Anal. Chem. **2000,** *72,* 5233−5238

Allele-Specific HLA-DR Typing by Mass Spectrometry: An Alternative to Hybridization-Based Typing Methods

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The primer oligomer base extension (PROBE) reaction, combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, is used to characterize HLA-DR2 polymorphism. Alleles are distinguished rapidly and accurately by measuring the mass of primer extension products at every known variable region of HLA-DR2 alleles. Since differentiation of alleles by PROBE relies on measuring differences in extension product mass rather than differences in hybridization properties, mistyped alleles resulting from nonspecific hybridization are absent. The method shows considerable potential for high-throughput screening of HLA-DR polymorphism in a chip-based format, including rapid tissue typing of unrelated volunteer donors.

Human leukocyte antigens (HLA) are cell surface proteins that mediate restricted recognition of pathogens by specialized T cell receptors (TCRs).[1] There are two major classes of HLA that are distinguished based on immunological function and biochemical structure. Class I HLA, which are expressed on the surface of all cells, present peptide fragments of proteolyzed cellular proteins and intracellular pathogens. Class II HLA, which are expressed on the surface of B cells, macrophages, and dendritic cells, present peptides derived from extracellular sources. Restricted recognition of the HLA−peptide complex by TCRs on cytotoxic and helper T lymphocytes provides the molecular basis of cell-mediated immunity.[2]

The genes encoding HLA are the most polymorphic known, and tissue typing prior to transplantation has become the major clinical application of high-resolution DNA diagnostic screening for HLA polymorphism.[3,4] Transplantation between HLA-unmatched donors and recipients dramatically increases the likelihood of outright organ rejection or, in the case of bone marrow transplantation (BMT), graft versus host disease (GVHD). Diagnostic assays for allelic polymorphism in HLA focus on the structural genes encoding the class I HLA-A and HLA-B antigens and the class II HLA-DR antigens. HLA-DRB genes in particular are routinely characterized with allelic specificity in tissue-typing laboratories; most clinically useful polymorphism in class II antigens occurs in the second exon of the gene encoding the β chain of class II HLA heterodimers.[5]

Due to advances in molecular methods such as PCR and refined hybridization methods, molecular (DNA-based) tissue-typing strategies have become established in clinical laboratories.[6,7] PCR amplification using sequence-specific primers (SSP) that complement variable regions at the ends of the HLA-DR second exon can be used to amplify subgroups of HLA-DR alleles. SSP is used routinely as a low-resolution (nonallele-specific) typing method. Alternatively, general primers complementary to conserved regions bordering the variable exon can be used to amplify all DR2 alleles. Radio- or fluorolabeled sequence-specific oligonucleotide probes (SSOP) complementary to variable bases in the amplified region are then used to characterize HLA-DR polymorphism with high resolution (allelic precision). Hybridization conditions in SSOP typing strategies must be extremely stringent to prevent false positive or false negative hybridization, especially when characterizing point mutations. False positive and negative hybridization is a major source of error in hybridization-based tissue-typing methods, especially in chip-based approaches where hybridization conditions are the same for different primers.[8]

Over the past decade, mass spectrometry has made contributions to a wide range of analytical and diagnostic problems in molecular biology and biochemistry. This is largely due to the invention of electrospray ionization[9] and matrix-assisted laser desorption/ionization (MALDI),[10] which are capable of volatilizing and ionizing high-mass biopolymers, thereby enabling their accurate mass measurement. Since mass spectrometry measures an intrinsic molecular property (molecular mass), the ability to measure the mass of large biopolymers, including polynucleotides,

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10.1021/ac991432z CCC: $19.00
Published on Web 09/26/2000

has spurred significant interest in the use of mass spectrometry as a broadly applicable DNA diagnostic method.

One early approach to differentiating single-base substitutions by mass spectrometry relied on the direct measurement of small mass shifts (e.g., Δm A→T = −9.0 Da) in amplified products, which required high-performance instrumentation.[11] Other efforts have been directed at detecting base substitutions that fall in restriction endonuclease recognition sites. The resulting altered restriction patterns (relative to wild type) can be identified by mass spectrometry.[12,13] This method is not promising as a universal diagnostic approach, since incomplete restriction site cleavage results in mistyped alleles in heterozygous samples. In addition, many mutations lie far outside restriction sites. MALDI-TOF mass spectrometry has also been used to characterize HLA-DQA polymorphism by detecting hybridization probes to amplified HLA templates.[14] This approach distinguishes between alleles based on differences in hybridization patterns; problems associated with promiscuous hybridization are still a primary source of error, and the high-mass resolution afforded by mass spectrometry provides no significant advantage.

