Simultaneous Genotyping of *DRB1/3/4/5* Loci by Oligonucleotide Microarray

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Matching of the HLA antigens for donor-recipient in transplantation, disease predisposition or protection, population studies, and forensic testing requires accurate but simple typing methods. Here, we describe a DNA-based tissue-typing assay that determines the haplotype of the *DRB1/3/4/5* loci in hybridization of oligonucleotide array after sample amplification. Using this multianalyte DNA hybridization system, we analyzed seven regions of exon 2 of *DRB* loci that have single-base discrimination. Thirty-six oligonucleotide probes complementary to the alleles of interest were immobilized on each microslide. The efficiency and specificity of identifying *DRB* genotypes using the oligonucleotide arrays was evaluated by blinded analysis of 147 samples from reference standards and subjects. The established method provides a rapid and inexpensive *DRB* “low-resolution” typing tool for prescreening a large number of samples. (*J Mol Diagn* 2005, 7:592–599)

The human leukocyte antigen (HLA) class II molecules of the human major histocompatibility complex are encoded on the short arm of human chromosome 6p21.3 in the HLA-D region. These glycoproteins consist of an α - and a β -chain associated as heterodimers on the cell surface of antigen-presenting cells such as B cells and macrophages. They play a central role in the regulation of the immune system¹ in transplantation biology,^{2,3} as well as in susceptibility to a number of diseases, including autoimmune disorders^{4,5} and certain cancers.^{6,7} The HLA-D region contains several class II genes and has three subregions: HLA-DR, -DQ and -DP according to the position of gene. For HLA-DR, one gene coding for the DR α -chain, *DRA*, and one gene coding for β -chain, *DRB1*, is always present. Depending on the *DRB1* type, a *DRB3*, *DRB4*, or *DRB5* may also be present and may be accompanied by pseudogenes. With the exception of the *DRA* molecule, the genes encoding the functional class II molecules are highly polymorphic with virtually all of the variability localized to the second exon. This exon en-

codes the amino-terminal extracellular domain, which functions as the antigen binding site for processed peptides.

With respect to the multiple functions of the HLA antigens, adequate typing analyses are extremely important. Matching for donor-recipient in transplantation and typing for disease predisposition or protection, for population studies, as well as for paternity or other forensic testing are all applications demanding an accurate but simple typing method. Traditionally, HLA antigens were tested by serological typing. Recent advances in DNA technology have vastly improved the detail to which HLA antigens can be characterized. Several different DNA-based techniques have been developed to improve HLA-class II typing such as polymerase chain reaction (PCR)-restriction fragment length polymorphism,⁸ PCR sequence-specific oligonucleotide probe (PCR-SSOP),⁹ reverse dot blot hybridization,¹⁰ PCR with sequence specific primers (PCR-SSP),^{11,12} oligonucleotide array,^{13,14} multiplex primer extension,¹⁵ and sequencing based typing (SBT).^{16,17} Two-step typing of *DRB1* locus has also been described previously. The system is based on protocol by using allele-specific PCR followed by hybridization of oligonucleotide probes.^{11,18} They differ in cost, time, and the provided degree of information.

It is not necessary that the “high-resolution” genotyping analysis is directly operated for samples in forensic casework and in routine clinical practice including donor-recipient matching. This paper presents a strategy by which low-resolution *DRB1/3/4/5* genotyping was achieved by microarray. This approach is presented here on 32 DNA samples of cell lines and 115 DNA samples of randomly selected individuals.

Materials and Methods

Chemicals

The silylated slides (CSS-100) were purchased from CEL Associates (Sunnyvale, CA). PCR kit (*rTaq*) was from TaKaRa (Kyoto, Japan). All chemicals and solvents were purchased from Sigma (St. Louis, MO) and Gibco-BRL (Carlsbad, CA), unless stated otherwise, and used with-

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Table 1. Cell Lines and Individuals Investigated by "Low-Resolution" Microarray Typing, SSP Analysis, SSOP Analysis, and SBT

Samples	Microarray typing	SSP analysis	SSOP analysis	SBT
Cell lines	32	5	5	
Stem cells in cord blood	50	30	25	15
Clinical samples	65	40	40	20
Total	147	75	70	35

out additional purification. Oligonucleotides were synthesized by standard phosphoramidite chemistry. Probes to be immobilized on microslides containing 5' amino group were synthesized with C6 linker. All oligonucleotides were purified by high performance liquid chromatography (HPLC) after synthesis.

