Nonelectrophoretic method for high-throughput HLA-DRB1 group genotyping

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Disorders with linkage to the human leukocyte antigen (HLA) region include inflammatory bowel disease, psoriasis, sarcoidosis, multiple sclerosis, type I diabetes mellitus, and others (1,2). Differentiation of gene effects from nearby HLA loci is a common problem in the mapping of the susceptibility variants in this region. The HLA molecules may be risk factors for the disease themselves, or they may serve as markers tagging risk haplotypes. In either situation, large numbers of patients need to be genotyped for one or more HLA loci. Full resolution of rare alleles is not required in these situations. Therefore, nonoverlapping groups of alleles are typed and used instead of the actual HLA alleles. Current HLA genotyping methodologies include group-specific PCR, genomic or cDNA sequencing, and hybridization methods, employed either individually or in combination. None of these methods scales well, if thousands of patients must be characterized. The

standard methods of single nucleotide polymorphism (SNP) genotyping for non-HLA loci like Taqman face significant problems in highly polymorphic regions like the informative HLA molecule exon sequences: (i) there is an extremely high density of SNPs, making probe and primer design very difficult. This high diversity is complicated by a complex haplotype structure making successful allele resolution, even with multiple probes, impossible; and (ii) the standard techniques require a universal and stable underlying PCR. For many HLA loci, the design of such primers is impossible, because either undesirable co-amplification of pseudogenes or lack of amplification of relevant alleles at the locus of interest occurs.

The *DRB1* locus has all these—from a genotyping point of view—undesirable characteristics (3,4) (HLA Database at www.ebi.ac.uk/

[imgt/hla](#)). Therefore, for the example of *DRB1* genotyping, we have developed a method combining allele-specific PCR with nonelectrophoretic PCR product detection. The requirements for this method included the ability to genotype thousands of patients at a reasonable cost, the potential for automation, and integration into an electronic laboratory information system.

Because *DRB1* is a multi-allelic system, several PCRs must be performed to construct the final genotype. Importantly, the primers for the group-specific PCR at the *DRB1* locus must avoid overlapping amplification of alleles between groups, because this would lead to ambiguous allele assignments.

Primers for the detection of the seven major *DRB1* allele groups were adopted from published reports (5,6) and are listed in Table 1. The allele groups include DRB1*01 (for group 1); DRB1*15, *16 (group 2); DRB1*03, *08, *11 (not *1122/30), *12, *13, *14 (not *1410/39) (group 3), DRB1*04, *1122, *1410 (group 4), DRB1*07 (group 5), DRB1*09 (group 6), and DRB1*10 (group 7). All reactions were carried in 10-μL reaction mixtures with 1 μL (10×) buffer, 0.2 mM dNTPs (each), 0.2 μM primer (each), and different MgCl₂ and *Taq* DNA polymerase

Figure 1. Screenshot of the accompanying “DRB tool” software. The software displays the final composite genotypes for the same plate as shown in Figure 2. Discrepancies that occurred during the verification of Mendelian inheritance are highlighted automatically in italics. Here, for wells D3, D10, H6, and H12, seven positive amplifications are detected and indicated as an error, because the corresponding control DNA with known *DRB1* allele was pipetted into each of the seven plates used for group amplification.

Figure 2. Results of a fluorescence reading from a 96-well plate for the amplification group 1. The y-axis denotes the signal intensity of the fluorescence measurement of the TECAN SpectraFluor plus in device-specific artificial units. The increase in reading in the positive samples corresponds to the double-stranded DNA (dsDNA) generated as a PCR product. Measurements are ordered on the basis of the signal intensity. The positions of the positive and negative controls on the plate are denoted with solid and open arrows, respectively. A clear, sudden increase in signal intensity is observed for samples positive in amplification group 1, allowing the setting of cut-offs of 300 and 600 for the discrimination of DRB1*01 negative and positive samples. Results of this plot were verified by gel electrophoresis (data not shown).

Table 1. Primers Used for the Amplification of *DRB1* Allele Groups

Amplification Group	Primer	<i>DRB1</i> Alleles Detected
1	5'-TTCTTGTTGGSAGCTTAAGTT-3'	DRB1*01
2	5'-TTCCTGTGGCAGCCTAAGAGG-3'	DRB1*15, *16
3	5'-CACGTTTCTTGAGTACTCTAB-3'	DRB1*03, *08, *11 (not *1122/30), *12, *13, *14 (not *1410/39)
4	5'-GTTTCTTGAGCAGGTAAAC-3'	DRB1*04, *1122, *1410
5	5'-CCTGTGGCAGGGTAARTATA-3'	DRB1*07
6	5'-CCCAACCACGTTTCTTGA-3'	DRB1*09
7	5'-AGACCACGTTTCTTGAGG-3'	DRB1*10

A common reverse primer 5'-CCGCTGCACTGTGAAGCTCT-3' was used for all amplifications (12). Allele group-specific primers were adapted according to References 13 and 14. Primers were selected for use in a Caucasian sample. Certain rare *DRB1* alleles will not be amplified with these primers, but their combined frequencies are below 0.05% and thus not relevant for the application needed in this context.

