

L. Zhou
J. Vandersteen
L. Wang
T. Fuller
M. Taylor
B. Palais
C.T. Wittwer

High-resolution DNA melting curve analysis to establish HLA genotypic identity

Key words:

DNA melting curve; genotype; HLA; LCGreen I; sibling transplant

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Abstract: High-resolution melting curve analysis is a closed-tube fluorescent technique that can be used for genotyping and heteroduplex detection after polymerase chain reaction. We applied this technique at the HLA-A locus and suggest that this method can be used as a rapid, inexpensive screen between siblings prior to living-related transplantation. At any locus, there are seven general cases of shared alleles among two individuals, ranging from identical homozygous genotypes (all alleles shared) to two heterozygous genotypes that share no alleles. We studied each case using previously typed cell lines to show that identity or non-identity can be determined in all cases by high-resolution melting curve analysis. HLA genotype identity is suggested when two individuals have the same melting curves. Identity is confirmed by comparing the melting curve of a 1:1 mixture with the individual melting curves. Non-identity at the amplified locus changes the heteroduplexes formed in the mixture compared with the original samples and alters the shape of the melting curve. The technique was tested on DNA from a 17-member CEPH family. High-resolution melting curve analysis revealed six different genotypes in the family. The genotype clustering was confirmed by sequence-based typing. Although this technique does not sequence or determine specific HLA alleles, it does rapidly establish identity at highly polymorphic HLA loci. The technique may also prove useful for confirmation of HLA genotypic identity between unrelated individuals prior to allogeneic hematopoietic stem-cell transplantation.

Authors' affiliation:

L. Zhou¹,
J. Vandersteen¹,
L. Wang¹,
T. Fuller¹,
M. Taylor¹,
B. Palais²,
C.T. Wittwer¹

¹Department of Pathology,
University of Utah, Salt Lake
City, UT, USA

²Department of Mathematics,
University of Utah, Salt Lake
City, UT, USA

Correspondence to:

Carl Wittwer, MD, PhD
Department of Pathology
University of Utah, School
of Medical
30 North 1900 East
Salt Lake City, UT 84132,
USA
e-mail: carl.wittwer@path.
utah.edu

The importance of HLA matching in transplantation has long been recognized. Current studies continue to show a correlation between degree of matching and graft survival (1, 2), especially in hematopoietic stem-cell transplantation (3). Techniques for HLA typing have become increasingly sophisticated, with high-resolution DNA typing explaining compatibility differences not previously understood by serologic typing. However, high-resolution typing comes at a cost, in terms of both expense and the time required for analysis.

HLA matching of related donors can be less complex than unrelated matching. Because of the strong linkage between various HLA loci, HLA haplotypes are inherited as a block over 98% of the time

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(4). A simple, rapid screening method to establish HLA identity between siblings, rather than current molecular or serologic typing, would be useful.

Heteroduplex analysis of polymerase chain reaction (PCR) products was used in the past in order to establish identity at HLA loci (5, 6). However, these subjective gel techniques have lost favor to high-resolution probe techniques and sequencing methods that are more robust. Recently, we have identified a DNA-binding dye, LCGreen I, that is compatible with PCR and that detects heteroduplexes in a closed-tube system (7). In contrast to conventional melting curve analysis, new instrumentation for high-resolution fluorescence melting can reliably detect single-base mismatches in 1–2 min after PCR (8). When combined with rapid-cycle PCR, amplification and analysis in a closed-tube system routinely require less than an hour (9–11).

In this study, we explore the feasibility of rapid HLA matching by closed-tube heteroduplex analysis of the highly polymorphic exons of HLA-A in known cell lines and a large CEPH family.

Materials and methods

DNA samples

Epstein–Barr virus-transformed, B-lymphoblastoid cell lines of known HLA-A genotype were obtained from the NMDP/ASHI Cell Repository (Mt. Laurel, NJ, USA). Samples (and genotypes) used were BM15 (A*0101, 0101), E4181324 (A*0101, 0101), CF996 (A*0201, 0301), EMJ (A*0201, 0301), BM16 (A*0201, 0201), PMG075 (A*0301, 3301), KT14 (A*2402, 2602), and RML (A*0204, 0204). Each haplotype sequence is available in the IMGT/HLA sequence database (<http://www.ebi.ac.uk/imgt/hla/>). DNA was extracted using the DNA-isolation kit from Puregene (Gentra Systems, Minneapolis, MN, USA). Genomic DNA from the CEPH/Utah family 1331 was obtained from the Coriell Institute (Camden, NJ, USA) with 17 individuals among three generations (four grandparents, two parents, and 11 children).