Subjects

DNA from 32 HLA class II-defined lymphoblastoid cell lines of the 13th International Histocompatibility Workshop (IHW) representing almost all defined *DRB* 1/3/4/5 subtypes served as reference samples: IHW9006 (DRB1*0101), IHW9007 (DRB1*0401/1602, DRB4*01, DRB5*02), IHW9011 (DRB1*1502, DRB5*01), IHW9016 (DRB1*1602, DRB5*02), IHW9022 (DRB1*0301, DRB3*01), IHW9028 (DRB1*0404, DRB4*01), IHW9035 (DRB1*1101, DRB3*02), IHW9040 (DRB1*1102, DRB3*02), IHW9045 (DRB1*1104/1201, DRB3*02), IHW9046 (DRB1*0701, DRB4*01), IHW9053 (DRB1*1302, DRB3*03), IHW9054 (DRB1*1401, DRB3*02), IHW9070 (DRB1*0803), IHW9075 (DRB1*09012, DRB4*01), IHW9081 (DRB1*1501, DRB5*01), IHW9085 (DRB1*3011, DRB3*02), IHW9092 (DRB1*0404, DRB4), IHW9093 (DRB1*0701, DRB4*01), IHW9103 (DRB1*09012, DRB4), IHW9215 (DRB1*0301/0701, DRB3*02, DRB4), IHW9220 (DRB1*0701/09012, DRB4), IHW9237 (DRB1*1501/1405, DRB3*02, DRB5), IHW9253 (DRB1*0406/12021, DRB3*02, DRB4), IHW9263 (DRB1*1404/15021, DRB3*02, DRB5), IHW9368 (DRB1*0901/1501, DRB4, DRB5), IHW9372 (DRB1*0901/1405, DRB3*02, DRB4), IHW9373 (DRB1*1302/0102, DRB3*03), IHW9376 (DRB1*07/11, DRB3*02, DRB4), IHW9380 (DRB1*1001/1601, DRB5), IHW9381 (DRB1*0301/0407, DRB3*01, DRB4), IHW9382 (DRB1*07/1503, DRB4, DRB5), IHW9398 (DRB1*0804/1303, DRB3*02). The cell lines represented the following DRB alleles: DRB1*01, DRB1*03, DRB1*04, DRB1*07, DRB1*08, DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*13, DRB1*14, DRB1*15, DRB1*16, DRB3*, DRB4*, DRB5*.

A total of 115 randomly selected healthy individuals were investigated (Table 1). All of these samples had previously been analyzed by SSOP kit (DYNAL, Oslo, Norway) and SSP kit (BIOTEST, PELFREEZ); part of them were confirmed by direct sequencing.

Microarray Construction

The 5' amino-modified probes of 14 to 17 bp were suspended in 3× standard saline citrate (SSC) at a concentration of 200 pmol/μl and printed on silylated slides by ProSys 5510 spotting workstation (Cartesian Technologies, Ann Arbor, MI). The 5' amino-linker allows selective binding of the amino-containing DNA to silylated slides through a Schiff's base reaction with aldehyde groups on the chip surface. Each oligonucleotide solution was spotted in triplicate.

PCR Amplification and Labeling of PCR Product

The oligonucleotide primers (GH46, CCGGATCCTTCGT-GTCCCCACAGCACG; RBAMP-B, CCGCTGCACTGT-GAAGCTCT) are used in this study to amplify DRB1/3/4/5 loci. Each 50-μl reaction contained 20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 μmol/L of each primer, 0.2 mmol/L of each dNTP, 2.5 units of polymerase (*rTaq*; TaKaRa, Japan), and 100 ng of genomic DNA. Reactions were carried out on a thermal cycler (PTC-225; MJ Research, Inc, MA), with an initial 3-minute denaturation at 95°C, 30 cycles of 94°C for 40 seconds, 63°C for 40 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes.

The labeling primers were designed to generate some short single-stranded labeled DNA. A 5-μl aliquot of the primary amplification product was used as target for a 50-μl linear PCR reaction using the reaction conditions and cycling conditions: 50-μl reaction, containing 20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 μmol/L labeling primer (RB-L: Cy5-CCGCTGCACTGTGAAGCTCT), 0.2 mmol/L of each dNTP, and 2.5 units of polymerase, was carried out with an initial 3-minute denaturation at 95°C, 20 cycles of 94°C for 40 seconds, 63°C for 40 seconds, and 72°C for 20 seconds. The linear PCR product was used in slide hybridization experiments. After adding 5 μl of 3.0 mol/L sodium acetate buffer (pH 5.2) and 2.5 volumes of cold 100% ethanol and cooling for 30 minutes at -20°C, PCR product was precipitated by spinning down in microcentrifuge for 10 minutes at 13,000 rpm. The pellet was washed twice with 70% ethanol, air dried, and dissolved in 8 μl of hybridization buffer (5× SSC, 0.2% sodium dodecyl sulfate [SDS]).

