Direct haplotype determination by double ARMS: specificity, sensitivity and genetic applications

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ABSTRACT

We have developed a novel double Amplification Refractory Mutation System (double ARMS) using a highly polymorphic region 5' to the human δ -globin gene as a model system. The double ARMS approach involves using two allele-specific ARMS primers simultaneously during DNA amplification by the polymerase chain reaction (PCR). The resulting system is highly sensitive and more specific than single ARMS. In addition, this approach enables the elucidation of the relationship of polymorphic sites on the same chromosome and thus allows the direct determination of haplotypes. We have also demonstrated that this system can be used in conjunction with inverse PCR, the resulting double ARMS inverse PCR (DARMSI-PCR) may allow haplotype determination on polymorphic sites which are separated further apart than the length limit imposed by PCR. The double ARMS approach has numerous other applications in molecular biology including HLA typing, virology, forensic pathology and the investigation of the phenomenon of chimerism following bone marrow transplantation.

INTRODUCTION

The determination of haplotypes at particular loci has been critical to the development of human genetics over the last few years. The linkage of specific mutations to defined haplotypes has greatly facilitated the molecular elucidation of genetic diseases such as β -thalassaemia (1,2). Studies on human evolution have also been performed by haplotype analysis which is generally more informative than analysis based on single site polymorphisms (3). A major practical problem is the determination of haplotypes in individuals who are heterozygous at a number of polymorphic sites without pedigree analysis (4). A general approach to resolving haplotypes by sequencing from a number of unrelated individuals has recently been described (5). With the advent of the polymerase chain reaction (PCR) (6) direct analysis of haplotype has become feasible by amplification from single sperm (7) and from dilution to a single molecule of genomic DNA (8,9).

These methods, though feasible, are technically demanding and protocols based on complex statistical calculations are required to maximise the accuracy of the analysis (10,11).

An important advance in DNA amplification technology is the development of allele-specific PCR or the Amplification Refractory Mutation System (ARMS) (12–14). ARMS allows DNA amplification which is specific enough to distinguish a single nucleotide difference. The ARMS concept relies on the fact that *Taq* polymerase, the DNA polymerase commonly used for PCR, lacks a 3' to 5' exonuclease activity. Thus, if a PCR primer has a mismatch at its 3'-terminal with regard to the template, the efficiency of extension by *Taq* polymerase will be reduced. However, the extent of the reduction in efficiency depends on the type of mismatch (12–14). In instances where the mismatch is not sufficiently refractory to extension, further deliberate mismatches near the 3'-end will often deliver the specificity required (12). Direct haplotype determination has been achieved by allele-specific amplification using a single ARMS primer followed by restriction enzyme analysis of the PCR product (15). This technique, however, cannot be generalised and is not suitable for haplotyping regions much larger than a few kilobases.

We reasoned that by using two ARMS primers simultaneously in a single PCR, direct haplotype determination can be achieved in one step. By using inverse PCR (16,17), this approach can be generalised to determine the linkage phase of any set of polymorphic markers. Furthermore, due to its exquisite sensitivity and specificity, this double ARMS approach has potential applications in many branches of molecular biology.

To test our system, we chose a highly polymorphic region 5' to the human δ -globin gene. Maeda et al (18) showed that the sequence in this region can be one of two types in human populations, termed R and T. The differences between the R and T sequences consist of 16 base substitutions and 2 deletions. These two chromosomes have remained discrete and have persisted in several different ethnic populations. The sequence differences between R and T allow multiple ARMS primers to be constructed. We have used the RT region as a model system to prove the feasibility of double ARMS for direct haplotype determination.

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Figure 1. Schematic representation of the polymorphic region 5' to the δ -globin gene. The relative locations of the primers are illustrated. Solid arrows indicate ARMS primers while striped arrows represent non-ARMS primers. T, R and S denote recognition sites for *TaqI*, *RsaI* and *Sau3AI*, respectively. Only the 4 *Sau3AI* sites relevant to inverse PCR are shown. Polymorphic sites are denoted by *.

RESULTS

Amplification and typing of individuals

Maeda et al (18) have sequenced a 3.1 kb region 5' to the human δ -globin gene and found 18 polymorphic sites, but only as two haplotypes, R and T. The two types of chromosomes are distinguished by the presence of a *RsaI* site at position 453 and the absence of a *TaqI* site at position 1152 for the R type and the reverse for the T type. We designed two non-ARMS primers COMM 179 and COMM 1223 which amplify a 1.1 kb region containing both polymorphic sites (Figure 1 and Table). Restriction of this PCR product with *RsaI* and with *TaqI* gave characteristic patterns for the RR, RT and TT individuals (Figure 2).