Amplification of the HLA-A gene

Specific amplification of HLA-A exons 2 and 3 was achieved by nested rapid-cycle PCR in a LightCycler with standard LightCycler capillaries (Roche Applied Science, Indianapolis, IN, USA). The outer PCR amplified a large (948 bp) fragment of the *HLA-A* gene using primers that hybridized to HLA-A intron 1 (GAAAC(C/G)GCCTCTG-(C/T)GGGGAGAAGCAA) and intron 3 (TGTTGGTCCCAATTGT-CTCCCTC) (12) in order to exclude other class I genes. The PCR

contained 0.5 μ M of each primer, 50 ng of genomic DNA, 3 mM MgCl₂, 50 mM Tris (pH 8.3), 0.2 mM of each dNTP, 500 μ g/mL of bovine serum albumin, and 0.4 U of Taq (recombinant in *Escherichia coli*) DNA polymerase (Roche Applied Science) in 10 μ l. The PCR solutions were loaded into the capillaries by brief centrifugation. When mixtures of two different DNA samples were tested, 25 ng of each of the two genomic DNAs was added to the PCR unless otherwise indicated. Cycling conditions were 94°C for 20 s followed by 40 cycles of 94°C for 1 s, 62°C without a temperature hold, and 72°C for 1 min with 20°C per s transition rates.

Nested PCR was used in order to amplify either HLA exon 2 or exon 3. A 340-bp fragment of HLA-A exon 2 was amplified with inner primers AGCCGCGCC(G/T)GGAAGAGGGTCG (intron 1) (13) and GGCCGGGGTCACTACCG (intron 2). A 366-bp fragment of HLA-A exon 3 was amplified with inner primers CCC(G/A)GGTTGGTCGGGGC (intron 2) and ATCAG(G/T)GAGGCGCCCGTG (intron 3). The nested amplifications used the same reagents except for 0.25 μ M of each primer, 1/10,000 of the first PCR product instead of genomic DNA, 2 mM MgCl₂, and 1 \times LCGreen I (a 1:10 dilution of 10 \times LCGreen I, Idaho Technology, Salt Lake City, UT, USA). Cycling conditions were 94°C for 5 s, followed by 25 cycles of 94°C for 1 s, 65°C without a temperature hold, and 72°C for 8 s. Following amplification, the PCR products were denatured at 94°C and were rapidly annealed by cooling to 60°C at 20°C per s.

High-resolution melting curve analysis

The glass capillaries containing the nested amplification products were directly transferred from the LightCycler to the high-resolution melting instrument HR-1 (Idaho Technology). The samples were heated from 60 to 95°C at a rate of 0.3°C/s as previously described (7, 8). Briefly, fluorescence (450 nm excitation/470 nm emission) and temperature measurements were acquired every 40 ms over 2 min. The data for each sample were displayed as a melting curve normalized to percent of fluorescence between linear fits of the raw fluorescence before the melting transition (the 100% line) and after the transition (the 0% line). Different melting curves were shifted horizontally to overlap between normalized fluorescence values of 3 and 7% (7).

Sequencing

Nested PCR products were sequenced by automated fluorescent sequencing at the University of Utah Core Facility (ABI 3700, PRISM BigDye terminator v3.1 cycle sequencing kit, and Sequencher version 4.0, Applied Biosystems, Foster City, CA, USA).

***In silico* T_m estimation of homoduplexes and heteroduplexes**

Melting temperatures of homoduplexes and heteroduplexes were calculated using a nearest-neighbor thermodynamic model (14) using custom software. This all-or-none model is strictly true only for short duplexes that have one melting domain. The melting temperature (T_m) at which half of the strands are in the double-helical state occurs at the phase transition where $dG=0$. At this point, $T_m = (\Sigma\Delta H/\Sigma\Delta S) + R \times \ln(C/2) + 0.368 N \times \ln[Na^+]$, where ΔH and ΔS are the contributions to free energy and entropy, respectively (interior tetrads and end pairs), R is the gas constant, C is the PCR product concentration, and N is one-half of the total number of phosphates in the duplex. Consensus parameters for the 10 matched tetrads were used (14). In addition, parameters for mismatches, including G-T (15), G-A (16), A-C (17), C-T (18), and AA, CC, GG, and T-T (19), dangling ends (20) and blunt ends (14) were included. Best-fit values of $0.2 \mu M$ for the amplicon concentration at the end of PCR and Mg^{++} equivalence (74-fold that of Na^+) were obtained using a data set of 475 duplexes (21). The effective concentration of Mg^{++} was decreased by the total dNTP concentration, assuming stoichiometric chelation. The effect of $Tris^+$ was assumed equal to Na^+ . The $[Tris^+]$ (20 mM) was calculated from the buffer concentration $[Tris]$ and pH.

