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High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR–SSOP–Luminex method in the Japanese population

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Abstract We have developed a new high-throughput, high-resolution genotyping method for the detection of alleles at the human leukocyte antigen (HLA)-A, -B, -C, and -DRB1 loci by combining polymerase chain reaction (PCR) and sequence-specific oligonucleotide probes (SSOPs) protocols with the Luminex 100 xMAP flow cytometry dual-laser system to quantitate fluorescently labeled oligonucleotides attached to color-coded microbeads. In order to detect the HLA alleles with a frequency of more than 0.1% in the Japanese population, we created 48 oligonucleotide probes for the HLA-A locus, 61 for HLA-B, 34 for HLA-C, and 51 for HLA-DRB1. The accuracy of the PCR–SSOP–Luminex method was determined by comparing it to the nucleotide sequencing method after subcloning into the plasmid vector using 150 multinational control samples obtained from the International HLA DNA Exchange University of California Los Angeles. In addition, we performed the PCR–SSOP–Luminex method for HLA allele typing on DNA samples collected from 1,018 Japanese volunteers. Overall, the genotyping method exhibited an accuracy of 85.91% for HLA-A, 85.03% for HLA-B, 97.32% for HLA-C, and 90.67% for HLA-DRB1 using 150 control samples, and 100% for HLA-A and -C, 99.90% for HLA-B, and 99.95% for HLA-DRB1 in 1,018 Japanese samples. The PCR–SSOP–Luminex method provides a simple, accurate, and rapid approach toward multiplex genotyping of HLA alleles to the four-digit or higher level of resolution in the Japanese population. It takes only approximately 5 h from DNA extraction to the definition of HLA four-digit alleles at the HLA-A, HLA-B, HLA-C, and HLA-DRB1 loci for 96 samples when handled by a single typist.

Keywords HLA genotyping · PCR · SSOP · Luminex method · Japanese

Abbreviations HLA: Human leukocyte antigen · MHC: Major histocompatibility complex · PCR: Polymerase chain reaction · SSCP: Single-strand conformational polymorphism · RFLP: Restriction fragment length polymorphism · SSP: Sequence-specific primer · SSOP: Sequence-specific oligonucleotide probe · UCLA: University of California Los Angeles · SA–PE: Streptavidin–phycoerythrin · EDC: Ethylene dichloride · MFI: Median fluorescence intensity · IC: Internal control

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Introduction

The human leukocyte antigen (HLA) complex, also called the major histocompatibility complex (MHC), is a genetic system that plays a key role in the recognition of self and

nonself in immune surveillance. Consequently, HLA typing of the highly polymorphic classical class I and class II genes within the HLA complex has become an indispensable laboratory procedure for determining and matching donor–recipient HLA compatibility in patients undergoing bone marrow or organ transplantations (Calmus 2004; Hurley et al. 2003; Kamoun and Sellers 2004; Matinlauri et al. 2004), as well as assisting with disease association studies (Mizuki et al. 1992a,b; Pugliese et al. 1994; Wooley et al. 1981), anthropological studies of genetic relationships within and between populations (Comas et al. 1998; Hawkins et al. 1988), and in the identification of individuals in paternity testing, forensics, and in legal medicine (Comey et al. 1993).

Historically, HLA typing was first performed by serological typing of antigens using antiserum (Claas et al. 1985; Kojima et al. 2001), but this procedure is now considered to lack the resolving power of DNA methods and to be rather cumbersome and resource-intensive because it requires different kinds of antiserum to be obtained by screening a large number of multipara bloods. The HLA genotyping methods that were developed during the past 25 years have provided more reliable results from preserved and nonpreserved cellular and tissue samples to which serological typing cannot be easily applied, as well as a more detailed classification system based on DNA sequence variations. The polymerase chain reaction (PCR) assay, developed by Saiki et al. in 1985 to amplify a segment of the targeted DNA by a million times or more (Saiki et al. 1985), was a major breakthrough technology not only for HLA genotyping but also for DNA diagnosis of inherited and infectious diseases, cancer, and for many other applications in clinical practice. The DNA-based HLA typing method using PCR-amplified DNA is now a common laboratory procedure, and a number of different HLA genotyping methods are available to detect the HLA alleles from the amplified DNA, such as by direct sequencing (Mizuki et al. 2001; Ramon et al. 2003; Santamaria et al. 1993), single-strand conformational polymorphisms (SSCPs) (Bannai et al. 1996; Huang et al. 2004; Teraoka et al. 2000), restriction fragment length polymorphisms (RFLPs) (Mizuki et al. 1992a,b; Olerup 1990; Ota et al. 1992), sequence-specific primers (SSPs) (Ando et al. 1996; Zetterquist and Olerup 1992), and direct or reverse sequence-specific oligonucleotide probes (SSOPs) (Levine and Yang 1994; Mizuki et al. 1992a,b) employing either labeled enzymes and colorimetric substrates or fluorometric labels. However, all these methods have their limitations in one way or another, such as lack of speed, low throughput of sample number, high cost, low specificity, low sensitivity, or a poor level of allelic resolution. For example, although SSOPs for the identification of HLA classical class I and class II alleles present in PCR-amplified samples have low resolution (two digits) and are time consuming, they are relatively economical and potentially easy to perform.

Recently, PCR and the Luminex microbead system for the simultaneous multiplex assay of amplicons hybridized to SSOP in a single detection solution have been described or sold commercially for high-throughput single nucleotide polymorphism (SNP) typing of HLA, disease genes, or detection of microorganisms (Diaz and Fell 2004; Dunbar et al. 2003; Ye et al. 2001). Here, we describe in detail the multilocus HLA genotyping method that we developed by combining the PCR amplification–SSOP protocol with Luminex technology to enable a high-throughput assay of HLA-A, -B, -C, and -DRB1 alleles with a high resolution (up to four digits or more in most cases) in the Japanese population. This is the first article to describe the verification of typing accuracy for the PCR–SSOP–Luminex method and HLA allele typing to the four-digit level of allele resolution in 1,018 DNA samples of the Japanese population by correlating the genotyped outputs to a Japanese high-resolution allele database.

Materials and methods

Materials

Genomic DNAs for 150 control multinational samples of known HLA genotypes were obtained from the University of California Los Angeles (UCLA) International HLA DNA Exchange (Los Angeles, CA, USA) to verify the accuracy of the PCR–SSOP–Luminex method. Peripheral blood cell samples were collected from 1,018 unrelated Japanese healthy volunteers with their informed consent, and ethics approval was obtained from the Ethics Committee of Tokai University Medical School.

Methods

DNA sample preparation

Genomic DNAs were extracted and purified from the 1,018 Japanese normal volunteers using QIAamp blood kit (Qiagen Inc., Chatsworth, CA, USA). The purity of the genomic DNA for each sample was determined by measuring the absorbance at 260 and 280 nm, with the A260/A280 values being in the range of 1.5–1.9, and the concentration of the DNA was adjusted to 10–20 ng/μl.