ARMS typing of individuals

Several groups have shown that the allele-specificity of PCR primers is conferred by the 3'-terminal nucleotide (12–14, 23).

Table. Sequences of primers

Sequence Name	Sequence
ARMS 643R	5' CCTTTTGTTCAGCTTTTCACTGTGTGTC 3'
ARMS 757R	5' TACTGAGTTCTAAAATCAGCT 3'
ARMS 757T	5' TACTGAGTTCTAAAATCATCG 3'
ARMS 894R	5' ATTCACAGTTATATCTAAACTCTTATAGAT 3'
ARMS 894T	5' ATTCACAGTTATATCTAAACTCTTAAAGAC 3'
ARMS 919R	5' AGGTTGGAGGTCAGAGGTTAGAAATCAGAC 3'
ARMS 919T	5' AGGTTGGAGGTCAGAGGTTAGAAATCACAG 3'
ARMS 1154R	5' TAATAGATAGGGACAAATTGAAGCAGAAGT 3'
ARMS 1154T	5' TAATAGATAGGGACAAATTGAAGCAGAAGC 3'
ARMS 1154aR	5' ATCATTTCCCTACCTCTAATCAACAGTGCA 3'
ARMS 1154aT	5' ATCATTTCCCTACCTCTAATCAACAGTGCG 3'
COMM 179	5' GCTCAAATAATTTATTTTTCAGGTTAGCA 3'
COMM 1223	5' GGAAAGAATAGCATCTACTCTTGTTCAGGA 3'
COMM 2735	5' CCCCTTTGGAAAACCTTTAATAGTATCCAC 3'
COMM 2807	5' CAGTGTACCAACAGAGTTTGAGTTTCTAT 3'
AAT-1	5' CCCACCTTCCCCTCTCTCCAGGCAAATGGG 3'
AAT-2	5' GGGCCTCAGTCCCAACATGGCTAAGAGGTG 3'

In some instances, additional deliberate mismatches near the 3'-end of certain primers are required to provide allele-specificity. We constructed a series of ARMS primers specific for either the R or T chromosomes (Figure 1 and Table). To test the allele-specificity of these ARMS primers, we tested each primer, together with a non-ARMS common primer (either COMM 179 or COMM 1223), on DNA from a homozygous RR and a homozygous TT individual. As shown in Figure 3, these primers gave visible signals only with DNA of the appropriate type.

To investigate if two ARMS primers could be used together in a single amplification, we performed PCR using two combinations of ARMS primers: ARMS 757/ARMS 919 and ARMS 894/ARMS 1154. In each case, the two primers were either both specific to the R type or both to the T type. As shown in Figure 3, double ARMS produced visible PCR products only on DNA of the appropriate type.

Figure 2. Typing of individuals by restriction enzyme analysis of the 1.1 kb PCR product. The same 9 individuals were used for *RsaI* and *TaqI* analysis. Thirty cycles of PCR were carried out prior to restriction. M denotes ϕ X174 DNA cut with *HaeIII* (marker).

Figure 3. ARMS and double ARMS analysis of DNA samples. The primers used are indicated on the top of the corresponding gels. RR and TT represent DNA from a homozygous RR and a homozygous TT individual, respectively. Twenty-five cycles of PCR were carried out. The figures 516, 388, 799, 1034, 212 and 319 indicate the sizes (in base-pairs) of the expected single and double ARMS products. C denotes the position of the internal positive control produced by AAT-1 and AAT-2. M denotes ϕ X174 DNA cut with *HaeIII* (marker).

Figure 4. Comparison of specificity of double ARMS vs single ARMS. In the left panel, the figures 516, 799 and 212 indicate the expected sizes (in base-pairs) of the PCR products from ARMS 757R/COMM 1223, COMM 179/ARMS 919R and ARMS 757R/ARMS 919R, respectively. In the right panel, the figures 388, 1034 and 319 represent the expected sizes (in base-pairs) of the PCR products from ARMS 894R/COMM 1223, COMM 179/ARMS 1154R and ARMS 894R/ARMS 1154R, respectively. Twenty-four hour exposure at room temperature without intensifying screen.