Results

Homoduplex and heteroduplex T_m estimation

The nearest-neighbor model of duplex melting was used in order to predict homoduplex and heteroduplex T_m s of the 340-bp amplicon of HLA-A exon 2 for all duplexes of alleles present in the B-lymphoblastoid cell lines. Six haplotypes were considered (A*0101, A*0201, A*0301, A*2402, A*2602, and A*3301) in all combinations, resulting in six homoduplexes and 15 heteroduplexes. The predicted homoduplex T_m s varied over only a $0.55^\circ C$ range from 93.68 to $94.23^\circ C$. The predicted heteroduplex T_m s (ranging from 90.18 to $92.57^\circ C$) were between 1.11 and $4.05^\circ C$ less than the homoduplex T_m s. The various heteroduplex pairs contained between seven and 20 mismatches. The correlation between the percentage of mismatched bases and the predicted difference between homoduplex and heteroduplex T_m s is shown in Fig. 1. The predicted T_m difference between heteroduplexes and homoduplexes is small, and the differences within either the heteroduplex group or the homoduplex group are even smaller. The ability to distinguish various genotypes by T_m depends on the resolution of the instrument used for acquiring the melting curves (8).

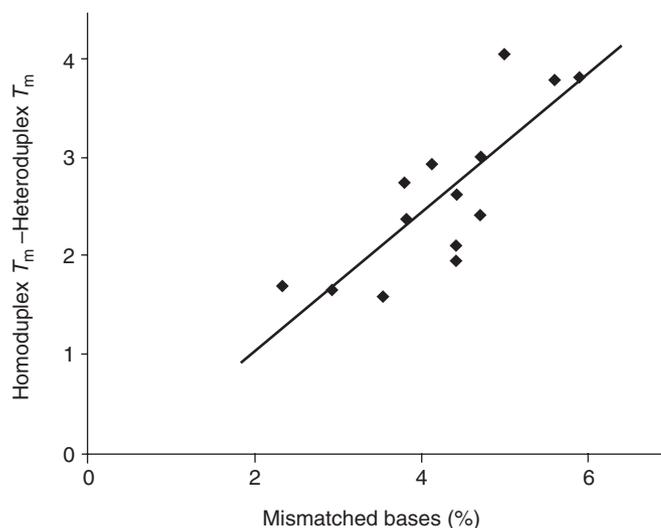


Fig. 1. The difference between homoduplex and heteroduplex melting temperatures (T_m s) plotted against the percentage of mismatched bases between various HLA-A exon 2 alleles. For each possible heterozygote combination of HLA-A genotypes A*0101, A*0201, A*0301, A*2402, A*2602, and A*3301, two homoduplex and two heteroduplex T_m s were calculated. For each heterozygote, the average heteroduplex T_m was subtracted from the average homoduplex T_m and plotted against the percentage of mismatched bases in the 340-bp HLA-A exon 2 amplicon.

Melting curve analysis of paired samples at HLA-A

The identity of two DNA samples at a polymorphic locus can be established using PCR followed by high-resolution melting curve analysis. The melting curves of each sample alone and a 1:1 mixture of both samples are compared. When the two samples are identical, all three melting curves will be same. If the samples are different, the melting curves from each individual are different, except in some cases when both are homozygous. The melting curve of the mixture will always be different from the original melting curves if the genotypes are different.

At any locus, there are seven general cases of shared alleles among two individuals (Table 1). The total number of alleles present among individuals varies from one to four. After PCR of a 1:1 mixture, denaturation, and annealing, the expected percentage of heteroduplexes assuming random association varies from 0 to 75. Both the percentage of heteroduplexes and the T_m s of the two homoduplexes and the two heteroduplexes determine the shape of the melting curve. The T_m of each heteroduplex depends on the number of nucleotide mismatches and what the specific mismatches are.

Specific examples of the general cases listed in Table 1 are shown in Fig. 2 for exon 2 of HLA-A. Qualitatively, similar results were obtained for exon 3 of HLA-A (data not shown). Each individual can be homozygous or heterozygous. In case 1 (Fig. 2A), both samples are

Seven general cases of shared HLA-A alleles among two individuals and the expected frequency of heteroduplexes

Case	Individual 1	Individual 2	Total number of alleles in mixture	Expected heteroduplex percentage in mixture
1	ww	ww	1	0.0
2	ww	wx	2	37.5
3	ww	xx	2	50.0
4	wx	wx	2	50.0
5	ww	xy	3	62.5
6	wx	wy	3	62.5
7	wx	yz	4	75.0

Table 1

homozygous for the same HLA-A allele. Only homoduplexes are formed when denatured PCR products are annealed. The melting curves of each sample alone and a 1:1 mixture of the samples are all identical.