Color-coded microbeads

Different sets of polystyrene color-coded microbeads (Multi-Analyte Microsphere Carboxylated; Luminex, Austin, TX, USA), approximately 5.5 um in diameter, were used for labeling with oligonucleotide annealing probes as the fundamental component of the xMAP technology. Each set of beads was prepared and color-coded by the manufacturer with a particular ratio of two different fluorescent dyes (red and infrared) embedded within the beads (Dunbar et al. 2003). By adjusting the concentrations of each fluorochrome, up to 100 different fluorescently labeled microbeads can be coded and identified by the Luminex 100 flow cytometer. One of the dual lasers equipped on Luminex

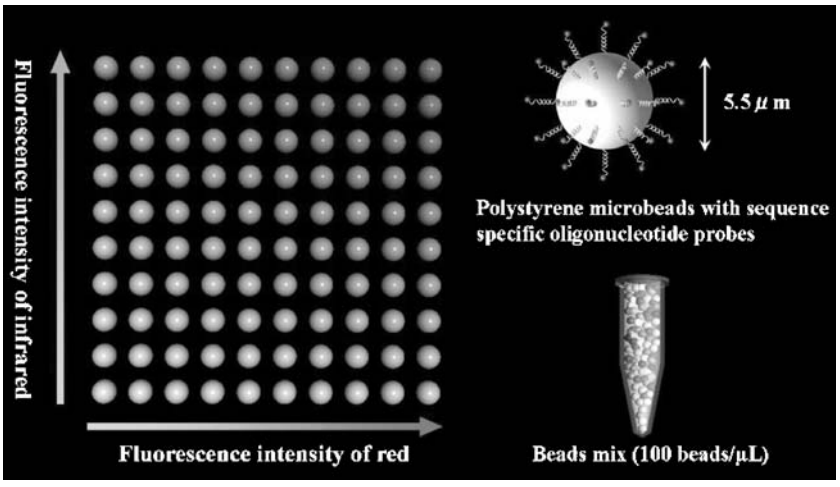

Fig. 1 Microbead structure, characteristics, and detection by Luminex 100 flow cytometer. By adjusting the concentrations of the red and infrared fluorochromes within the individual beads, up to 100 spectrally coded oligobead sets can be identified. Sequence-specific oligonucleotide probes were covalently bound to carboxylated microbeads (oligobeads) using EDC. An oligobead mix was prepared by mixing the oligonucleotide-labeled microbeads (oligobeads) to a final concentration of 100 beads/μl for hybridization

100 excites the internal dyes embedded within the beads and identifies the exact code number of the fluorescent microbeads by determining the preset ratio of internalized dyes (Fig. 1).

PCR–SSOP–Luminex method

The overall method for HLA genotyping by the PCR–SSOP–Luminex method, involving PCR amplification, hybridization, streptavidin–phycoerythrin (SA–PE) reaction, and the measurement of the analytes, is summarized in Fig. 2.

PCR amplification

We designed 9 primers for the HLA-A locus, 12 for the HLA-B locus, and 7 for the HLA-C locus to perform gene amplification by PCR and amplified exon 2 and exon 3

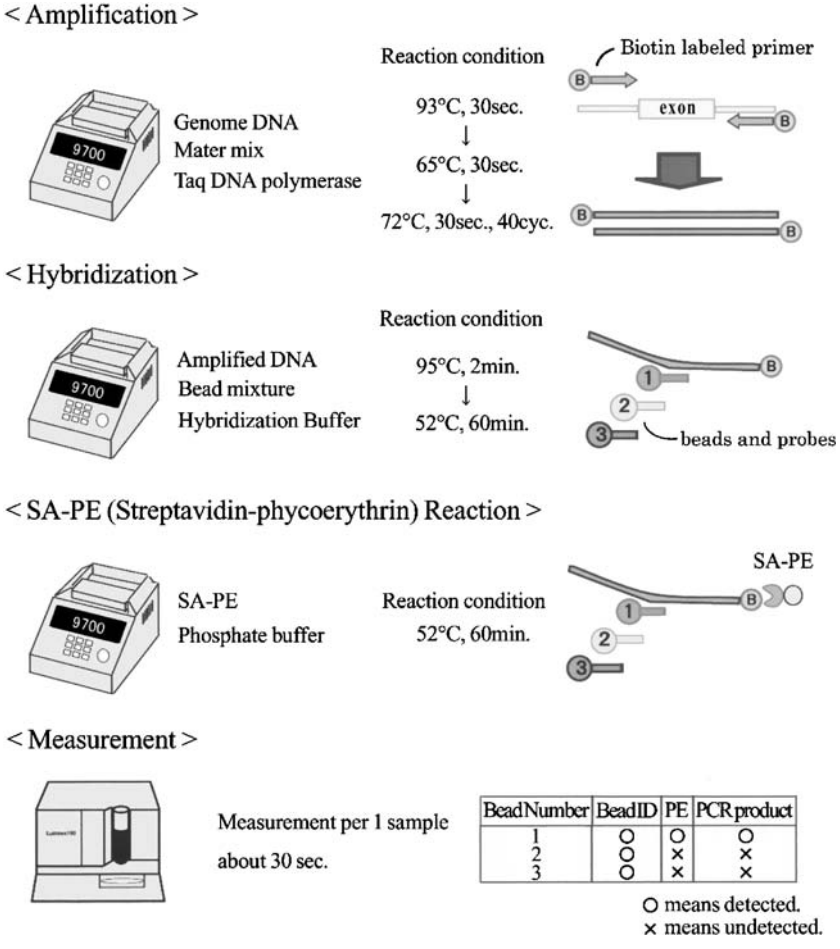

Fig. 2 Schematic summary of the PCR–SSOP–Luminex method. *Amplification*: target DNA is PCR-amplified using 5′-biotin-labeled primers that are highly specific to certain sequences of HLA genes. *Hybridization*: after denaturization at 95°C, amplified DNA is allowed to hybridize to complementary DNA probes coupled to microbeads. *SA–PE reaction*: the hybridized PCR product on the oligobeads is labeled with SA–PE. *Measurement*: Luminex apparatus identifies the fluorescent intensity of PE on each coded oligobead that has hybridized with the biotin-labeled PCR product. Genosearch typing software assists in determining the HLA genotype (alleles) of the sample DNA

regions using these primers (Table 1). Two types of amplification systems were required for the assay of the HLA-DRB1 locus, with five primers for the primer set 1 (PS1) and nine primers for the primer set 2 (PS2) to amplify the exon 2 region (Table 1). These PCR primers were biotinylated at their 5′-ends. The amplicon sizes were 400–500 bp (exon 2) and 300–400 bp (exon 3) for the HLA-A, -B, and -C loci and 250–300 bp (exon 2) for the HLA-DRB1 locus. As the first part of the PCR–SSOP–Luminex method shown in Fig. 2, amplification reactions were carried out in 1× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3) with 1.5 mM $MgCl_2$, 2% dimethyl sulfoxide (DMSO), 0.2 mM dNTPs, and then 0.1–1.0 μM primers. *Taq* DNA polymerase (50 U/ml) and 50–100 ng of genomic DNA were added to a final total volume of 25 μl. PCR reactions were performed for 40 cycles, with denaturation at 93°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s using a GeneAmp9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Preparation and hybridization of oligonucleotide probes

For this study, we designed 48 SSOPs for HLA-A, 61 for HLA-B, 34 for HLA-C, and 51 for HLA-DRB1 on the basis that the detection of these sequence (allele) frequencies was ≥0.1% in the Japanese population. Table 2 shows all of the oligonucleotide probe sequences. These synthesized oligonucleotide probes were modified at the 5′-end with a terminal amino group and covalently bound to the carboxylated fluorescent microbeads using ethylene dichloride (EDC), following the recommended procedures by Luminex Corporation (Fulton et al. 1997). The oligonucleotide-labeled microbeads (oligobeads) were mixed together to make an oligobead mixture of 100 oligobeads/μl for hybridization. The 5′-biotin-labeled PCR amplicons were hybridized to the oligobeads in a total volume of 50 μl per well in a 96-well plate by adding 5 μl of the appropriate oligobead mixture and 5 μl of the PCR amplicon to 40 μl of hybridization buffer [3.75 M TMAC, 62.5 mM TB (pH 8.0), 0.5 mM EDTA, 0.125% *N*-lauroylsarcosine]. This reaction mixture was first denatured at 95°C for 2 min and then hybridized at 52°C for 60 min using the GeneAmp9700 thermal cycler.

