for 45 sec, and 72°C for 30 sec; followed by 27 cycles of 95°C for 25 sec, 63°C for 45 sec, and 72°C for 30 sec; followed by a final cycle of 72°C for 10 min.

**AMDI Fluorescence Detection.** Stock SYBR green I (10,000×) was diluted 1:5,000 in 200 mM CABS buffer (pH 12.7). At the end of the PCR, 15 $\mu$l of the above dilution was added to each final reaction volume of 15 $\mu$l by using the onboard liquid dispenser. The reagents were mixed and the fluorescence recorded at appropriate excitation and emission wavelengths.

## Results

Initially we used a relatively broad typing for HLA-DRB alleles by using 32 sequence-specific primer combinations to test 80 individual lymphoblastoid cell lines. The reactions were performed in triplicate in a 96-well plate with 100 ng of DNA for each amplification in a total volume of 15 $\mu$l. The AMDI detection system, facilitating direct fluorimetric analysis, was used to detect the presence or absence of amplicon. An equal volume of SYBR green I diluted 1:5,000 in 200 mM CABS (pH 12.7) was added to each reaction automatically by using the onboard injector attached to the fluorimeter. The fluorescence was recorded at appropriate wavelengths after programmed mixing inside the apparatus.

A typical set of reactions showing the actual fluorescence readings, using DNA from a lymphoblastoid cell line of known HLA type, is show in Fig. 4. There is good agreement between the triplicates, and positive readings are very significantly above the background. The standard deviation of the background is calculated from known negative reactions on the plate, and

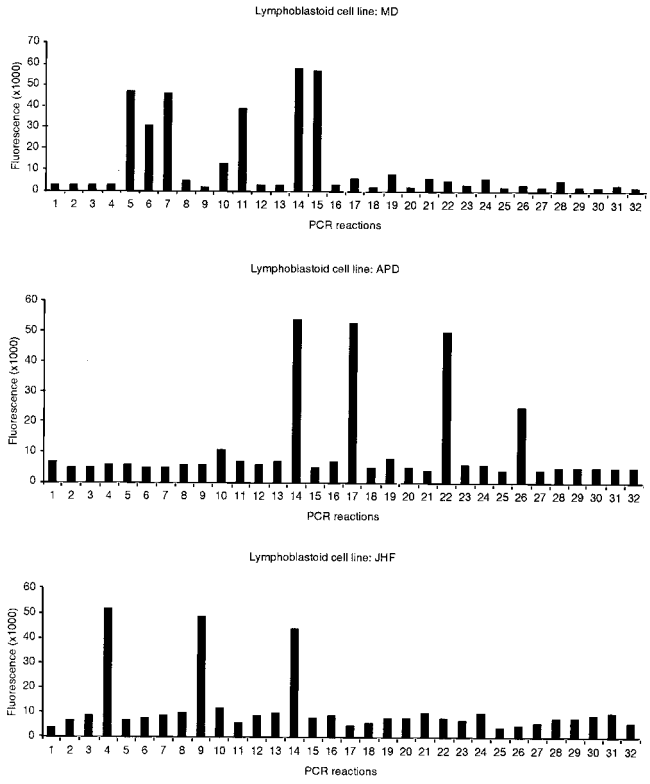


**Fig. 5.** HLA-DR typing reactions for three different DNA samples. The conditions for the assays are as described in *Methods*, and the 32 primer pairs are as described in Fig. 3. Each column is the mean of triplicate readings, and in each case the clear positive reactions are those expected from the known HLA type of the cell line. Primer pair 10 gives a consistently high background, probably because of crossreaction with other loci, and is negative in each case.

positive is measured by the number of standard deviations of a reading above the mean background. This value is generally at least 5 and can be up to 50, so that the difference between positive readings and the background is always highly significant. This procedure and modifications of it are easily programmed to give automatic assessment of positive readings (at different levels if required) and negatives, from which a genotype can then be deduced that can be transferred directly into an appropriate database. An indication of the consistency of the typing results carried out in this way is illustrated in Fig. 5, which shows the results of tests on three different lymphoblastoid cell lines using the same primer pair combinations, where what is plotted is the mean of the triplicates. So far, our typing results with a series of more than 80 cell lines show complete concordance between conventional reading with a gel and the AMDI assay. An attempt has been made to test each primer combination for a positive reaction at least three times; with the rare alleles this is not always possible. Some of the more common alleles, on the other hand, have been tested numerous times.