In case 2 (Fig. 2B), one sample is homozygous and the other is heterozygous, with the homozygous allele also present as one of the alleles in the heterozygous sample. In a 1:1 mixture of the samples, the two alleles are present at a 3:1 ratio, resulting in 37.5% heteroduplexes. All melting curves are different from each other, and the melting curve of the mixture is between the homoduplex and heteroduplex melting curves. This is expected because more heteroduplexes are predicted in the heterozygous sample (50%) than in the mixture (37.5%).

In case 3 (Fig. 2C), both samples are homozygous but with different alleles. The predicted T_m s of the homozygotes are very close at 93.68 and 93.71°C. Although the melting curves appear slightly different, the call of identity or non-identity cannot be made with certainty in this case from the individual samples. However, the melting curve of the mixed sample is obviously shifted to lower melting temperatures, indicating that the individual samples are indeed different. The mixture contains 50% heteroduplexes.

In case 4 (Fig. 2D), both samples are heterozygous and each contains the same two alleles. The heteroduplex percentage expected in both individual samples and their mixture is 50. The heteroduplexes present and the melting curves of both samples and their mixture are identical.

In case 5 (Fig. 2E), one sample is homozygous, the other is heterozygous, and no alleles are shared. The melting curve of the heterozygous sample is shifted to lower temperatures compared to the homozygous sample. The mixed sample shows melting at still lower temperatures with an expected heteroduplex percentage of 62.5. The melting curves of each sample and the mixture are clearly different.

In case 6 (Fig. 2F), both samples are heterozygous and one allele is shared. Each sample has a melting curve with a characteristic shape.

The melting curve of the mixture shows more low temperature melting and has a different shape than either sample alone. The expected heteroduplex percentage is 62.5.

Case 7 (Fig. 2G), in which both samples are heterozygous and no alleles are shared, is the most common case for highly polymorphic loci. For each sample alone, 50% heteroduplexes are expected. In the mixture, four different homoduplexes and 12 heteroduplexes are formed with an overall expected heteroduplex percentage of 75. All three melting curves are distinctly different.

When the samples are not identical, the melting curve of the mixture is usually below the melting curves of the individual samples because the percentage of heteroduplexes is increased over either sample alone. The only exception is case 2 (Table 1 and Fig. 2B) where the melting curve of the mixture is between the individual curves, as is the heteroduplex percentage. Using this most difficult case, the relative amount of each sample was varied to study the effects on the melting curves of the mixtures (Fig. 3). Minor DNA fractions of down to at least 20% were easily distinguished from pure individual samples. The allele fraction sensitivity should be even greater for the more common cases with three or four alleles in the mixture (cases 5–7, Table 1).

The alleles studied in Fig. 2 differed at the broad allele level and had between seven and 20 mismatches within the amplicon. The ability of high-resolution melting to reliably distinguish alleles that differ by only one nucleotide in the 366-bp exon 3 amplicon is shown in Fig. 4. Even though the T_m shift is small, the resolution of the method clearly distinguished 10 replicates of the mixture from 10 replicates of a homozygous individual.

Determining HLA-A identity in an extended family

DNA from a large CEPH family (Fig. 5) was used in order to establish genotype inheritance and HLA-A identity among siblings. Analysis of HLA-A exon 2 PCR products amplified from the 17 members of the family clustered into six different melting curve groups (Fig. 6)