SA–PE reaction and measurement of analytes

After hybridization, the oligobeads were washed by adding 100 μl of PBS–Tween [1× PBS (pH 7.5), 0.01% Tween-20] to the hybridization solution in each well and then pelleted by microcentrifugation at 1,000×*g* (3,300 rpm) for 5 min using a centrifuge equipped with a swing-out microwell plate rotor. The supernatant was carefully removed and discarded, and the pelleted oligobeads were reacted with a 70-μl aliquot of a 100×-diluted solution of SA–PE (G&G Science Co., Ltd., Fukushima, Japan) in PBS–Tween. After careful mixing with a pipette, the hybridized amplicons were labeled with SA–PE at 52°C for 5 min using the GeneAmp9700 thermal cycler. Reaction outcomes were measured by the Luminex 100 flow cytometer that is equipped with two types of lasers. The bead populations were detected and identified using the 635-nm laser. The PE fluorescence of the SA–PE–biotin labeled amplicons that had hybridized to the oligobeads was quantitated using the 532-nm laser. The median fluorescence intensity (MFI) of PE was used to quantify the amount of DNA bound to the oligobeads. The measured data were read using dedicated software (Genosearch HLA typing software) obtained from the G&G Science Co., Ltd. The fluorescence intensity of negative controls was subtracted as background from each of the MFI values to determine the true intensity. The preset cutoff value for each fluorescing oligobead set was used to discriminate between positive and negative controls. We matched the SSOP hybridization results with the Pattern File database using Genosearch HLA typing software to determine the HLA alleles. To obtain high-resolution alleles (four digits or more), we used the Japanese High-resolution Pattern File (JH Pattern File) database, which correlates the frequency and haplotype pattern with all the known allele frequencies that are ≥0.1% in the Japanese population and the major alleles in countries other than Japan. The small numbers of ambiguous samples were redetermined using the Full Type Pattern File (FT Pattern File) that corresponded to all existing alleles. However, the FT Pattern File often resulted in low-resolution allele typing (two-digit alleles) because of ambiguities due to the large number of different alleles in the database. An example of the probe-determination patterns obtained for some allele types is presented in Table 3.

Statistical analysis

Allele frequencies were estimated by direct counting. The significance of the distribution of alleles between the result for the PCR–SSOP–Luminex method and the Japanese allele frequencies previously reported (Hashimoto et al. 1994; Saito et al. 2000; Tanaka et al. 1996, 1999; Tokunaga et al. 1997) was analyzed by Fisher's exact probability test. Furthermore, the *P* value was corrected by multiplication by the number of HLA alleles (corrected *P* value, P_c value). A P_c value of less than 0.05 was evaluated as statistically significant.

Results

Verification of typing accuracy for the PCR–SSOP–Luminex method

One hundred and fifty genomic DNA control samples of multiethnic origins (Caucasian, 32.7%; Hispanic, 20.0%; Black, 6.7%; Asian, 4.7%; Oriental, 4.0%; Filipino, 2.0%; Vietnamese, 2.0%; American Indian, 1.3%; Korean, 1.3%; Indian, 0.7%; Japanese, 0.7%; Latin American, 0.7%; un-

Table 1 Sequence for PCR primers

HLA-A exon 2 primer set	
A ex2-1 F	AAACCGCCTCTGCGGGGAGAAGCAA
A ex2-2 F	AAACGGCCTCTGTGGGGAGAAGCAA
A ex2-3 F	AAACGGCCTCTGCGGGGAGAAGCAA
A ex2-1 R	GATCTCGGACCCGGAGACTGT
HLA-A exon 3 primer set	
A ex3-1 F	GGGCTGACCGCGGGGT
A ex3-2 F	CGGGCTGACCTCGGGGT
A ex3-3 F	CTGGGCTGACCGTGGGGT
A ex3-1 R	GTGGCCCCTGGTACCCGT
A ex3-2 R	GTGGCCTCTGGTACCCGT
HLA-B exon 2 primer set	
B ex2-1 F	GGGCGGGCAGGAGAGAGGGGACCGCAG
B ex2-2 F	GGGCGGGCAGGAGCAAGGGGACCGCAG
B ex2-3 F	GGGCGGGCAGGAGCGAGGGGACCGCAG
B ex2-1 R	GATCTCGGACCCGGAGACTCG
B ex2-2 R	CGATCTCGGACCTAGAGACTCG
HLA-B exon 3 primer set	
B ex3-1 F	CGGGGCTGACCGCGGGGC
B ex3-2 F	CGGTGCTGACCGCGGGGC
B ex3-3 F	CTGGGCTGACCGCGGGGC
B ex3-4 F	CGGGGCTGACCGCGGGGG
B ex3-5 F	TGGGCTGACCGCGGGGG
B ex3-1 R	AGGCCATCCCCGCCGACCTAT
B ex3-2 R	AGGCCATCCCCGGCGACCTAT
HLA-C exon 2 primer set	
C ex2-1 F	GAGGTGCCCGCCCGGCGA
C ex2-2 F	GAGGGGCCCGCCCGGCGA
C ex2-3 F	GAGGGGCCCTCCCGGCGA
C ex2-1 R	GGGTCTGGGCGGGTTCCGCA
HLA-C exon 3 primer set	
C ex3-1 F	TCGACCGGAGAGAGCCCCAGT
C ex3-2 F	TCGACCGGGGAGAGCCCCAGT
C ex3-1 R	GCTGATCCCATTTTCCTCCCCTCCTC
HLA-DRB1-PS1 primer set	
DR PS1-1F	GTTCGTGTCCCCACAGCACGT
DR PS1-1R	CTGCACTGTGAAGCTGTCACC
DR PS1-2R	CTGCACCGTGAAGCTGTCACC
HLA-DRB1-PS2 primer set	
DR PS2-1 F	CCCACAGCACGTTTCTTGGAGTACTC
DR PS2-2 F	CAGCACGTTTCTTGGAGCAGGTTAAACA
DR PS2-3 F	GCACGTTTCCTGTGGCAGCCTAAGAGG
DR PS2-4 F	GCACGTTTCTTGTGGCAGCTTAAGTT
DR PS2-5 F	GCACGTTTCTTGTGGCAGCTAAAGTT
DR PS2-1 R	CGCTGCACTGTGAAGCTTTCCA
DR PS2-2 R	GCTGCACTGTGAAGCTTTCCA
HLA-DRB1 IC primer set[a]	
IC F	TTGGCCAATCTACTCCCAGGAGCAG
IC R	GGTAGACCACCAGCAGCCTAAGGGT

[a]Internal control (IC) primer set was added to PS1 and PS2 primer sets

known, 23.3%) with known HLA genotypes were obtained from the UCLA HLA DNA Exchange and analyzed using the PCR–SSOP–Luminex method. One hundred and forty nine samples for HLA-A, 147 samples for HLA-B, 149 samples for HLA-C, and 150 samples for HLA-DRB1, excluding defective samples for the PCR amplification, were analyzed by the JH Pattern File. The typing accuracy of the PCR–SSOP–Luminex method was verified to be