Slide Array Hybridization

Hybridization reactions were performed using a plastic chamber attached to the slide by a rectangular adhesive spacer (press-seal hybridization chambers, Hybri-well, Sigma, MO). Hybridization mixtures (8 μl) contained linear PCR product in slide hybridization buffer (5× SSC, 0.2% SDS). The mixture was denatured in boiling water for 3 minutes. Hybridization was carried out overnight at 42°C. After hybridization, the hybridization chamber was removed, and the slide was washed in 200 ml of 2× SSC, 0.2% SDS buffer for 5 minutes at 42°C, 1 × SSC buffer for

Figure 1. A: The principle of PCR amplification and labeling of DRB gene, schematic representation of the exon 2. Figure indicates the relative positions of primers (the sequences were shown as underlined segments of the lowercase). The sequence of exon 2 is in gray. **B:** PCR products (289-bp) of exon 2 of DRB1/3/4/5 genes using the generic DRB primers (GH46 and RBAMP-B) were visualized by agarose gel electrophoresis.

5 minutes at 42°C. Washed slides were centrifugated at 500 rpm × 5 minutes for removing excess liquid.

Scan and Data Analysis

The microarrays were scanned using a ScanArray 5000 system (General Scanning, PE, Shelton, CT) at appropriate sensitivity levels of photomultiplier. The instrument generates fluorescence images by scanning a laser beam (634 nm, 50 μm) over the sample surface. The data were saved as a 16-bit TIF file. The signal on each spot was analyzed using the DRBtyping software (a simple computer algorithm allows the genotype to be determined by analysis of the pattern of hybridization). For each spot, pixel intensities within the spot image were summed. The average value and SD of pixel intensities for each spot were calculated, and the local background level was subtracted from the sum of the signal intensity.

Results and Discussion

PCR Amplification and Labeling by Linear PCR

Certain DRB haplotypes express more than one DRB locus. To amplify all functional DRB alleles of an individual, The generic DRB primers (GH46, CCGGATCCTTCGTGTCCCCAC AGCACG; RBAMP-B, CCGCTGCACTGTGAAGCTCT) were used. These primers produce a 289-bp fragment. PCR products were visualized by agarose gel electrophoresis (Figure 1B).

The products of amplification of DRB locus are 289-bp double-stranded DNAs. The hybridization of the double-stranded DNA to the support-bound oligonucleotide ar-

Figure 2. The double-stranded PCR products and single-stranded products generated from linear PCR of the sample (IHW9382) were applied to the oligonucleotide array. The results demonstrated that single-stranded products generated much higher signals than double-stranded DNA. Subgroup A1: A01, A02, A03, A04, A04', A05, A06, A08, and A12; subgroup A2: A07, A09, A10, and A11. **White bars:** The fluorescence signals of hybridization with single-stranded DNA. **Gray bars:** The fluorescence signals of hybridization with double-stranded DNA.

ray will necessarily suffer from competition of the complementary strand with the oligonucleotide probes. In an attempt to improve efficiency of hybridization, we have used fragmentation and labeling methods that use linear PCR with 5'-Cy5-labeled primers to generate short single-stranded DNA target (Figure 1A).

Five microliters of PCR product was used for linear amplification with fluorescently labeled primers: RB-L, Cy5-CCGCTGCACTGTGAAGCTCT for a 289-base-long fragment of exon 2 of DRB genes. Short single-stranded products generated from linear PCR demonstrated very high hybridization efficiency when applied to the oligonucleotide array, compared with double-stranded PCR products. The double-stranded DNA from PCR product of the sample (IHW9382) with primers (GH46/RB-L) and single-stranded DNA from linear PCR with RB-L were hybridized, respectively. The results demonstrated that single-stranded products generated much higher signals than double-stranded DNA (Figure 2).

Design of Probes for Array

A key feature of the oligonucleotide array assay is the high redundancy of probes. Thirty-six 14- to 17-mer oligonucleotide probes were designed for all polymorphisms in exon 2 of DRB genes (alignment based on Release 1.12 (<http://www.ebi.ac.uk/imgt/hla/docs/release.html>; update, October 31, 2001). Probes were grouped into seven regions for exon 2 (Table 2). The 14- to 17-mer probes in each group were descriptive for the identical base positions in exons 2. Of 36 probes, 9 probes were descriptive for a single nucleotide change. The probes should be carefully selected to achieve significant discrimination of single-base mismatch. We investigated whether discrimination of single base mismatches among amplicons is feasible with all groups of probe. For example, we synthesized five pairs of B01/B02 probes (12-mer, 13-mer, 14-mer, 15-mer, and 16-mer).

Figure 3. The amplicons of DRB loci of IHW9006 were hybridized with B01/B02 probes with different oligonucleotide length immobilized on slides. The B01/B02 probes of group B were selected to achieve significant discrimination of single-base mismatch, considering the strength and ratio of fluorescence signal of B01/B02 probe pair.