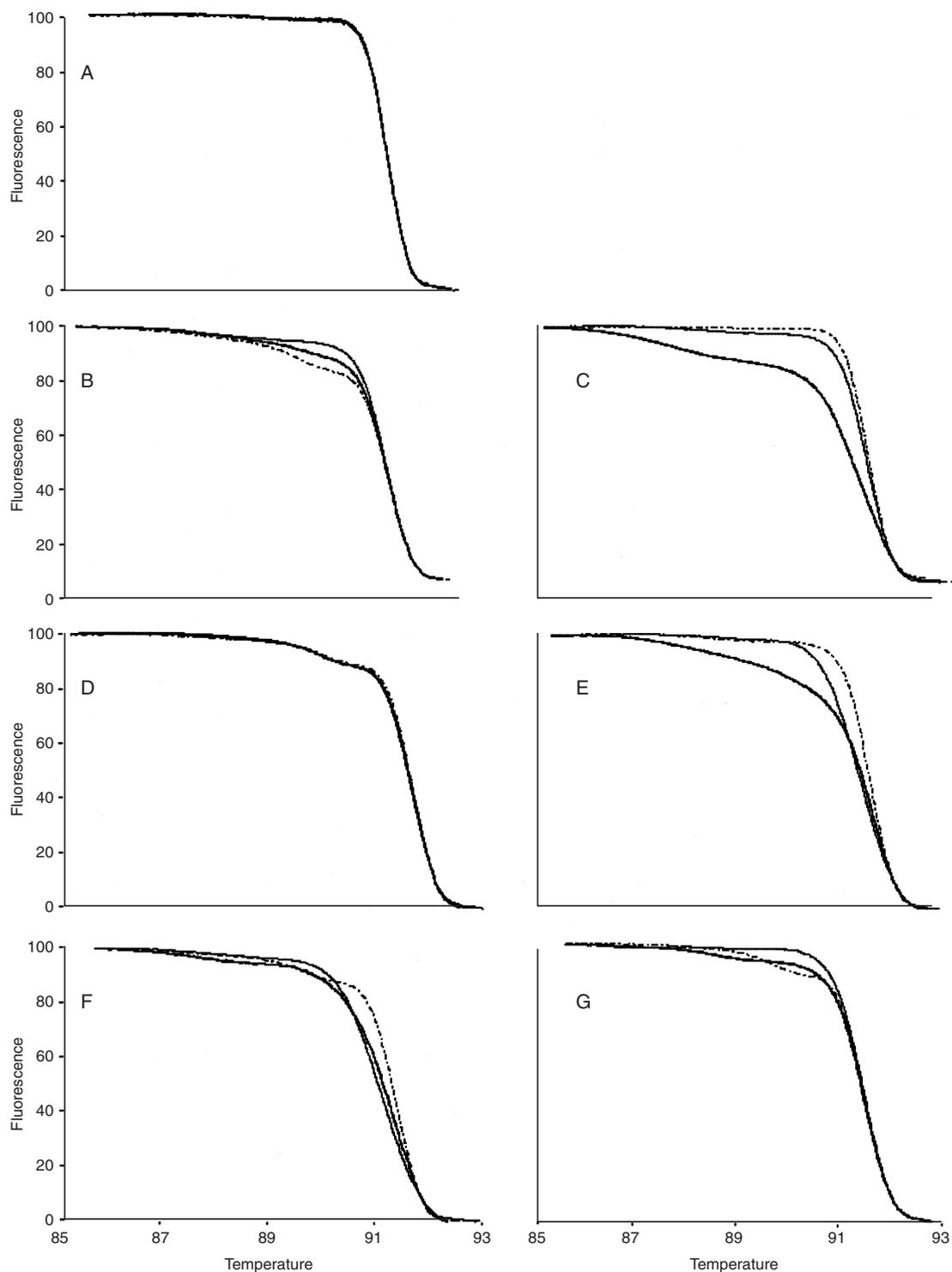


Fig. 2. DNA melting curves of the seven general cases of shared alleles among two individuals (Table 1) using HLA-A exon 2 as an example. For each case, three melting curves are shown, one for each of the two individual DNA samples (thin, solid, and dashed lines), and a third curve of a 1:1 DNA mixture of both individuals (thick line). (A) Samples BM15 and E4181324 are homozygous for the same allele (A*0101, 0101). (B) Sample BM16 is homozygous (A*0201, 0201), and the other sample (CF996) is heterozygous (A*0201, 0301), sharing one allele. (C) Sample BM15 (A*0101, 0101) and BM16 (A*0201, 0201) are both homozygous but share no alleles. (D) Both samples CF996 and EMJ have the same heterozygous genotype (A*0101, 0202). (E) Sample BM16 is homozygous (A*0201, 0201), whereas sample PMG075 is heterozygous (A*0301, 3301) with no alleles shared. (F) Both samples EMJ (A*0201, 0301) and PMG075 (A*0301, 3301) are heterozygous, and one allele is shared. (G) Both samples CF996 (A*0201, 0301) and KT14 (A*2402, 2602) are heterozygous, and no alleles are shared.