Table 2 Sequence for oligonucleotide probes

HLA-A probe set		HLA-C probe set	
A01	GGATGTGAAGAAATACCTCATG	C01	CACGGATGTGAAGAAATACTT
A02	GGAGGTGTAGAAATACCTCATGGAA	C02	GGACACAGCGGTGTAGAAATACC
A03	GGATGTGGAGAAATACCTCATG	C03	CACGGATGTGGAGAAATACCT
A04	TGTGGTGAAATACCTCATGGAG	C04	CCGGGACACGGCGGTGTAGAAATAC
A05	TGGAGAAATACCTCATGGATTG	C05	CACGGCGGTGTCGAAATACCTCA
A06	GGAAGTGTAGAAATACCTCATG	C06	GAGCCCCACTTCATCGCAG
A07	AAGCGGGGCTTCCCGCGGCCGG	C07	CACTGCGATGAAGCGGGGC
A08	AAGCGGGGCTCCCCGCGGCCGG	C08	GAGACACAGAAGTACAAG
A09	GAAGCGGGGCTCTCCACTGCC	C09	GAGACACAGAACTACAAGC
A10	GACCAGGAGACACGGAATGTG	C10	GTGAGCCTGCGGAACCTG
A11	TTGGGACCGGAACACACGGAA	C11	GTGAACCTGCGGAAACTGC
A12	TGGGACGGGGAGACACGGAAA	C12	GAGGCCGGTGAGTGACCC
A13	GACGAGGAGACAGGGAAAGT	C13	AGCGAGGCCAGTGAGTGA
A14	TATTGGGACCTGCAGACACGG	C14	GTAGGCGTACTGGTCATACC
A15	CAGACTCACCGAGTGGACCT	C15	GTAGGCGAACTGGTTATACC
A16	CAGACTGACCGAGTGGACCT	C16	GTCGTAGGCGGACTGGTCAT
A17	CAGATTGACCGAGTGGACC	C17	TAGGCTAACTGGTCATGCCC
A18	CAGACTGACCGAGCGAACCTG	C18	TAGGCGAACTGGTCATGC
A19	CAGACTGACCGAGAGAACC	C19	TCCTGGACCGCTGCGGACAC
A20	GACCGAGAGAGCCTGCGGATC	C20	CGCTCCTGGACTGCCGCGGA
A21	CTGCGGATCGCGCTCCGCTAC	C21	CCGCCGCGGACAAGGCGGCT
A22	GTCGCAGCCACACATCCTCTGG	C22	GAGGCGGCCCGTGCGGCGG
A23	TCGCAGCCATACATCCTCTGGA	C23	GGAGCAGCGGAGAGTCTACC
A24	GTCGCAGCCAAACATCCTCTG	C24	GAGCAGTGGAGAGCCTACC
A25	CGTAAGCGTTCTGCTGGTAC	C25	GAGCAGCTGAAAGCCTACCTGG
A26	CGTAAGCGTCCTGCTGGTAC	C26	AGCAGCAGAGAGCCTACCTG
A27	GGACAAGGCAGCTCAGACCAC	C27	GAGCAGGACAGAGCCTACCT
A28	TGGGAGACGGCCCATGAGG	C28	CTGGAGGGCACGTGCGTGGA
A29	GAGGCGGCCCGTGTGGCG	C29	CTGGAGGGCGAGTGCGTG
A30	GAGGCGGCCCATGTGGCG	C30	CTGGAGGGCCTGTGCGTG
A31	GGAGCAGCGGAGAGTCTACC	C31	AGAACGGGAAGAAGACG
A32	GCAGCAGAGAGCCTACCTG	C32	GAGAACAGGAAGAAGACG
A33	GGAGCAGTTGAGAGCCTA	CP2	GCGCGGCTACTACAACCAG
A34	GGAGCAGTGGAGAGCCT	CP3	GTGCGTGGAGTGGCTCCGCA
A35	AGAGCCTACCTGGATGGC		
A36	CTGGAGGGCGAGTGCGTGG	HLA-DRB1 probe set	
A37	CTGGAGGGCCGGTGCGTGGA	D01	AATGACATTCAAACTTA
A38	GTGCGTGGACGGGCTCCGCA	D02	ATGACACTCATGTTTAAC
A39	GTGCGTGGAGTGGCTCCGCA	D03	TGACACTTATACTTACCCT
A40	TGGAGAAATACCTCATGGAGT	D04	ATAACACTCACCCGTAGA
A41	CAGGAGAGTCCGGAGTATT	D05	CACAAACTTATCCTGCTT
A42	GAGCAGGAGGGTCCGGAG	D06	CACAAACTTAACCTCCTC
A43	GTAAGCGTCCCGCTGGTAC	D07	ACACTCAGACGTAGAGTA
A44	GGTGGTCTGAGCTGCCATGTC	D08	AAATGACACTCCCTCTT
A45	CTGGAGGGCACGTGCGTGGA	D09	CTTGGTTATAGAAGTATCTGT
A46	CTGCGCGGCTAGTACAA	D10	CACGTTCTCCTCCTGGTTATGG
AP2	GCTACTACAACCAGAGCGAG	D11	GAAGCGCACGAACTCCTCCTGGT
AP3	GGAAGGAGACGCTGCAGCGC	D12	CACCGCCCGGAACTCCCCC
		D13	CGGCCTGATGCCGAGTAC
HLA-B probe set		D14	GGGCGGCCTAGCGCCGAGTAC
B01	CATGGCGGTGTAGAAATACCTCA	D15	CGGCCTGATGAGGAGTACT
B02	CACGGAGGTGTGGAAATACCTCA	D16	CGGCCTGCTGCGGAGCACTGG
B03	CACGGAGGTGTAGAAATACCTCA	D17	TCCTGGAAGACGAGCGG
B04	CATGGCGGTGTGGAAATACCTC	D18	GAAGGACCTCCTGGAAGACAG

Table 2 (continued)