Assays relying on product differentiation based on high-fidelity, allele-specific extension reactions (as opposed to allele-specific hybridization), combined with detection by mass spectrometry, have been developed. The primer oligo base extension (PROBE) reaction was originally demonstrated as a diagnostic method to characterize deletions, substitutions, and point mutations in the cystic fibrosis transmembrane regulator (CFTR) deletions,[15] apoE polymorphisms,[16] and microsatellite repeat lengths.[17] The ability to analyze 100 oligonucleotide samples by mass spectrometry in a chip-based miniature array format has also been demonstrated.[18] When optimized, fewer than 10 s is necessary to acquire spectra of oligonucleotides from less than 1 fmol of sample;[18] therefore, mass spectrometry would be a rapid, cost-effective method for detecting a large number of allelic variations in HLA for a large number of patients.

General Strategy for Characterizing HLA-DRB Alleles Using PROBE. There are over 225 known HLA-DR alleles, which can be separated into 8 amplified subgroups using SSP. Our general method for characterizing HLA polymorphism is a two-step process, comprising subgroup amplification by SSP followed by a series of PROBE reactions for each amplified subgroup. First, one of the eight subgroups of HLA-DR is amplified by SSP. Second, primers are annealed to the template just downstream of a variable base on the template strand. The hybridized primer is extended using a DNA polymerase and a specific dNTP/ddNTP mixture. Following sample conditioning (i.e., desalting and removal of extension products from the template), the masses of the extension products detected by mass spectrometry reveal the presence or absence of specific bases.

We developed a series of PROBE reactions for one of the eight sequence motifs at codons 6−14. We first used SSP to amplify DR2 alleles. Subsequently, PROBE reactions identified mass differences at each variable base with specificity and redundancy. Eleven PROBE reactions identify all known variable bases in the amplified DR2 haplotype. These reactions are listed in Figure 1. While fewer reactions are necessary in many cases to determine the correct allele, a battery of all 11 provide unambiguous characterization in every case, usually with redundancy. Figure 1a shows the seven reactions conducted on the sense strand primer, and Figure 1b shows the four reactions conducted on the antisense strand primer. Each reaction is designated by the codon and base in the sense strand template. Extended bases in PROBE reaction products are shown in boldface type. Masses of PROBE products are shown to the right of extended products. Allelic differences caused by silent mutations were not assayed, since they result in no difference in the sequence of expressed HLA.

MATERIALS AND METHODS

Purification of Genomic DNA. Genomic DNA was obtained from a series of reference B and T cells of healthy individuals. Blood plasma was separated and discarded by centrifugation. The remaining cell pellet was dissolved in 0.5 mL of sucrose−Triton buffer and vortexed for 30 s to lyse the cell membranes. The solution was centrifuged for 40 s, and the supernatant was discarded. a 0.5-mL aliquot ofPCR-DK buffer and 3 μL of proteinase K (10 mg/mL) were added to the nuclear pellets. The mixture was incubated at 55 °C for 1 h and vortexed once. The proteinase K was heat inactivated at 95 °C for 1 h. The samples were stored at −80 °C.

PCR Amplification. Oligonucleotides were obtained from Operon Technologies, Inc. (Alameda, CA). PCR primers, biotinylated (bio-) as indicated, were delineated from HLA-DR exon sequences published by the American Society of Histocompatibility and Immunogenetics (ASHI). To generate a sense strand template for probe reactions, 5′-bio-dTTC CTG TGG CAG CCT AAG AGG and 5′-dCCG CTG CAC TGT GAA GCT CT-3′ primers were used. To generate an antisense strand template for probe reactions, 5′-bio-dCCG CTG CAC TGT GAA GCT CT-3′ and 5′-dTTC CTG TGG CAG CCT AAG AGG-3′ primers were used. The total reaction volume was 100 μL, with 16 pmol of biotinylated primer and 20 pmol of unbiotinylated primer per reaction. Amplitaq polymerase including 10× buffer (1× buffer: 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 20 mmol/L KCl, pH 8.3) and dNTP reagents was purchased from Perkin-Elmer (Branchburg, NJ). Cycling conditions were as follows: 5 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s ramp to 60 °C, 8 s at 60 °C, and 15 s at 72 °C; 7 min at 72 °C. Products were stored at −20 °C.