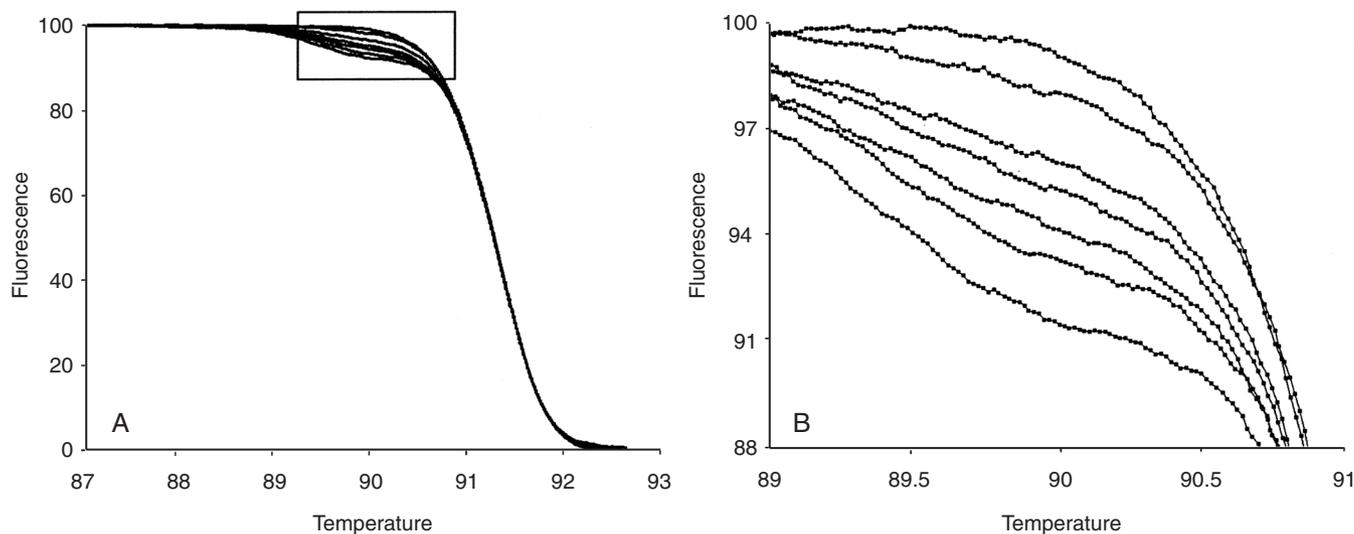


Fig. 3. The effect of the relative amount of each sample on the melting curves of mixtures. Various amounts of the cell lines BM16 (A*0201, 0201) and EMJ (A*0201, 0301) were amplified and melted as in Fig. 2B. The entire melting curve is shown in (A), whereas the boxed area is magnified in (B). The total amount of genomic DNA amplified was 50 ng, and the relative percentages of EMJ DNA (top to bottom) were 0, 20, 40, 50, 60, 80, and 100.

indicating that there are at least six different HLA-A genotypes in the family. In order to verify that only six genotypes are present, one sample from each group was mixed pair-wise with all other members of the same group. In every case, the mixture had the same melting curve as the individual DNA samples. In contrast, mixing samples from different groups generated melting curves distinct from both

individual DNA samples (data not shown). A similar analysis using exon 3 of HLA-A generated the same groups (data not shown).

The PCR products of HLA-A exons 2 and 3 of the CEPH family were sequenced. The results confirmed the melting curve analysis, identifying the six HLA-A genotypes as: A*02011, 3101 (ab); A*3101, 2402101 (bc); A*02011, 2402101 (ac); A*02011, 03011 (ad); A*02011, 02011 (aa); and A*2402101, 01011 (ce).

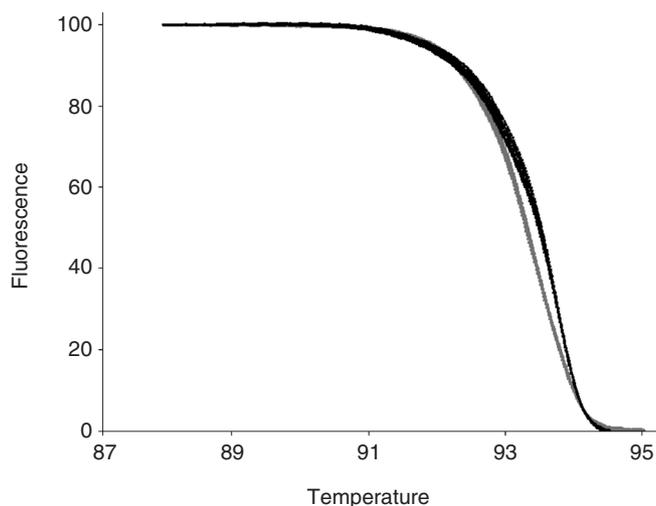


Fig. 4. High-resolution melting analysis can identify a mixture when only a single-base difference is present. BM16 (A*0201, 0201) and RML (A*0204, 0204) differ by only a single base (G vs T at position 362) in the 366-bp amplicon of HLA-A exon 3. Some 50 ng of BM16 and a 1:1 mixture of BM16 and RML DNA were amplified, each in 10 separate polymerase chain reaction (PCR) reactions. High-resolution melting analysis of all 20 reactions clearly showed separation between the homozygous samples (dark lines) and the mixtures (gray lines).