The probes were designed such that mismatches occur in the middle of the probe. The amplicons of DRB locus of IHW9006 and IHW9011 were hybridized with these GC-rich probes immobilized on slides. The laser-scanned image of IHW9006 hybridization was shown in Figure 3. The result of IHW9006 hybridization was not shown. The probes of 14-mer were determined, considering the strength and ratio of fluorescence signal of B01/B02 probe pair. The probes of other groups were also determined according to this same principle with 32 known different cell lines (each probe can obtain positive signal once at least).

The probes of group A play an important role in a general genotyping analysis at the *DRB1*/3/4/5 loci because the extensive allelic diversity at HLA-DRB locus is localized to this region. Group A has the enough information for the initial identification of the majority of the *DRB1*/3/4/5 types (Table 3). Group A is divided into two subgroups. The subgroup A1 (A01, A02, A03, A04, A04', A05, A06, A08, and A12) hybridizes to the *DRB1* locus, whereas the subgroup A2 (A07, A09, A10, and A11) hybridizes to the *DRB3*/4/5 loci. For heterozygous samples, two oligonucleotide probes in a subgroup could be perfectly matched with the PCR product, each matched with a different allele. For homozygous samples, only one probe in each subgroup would be perfectly matched with the PCR product. Hybridization signal intensities were quantified by summing the intensities of all pixels located inside the image spot. Each signal was ranked from highest to lowest in group A according to their intensities. The highest ranked signal(s) was chosen as the positive signal(s) (Figure 4). When hybridized to homozygous samples (*DRB1*), only one probe in subgroup A1 produced a positive signal as the perfect match. In heterozygous samples for two different *DRB1* alleles, two probes with highest signal intensities in subgroup A1 were selected as positive, unless only one positive signal was obtained in that group. The information from the positive hybridization signals of other groups then was added to refine the results from allele families. We used an intensity

Figure 4. Procedure for analysis of hybridization data are illustrated for the sample (*DRB1**04, *DRB4*). All oligonucleotide probes were spotted and named (refer to Table 2 for probes). After hybridization, signal intensities were quantified.

ratio of 3:1 as the threshold to categorize the positive and negative signals. In most cases, the ratio between the intensities of positive signals and those of negative signals in each pair/group exceeded 3:1, depending on the physical characteristics of probes, condition of hybridization, and washing. All probes in each pair/group served as the control for each other, offering unequivocal and specific discrimination of each genotype.

Genotyping of *DRB1*/3/4/5 Loci

The fluorescently labeled linear PCR products of samples were hybridized to the microarrays. After hybridization and washing, the slide was scanned on ScanArray5000 to reveal the distribution of hybridized target. The fluorescent signal on each spot was calculated using the *DRB*typing software. Hybridization signal intensities were quantified by summing the intensities of all pixels located inside the image spot. That image shows high fluorescent signal on the spots containing the probes, which perfectly match to different positions within the linear PCR products. Very weak fluorescent signal is found on the spots containing related probes, which even only single-base mismatch to corresponding positions. In most cases, hybridization signals from spots containing the matched probe exceeded signals from the spots containing corresponding single-base mismatch probe by at least threefold.

In the first step, the hybridization signals were separated into groups according to the locations with which they hybridized (Table 2), and their intensities were integrated. In the second step, group A with the most probes was analyzed first (subgroup A1 and subgroup A2). Each signal was ranked from highest to lowest in this probe