Affinity Capture and Denaturation of PROBE Templates. Aliquots (10 μL) of the amplified product (∼1 pmol) were added to 10 μL of streptavidin-coated beads (Dynal, Oslo, Norway) in 5× incubation buffer (1 M NH_4Cl, 60 mM NH_4OH) and 30 μL of water. After incubation for 30 min at 37 °C, the supernatant was removed and the bead-bound template was incubated for 5 min in 50 μL of 100 mM NaOH to remove the nonimmobilized strand. The beads were washed once with 50 μL of 50 mM NH_4OH and

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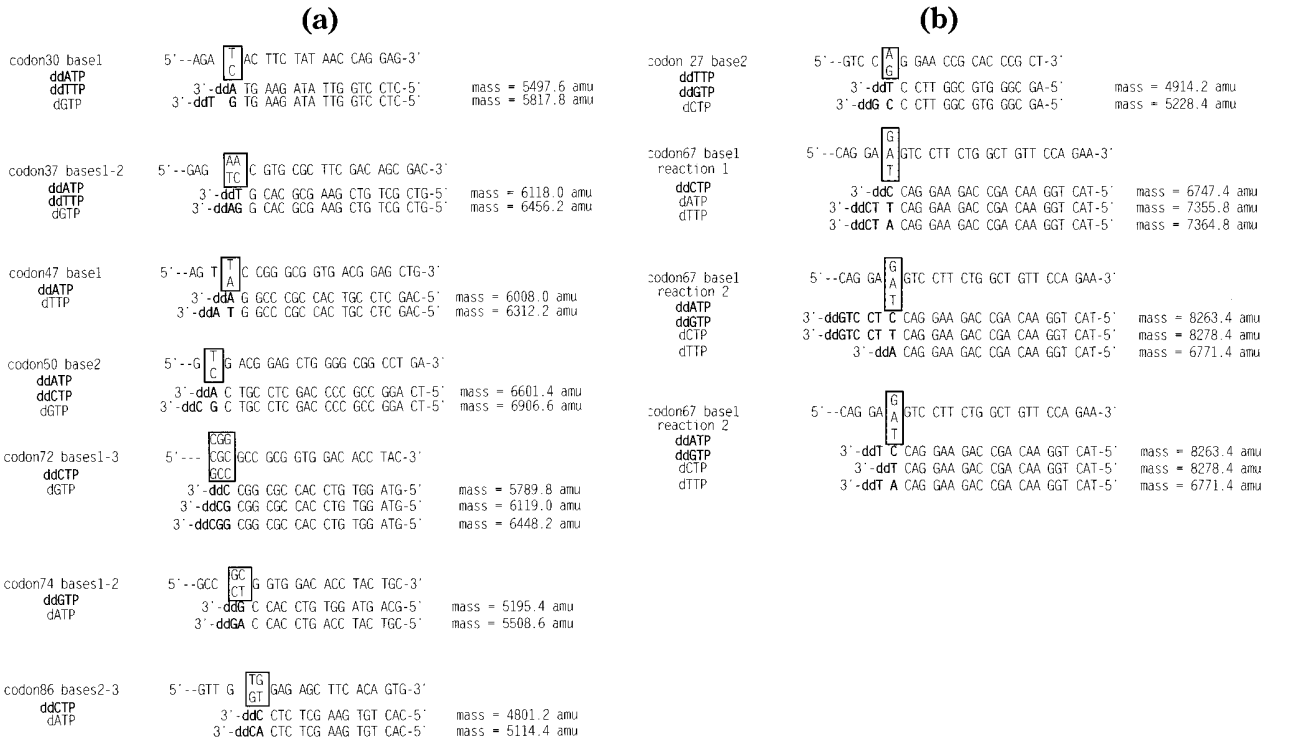

Figure 1. Extension reactions using (a) the sense strand template and (b) the antisense strand template. PROBE reactions for each variable base are designated by codon and base number. The dNTP/ddNTP mix used for each reaction is listed below each codon/base designation. The 5′-3′ oligonucleotide in each reaction is the template strand; possible extension products are shown 3′-5′ below the template strand. Extended bases are in boldface type. Masses of extension products appear to the right of each extension product.