Discussion

Conventional real-time PCR instruments do not have the resolution necessary to discern small differences in melting temperature (8, 22). However, high-resolution melting of PCR products with DNA dyes that detect heteroduplexes can identify a single heterozygous base pair in amplicons as large as 544 bp (7). In the current study of HLA-A genotypic identity, product sizes were 340 bp (exon 2) and 366 bp (exon 3). Several mismatches were usually present between alleles, and non-identical individuals were easy to identify. Even, mixtures with only a single-base mismatch could be identified (Fig. 4). However, sequence differences become more difficult to detect as the amplicon size increases. With targets other than HLA, we have found that as long as the amplicon is less than 400 bp in length, the sensitivity of detecting single-base differences in a mixture approaches 100% (data not shown).

High-resolution melting analysis of highly polymorphic loci can determine genotypic identity between individuals. If the melting curves are the same, the genotypes are the same. If the melting

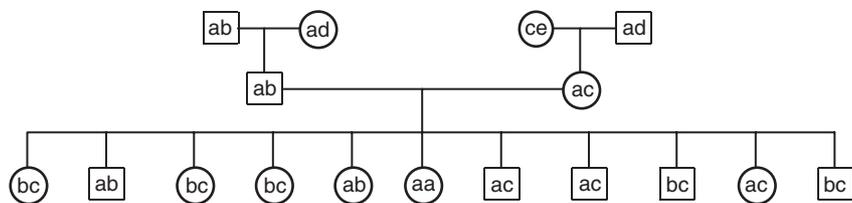


Fig. 5. Pedigree of CEPH family 1331. The HLA-A genotypes at exon 2 are as follows: a, A*02011; b, A*3101; c, A*2402101; d, A*03011; and e, A*01011.

curves are different, the genotypes are different. Although not common in highly polymorphic regions, different homozygous genotypes can be difficult to distinguish if they have similar melting temperatures. In these cases, the melting curve of a mixed sample clearly shifts the melting curve to lower temperatures because of the heteroduplexes generated. The samples can be mixed either before PCR or after amplification, as long as the mixture is denatured and annealed before melting.

Amplification of a heterozygous mixture of DNA results in both homoduplex and heteroduplex products in ratios that depend on how many alleles are shared between the two samples. Assuming random association after PCR of a 1:1 mixture of two DNA samples, the expected heteroduplex percentage varies from 37.5 to 75 (Table 1). Heteroduplex products melt at lower temperatures than perfectly matched homoduplexes and result in a shift in the melting curve to lower temperatures. Even though equal amounts of heteroduplexes and homoduplexes are expected from a heterozygous sample, the magnitude of the fluorescence change observed by the heteroduplex transition is smaller. This is most likely a result of redistribution of heteroduplexes into homoduplexes during melting (8) and is minimized with the dye, LCGreen I (7). Different heterozygotes can be distinguished from each other primarily because the heteroduplex

products of each are different, resulting in different contributions to the overall melting curve.

High-resolution melting analysis requires specific amplification of only one HLA locus and non-specific amplification of all alleles at that locus. A nested PCR design was devised to first achieve locus-specific amplification of HLA-A with conserved sequences for outer primers in the first and third intron as previously reported (12). Inner consensus primers for exon 2 and exon 3 were then designed to bracket each intron–exon boundary. Alternative bases were incorporated into the primers when necessary. Only a few primer–template mismatches were present when available sequences of 44 HLA-A alleles on the IMGT/HLA database (<http://www.ebi.ac.uk/imgt/hla/>) were analyzed, and none were present within five bases of the primer 3'-end. Nevertheless, unexpected/undesired primer mismatches with specific HLA alleles could result in unequal amplification. Furthermore, PCR inhibitors, DNA quality, and errors in quantification can result in some samples producing more amplicon than others. These factors can be monitored by real-time PCR because the dye can be observed in the SYBR[®] Green I/fluorescein channel (data not shown). Moreover, high-resolution melting analysis can tolerate some degree on unequal amplification because mixtures of DNA varying by at least four-fold can still be identified (Fig. 3). All genotyping