HLA-A probe set		HLA-C probe set	
B05	CATGGCGGTGTCGAAATACCTC	D19	AGCAGAGGCGGGCCGCGGTGGA
B06	TAGCCCACGGTGATGAAGCG	D20	CAGAAGCGGACCGCGGTGGAC
B07	CAGTTCGTGAGGTTCGACA	D21	AGGCGGGCCGAGGTGGACA
B08	CGAGTCCGAGGATGGCGCCCCGGGCG	D22	AGGCGGGCCCTGGTGGAC
B09	TTGAGGACGGAGCCCCGGGCGC	D23	ATTGAAGAATTGACACTCAGA
B10	CTCCTGCTCCACCCACGGCGCC	D24	CTCCTCTTGGTGATAGAAGTATC
B11	GAGGGGCCGGAGTATTGGG	D25	CACGTTCTCCTCCTGGTTATG
B12	GAGGGGCCGGAATATTGGGAC	D26	GAAGCGCTCGTACTCCTCTTG
B13	GACCGGGAGATACAGATC	D27	GTCGAAGCGCACGGACTCCT
B14	GGGACCGGGAGACACAGA	D28	CCTGTCGCCGAGTCCTGGAAC
B15	CACAGAAGTACAAGCGCCAGG	D29	CGGCCTGATGCCGAGTAC
B16	ATCTACAAGGCCCAGGCA	D30	GGGCGGCCTAGCGCCGAGTAC
B17	ATCTGCAAGGCCAAGGCA	D31	CGGCCTGCTGCGGAGCACTGG
B18	GATCTTCAAGACCAACA	D32	CCTGACGCTGAGTACTGGAGG
B19	CACAGATCTCCAAGACCA	D33	GAAGGACATCCTGGAAGAC
B20	CACGGAACATGAAGGCC	D34	AGAAGGACTTCCTGGAAGAC
B21	CAGATCTGCAAGACCAAC	D35	GAAGGACCTCCTGGAAGACAG
B22	CAGATCTACAAGACCAA	D36	CTCCTGGAACAGAGGCGGGCC
B23	ACTGACCGAGAGAGCC	D37	TCCTGGAAGACGAGCGG
B24	CGAGAGGACCTGCGGACCCTG	D38	AAGCGGGGCCGGGTGGACA
B25	CGAGAGAACCTGCGGATCGCG	D39	AGGCGGGCCGAGGTGGACACCT
B26	CGAGAGAACCTGCGCACCGC	D40	GAGCAGAGGCGGGCCGCGGTG
B27	GCGCGGCTACTACAAC	D41	AGGCGGGCCCTGGTGGACA
B28	GCTCCGCTACTACAACC	D42	GGGAGTACCGGGCGGTGACGGAGCT
B29	TCGTCTGCCAAGTGTGAGAC	D43	GGCCGCGGTGGACACCTA
B30	TCCTCTGCCAAGTGTGAGAC	D44	CACAACTACGGGGCTGTG
B31	CATCCTCTGGATGATGTGAGA	D45	ACAACTACGGGGTTGTGG
B32	GCCGTACATCCTCTGGAGGGT	DB3	GGGCGGTGAGGGAGCTGGG
B33	GCCGTACATGCTCTGGAGGG	DB5	CTCTGTGCAGGAACCGCAC
B34	TAGGCGTACTGGTCATGCCC	DP1	TGGAACAGCCAGAAGGAC
B35	CTGGTTATGCCCGCGGAGGA	DP2	TGGAACAGCCAGAAGGAC
B36	GCGAACTGGTTATACCCGC	DIC1	GCCCAGCCCTGGCTCCT
B37	CGTCGTAGGCTAACTGGTTAT	DIC2	GCCCAGCCCTGGCTCCT
B38	ATGACCAGTCCGCCTACGAC		
B39	ATCACCCGGCGCAAGTGGGA		
B40	GAGCAGGACAGAGCCTACCT		
B41	CAGCTGAGAGCCTACCTGG		
B42	GCTGAGAACCTACCTGGAG		
B43	GAGCAGTGGAGAGCCTACC		
B44	CTGGAGGGCCTGTGCGTG		
B45	CCTGGAGGGCGAGTGCGTG		
B46	TACCTGGAGGGCACGTGC		
B47	CTCCGCAGATACCTGGAG		
B48	CTCCGCAGACACCTGGAG		
B49	AAGGACAAGCTGGAGCGC		
B50	GCCAAACATCCTCTGGAGGGT		
B51	GCCTTACATCCTCTGGAGGGT		
B52	TGGGACCGGAACACACAG		
B53	GGAGCAGCGGAGAGTCTACC		
B54	ATCTCCCAGCGCAAGTTGGAG		
B55	ACTGACCGAGTGGGCCTG		

Table 2 (continued)

HLA-A probe set		HLA-C probe set
B56	CCGGGACACGGCGGTGTAGAAAT	
B57	CCGAGGAAGGAGCCGCGGGCG	
B58	ACTTACCGAGAGAGCC	
B59	TGCGTGGAGGGGCTCCGCAG	
BP2	TACTACAACCAGAGCGAGGCC	
BP3	ACCTGGAGAACGGGAAGGA	

high using 128 samples for HLA-A (85.91%), 125 samples for HLA-B (85.03%), 145 samples for HLA-C (97.32%), and 136 samples for HLA-DRB1 (90.67%) when compared to the HLA genotype information provided by UCLA for each of their samples and the sequence data that we obtained for these samples by the nucleotide sequencing method after subcloning into the plasmid vector. The samples that were not determined accurately with the JH pattern file were redetermined using the FT pattern file. As a result, the genotypes were redetermined correctly for 21 samples at the HLA-A locus, 21 samples at the HLA-B locus, 2 samples at the HLA-C locus, and 14 samples at the HLA-DRB1 locus, and they were also confirmed to be the correct on the basis of the genotype information provided by UCLA for each sample and the sequence data that we obtained by sequencing each sample DNA. Therefore, the final analysis of the control DNA samples showed that the PCR–SSOP–Luminex method was accurate to the level of 100% (149/149) for HLA-A, 99.32% (146/147) for HLA-B, 98.66% (147/149) for HLA-C, and 100% (150/150) for HLA-DRB1. One sample in the analysis of HLA-B and two samples in the analysis of HLA-C were ambiguous. These ambiguous samples were revealed by DNA sequencing to represent new alleles. In fact, these three alleles, which were the most homologous to HLA-B*5301, -Cw*0401, and -Cw*1402 at the nucleotide sequence level, have recently got official names, i.e., HLA-B*5310, -Cw*040103, and -Cw*1406, respectively, by the World Health Organization (WHO) Nomenclature Committee for Factors of the HLA System.

We also performed allele typing using the PCR–SSOP–Luminex method on the HLA-A, HLA-B, HLA-C, and HLA-DRB1 loci of 1,018 Japanese DNA samples. The alleles were determined using the JH Pattern File. The results for allele typing of the HLA-A and -C loci were to the four-digit level in all samples, even when they were in heterozygous combinations, and no ambiguity was observed (Table 4). There were ambiguities for HLA-B in two samples and for HLA-DRB1 in one sample because their alleles had not been previously registered in the JH pattern file. These three ambiguous samples were redetermined using the FT Pattern File and were found by DNA sequencing to be the rare alleles HLA-B*1505, -B*3551 and -DRB1*1312. Thus, genotyping 1,018 Japanese DNA samples by the PCR–Luminex method revealed it was accurate to 100% (2036/2036) for HLA-A and -C, 99.90% (2034/2036) for HLA-B, and 99.95% (2035/2036) for HLA-DRB1.

HLA allele frequencies in Japanese population

Table 4 shows a summary of the HLA allele frequencies determined by the PCR–SSOP–Luminex method in 1,018 DNA samples in the Japanese population. Hardy–Weinberg equilibrium analyses of HLA polymorphisms in Table 4 revealed no deviation, which strongly suggests that this method is accurate, with a low rate of genotyping error. The analyses were performed at the four-digit or higher allele level, resulting in the identification of 21 alleles for HLA-A, 42 for HLA-B, 17 for HLA-C, and 32 for HLA-DRB1. HLA-A*2402 showed the highest allele frequency (36.20%) at the HLA-A locus. Alleles with the frequency of 5% or more included HLA-A*0201 (11.64%), -A*0206 (8.74%), -A*1101 (10.22%), -A*2601 (7.66%), -A*3101 (9.14%), and -A*3303 (6.68%). The highest allele frequency for HLA-B was HLA-B*5201 (10.90%). Alleles with the frequency of 5% or more included HLA-B*0702 (5.70%), -B*1501 (7.51%), -B*3501 (7.76%), -B*4001 (5.40%), -B*4002 (8.25%), -B*4006 (5.26%), -B*4403 (6.24%), -B*5101 (8.35%), and -B*5401 (8.79%). In comparison with the 42 alleles for HLA-B, only 17 alleles were detected for HLA-C, with HLA-Cw*0102 having the highest frequency (18.17%). Alleles with the frequency of 5% or more included HLA-Cw*0303 (13.02%), -Cw*0304 (12.97%), -Cw*0702 (12.67%), -Cw*0801 (8.15%), -Cw*1202 (11.00%), -Cw*1402 (6.53%), and -Cw*1403 (6.19%). The highest allele frequency for HLA-DRB1 was HLA-DRB1*0901 (14.88%), and the second highest was HLA-DRB1*0405 (14.05%). Alleles with the frequency of 5% or more included HLA-DRB1*0101 (5.60%), -DRB1*0803 (9.43%), -DRB1*1302 (6.43%), -DRB1*1501 (5.75%), and -DRB1*1502 (9.87%).

The allele frequencies obtained in our study were compared with those of the previous reports on the Japanese populations (Hashimoto et al. 1994; Saito et al. 2000; Tanaka et al. 1996, 1999; Tokunaga et al. 1997), and only HLA-A*2420 showed a significant difference in allele frequency (P_c=0.015).