3 times with 50 μL of 10 mM Tris-Cl pH 8.0. Following the last wash, the supernatant was removed from the solution.

PROBE. A PROBE mixture was prepared by combining 10 pmol of primer, 2.5 units of Thermosequenase, 1.5 μL of 10× buffer (1× buffer: 10 mmol/L Tris-HCl, 1.5 mmol/L $MgCl_2$, 20 mmol/L KCl, pH 8.3), 50 μmol/mL each dNTP (Pharmacia, Pascataway, NJ) and ddNTP (Life Technologies, Rockville, MD), and water for a total volume of 15 μL. The solution was added to the bead-bound template. Cycling conditions were as follows: 1 min at 80 °C, 10 cycles of 10 s at 50 °C and 5 s at 72 °C, followed by 3 min at 72 °C. The solution was washed for 2 min with 50 μL of 700 mmol/L ammonium citrate at 50 °C, followed by 2 × 50 μL of 70 mmol/L ammonium citrate. The wash buffer was removed, and extended products were removed by incubating with 2 μL of 50 mmol/L NH_4OH at 80 °C for 5 min. After rapid cooling, the extension products were removed in the supernatant.

Mass Spectrometry. A 0.5-μL aliquot of matrix solution (saturated solution of 3-HPA in 1:1 water/acetonitrile) was combined with 0.5 μL of extension sample, mixed on a stainless steel sample target, and allowed to air-dry. The sample was introduced into either a Kratos (Manchester, U.K.) MALDI-4 pulsed extraction time-of-flight mass spectrometer or a Perseptive Biosystems (Boston, MA) Voyager-DE MALDI-TOF mass spectrometer. Mass spectra were acquired in negative ion mode at 22.5- and 30-kV extraction potentials, respectively. The expected extension product masses were calculated from atomic compositions, and experimental values were all determined using external calibration and are reported in their single negative charge deprotonated forms.

RESULTS AND DISCUSSION

HLA-DR2 alleles were typed for a series of three homozygous and four heterozygous individuals expressing a single DR2 allele. The heterozygous individuals expressed one DR2 allele and one or more non-DR2 allele. Only the DR2 allele was amplified in the initial PCR step. Each PROBE reaction was conducted separately. For most variations, only one PROBE reaction was necessary to distinguish between alleles. The one exception was codon 67 base 1, where there are three possible variable bases. Two of the three separate PROBE reactions determine the presence of the correct variable base.

All 11 PROBE reactions were successful in each sample. Representative mass spectra are shown in Figures 2−4. The possible PROBE products and their calculated masses are listed at the top of each spectrum. The observed extension product is underlined and shown in bold. Since this individual expressed only one allele in the amplified subgroup, only one PROBE product was observed for each reaction.

Table 1 compares the detected masses of extension products with the calculated masses of the expected reaction PROBE products for each possible allele in the subgroup. The allele names are listed in the left-hand column, and the location of the variable bases in the amplified region are shown across the top row. The masses are shown in reference to the DRB1*15011 allele; observed masses of PROBE products for each sample are in italic type beneath the allele they uniquely identify. Silent mutations, while not necessary for tissue-typing purposes, distinguish 16011 from 16012 and 16021 from 16022 using this assay. The pattern of PROBE products unambiguously identifies the DR2 allele.