## Discussion

The use of the double-stranded DNA-specific dye, SYBR green I, together with reading the results of a PCR reaction at a buffered high pH makes AMDI a simple, robust, automatable, and inexpensive assay for SNP typing. The dye and buffer make a very minor contribution to the cost of the assay, which is largely determined, in material terms, by the cost of the *Taq* Gold polymerase, the oligonucleotides, and the plate, which would in any case be needed for any PCR-based typing assay. The addition of the dye in high-pH buffer can be automated by using readers

such as the BMG Fluostar (Aylesbury, U.K.), which has an onboard injector so that the mixing and reading can be done with a single machine, within a few minutes for a 96-well plate. The fluorescence values are then immediately available on a dedicated computer for analysis and subsequent transfer to an individual genotype database. The plates are set up with the oligonucleotide mixtures and with DNA, enzyme, and other reagent addition by using standard automated liquid handling equipment, so that at this stage the only part of the assay procedure that is not automated is moving a plate from the liquid-handling equipment to the PCR machine and then to the reader.

The assays developed so far cannot be multiplexed, and neither is it possible to include built-in negative or positive controls. However, the ease with which high throughput can be achieved and the clear-cut differences between positive and negative reactions suggest that this is not a major disadvantage. As set up, using one plate to type a given DNA sample for many different SNPs (or HLA alleles) there is a requirement that each SNP assay using ARMS-PCR works satisfactorily under one standard set of conditions. HLA typing is very demanding in comparison with straightforward SNP typing because of the complexity of individual allele definitions by combinations of reactions, and because of the problem of multiple duplicated genes with very similar sequences. We have, nevertheless, found it possible to use the assay for a wide range of oligonucleotide combinations on a single plate both for HLA class II typing as already described and, in preliminary results, with a comparable set of oligonucleotides for a broad spectrum HLA class I typing. We have found that the results of an ARMS-PCR reaction with a given pair of oligonucleotides, as assessed on a gel, match closely the results obtained with the AMDI assay. Thus, each SNP assay can first be optimized, with respect to oligonucleotide composition and cycling conditions as appropriate, the results can be assessed on a gel, and then subsequently these optimum conditions can be used for the AMDI assay. The ease with which this assay can be performed has led to further use in this laboratory. Populations have recently been typed successfully for the HLA-A, DRB, and DQ loci in addition to the Y chromosome SNPs. We also have applied the assay to typing for missense variants in the *APC* gene. Over 100 primer combinations have now been tested successfully, and we see no reason why it cannot be applied quite generally to any SNP that can be assessed with ARMS-PCR.

Published data on the use of SYBR green I suggest highly specific activation of fluorescence by binding to double-stranded DNA with at least 15-fold less reactivity than with single-stranded DNA (product information sheet). Although the initial trials, for example, with ethidium bromide homodimer, involved a dye that was clearly intercalating, the mechanism by which SYBR green I binds double-stranded DNA is not well established. This matches our experience in preliminary tests that show limited binding of SYBR green I to poly(A) or poly(T) oligonucleotides separately but substantial fluorescence if combined. The fact that it does not cause classical frameshift mutations in bacterial mutagenesis systems (31) suggests that it is not a conventionally intercalating dye and may, for example, be binding into the minor groove of DNA. The basis for the background binding that is removed by high pH after a PCR reaction is also not entirely clear. At least some of this is attributable to primer/dimer formation. However, the indication is that excess oligonucleotides at the end of a series of PCR cycles may form some tertiary structures that bind SYBR green I but that are readily disrupted at a high pH. We also have found that other high-pH buffers, such as [3-cyclohexylamino]-1-propane sulfonic acid (CAPS), which has a slightly lower pKa than CABS, can be used with appropriate adjustment of the pH, and that a slight increase of the temperature at which the reading is done may enhance the differential between positive and negative readings. Other double-strand DNA-specific dyes such as Pico green also may be used, but these are more expensive and do not give significantly better results. The assay works with specific PCR products as small as 100 bp and can generally be scaled down so that significantly less than 100 ng of DNA is used per assay.

In conclusion, we suggest that AMDI, an ARMS-PCR-based detection system for SNPs with a readout based on the double-strand DNA-specific fluorescent dye SYBR green I and at a high-buffered pH, provides a simple, robust, relatively inexpensive, and highly automatable SNP typing procedure that may be widely applicable.




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