Table 3 An example of the probe-determination patterns obtained for some allele types at the HLA-A, -B, -C, and -DRB1 gene loci

HLA-A

Sample No.	Type		A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24	A25
1	A*0201	A*2402	963	20	858	0	0	57	43	1260	26	6	7	1224	887	4	1714	0	5	11	1175	43	1203	0	544	8	0
2	A*2402	-	30	7	1055	0	17	47	40	1254	39	19	0	12	1849	1	15	0	10	12	1552	77	1506	13	10	11	0
3	A*2601	A*3101	9	801	0	1379	0	99	0	797	29	1778	1805	9	4	12	12	54	776	825	29	4	7	0	424	6	0
4	A*0206	A*2420	5	808	915	0	837	104	52	1170	24	0	4	1384	840	3	1760	0	7	16	1166	41	1171	0	541	10	0
5	A*1101	A*3303	9	825	22	1263	8	100	0	745	32	1668	1959	15	9	13	34	1284	874	10	13	15	11	12	8	0	0

Sample No.	Type		A26	A27	A28	A29	A30	A31	A32	A33	A34	A35	A36	A37	A38	A39	A40	A41	A42	A43	A44	A45	AP2	AP3
1	A*0201	A*2402	6	13	0	96	1137	20	1370	1164	0	8	22	13	1382	1032	11	1508	300	710	4	315	2143	2548
2	A*2402	-	0	1	0	117	1241	28	1707	0	12	8	23	17	1735	31	1	1226	349	78	5	3	2191	2674
3	A*2601	A*3101	787	0	625	1077	0	18	27	968	368	12	12	1452	37	1485	0	1014	9	5	19	3	2316	2473
4	A*0206	A*2420	5	11	2	123	1314	25	1425	1151	6	12	28	27	1357	1040	8	1547	0	774	11	380	2155	2839
5	A*1101	A*3303	27	0	1	1100	0	22	1182	1005	10	15	25	1423	44	1616	5	1087	1	23	0	43	2286	2745

HLA-B

Sample No.	Type		B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24	B25
1	B*5101	B*5201	911	0	2	0	4	9	1269	20	1003	8	460	0	0	1125	2	0	3	1578	1500	0	0	23	0	1	1758
2	B*5201	B*5401	1038	0	4	0	4	21	893	0	715	601	578	0	0	1309	0	1815	5	24	1351	0	3	21	1651	2	1491
3	B*4002	B*5601	820	1015	6	7	1	1550	821	0	0	13	545	7	0	1029	0	1501	1	22	779	2	9	8	1620	5	13
4	B*3501	B*5401	923	0	2	0	1	14	810	0	555	495	615	0	0	12	0	1630	1	668	5	0	0	4	1659	0	0
5	B*4403	B*5401	909	3	7	0	4	1332	187	1	0	611	489	1	0	755	0	1430	0	17	605	2	1	0	1311	0	12

Sample No.	Type		B26	B27	B28	B29	B30	B31	B32	B33	B34	B35	B36	B37	B38	B39	B40	B41	B42	B43	B44	B45	B46	B47	B48	B49	B50
1	B*5101	B*5201	12	24	1438	1536	0	2	0	0	2	779	12	8	0	20	0	1317	14	20	1687	19	25	0	1774	0	3
2	B*5201	B*5401	9	1197	1130	1579	0	6	3	0	5	1184	11	777	0	24	5	1289	25	17	1329	37	1676	996	1724	0	2
3	B*4002	B*5601	6	1364	14	1487	0	6	0	893	4	1394	12	1054	0	14	0	1142	25	19	1401	2094	25	1303	0	1	4
4	B*3501	B*5401	2	1620	6	1545	0	397	0	0	8	1014	17	899	595	30	0	1264	21	15	1357	15	1695	1308	0	2	8
5	B*4403	B*5401	809	900	517	1257	0	392	5	0	0	1002	7	849	0	17	0	1247	20	4	1246	0	1683	1287	0	0	7

Sample No.	Type		B51	B52	B53	B54	B55	B56	B57	B58	B59	B60	B61	BP2	BP3
1	B*5101	B*5201	21	49	35	662	0	4	14	2569	293	59	33	4622	1800
2	B*5201	B*5401	40	45	46	746	7	7	14	2170	180	58	39	4709	1938
3	B*4002	B*5601	27	39	20	807	0	4	833	2157	1143	81	51	4270	1864
4	B*3501	B*5401	34	46	43	495	7	11	16	2930	1215	82	49	4493	1929
5	B*4403	B*5401	31	30	37	443	21	5	651	2004	18	26	26	4172	1992

HLA-C

Sample No.	Type		C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25
1	Cw*1202	Cw*1502	0	823	2	472	0	464	421	643	925	1528	2253	1259	30	13	0	392	965	0	623	8	6	22	0	528	467
2	Cw*0102	Cw*1202	514	0	0	531	0	4	474	1468	3	2272	7	1082	4	815	1	347	4	0	574	4	8	9	236	451	0
3	Cw*0304	Cw*0401	0	923	1251	0	0	533	455	2066	9	1985	2519	759	0	809	1016	0	5	0	8	22	17	16	310	0	358
4	Cw*0102	Cw*0303	471	793	0	0	0	617	0	1886	4	2342	5	474	2729	1211	1	0	5	0	8	16	11	21	313	0	315
5	Cw*0102	Cw*1403	428	6	940	0	0	480	0	1931	11	2198	0	887	9	819	5	448	3	0	6	13	10	8	411	0	8

Sample No.	Type		C26	C27	C28	C29	C30	C31	C32	CP2	CP3
1	Cw*1202	Cw*1502	0	7	1025	12	3	20	9	1352	1700
2	Cw*0102	Cw*1202	0	2	937	13	4	28	9	1101	1791
3	Cw*0304	Cw*0401	0	1	725	9	898	16	8	1641	2117
4	Cw*0102	Cw*0303	0	2	690	5	878	16	11	1257	1921
5	Cw*0102	Cw*1403	0	1	994	15	1	22	13	1008	1741

HLA-DRB1

Sample No.	Type		D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24	D25
1	DRB1*1501	DRB1*1502	7	6	13	1	0	5	0	1462	523	9	0	297	3	0	7	8	5	12	0	24	37	7	0	9	8
2	DRB1*0405	DRB1*1502	8	814	2	0	2	1	6	1443	480	3	0	326	9	653	1	7	0	20	740	0	26	19	0	6	9
3	DRB1*0901	-	6	0	0	0	1008	6	9	3	0	2	1	14	6	4	2	2	4	14	2	0	894	10	0	1	8
4	DRB1*0405	DRB1*1101	11	716	5	0	1	5	890	6	494	3	2	189	900	679	692	3	5	19	780	0	0	32	0	8	14
5	DRB1*0901	DRB1*1302	14	8	4	4	890	0	1135	8	10	428	2	319	845	11	7	2	2268	16	0	0	582	29	0	4	9

Sample No.	Type		D26	D27	D28	D29	D30	D31	D32	D33	D34	D35	D36	D37	D38	D39	D40	D41	D42	DB3	DB5	DIC1	DIC2	DP1	DP2
1	DRB1*1501	DRB1*1502	7	469	5	2	0	0	1328	2	0	19	0	8	2	0	18	0	396	41	1330	1276	1013	2573	2493
2	DRB1*0405	DRB1*1502	0	0	0	0	2	0	22	7	0	2	0	3	2	0	11	0	415	47	1211	1319	950	2704	12
3	DRB1*0901	-	10	0	7	0	0	0	4	0	3	9	0	3	6	0	0	3	479	63	10	1462	981	1644	1
4	DRB1*0405	DRB1*1101	0	9	4	0	5	0	8	0	2	6	5	1	6	1	0	0	354	301	19	1461	1101	2662	5
5	DRB1*0901	DRB1*1302	4	0	5	6	4	2	8	0	0	7	0	8	0	0	3	3	414	94	20	1380	1116	2271	6

The sample numbers (1 to 5) are in the first column, the genotype (alleles) in the second column and the fluorescence intensity of the PE-labelled beads in the third column. The oligobead numbers are indicated for each of the rows in the third column. The rows labeled as P in the third column are PCR positive controls. The positive fluorescence intensities above the given cut-off values that together indicate the allelic pattern are shaded as a gray block.