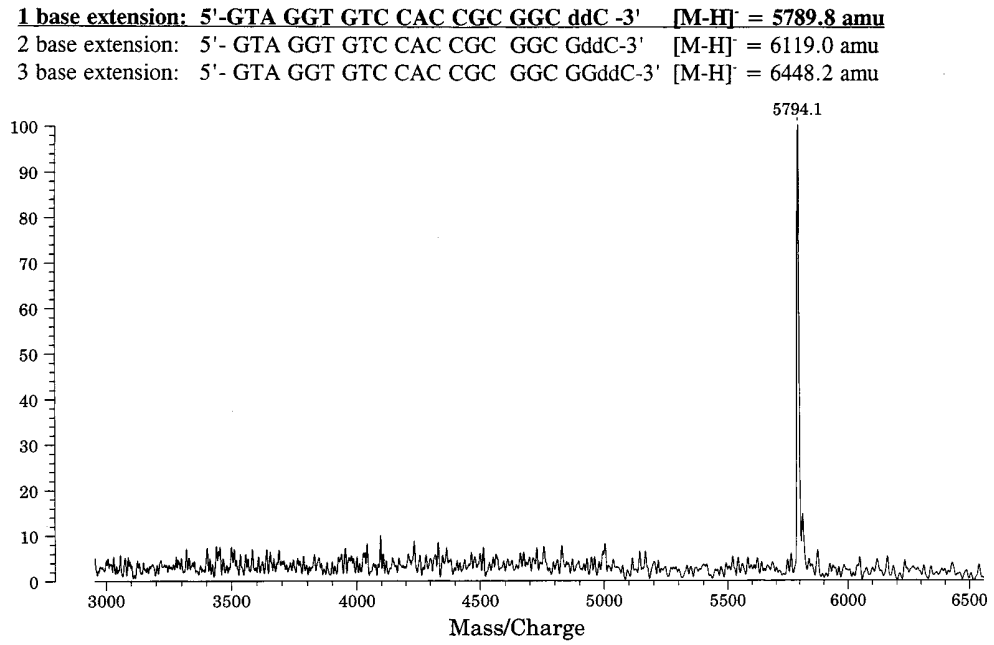

Figure 2. MALDI mass spectrum resulting from the PROBE reaction for codon 72 base 1−3. Possible reaction products are shown above the mass spectrum with the correct product in bold type.

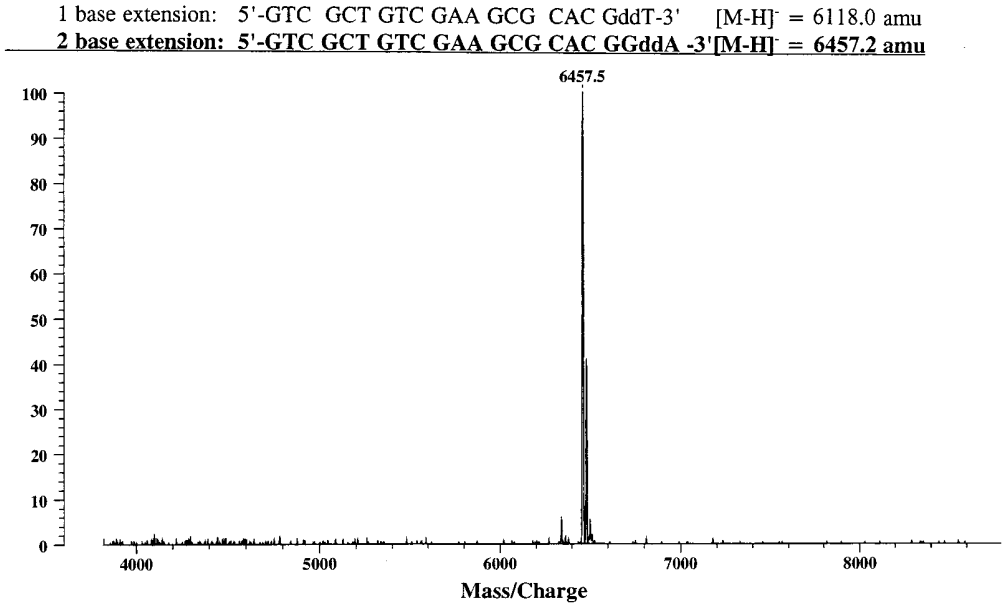

Figure 3. MALDI mass spectrum resulting from the PROBE reaction for codon 37 base 2. Possible reaction products are shown above the mass spectrum with the correct product in bold type.