Table 2. Sequences of Oligonucleotide Probes for DRB

Groups	Probes	Sequences*	Positions [†]
A1	A01	GGC AGC TTA AGT TTG AA	112 to 129
	A02	CTC TAC GGG TGA GTG T	117 to 132
	A03	CTC TAC GTC TGA GTG T	117 to 132
	A04	TTG GAG GAG GTT AAG TT	109 to 125
	A04'	A AGA CGC GTC CAT AAC	171 to 186
	A05	AGG GTA AGT ATA AGT GT	116 to 132
	A06	CAG CCT AAG AGG GAG T	115 to 130
	A08	CAG GTT AAA CAT GAG TG	115 to 131
	A12	CTT GAA GCA GGA TAA G	108 to 123
A2	A07	GCA GGA TAA GTA TGA G	114 to 129
	A09	TGG AGC TGC TTA AGT CT	110 to 126
	A10	TGG AGC TGC GTA AGT CT	110 to 126
	A11	G CAG GCT AAG TGT GAG	114 to 129
B	B01	GGG GAG TTC CGG GC	220 to 233
	B02	GGG GAG TAC CGG GC	220 to 233
C	C01	GCG GTG AGG GAG CT	232 to 245
	C02	GCG GTG ACG GAG CT	232 to 245
D	D01	CTG CTG CGG AGC ACT	254 to 268
	D02	CGG CCT AGC GCC GAG	250 to 264
	D03	CCT GAT GAG GAG TAC T	253 to 268
	D04	GCC TGA CGC TGA GTA C	252 to 267
	D05	CGG CCT GTC GCC GAG T	250 to 265
	D06	GCC TGA TGC CGA GTA	252 to 266
E	E01	GGA AGA CGA GCG GGC	291 to 305
	E02	GGA GCA GGC GCG GG	291 to 304
	E03	GGA AGA CAG GCG SGC S = G/C	291 to 305
	E04	GAG CRG AGG CGG GC R = A/G	292 to 305
	E05	GAG CAG AAG CGG GGC	292 to 306
F	F01	GCG GGC CCT GGT GGA	300 to 314
	F02	GCG GGC CCR GGT GGA R = A/G	300 to 314
	F03	GCG GGC CGC GGT GGA	300 to 314
	F04	GCG GGC CGA GGT GGA	300 to 314
G	G01	GAC ACC GTG TGC AGA	313 to 327
	G02	GTG GAC ACC TAC TGC	310 to 324
	G03	GTG GAC AAC TAC TGC	310 to 324
	G04	GTG GAC AAT TAC TGC	310 to 324

*Each oligonucleotide probe has a 8 dT linker and amino group at its 5' end.

[†]The positions of exon 2: IMGT/HLA Database (<http://www.ebi.ac.uk/imgt/hla/align.html>).

group according to their intensities. The highest ranked signal(s) in the group was chosen as the positive signal(s). The hybridization signals were categorized into positive signals and negative signals by comparing signal intensities in group A. The strongest one or two signals in subgroup A1 were considered positive and the rest of signals were considered negative. When the signals for probes in group A was compared with their corresponding theoretical hybridizations (Table 3), most of the allele family could be assigned from this information alone.

In the third step, The information from other positive hybridization signals then was added to refine the results from allele families into single allele types. A theoretical hybridization pattern was generated for each allele by counting the probes matched with its sequence. Table 4 demonstrates the procedure for analysis of hybridization data of IHW9382 sample shown in Figure 2. *DRB1* allele families (*DRB1*07*, *DRB1*15*, or *DRB1*16*) are selected with positive signals (A05 and A06) in subgroup A1; *DRB3/4/5* alleles (*DRB4* and *DRB5*) are confirmed with positive signals (A07 and A11) in subgroup A2. Ambiguous results (*DRB1*15* or *DRB1*16*) are further analyzed with hybridization data from the remaining groups: some alleles may be excluded with the negative signals; some alleles (*DRB1*1507/11*,

*DRB1*16*) may be excluded by the distribution of positive signals in group (because B01 and B02 are both positive, each class contains an allele of *DRB1*07/15/16*, *DRB4* and *DRB5* at least). So the genotype of IHW9382 can be deduced to *DRB1*15/07*, *DRB4*, *DRB5*.

DRB Genotyping for Reference Samples

The microarray-based genotyping for *DRB1/3/4/5* loci was tested for 32 IHW cell lines. All probes in each group served as the control for each other, offering unequivocal and specific discrimination of each allele. No false-positive/negative signal spots were obtained in the IHW cell lines investigated (Figure 5).

Reproducibility

Twenty cell lines were investigated on five separate tests by this analysis method and interpreted blindly. The reproducibility among the repeated typing was 100%.

Applications of Microarray to Screening

To demonstrate how the oligonucleotide array could be used to genotype blinded samples, we conducted an