Table 4 Allele frequencies for HLA-A, -B, -C, and -DRB1 gene loci in 1,018 Japanese individuals

Alleles	Count	Frequency (%)[a]	Frequency (%)[b]	P_c value	Alleles	Count	Frequency (%)[a]	Frequency (%)[b]	P_c value
HLA-A	*n*=2036				HLA-C				
A*0101	5	0.2	0.2–1.8		Cw*0102	370	18.2	14.8–17.0	
A*0201	237	11.6	10.9–11.5		Cw*0103	8	0.4	r–0.4	
A*0203	2	0.1	r		Cw*0302	12	0.6	r–0.4	
A*0206	178	8.7	7.7–10.4		Cw*0303	265	13	7.8–12.1	
A*0207	69	3.4	2.2–4		Cw*0304	264	13	11.3–13.7	
A*0210	9	0.4	r–0.7		Cw*0401	88	4.3	4.6–6.5	
A*0218	1	0.05	r–0.1		Cw*0501	6	0.3	r–0.4	
A*0301	5	0.2	r–0.8		Cw*0602	14	0.7	1.6–6.2	
A*0302	2	0.1	r–0.1		Cw*0702	258	12.7	11.3–14.6	
A*1101	208	10.2	8.1–11.1		Cw*0704	23	1.1	0.9	
A*1102	5	0.2	r–0.1		Cw*0801	166	8.2	7.4–10.9	
A*2402	737	36.2	32.7–37.9		Cw*0803	24	1.2	2.0–2.6	
A*2404	3	0.1	r–0.1		Cw*1202	224	11	10.4–10.5	
A*2420	17	0.8	r	0.015	Cw*1203	3	0.1	r	
A*2601	156	7.7	6.2–9.8		Cw*1402	133	6.5	4.9–5.7	
A*2602	47	2.3	1.3–2.3		Cw*1403	126	6.2	8.9–12.2	
A*2603	30	1.5	1.3–2.4		Cw*1502	52	2.6	1.7–2.7	
A*2605	1	0.05	r–0.1						
A*3001	2	0.1	0.1–0.4		HLA-B				
A*3101	186	9.1	7.1–9.3		B*0702	116	5.7	5.2–6.5	
A*3303	136	6.7	7.9–12.8		B*1301	26	1.3	0.9–1.5	
					B*1302	5	0.2	0.1–0.4	
HLA-DRB1					B*1501	153	7.5	6.5–8.7	
DRB1*0101	114	5.6	4.8–6.5		B*1502	1	0.05	r–0.1	
DRB1*0301	1	0.05	0.2–0.4		B*1505	1	0.05	NT	
DRB1*0401	26	1.3	0.7–1.6		B*1507	9	0.4	r–0.7	
DRB1*0403	61	3	1.9–4.0		B*1511	17	0.8	0.4–1.0	
DRB1*0404	7	0.3	0.1–0.2		B*1518	35	1.7	0.9–1.72	
DRB1*0405	286	14	11.5–15.5		B*1527	6	0.3	r–0.1	
DRB1*0406	71	3.5	3.2–3.5		B*1528	1	0.05	r	
DRB1*0407	10	0.5	0.4–0.9		B*2704	4	0.2	r–0.3	
DRB1*0410	37	1.8	1.8–1.9		B*2705	1	0.05	r–0.1	
DRB1*0701	4	0.2	0.3–0.8		B*3501	158	7.8	7.6–8.6	
DRB1*0802	100	4.9	3.6–5.0		B*3551	1	0.05	NT	
DRB1*0803	192	9.4	7.6–8.1		B*3701	9	0.4	0.2–1.3	
DRB1*0809	1	0.05	r		B*3802	5	0.2	0.1–0.4	
DRB1*0901	303	14.9	12.4–15.0		B*3901	70	3.4	3.5–4.4	
DRB1*1001	8	0.4	0.6–0.9		B*390201	5	0.2	0.2–0.9	
DRB1*1101	57	2.8	2.2–3.4		B*390202	3	0.1	NT	
DRB1*1201	73	3.6	3.5–3.9		B*3904	4	0.2	0.1–0.2	
DRB1*1202	35	1.7	1.3–2.7		B*3923	1	0.05	r	
DRB1*1301	9	0.4	0.4–0.9		B*4001	110	5.4	4.2–6.1	
DRB1*1302	131	6.4	5.3–7.7		B*4002	168	8.3	6.1–8.6	
DRB1*1307	2	0.1	0.1		B*4003	5	0.2	r–1.1	
DRB1*1312	1	0.05	NT		B*4006	107	5.3	3.9–5.3	
DRB1*1401	78	3.8	1.9–4.5		B*4402	7	0.3	r–1.0	
DRB1*1402	1	0.05	r–0.2		B*4403	127	6.2	6.9–12.2	
DRB1*1403	26	1.3	1.5–1.6		B*4601	101	5	3.6–6.1	
DRB1*1405	44	2.2	1.1–2.6		B*4801	54	2.7	2.7–4.3	
DRB1*1406	26	1.3	1.1–1.8		B*5101	170	8.3	7.0–7.9	
DRB1*1407	2	0.1	0.1–0.3		B*5102	6	0.3	r–0.1	
DRB1*1412	1	0.05	r–0.1		B*5201	222	10.9	10.4–13.7	
DRB1*1501	117	5.7	6.1–8.5		B*5401	179	8.8	6.5–7.7	

Table 4 (continued)

Alleles	Count	Frequency (%)[a]	Frequency (%)[b]	P_c value	Alleles	Count	Frequency (%)[a]	Frequency (%)[b]	P_c value
DRB1*1502	201	9.9	8.7–12.2		B*5502	39	1.9	1.9–3.2	
DRB1*1602	11	0.5	0.5–0.9		B*5504	1	0.05	r–0.3	
					B*5601	30	1.5	0.5–1.3	
					B*5603	1	0.05	r–0.2	
					B*5801	10	0.5	r–0.6	
					B*5901	40	2	0.9–1.8	
					B*670101	19	0.9	1.0–1.7	
					B*670102	9	0.4	NT	

The P_c value is shown only for those alleles with $P_c<0.05$
r Rare frequency, *NT* not tested
[a]Allele frequencies of this study were defined as the percentage of the corresponding allele in the total of 2,036 alleles. Alleles in the homozygous combinations were counted twice
[b]Allele frequencies of HLA-A, -B, -C, and -DRB1 were referred from previous reports (Hashimoto et al. 1994; Saito et al. 2000; Tanaka et al. 1996, 1999; Tokunaga et al. 1997)

Discussion

We have developed a PCR–SSOP–Luminex method to define the HLA alleles with the frequency of more than 0.1% in the Japanese population. This method is a novel, rapid, high-throughput HLA genotyping technique employing PCR–SSOP protocols and the xMAP technology developed by Luminex Corporation as the analytical technique. xMAP technology is a simultaneous multiplex bioassay using a rapid flow cytometer that allows multiple analyses attached to color-coded microbeads to be detected in solution of a single tube or a single well of a 96-well microplate (Dunbar et al. 2003; Spiro et al. 2000). Currently, this technology has been used to recognize genetic polymorphism in human disease genes and homologous genes in bacteria (Diaz and Fell 2004; Ye et al. 2001). The HLA PCR–SSOP–Luminex method used here in conjunction with the JH Pattern File and the FT Pattern File provided highly accurate HLA typing results in 150 genomic DNA samples obtained from the UCLA International HLA DNA Exchange.