PROBE reactions distinguish between products that differ in mass by an entire nucleotide. All measured masses of PROBE products were measured to within ±15 amu of calculated masses using external calibration. The average difference in mass of a single base is 305.2 amu. The observed error therefore does not result in mistyped alleles, since mass differences between PROBE reaction products are over 1 order of magnitude greater than observed experimental error. While this is acceptable error for detecting unknown oligonucleotides, a smaller error is usually observed in MALDI-TOF mass spectra of peptide samples. The relatively large size of 3-HPA matrix crystals is the major reason a larger error is observed for oligonucleotide samples; since large 3-HPA crystals extend into the source region, ions of the same mass are accelerated at energies slightly different from the external calibrant.

CONCLUSION

The PROBE reaction, combined with mass spectrometry, is a rapid diagnostic method to detect HLA-DR polymorphism unambiguously. The PROBE reaction is well suited for the rapid detection of variable bases in the HLA-DR locus, since only one exon encodes most known variation, and only this exon must be amplified to characterize polymorphism with allelic specificity. In addition, variable bases are localized to specific hypervariable

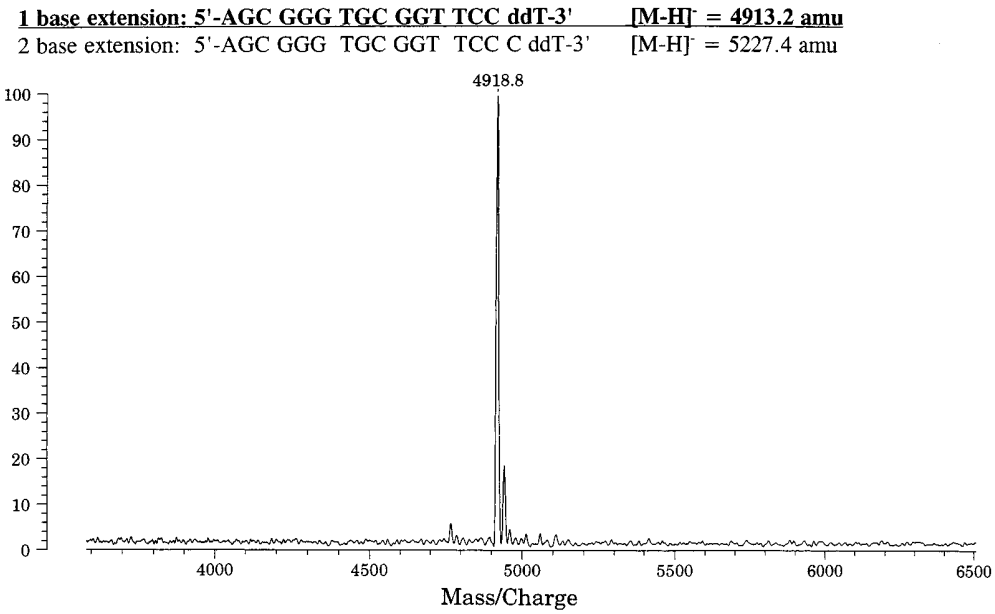

Figure 4. MALDI mass spectrum resulting from the PROBE reaction for codon 27 base 2. Possible reaction products are shown above the mass spectrum with the correct product in bold type.

Table 1. Comparison of Observed PROBE Product Masses with Calculated PROBE Product Masses for a Series of Unknown Samples Having a DR2 Allele[a]