Table 3. Alleles for DRB Probes

Probes	Alleles
A01	DRB1*01xx
A02	DRB1*08xx,1*12xx,1105,1317,1404/11/15/28/31
A03	DRB1*03xx,1*11xx,1*13xx,1*14xx
A04	DRB1*10xx
A04'	DRB1*10xx
A05	DRB1*07xx
A06	DRB1*15xx,1*16xx
A08	DRB1*04xx,1122,1410
A12	DRB1*09012
A07	DRB5*xxxx
A09	DRB1*1130,DRB3*02xx, DRB3*03xx
A10	DRB3*01xx
A11	DRB4*xxxx
B01	DRB1:other excluding alleles of B02 and DRB4*xxxx
B02	DRB1:01xx,0302,0303,0306,0317,04xx(no 0428/35/42/ 43),07xx,08xx(no 0817),09xx,10xx,1117/37/41,1303/07/08/12/13/19/26/32/33/36/37/38/40/47/48/49,14xx(no 1417/21/30/33/35),1507/11,16xx, DRB3*xxxx,DRB5*xxxx
C01	DRB1:0317,1139 DRB3*0107/0201/0202/0203/0204/0205/0206/0207/0208/0211/0212/0213/0214
C02	Other excluding alleles of C01
D01	DRB1:0310,0808,1343,1345,1401/04/07/10/16/22/25/26/28/31/32/35/38/39
D02	DRB1:0312,0405/09/10/11/12/17/24/28/29/30,0801/03/05/06/10/12/14/16/17/18/22/23,1303/04/12/13/21/30/32/33/38/48/49,1413,1512, DRB3*0208
D03	DRB1:0308,0415,11xx,1204,1411
D04	DRB1:04032,13072,13142,1326,15xx(no 15022 and 1512), 16xx, DRB4*xxxx,DRB5*xxxx
D05	DRB1:07xx,09012,12xx(no 1204),1331,1346, DRB3*01xx (no 0107),DRB3*0207/09,DRB3*03xx
D06	Other excluding alleles of D01 to 05
E01	DRB1:0103,0402/14/37,1102/03/11/14/16/20/21/36/40/41,1301/02/04/08/15/16/17/19/20/22/23/24/27/28/29/31/32/34/35/36/38/39/40/41/43/45/48/,1416,1510
E02	DRB1:0106,1309,1424/37,15xx(no 1510), DRB5*0106, DRB5*0202/03/04
E03	DRB1:0412/15/18/25/36/,08xx,11xx(no 1102/03/07/11/13/14/16/17/20/21/26/34/36/40/41),12xx,1305/06/07/11/12/13/14/18/21/25/26/30/42/46/47/49,1403/12/15/22/25/27/40,16xx, DRB5*01xx(no 0103/06)
E04	DRB1:01xx(no 0103/06),0403/04/05/06/07/10/11/17/19/20/23/27/28/29/30/31/39/40/41/42/43,1126/34,1344,1402/06/09/13/17/29/30/33/41, DRB4*xxxx, DRB5*0205
E05	DRB1:03xx,0401/09/13/16/21/22/26/33/34/35/38,1107,1419/21, DRB3*xxxx
F01	DRB1:0412/18/25/31,08xx(no 0805/18/24),1123/25,1313 /18/47,1403/12/15/27/40,1604, DRB5*0104
F02	DRB1:03xx,0422,07xx,1107, DRB3*xxxx,
F03	DRB1:01xx,0401/02/04/05/08/09/10/13/14/15/16/19/21/23/24/26/28/29/30/32/33/34/35/36/37/38/40/42/43,0805/18/24,10xx,11xx(no 1107/17/23/25/32),12xx,13xx(no 13012/13/18/47),1402/06/09/13/16/17/19/20/21/22/24/25/29/30/31/32/34/37/41,15xx,16xx(no 1604), DRB5*xxxx(no 0104)
F04	DRB1:0403/06/07/11/17/20/27/39/41,09012,1117,1401/04/05/07/08/10/11/14/18/23/26/28/33/35/36/38/39, DRB4*xxxx
G01	DRB1:0701/03/05,09012
G02	DRB1:01xx(no 0104),0314/15,04xx(no 0422),08xx,10xx, 11xx (no 1127),12012,1202,13xx(no 1333),14012/02/03/06/09 /12/13/15/16/17/19/20/21/22/24/25/27/29/30/32 /33/37/40/41,15xx(no 15013),16xx, DRB4*xxxx(no 0102),DRB5*xxxx
G03	DRB1:03xx(no 03012/022/14/15/17),0422,1107,11272,1333, DRB3*02022/04,DRB4*0104,
G04	DRB1:03012/022/17,0704,11271,1438, DRB3*xxxx(no 02022,0204)

equivalency study of 115 unrelated individuals whose *DRB1/3/4/5* alleles had been typed previously. The part of some were confirmed by sequencing. In these subjects, 50 were samples from the Cord Blood Bank, and other 65 were clinical samples from the hospital. The

assay was thoroughly evaluated and has been used to type these samples at allele level-resolution. All 115 samples of the study were able to be typed by PCR-SSP, SSOP DR typing, and microarray typing. The concordance between two methods was 100%. The data of 147