Each classical class I and class II HLA locus in the HLA region is located within a 4-Mb genomic region on the short arm of chromosome 6 (6p21.3), and together, these genes are extremely rich in polymorphisms, probably providing the greatest number of human gene alleles than any other gene combinations within a relatively small region (<5 Mb) of the human genome. For example, the following number of antigens and alleles were named officially by the WHO Nomenclature Committee in 2004: 28 serological antigens and 349 DNA alleles within HLA-A, 59 antigens and 627 alleles within HLA-B, 10 antigens and 182 alleles within HLA-C, 21 antigens and 394 alleles within HLA-DRB1 (Marsh et al. 2005). The results of a retrospective study on bone marrow transplantations from unrelated donors in Japanese patients demonstrated that donor–recipient compatibility for HLA class I antigens, especially HLA-A compatibility at the allele level, might greatly influence the success of the transplant (Spencer et al. 1995). HLA gene polymorphism is considered to be one of the factors that can be used to identify susceptibility loci for autoimmune diseases, and more than 100 genetic or infectious diseases have been associated with the MHC, including type I diabetes and rheumatoid arthritis (Pugliese et al. 1994; Shiina et al. 2004; Wooley et al. 1981). In this regard, the HLA genotyping PCR–SSOP–Luminex method that we have developed will be particularly useful for disease association studies, where a rapid high-throughput method is required for genotyping a large number of samples for statistical purposes. In addition, our HLA genotyping method at four loci, HLA-A, -B, -C, and -DRB1, will help in HLA haplotype mapping, as allele variations at multiple gene loci are considered to be better markers for the study of some chronic or complex diseases than allele markers at single gene loci.

Although HLA genotyping kits employing various methods are commercially available, a lot of the alleles that are absent from the Japanese population may be contained within the commercial kits that are prepared by overseas manufacturers for use in Japan. When these commercial kits in the allele analysis are used at the four-digit level, particular problems may occur because of ambiguities due to the increased number of alleles. We found that the resolution of our method could be increased to the four-digit or higher level by incorporating into our analytical software a high-resolution database of all the Japanese population's HLA alleles that have a frequency of ≥0.1% in the population. We have called this database the JH Pattern File for the Japanese population, and by incorporating it into our analytical file, we have increased the accuracy of our HLA genotyping method at the four-digit level or more to 100% for HLA-A and -C, 99.90% for HLA-B, and 99.95% for HLA-DRB1. However, in more diverse populations groups, or if the HLA database is incomplete, then it is likely that the PCR–SSOP–Luminex method will have low-to-medium resolution with numerous candidate alleles because of the many alleles and ambiguities created by the four-digit level. The preset cutoff value for each fluorescing oligobead set used to discriminate between positive and negative controls was

empirically determined for each probe, depending on the existing data of samples with known HLA alleles. We have already collected fluorescence intensity patterns of the SSOP hybridization results obtained by our Luminex method from more than 20,000 samples including Japanese, Caucasians, and Africans, which enabled us to determine the definite cutoff values for accurate HLA typing.

The HLA alleles at the four-digit level reported in the Japanese population include 33 alleles for HLA-A, 55 for HLA-B, 28 for HLA-C, and 34 for HLA-DRB1 (Hashimoto et al. 1994; Saito et al. 2000; Tanaka et al. 1996, 1999; Tokunaga et al. 1997). We applied the PCR–SSOP–Luminex method to the four-digit level or higher, including the analysis of heterozygous combinations in 1,018 Japanese DNA samples. The following number of alleles was identified by the PCR–SSOP–Luminex method, 21 for HLA-A, 42 for HLA-B, 17 for HLA-C, and 32 HLA-DRB1. The alleles which were not identified in this study, but that had been previously reported in the Japanese population included rare alleles whose frequencies were less than 0.1%. The frequency distribution of HLA alleles is different among the races and ethnicities, and most frequencies of HLA alleles in the samples collected from Japanese have already been reported (Hawkins et al. 1988; Imanishi et al. 1992). The allele frequencies obtained in our study showed no significant difference with those previously reported for the Japanese population, with the exception of HLA-A*2420. Although HLA-A*2420 was deemed a rare Japanese allele in the past reports (Hashimoto et al. 1994; Saito et al. 2000; Tanaka et al. 1996, 1999; Tokunaga et al. 1997), 17 alleles with the frequency of 0.83% were detected in this study (P_c=0.015). HLA-A*2420 and -A*2402, which has the highest frequency at the HLA-A locus of Japanese, can be mistyped because A*2420 differs from A*2402 by a single nucleotide substitution from cytosine to adenine at position 81 (Scheltinga et al. 2000). We created oligonucleotide probes HLA-A, -B, -C, and -DRB1, respectively, to determine accurately the genotypes of known Japanese alleles. Consequently, there is a possibility that A*2420 was genotyped as A*2402 in previous methods. As a result of genotyping 1,018 Japanese samples, only two allele samples for HLA-B and one for HLA-DRB1 failed to be determined using the JH pattern file. However, these three alleles were then identified by using the FT Pattern File and confirmed by nucleotide sequencing to be the rare alleles with a frequency of less than 0.1% (0.05%) in the Japanese population, HLA-B*1505, -B*3551, and -DRB1*1312. Therefore, the PCR–SSOP–Luminex method is able to determine the previously reported alleles accurately for Japanese samples with more than 99.9% probability. Ambiguities observed in these rare alleles were often seen in HLA typing using PCR–SSOP. To circumvent this problem, other than confirmation by PCR–SSP and/or nucleotide sequencing, data accumulation of HLA allele frequencies in as many populations as possible may again be indispensable as described above.

In the PCR–SSOP–Luminex method that we have described here, we used 96-well plates for genotyping each HLA locus. It took only approximately 5 h from the extraction of genomic DNA to the determination of HLA alleles at the four-digit alleles at the HLA-A, HLA-B, HLA-C, and HLA-DRB1 loci for 96 samples with Luminex 100 when handled by a single typist. Other Microplate Systems, such as PCR–rSSO (Mizuki et al. 1992a,b; Levine and Yang 1994), provide only one oligonucleotide probe per well, whereas our PCR–SSOP–Luminex method allows up to 100 different analytes to be measured simultaneously. Moreover, assays of the HLA-A, -B, -C, and -DRB1 loci were run under the same reaction conditions of PCR and hybridization, therefore allowing the measurement of 96 samples including negative and positive controls for HLA-A, -B, and -C loci using GeneAmp 9700 and Luminex 100. For genotyping HLA-DRB1, two types of amplification systems were required to amplify and measure the DRB1-PS1 and DRB1-PS2 reactions separately, resulting in the measurement of 48 samples (include negative and positive controls) using GeneAmp 9700 and Luminex 100. Thus, the PCR–SSOP–Luminex method is very useful for genotyping a large number of samples by reducing the number of manual procedures.

In conclusion, we developed and performed gene allele typing for HLA-A, -B, -C, and -DRB1 at the four-digit or higher level by a PCR–SSOP–Luminex method using DNA samples from the Japanese population and HLA pretyped control DNA samples obtained from the UCLA HLA DNA Exchange. This novel high-throughput method for HLA allele typing permitted the simple and rapid processing of large number of samples.

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