Specificity comparison between double and single ARMS

As illustrated above, both double and single ARMS reactions exhibited allele-specificity, when the PCR products were observed under UV transillumination on ethidium stained gels. In order to investigate the relative specificity of both systems, we performed Southern blotting of PCR products from mismatched ARMS reactions, i.e., cases where the primers used were of the 'wrong' type for the DNA concerned. As a test system we chose the primer combinations ARMS 757R/919R and ARMS 894R/1154R on 2 μ g of homozygous TT DNA. PCR products from 20, 30 and 40 cycles were blotted. As a positive control, PCR products from a matched reaction, i.e., the same ARMS primers on 2 μ g of RR DNA, were blotted after 20 cycles. The probe used was described in Materials and Methods.

As shown in Figure 4 double ARMS reactions were significantly more specific compared with single ARMS ones. In the mismatched reactions, a number of non-specific bands were observed for the single ARMS reactions, even as early as 20 cycles. These were probably formed due to the fact that transcripts from the common primers were continuously being generated during every PCR cycle. If by chance a second primer bound to these transcripts, a non-specific band would be produced. On the contrary, after 40 cycles on TT DNA, no trace of any non-specific reaction could be observed for the combination ARMS 757R/919R while the combination ARMS 894R/1154R produced only a very weak hybridisation signal. Due to its superior specificity, the combination ARMS 757R/919R was chosen for further experimentation.

Sensitivity of double ARMS

To estimate the sensitivity of double ARMS 3 μ g of genomic DNA from a RR homozygote were diluted by 10^2 , 10^3 , 10^4 , 10^5 , 10^6 and 10^7 times in water and subjected to 40 cycles of amplification using ARMS 757R/919R. The PCR products were blotted and hybridised with a 32 P-labelled probe (see Materials and Methods). As shown in Figure 5a the system was capable of detecting a single target molecule in water (i.e. up to the 10^6 dilution).

Figure 5. Sensitivity of double ARMS (40 cycles). (a) Dilutions were made in water. (b) Dilutions were made in 2 μ g of TT DNA. 'Blank' indicates 'no DNA' negative control. '0' indicates TT DNA only. Two-day exposure at -70°C with intensifying screens.

We further investigated the sensitivity of double ARMS by repeating the experiment, but with 2 μ g of TT DNA added to each dilution. As shown in Figure 5b the system was capable of detecting an equivalent of 10 target molecules amongst 2 μ g of irrelevant DNA (i.e. up to the 10^5 dilution).

Direct haplotype analysis using double ARMS

We reasoned that by performing double ARMS using different primer combinations, the haplotype of an individual could be determined directly. We decided to demonstrate this by establishing the linkage phase of two polymorphic sites at positions 757 and 919. We chose two individuals, one being a RR homozygote and the other a TT homozygote. Four double ARMS reactions were performed for each individual: ARMS 757R/919R, ARMS 757R/919T, ARMS 757T/919R and ARMS

Figure 6. Direct haplotype determination using double ARMS. Twenty-five cycles of PCR were used. C and 212 indicate the positions of the internal positive control and the 212 bp double ARMS product, respectively. M denotes ϕ X174 DNA cut with *Hae*III (marker).

757T/919T (Figure 6). For the RR individual only the ARMS 757R/919R reaction gave a positive signal, thus reflecting the linkage phase of the R chromosome. For the TT individual, the corresponding ARMS 757T/919T reaction was positive.

To further test the system we performed a direct haplotype determination on a heterozygous RT individual. According to Maeda et al (18) the R and T chromosomes have remained discrete during evolution and thus an RT heterozygote will possess an R and an T chromosome. As shown in Figure 6 the ARMS 757R/919R and ARMS 757T/919T reactions were positive while the other two reactions involving ARMS 757R/919T and ARMS 757T/919R were negative. This demonstrated the existence of two discrete chromosomes of the R and T varieties. A further 4 RT heterozygotes were haplotyped using this approach, with identical results (data not shown).

Double ARMS inverse PCR (DARMSI-PCR)

The direct haplotype determination system illustrated above is limited by the fact that PCR cannot be done when the primers are separated by longer than a few kilobases. We therefore investigated the possibility of performing inverse PCR (16,17) using the double ARMS approach. Through circularization, genomic targets which are far apart could be brought close enough together for inverse PCR to be carried out (Figure 7).

To test this double ARMS inverse PCR (DARMSI-PCR) approach we used primers ARMS 919(R or T) and ARMS 1154a(R or T). The latter primers were allele-specific for the same polymorphic site at position 1154 detected by ARMS 1154R and ARMS 1154T except that they were constructed in the opposite direction for inverse PCR (Figure 1 and Figure 7). Allele-specificity of ARMS 1154aR and ARMS 1154aT was tested by amplification from RR and TT DNA, in combination with COMM 1223 (Figure 8a). As shown in Figure 8a, ARMS 1154aR and ARMS 1154aT exhibited specificity for their respective alleles.