Table 2. PCR Conditions and Annealing Temperatures Used for the *DRB1* Allele Group Identification

Amplification Group	MgCl ₂ (mM)	Taq DNA Polymerase (U/μL)	Annealing Temperature A (°C)	Annealing Temperature B (°C)
1	2	0.024	61	53
2	2	0.030	65	57
3	3.5	0.036	61	53
4	2	0.024	65	57
5	2	0.018	65	57
6	3	0.036	56	48
7	2	0.036	63	55

concentrations listed in Table 2 (AmpliTaq™ Gold with GeneAmp®; Applied Biosystems, Foster City, CA, USA). The PCR programs for all primer pairs were started with denaturation at 95°C for 12 min followed by 16 cycles of denaturation at 95°C for 30 s, annealing (temperature A in Table 2 is specific for every primer and decreases 0.5°C with every following cycle) for 30 s, and extension at 72°C for 30 s. This was continued by 19 cycles of denaturation at 95°C for 30 s, annealing (temperature B in Table 2 is specific for every primer) for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min (GeneAmp PCR System 9700; Applied Biosystems). Successful establishment of PCR conditions was verified in each case on a panel of human DNAs with known genotypes from the German HLA quality control panel: DRB1*01 (for group 1), DRB1*15, *16 (group 2), DRB1*08, *12 (group 3), DRB1*04 (group 4), DRB1*07 (group 5), DRB1*03, *09 (group 6), and DRB1*03, *10 (group 7). Five microliters of PCR product were transferred into 96-well plates with 45 μL of

diluted PicoGreen® (Molecular Probes, Eugene, OR, USA) [1:400 with 0.1 Tris-EDTA (TE) buffer]. The plates were vortex mixed, incubated for 5 min, and then read in a SpectraFluor® plus Fluorometer (TECAN, Männedorf, Switzerland) with excitation wavelength 485 nm. The raw data files were exported for each plate separately.

The validity of amplification results of a group-specific PCR depends critically on uniform reaction conditions in all wells. While parameters like temperature profile, enzyme, and salt concentrations are highly standardized through the use of modern PCR machines, DNA concentration is the most critical parameter for uniform and reproducible genotyping results. DNA concentrations were therefore measured with a double-stranded DNA (dsDNA)-specific dye (PicoGreen) and diluted to 5 ng/μL in 0.1 TE buffer. The diluted DNA was then further diluted to 0.5 ng/μL and dispensed in amounts of 2.5 ng/well in 96-well plates (Corning Incorporated Life Sciences, Acton, MA, USA).

Amplification success in a group-specific PCR may be scored by elec-

trophoretic separation and staining. However, agarose gel electrophoresis does not scale well in a high-throughput setting. The alternative use of automated sequencing equipment with fluorescent detection requires costly fluorescent labeling of PCR products and substantive consumable cost entailed by the use of the required gel matrices in capillary sequencers. Alternatively, products can be detected using a fluorescent detection by inclusion of a Taqman™ probe (Applied Biosystems) in the reaction (7). This methodology requires the use of specialized instrumentation and the costly use of a separately labeled probe per reaction. We therefore adapted a nonelectrophoretic detection method that has been previously described for the genotyping of a GA transition in the p53 proto-oncogene of Chinook salmon (8).

For the analysis of the resulting raw fluorescence results, specialized software was created. This software allows: (i) empirical definition of cut-off values for the scoring of each PCR (negative, unknown, positive) on the basis of the allele-specific positive and negative controls included in each plate displayed in a graphic plot of the measured intensities; (ii) integration of all seven amplification results into a single genotype assignment (Figure 1); and (iii) testing for inconsistencies and Mendelian segregation and import into the laboratory information system (9).

The software is available from http://www.muco.sa.de/neu/index_bioinformatics.html. This method was employed in over 2295 individuals from 995 pedigrees and 538 unrelated individuals. A genotyping success rate of 94.11% was achieved. Genotyping failure was recorded in situations where signal intensities could not be clearly assigned either to the positive or negative groups (Figure 2). A percentage of 1.3 of the data had to be discarded from the family data due to Mendel errors. The term "Mendel errors" refers to violations of Mendelian inheritance within the families. This error rate compares favorably to other genotyping methodologies, as for instance the 1.6% reported recently for SNP data in Matise et al. (10). The analysis of genotype and allele frequencies revealed no departure from Hardy-Weinberg-Equilibrium ($P > 0.50$).

In summary, this nonelectrophoretic software-supported scoring of specially optimized allele group-specific PCRs is a rapid and easily automated method for the high-throughput genotyping of large patient samples. By design, only limited resolution of *DRB1* alleles is achieved, and separate PCRs have to be performed for each allele group. The method has several advantages over the alternatives and will complement other approaches (5,6,11): (i) it does not require agarose gel electrophoresis, which does not scale well for high-throughput applications; (ii) it is much less expensive than TaqMan assays (7) or fluorescently labeled PCR products for automated sequencing; and (iii) no specialized equipment is required. We anticipate that such methods may facilitate the gene identification of complex HLA-linked autoimmune disorders.

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