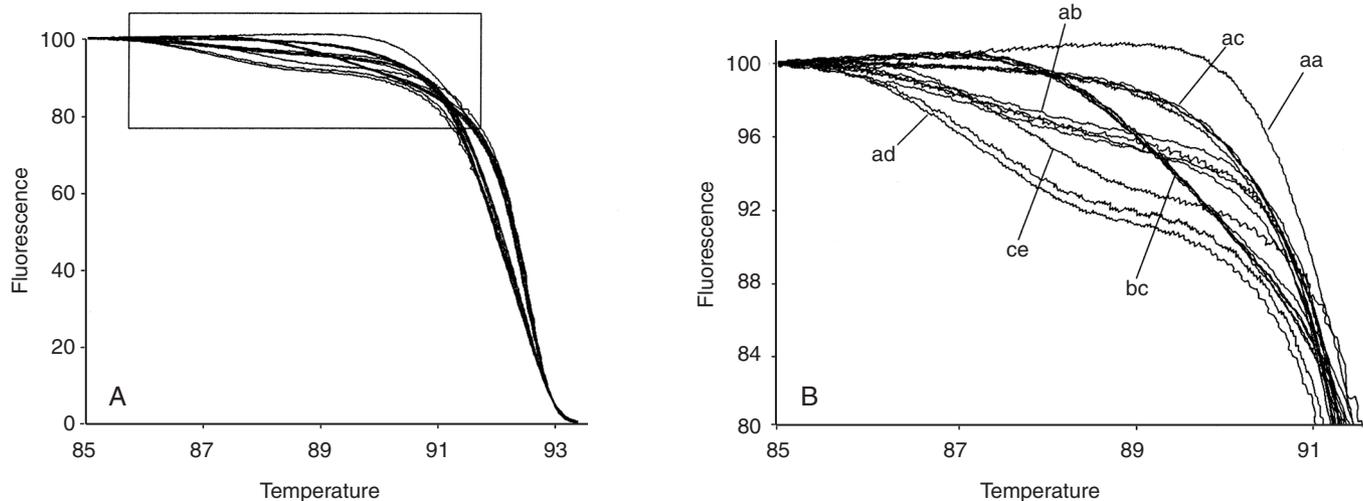


Fig. 6. HLA-A exon 2 melting curves of the pedigree shown in Fig. 5. There are six different groups of melting curves representing six genotypes among 17 family members. (A) The normalized melting curves. (B) Enlarged box shown in A, with the genotypes according to Fig. 5.

techniques that use PCR are limited by potential sequence variants under the primers.

The rapidity and simplicity of melting analysis for establishing genotypic HLA identity is attractive. The heteroduplex dye is added before PCR, and expensive real-time instrumentation is not needed. Rapid-cycle PCR can be performed in 10–30 min, although two amplifications are required for the nested PCR examples described here. After the final PCR amplification, only 1–2 min is necessary for high-resolution melting. The method is ‘closed-tube’ with the melting curve acquired in the same container used for amplification. No sample processing or separation steps are necessary. Current high-resolution instrumentation is limited to single-sample analysis, but this still allows a throughput of 30–45 samples per hour. Disadvantages of high-resolution melting for HLA identity include detection of silent mutations that do not affect amino acid sequence. In cases where a difference is detected, sequencing or another means of genotyping may still be needed to identify the nature of the variant.

We have used melting analysis to establish identity at the HLA-A locus. For establishing full HLA genotypic identity and to rule out the possibility of crossovers throughout the HLA region, analysis of additional HLA loci, for example, HLA-B, HLA-DR, and/or HLA-DQ, would be necessary. Current protocols require serotyping, genotyping, or sequencing to establish the HLA type of each individual, followed by a search for identical types. The resolution of serotyping

and probe genotyping is limited, and even sequencing can result in allelic ambiguities. In contrast, melting analysis of mixed DNA samples directly determines the sequence identity of the amplified regions. Genotyping for confirmation of HLA identity should not be necessary, although the samples can always be further typed molecularly or sequenced if desired. Although not tested here, ambiguous allele combinations should be resolvable because different haplotypes produce unique heteroduplexes that should result in distinguishable melting curves.

In the future, the potential utility of using melting curve analysis for establishing genotypic HLA identity of unrelated donor–recipient pairs for allogeneic hematopoietic stem-cell transplantation is extremely attractive (3). First, the need for resolving allelic ambiguities commonly observed even with HLA sequence-based typing would be eliminated because identity of melting curves for each locus would indicate sequence identity. Second, the rapid nature of these assays would mean that a multitude of potential donors for a given patient could be evaluated in a single day, obviously for much less expense than sequence-based typing. Finally, the cost of rapid-cycle PCR and high-resolution melting instruments is modest when compared with that of real-time PCR machines. Throughput could be increased by coupling this technology to a robotic system for centralized or regional centers that have high volume needs for unrelated donor genotyping.

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