Following circularization and reopening of the circles, direct haplotype determination was carried out using ARMS 919R/1154aR, ARMS 919R/1154aT, ARMS 919T/1154aR and ARMS 919T/1154aT. As an internal control for circularization and amplification, a control set of non-ARMS primers COMM 2735 and COMM 2807 (Figure 7) were used to amplify a region

Figure 7. Schematic representation of double ARMS inverse PCR. Solid arrows indicate ARMS primers; striped arrows indicate non-ARMS primers. R and S represent recognition sites for *Rsa*I and *Sau*3AI.

Figure 8. (a) Allele-specificity of ARMS 1154aR and ARMS 1154aT (twenty-five cycles). C and 128 represent the positions of the internal positive control (produced by AAT-1 and AAT-2) and the 128 bp single ARMS product. (b) Direct haplotype determination on an RT heterozygote using double ARMS inverse PCR. The 414 bp internal positive control signal (indicated by the arrow marked 414) was produced by COMM 2735 and COMM 2807. The arrow marked 690 indicates the position of the 690 bp double ARMS inverse PCR product. Forty cycles were carried out. M denotes ϕ X174 DNA cut with *Hae*III (marker).

3' to the two polymorphic sites studied. A positive signal from COMM 2735 and COMM 2807 was only possible if this region was circularised.

As shown in Figure 8b, direct haplotype determination of an RT heterozygote using DARMSI-PCR was possible. Figure 8b shows positive double ARMS signals for the combinations ARMS 919R/1154aR and ARMS 919T/1154aT and no signal for the combinations ARMS 919R/1154aT and ARMS 919T/1154aR. These results once again demonstrated the existence of two discrete chromosomes of the R and T types. To further confirm the identity of the inverse PCR products we restricted the products with *Sau3AI* and obtained the restriction pattern expected from sequence data (results not shown). These data suggested that the double ARMS approach could be used as a general method for haplotype determination.

Theoretical considerations on the number of double ARMS reactions necessary for haplotype determination

We have demonstrated that it is possible to determine the linkage phase of two polymorphic sites by a set of 4 double ARMS reactions. This number, however, is not the minimum number of reactions that is required to establish the linkage phase unambiguously.

For simplicity's sake, in the following analysis, we assume prior knowledge that the individual concerned is heterozygous at each of the polymorphic sites studied. This assumption is justified on the grounds that if the individual is not a compound heterozygote, linkage phase determination will not be necessary. Homozygosity and heterozygosity at individual polymorphic sites could easily be established by conventional methods, e.g. the Southern blot.

In the following analysis a two-allele system is used for illustration purposes. This analysis, however, can be extended to polymorphic systems with more than two alleles as any heterozygous diploid individual can only have two of the alleles. Thus, for any pair of polymorphic sites, let us denote the two alleles as + and -. Thus for any pair of polymorphic sites, the linkage phase of the two chromosomes can be ++ and -- OR +- and -+. The establishment of the phase of one chromosome will fix the phase of its partner. Thus if a double ARMS reaction is carried out with two ARMS primers specific for the + allele at site 1 and the + allele at site 2; and a positive signal is obtained, then the phase of one chromosome is established as ++. Its partner will thus be --. On the contrary, if no signal is obtained, then the linkage phase may be deduced to be +- and -+. The above analysis can be extended to determine the linkage phase of any number of polymorphic sites. This analysis shows that only one double ARMS reaction is sufficient to establish the linkage phase of each pair of polymorphic sites. Hence for n such sites, there are $n-1$ sequential pairs and thus $n-1$ is the minimum number of double ARMS reactions required to establish the linkage phase of n sites.

This 'minimalistic' approach suffers from the disadvantage that occasionally the haplotype may have to be deduced on the basis of a lack of PCR signal. The absence of signal may, on occasions, be the result of PCR failure. Although potential PCR failure may be detected by including an internal positive control in every ARMS reaction, a more robust system is preferred. We have therefore devised the following system which is a compromise between the technically robust '4-tube' approach which is the system this present work is based on, and the '1-tube' method described in the previous paragraph.