Table 4. Analysis of Hybridization Data of IHW9382 Sample

Group	Positive signals	Pick out	Results
Step 1: Analysis of group A (allele classification with positive signals)			
Subgroup A1	A05	/	DRB1*07xx
	A06	/	DRB1*15xx, DRB1*16xx
Subgroup A2	A07	/	DRB5*xxxx
	A11	/	DRB4*xxxx
Step 2: Narrow down the results from first step (allele exclusion with negative signals, allele distribution with positive signals)			
Group B	B01, B02	DRB1*1507/11, DRB1*16xx	DRB1*15 xx (no 07/12), DRB1*07xx, DRB4*xxxx, DRB5*xxxx
Group C	C02	/	DRB1*15 xx (no 07/12), DRB1*07xx, DRB4*xxxx, DRB5*xxxx
Group D	D04, D05	/	DRB1*15 xx (no 07/12), DRB1*07xx, DRB4*xxxx, DRB5*xxxx
Group E	E02, E03, E04	DRB1*1510, DRB5*02xx	DRB1*15 xx (no 07/10/12), DRB1*07xx, DRB4*xxxx, DRB5*01xx
Group F	F02, F03, F04	DRB5*0104	DRB1*15 xx (no 07/10/12), DRB1*07xx, DRB4*xxxx, DRB5*01xx
Group G	G01, G02	DRB1*0704	DRB1*15 xx (no 1507/10/12), DRB1*07xx (no 0704), DRB4*xxxx, DRB5*01xx

samples are summarized in Figure 5. In most cases, the ratio between the intensities of positive signals and those of negative signals in each pair/group varied from 32.5:1 to 3:1 (Table 5), Exceptions to this occurred with two probes (F03 and F04) of group F. The F03 and F04 probes involved a C/A change nine nucleotides from the 5' end. The positive-to-negative ratios for these pairs declined to 2.72:1 and 2.85:1, yielding a false-positive signal at low frequency (2 cases in 147 samples). The polymorphisms defined by the F03/F04 probe pairs described a single-base change in GC-rich probes (86.7%

GC). These probes were not essential for the assignment of the DRB alleles of the two samples; hybridization data from the remaining probes was more than sufficient to deduce the genotypes. When the genotypes of the 147 samples were unblinded and compared with the results (SSP, SSOP, and SBT), the correct interpretation of the array hybridization patterns was made for all samples. This equivalency study demonstrates that the oligonucleotide probe array does not generate ambiguous results to these heterozygous combinations and homozygous samples (for various combinations of DRB alleles, see Supplemental Table at <http://jmd.amjpathol.org/>).

This method does not perform *HLA-DRB1/3/4/5* loci high-resolution genotyping, but microarray methods could be developed for that application by adding more probes. High-resolution HLA typing is of more importance for complex genetic association and tissue typing before organ transplantation, and this method would require further development for these purposes. However, it is useful for screening of subjects for whom expensive and time-consuming high-resolution genotyping is not necessary. New alleles in the DRB continue to be discovered at a rapid pace. The addition of new probe sequences descriptive of novel polymorphisms is a necessary and anticipated modification of this first generation array. The flexible nature of our array system allows probes to be easily added or modified without significantly increased costs. This method proved to be flexi-

Figure 5. Hybridization results of the 32 IHW cell lines serving as reference samples. A gray box indicates a positive signal of hybridization, and a white box indicates a negative signal of hybridization.

Table 5. The Fluorescence Signal Ratio of Eight Probe Groups Corresponding to Hybridization Data of 147 Samples

Probe groups	$S_{\max}/S_{2nd}^*$	$S_{\max}/S_{3rd}$	$S_{\max}/S_{4th}$	S_{pos}/S_{neg}
Subgroup A1	1.06 to 1.58	/	/	5.32 to 21.73
Subgroup A2	1.14 to 1.46	/	/	5.11 to 14.48
Group B	1.03 to 1.37	/	/	4.52 to 18.03
Group C	1.06 to 1.45	/	/	3.77 to 15.59
Group D	1.18 to 1.33	1.12 to 1.52	1.25 to 1.71	3.63 to 32.52
Group E	1.07 to 1.21	1.05 to 1.67	1.18 to 2.15	4.16 to 23.17
Group F	1.01 to 1.28	1.04 to 1.44	1.13 to 1.76	2.72 [†] to 11.20
Group G	1.03 to 1.56	1.04 to 1.73	1.06 to 1.82	3.31 to 15.82

* $S_{\max}/S_{2nd}$, the ratio of the highest to the second ranked signal in positive signals of the group; $S_{\max}/S_{3rd}$, the ratio of the highest to the third ranked signal in positive signals of the group; $S_{\max}/S_{4th}$, the ratio of the highest to the fourth ranked signal in positive signals of the group; S_{pos}/S_{neg} , the ratio of the lowest signal in positive signals of the group to the highest signal in negative signals of the group.

[†]The ratio of F03/F04 or F04/F03 is smaller than 3 in some cases.

ble, easy to update for newly described alleles, easy to perform, fast, and safe. It is therefore an attractive alternative to serological typing in routine medical practice for tissue typing, disease susceptibility studies, and forensic medicine.

In conclusion, this method has advantages over other HLA-DRB genotyping methods in sample throughput and speed. Another advantage of this approach is that it allows the use of only one PCR reaction and one hybridization to simultaneously genotype all HLA-DRB genes (HLA-DRB1, DRB3, DRB4, and DRB5). The main limitation of this method is its low resolution, which could be overcome by adding more probes. A similar microarray approach using more probes may yield more accurate and specific *HLA-DRB1/3/4/5* genotyping results in the future.

