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SBT HLA-DRB1/3/4/5 High Resolution Typing Procedure

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Genomic DNA is isolated from various sample types according to the appropriate manufacturer's protocol: transformed cells and blood spotted filter paper (PuregeneDNA Isolation Kit, Gentra Systems, Minneapolis, MN); frozen whole blood, granulocytes, and mononuclear cells (Qiagen QIAmp DNA Blood Mini Kit, Valencia, CA).

High resolution typing of HLA-DRB1 is accomplished using the commercially available AlleleSEQR (Forensic Analytical, Hayward, CA) reagents and protocol. A pre-formulated PCR pre-mix contains buffer, dNTPs, MgCl₂, oligonucleotide primers, and water. Per the American Society of Histocompatibility and Immunogenetics (ASHI) guidelines, AmpliTaq Gold (Applied Biosystems, Foster City, CA) is provided as a separate reagent to be added to the pre-mix at the time of amplification reaction preparation so as to eliminate the possibility of non-specific amplification during storage. ExoSAP-IT (USB, Cleveland, OH) is used to remove unincorporated PCR components, primers and dNTPs. This reagent is a mixture of exonuclease I, which digests single stranded oligonucleotides (ie: unincorporated PCR primers), and shrimp alkaline phosphatase which digests unincorporated dNTPs. The sequencing mix for HLA-DRB1 is based on the Sanger dideoxy chain terminator DNA sequencing method. The pre-formulated mixes are based on sequencing chemistry from Amersham Biosciences (Piscataway, NJ). The DYEnamic ET Terminators contain fluorescent dye labeled ddNTPs and DNA polymerase. When combined with oligonucleotide sequencing primers that recognize sequence motifs within the PCR amplicon, the sequencing reactions generate fluorescent sequence ladders. Sequencing primers are chosen to generate a sequence for each exon in both directions for accuracy and simplified editing. Sequence ladders are purified and concentrated by ethanol precipitation, heat denatured in formamide, and electrophoretically separated and analyzed on an ABI 377 or 3100 automated DNA sequencer from Applied Biosystems.

The exon 2 sequence of DRB1, from codon 13 to 90, is generated. After preparation of genomic DNA, a region of the DRB1 gene is amplified using a pre-formulated, single-reaction, multiplex PCR that can amplify exon 2 of all major allele groups. The specificities included in this multiplex mixture are DR1, DR2, DR3/11/6, DR4, DR7,

DR8/12, DR9 and DR10. The allele group specificities of the primers are based on sequence polymorphism in the first hypervariable region at the 5' end of exon 2. Two 3' primers are located at the 3' end of exon 2 and extend into the 5' end of intron 2. All amplification primers contain oligonucleotide tails that, when incorporated into an amplicon, serve as sequencing primer recognition sites. Following PCR amplification according to the manufacturer's instructions, the amplicon is processed with ExoSAP-IT.

The enzyme-treated PCR is added to each of 3 different pre-formulated sequencing reaction mixtures: exon 2 forward and reverse, and a mixture containing a primer specific for the GTG motif of codon 86. The codon 86 primer specifically primes off the GTG motif and can therefore generate a hemizygous sequence from a PCR reaction containing two alleles with different motifs at codon 86. Use of this third sequencing reaction mix for all samples helps to reduce the level of ambiguous heterozygote typing results without needing to perform a second round of testing.

HLA-DRB3/4/5 loci are typed using amplification and sequencing reagents developed and/or validated at the University of New Mexico. Products are amplified from genomic DNA for subsequent Sanger dideoxy thermal cycle sequencing with resolution of ladders on the Applied Biosystems ABI 377 or 3100 instruments.

For HLA-DRB3, an exon 2 PCR product is prepared using a 5' HLA-DRB3 specific primer B3 (exon 2, codons 4-10, 309 bp product) paired with the generic 3' primer AmpB-T (codons 89-94). Amplification is in a 50 μ L reaction volume containing 0.25 μ M primers, 100 ng genomic DNA, 2.0 mM MgCl₂, 200 μ M dNTP, and 1.25 U Taq Gold polymerase for 35 cycles of the following: 95° C for 20 seconds, 60° C for 20 seconds. The PCR product is purified using Qiagen Columns to achieve a final concentration of approx. 5ng/ μ l. Sequencing reactions are performed with Big Dye Terminator cycle sequencing kits (Perkin Elmer ABI, Foster City, CA) using B3 or Amp B-T primers. The sequencing products are precipitated in ethanol, denatured and loaded on sequencing gels for analysis. HLA-DRB4 exon 2 PCR products (259 bp) are prepared using a 5' DRB4 generic primer B4 (exon 2, 22-28) paired with the generic 3' primer AmpB-T (codons 89-94). HLA-DRB4 exon 3 PCR products are prepared using a 5' 3eB45' (exon 3, 98-105) paired with a 3' B4 3e215A (exon 4, 215-221) and followed by a nested PCR with 5' 3eB45' paired with a 3' primer B43e (exon 3, 177-183). Amplification is performed in a 50 μ L reaction volume containing 0.3 μ M primers, 125 ng genomic DNA, 1.5 mM MgCl₂, 200 μ M dNTP, and 1.25 U Taq polymerase for 35 cycles of the following: 95° C for 30 seconds, 60C for 30 seconds, 72 for 30 seconds. The PCR product is purified using Qiagen Columns to achieve a final concentration of approximately 5ng/ μ l. Sequencing reactions are performed with Big Dye Terminator cycle sequencing kits (Perkin Elmer ABI, Foster City, CA) using DRB4, Amp B-T, 5'3eB45', or B43e primers. The sequencing products are precipitated in ethanol, denatured and loaded on sequencing gels for analysis.

The HLA-DRB5 exon 2 PCR product is prepared using a 5' HLA-DRB5 specific primer B5 (exon 2, codons 4-11, 306 bp product) paired with the generic 3' primer AmpB-T (codons 89-94). Amplification is in a 50 μ L reaction volume containing 0.25 μ M primers,

100 ng genomic DNA, 1.5 mM MgCl₂, 200 μM dNTP, and 1.25 U Taq Gold polymerase for 30 cycles of the following: 95° C for 20 seconds, 60° C for 20 seconds. The PCR product is purified using Qiagen Columns to achieve a final concentration of approximately 5ng/ul. Sequencing reactions are performed with Big Dye Terminator cycle sequencing kits (Perkin Elmer ABI, Foster City, CA) using DRB5 or Amp B-T primers. The sequencing products are precipitated in ethanol, denatured and loaded on sequencing gels for analysis.

All sequences are processed using MatchTools and MT Navigator HLA typing software from Applied Biosystems. These programs allow for allele assignment and sequence editing, respectively.

A multilayered approach is used for resolving ambiguous HLA-DRB1 heterozygous combinations. For DRB1, the commercial reagents obtained from the Forensic Analytical group have incorporated group specific sequencing primers taking advantage of codon 86 and polymorphisms. Doing so significantly reduces the number of ambiguities requiring resolution with secondary approaches. Also used are a series of group specific amplification primers which utilize the production of hemizygous sequence ladders. These include primers specific for DR groups such as DRB1*01 alleles as well as additional primer pairs which exploit the polymorphic positions outside the 5' hypervariable region in exon 2 including codon 32 (32C and 32T). Finally, the single allele amplification method developed by the University of New Mexico laboratory is also employed. This method relies on a dilution of genomic DNA prior to the PCR so that hemizygous PCR products can be prepared. HLA-DRB1 results obtained by this method have been independently confirmed in the Forensic Analytical lab using the AlleleSEQR PCR pre-mixes.