We reasoned that the ideal system would require that, for each pair of polymorphic sites, a double ARMS reaction giving a positive and one giving a negative reaction be performed simultaneously. These reactions should be designed such that a positive reaction in one case should be accompanied by a negative one in the other. Two positive or two negative reactions will signify technical problems. Using the same example as above, in addition to performing a double ARMS reaction specific for the + allele at site 1 and the + allele at site 2, we would perform a second double ARMS reaction using primers specific for the + allele at site 1 and the - allele at site 2. If the first double ARMS reaction is positive then the linkage phase of one chromosome is established as ++ and its partner is deduced to be --. On the other hand, if the second double ARMS reaction is positive then the linkage phase of one chromosome is established as +- and its partner deduced to be -+. In both of these cases, the other double ARMS reaction should be negative.

Thus, for any pair of markers studied two double ARMS reactions are sufficient to determine the linkage phase unambiguously and provide some degree of internal cross-checking for possible technical failure. When determining the linkage phase of n polymorphic sites the number of double ARMS reaction is thus $2(n-1)$.

DISCUSSION

We have illustrated the characteristics and applications of the double ARMS concept. We chose to use the well-characterised RT system (18) as the test system since the 2 types of chromosomes (R and T) have multiple sequence differences, thus allowing the construction of a number of ARMS primers. All our results are in agreement with the original observation that the two types of chromosomes have remained discrete throughout evolution since we have found no 'hybrid' RT chromosomes.

The application of double ARMS to direct haplotype determination has important implications. Existing general methods for direct haplotype determination include single sperm typing (7) and the single molecule dilution method (8,9). These methods, however, are technically demanding as the PCR has to be able to amplify from a single molecule. The degree of amplification requires obsessive care to avoid contamination (24,25). Furthermore, the use of single molecule dilution also requires the amplification of multiple aliquots, typically ten (8). In this paper, we demonstrated direct haplotype determination using four double ARMS reactions. As shown in our analysis, using double ARMS, the linkage phase of any pair of polymorphic sites can be established by a theoretical minimum of just one reaction. For greater robustness with internal controls for potential technical failure, a two-reaction system per pair of polymorphic sites is recommended. Thus, for the haplotyping of large number of samples, the double ARMS approach is by far the most practical. Methods based on single molecule analysis, however, are valuable in detecting DNA polymorphisms which are not readily amenable to ARMS analysis, e.g., minisatellite variant repeats (9).

The limitation of the double ARMS analysis is the size limit imposed by PCR. However, this may be overcome by performing a double ARMS inverse PCR (DARMSI-PCR). Though the exact conditions for sample preparation for inverse PCR may vary and have to be determined by experimentation based on Southern blotting and available sequence information; once established,

these conditions could be used repeatedly to generate haplotype data for a large number of samples. Although we have so far applied inverse PCR to relatively small genomic fragments (a 865 bp *Sau3AI* fragment for DARMSI-PCR and a 427 bp *Sau3AI* fragment for the corresponding internal control), others have successfully applied inverse PCR to genomic regions of over 10 kb (26). Thus, having demonstrated that DARMSI-PCR is feasible on relatively small regions, it is to be expected that the method should be applicable to regions as long as those amplified by conventional inverse PCR, i.e. 10 kb. We are currently investigating the use of DARMSI-PCR on larger genomic regions in the β -globin cluster.

Thus, the double ARMS method is a general approach to determining linkage phase of polymorphic markers. Its application extends to all the use of haplotype information including linkage analysis for the diagnosis of genetic diseases, e.g. β -thalassaemia. Population studies on the geographical distribution of haplotypes can be performed without pedigree analysis. The DARMSI-PCR method can be used to haplotype large chromosomal areas. This type of analysis may be used to study genetic recombination. We also predict that double ARMS will prove to be useful for direct determination of HLA haplotypes. Double ARMS may be readily modified for routine diagnostic purposes since it is easy to perform and is not dependent upon radioactivity.

We have also shown that under identical amplification conditions double ARMS possesses much higher specificity than the single ARMS approach. This exquisite specificity, combined with its sensitivity is thus ideal for detecting a minority DNA population amongst a background of related but non-identical DNA molecules. Potential applications include the study of the emergence of viral variants from viral isolates, e.g. the human immunodeficiency viruses. Similarly, the double ARMS approach may be used to study the phenomenon of chimerism following bone marrow transplantation. We also envisage that its sensitivity will allow samples to be collected in a variety of formats, e.g., hair, and thus may have implications in forensic pathology.

In conclusion, we have presented a novel double ARMS technique that is highly specific and sensitive. It can be used as a general method for direct haplotype determination and has a large number of potential applications in many branches of molecular biology.

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