References

1. Pullen AM, Kappler JW, Marrack P: Tolerance to self antigens shapes the T-cell repertoire. *Immunol Rev* 1989, 107:125–139
2. Moya-Quiles MR, Torio A, Muro M, Montes-Ares O, Marin L, Minguela A, Sanchez-Bueno F, Garcia-Alonso AM, Parrilla P, Alvarez-Lopez MR: Impact of HLA-C on acute rejection in liver transplantation. *Transplant Proc* 2003, 35:1892–1893
3. Clift RA, Storb R: Histoincompatible bone marrow transplants in humans. *Annu Rev Immunol* 1987, 5:43–64
4. Nepom GT, Erlich HA: MHC Class-II molecules and autoimmunity. *Annu Rev Immunol* 1991, 9:493–525
5. Wu H, Khanna D, Park G, Gersuk V, Nepom GT, Wong WK, Paulus HE, Tsao BP: Interaction between RANKL and HLA-DRB1 genotypes may contribute to younger age at onset of seropositive rheumatoid arthritis in an inception cohort. *Arthritis Rheum* 2004, 50:3093–3103
6. Klitz W: Viruses, cancer and the MHC. *Nature* 1992, 356:17–18
7. Rios A, Rodriguez JM, Moya MR, Galindo PJ, Canteras M, Alvarez MR, Parrilla P: Frequency of HLA-C alleles in differentiated thyroid carcinoma in southeastern Spain. *Cancer* 2004, 100:264–269
8. Ota M, Seki T, Fukusima H, Tsuji K, Inoko H: HLA DRB1 genotyping by modified PCR-RFLP method combined with group-specific primers. *Tissue Antigens* 1992, 39:187–202
9. Tiercy JM, Morel C, Freidel AC, Zwahlen F, Gebuhrer L, Betuel H, Jeannet M, Mach B: Selection of unrelated donors for bone marrow transplantation is improved by HLA class II genotyping with oligonucleotide hybridization. *Proc Natl Acad Sci USA* 1991, 88:7121–7125
10. Buyse I, Decorte R, Baens M, Cuppens H, Semana G, Emonds MP, Marynen P, Cassiman JJ: Rapid DNA typing of class II HLA antigens using the PCR and reverse dot blot hybridization. *Tissue Antigens* 1993, 41:1–14
11. Knipper AJ, Hinney A, Schuch B, Enczmann J, Uhrberg M, Wernet P: Selection of unrelated bone marrow donors by PCR-SSP typing and subsequent nonradioactive sequence-based typing for HLA DRB1/3/4/5, DQB1, and DPB1 alleles. *Tissue Antigens* 1994, 44:275–284
12. Sylvain K, Aurelie H, Marc M, Christophe R: Rapid screening for HLA-B27 by a TaqMan-PCR assay using sequence-specific primers and a minor groove binder probe, a novel type of TaqMan trade mark probe. *J Immunol Methods* 2004, 287:179–186
13. Zhen G, Mark SG, Lee H, John AH, Effie WP: Oligonucleotide array for high-throughput SNPs detection in the MHC Class I genes: hLA-B as a model system. *Genome Res* 2001, 12:447–257
14. Consolandi C, Frosini A, Pera C, Ferrara GB, Bordoni R, Castiglioni B, Rizzi E, Mezzelani A, Bernardi LR, De Bellis G, Battaglia C: Polymorphism analysis within the HLA-A locus by universal oligonucleotide array. *Hum Mutat* 2004, 24:428–434
15. Jaakkola E, Herzberg I, Crane AM, Pointon JJ, Laiho K, Kauppi M, Kaarela K, Wordsworth BP, Tuomilehto J, Brown MA: A novel human leucocyte antigen-DRB1 genotyping method based on multiplex primer extension reactions. *Tissue Antigens* 2004, 64:88–95
16. Kotsch K, Wehling J, Blasczyk R: Sequencing of HLA class II genes based on the conserved diversity of the non-coding regions: sequencing based typing of HLA-DRB genes. *Tissue Antigens* 1999, 53:486–497
17. Sayer D, Whidborne R, Brestovac B, Trimboli F, Witt C, Christiansen F: HLA-DRB1 DNA sequencing based typing: an approach suitable for high throughput typing including unrelated bone marrow registry donors. *Tissue Antigens* 2001, 57:46–54
18. Stephen JS, Robert LG, Henry AE: Rapid typing of DNA sequence polymorphism at the HLA-DRB1 locus using the PCR and nonradioactive oligonucleotide probes. *Human Immunol* 1991, 30:190–201