						c67b1					
allele	c27b2	c30b1	c37b1−2	c47b2	c50b2	rRxn1	rxn2	rxn3	c72b1-3	c74b1-2	c86b2-3
DRB1*15011	4913.2	5497.6	6456.2	6008.2	6601.4	7363.8	6770.4	7074.6	5789.8	5195.4	4801.2
DRB1*15012											
unknown 2	*4917.4*	*5507.7*	*6457.5*	*6016.3*	*6603.7*	*7366.8*	*6770.6*	*7066.9*	*5789.1*	*5203.4*	*4805.4*
unknown 7	*4911.7*	*5506.6*	*6452.3*	*6010.1*	*6598.0*	*7369.2*	*6775.3*	*7078.1*	*5792.8*	*5202.5*	*4803.7*
DRB1*15021											5114.2
DRB1*15022											5114.2
unknown 3	*4910.7*	*5508.9*	*6456.0*	*6018.5*	*6614.5*	*7365.8*	*6772.2*	*7068.5*	*5788.3*	*5208.5*	*5116.1*
DRB1*1503		5817.8									
unknown 1	*4915.4*	*5804.7*	*6452.2*	*6017.0*	*6609.8*	*7360.3*	*6770.6*	*7081.2*	*5794.7*	*5194.0*	*4811.3*
DRB1*1504						7355.8	8277.4	6761.4			
DRB1*1505						6746.4	8262.2	7050.6			
DRB1*1506					6906.6						
DRB1*16011				6312.4		7354.8	8277.4	6761.4	6119.0		5114.2
unknown 4	*4911.7*	*5502.2*	*6459.8*	*6301.5*	*6600.1*	*7356.1*	*8275.2*	*6771.2*	*6118.2*	*5197.0*	*5118.2*
DRB1*16012				6312.4		7354.8	8277.4	6761.4			5114.2
DRB1*16021				6312.4		6746.4	8262.2	7050.6	6119.0		5114.2
unknown 5	*4911.1*	*5502.6*	*6472.8*	*6300.0*	*6601.9*	*6752.8*	*8258.5*	*7046.6*	*6125.1*	*5203.3*	*5118.9*
unknown 6	*4913.1*	*5506.0*	*6457.5*	*6298.5*	*6593.4*	*6753.6*	*8256.8*	*7062.4*	*6119.2*	*5201.0*	*5109.4*
DRB1*16022				6312.4		6746.4	8262.2	7050.6			5114.2
DRB1*1603				6312.4		7354.8	8277.4	6761.4	6448.2		5114.2
DRB1*1604				6312.4		7354.8	8277.4	6761.4		5508.6	5114.2
DRB1*1605				6312.4					6119.0		5114.2
DRB1*1606				6312.4					6448.2		5114.2
DRB1*1607	5227.4			6312.4					6119.0		5114.2
DRB1*1608			6118.0	6312.4		7354.8	8277.4	6761.4	6119.0		5114.2

[a] The DRB1*15011 allele is used as a reference; only those masses that differ are shown for the other alleles. Unknowns and their masses are listed below their corresponding allele. DR2 alleles are listed in the left column; locations of PROBE reactions in the amplified subunit are listed at the top.

regions within the exon. This allows the same PROBE primer to be annealed within the conserved region just downstream of variable bases in all reactions.

Tissue typing by PROBE is extremely rapid. After the initial 90-min SSP amplification and surface immobilization of the biotinylated template, the battery of PROBE reactions for a single individual can be completed in 30 min. Analysis of diagnostic products is also quite rapid; a 50-laser pulse average mass spectrum is easily acquired in under 10 s.

The greatest strength of the PROBE method is that it does not rely on hybridization to distinguish between HLA-DR alleles. The PROBE method distinguished alleles by measuring mass differences between extended products, and the difference in mass is easily resolved by mass spectrometry. Hybridization methods,

particularly in a clinical setting, require high-stringency posthybridization washes to distinguish between alleles. If hybridization conditions are not precisely controlled, false positive or negative results can be observed. This has significant repercussions for chip-based hybridization schemes, which do not control hybridization conditions as acutely with posthybridization washes. Annealing a number of different probes with different base compositions and therefore different annealing temperatures under similar buffer conditions would result in false positive and false negative detection of polymorphisms. PROBE-based typing methods do not have these limitations, since variation is not measured on the basis of hybridization and therefore PROBE primers are hybridized and extended under the same conditions regardless of primer length, sequence, or base composition. As a result, the same extension conditions can be used to produce PROBE products from a large number of primers in the same reaction, including in a chip-based format.

ACKNOWLEDGMENT

The authors thank Daniel P. Little for his assistance in designing the experimental approach and anticipating experimental difficulties. The authors also thank Loretta Leppin of Sequenom Inc., and William H. Wood, III and Brenda Cissell of the Immunogenetics Laboratories of The Johns Hopkins University for excellent technical assistance.

Received for review December 13, 1999. Revised manuscript received August 3, 2000. Accepted August 7, 2000.

AC991432Z