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SBT/SSOP HLA-C High Resolution Typing Procedure

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High resolution typing of HLA-C is accomplished using state-of-the art robotic instrumentation, automated workstations and advanced bioinformatic tools developed in-house at Histogenetics. For HLA-C, the primary typing methodology is sequence based typing (SBT) with sequence specific oligonucleotide probe (SSOP) serving as a complimentary and confirmatory methodology.

The DNA isolation procedure, designed to extract DNA from whole blood, filter paper and buccal swabs, was also developed in-house and uses 96-channel automated liquid handlers.

All PCR reactions are set up with 8 and/or 96-channel pipetters. Work lists and work maps are generated automatically by HistoLab, our custom designed web-enabled software, and instructions are loaded into the computer which operates the robots.

Currently, HLA-C amplicons are produced from 4 sets of group-specific amplification primers. The polymerase chain reaction (PCR) products are tested in agarose gel and the results are scored and stored for selection for SBT. Typically, amplicons from two different groups are selected for each locus. For those samples which show only one positive reaction from the groups from a given locus, amplicons from generic amplification are also selected for SBT to monitor dropouts. Positive PCR-wells are re-arrayed using our in-house developed "cherry-picking" software and the re-arrayed amplicons are further processed by SBT. This software is interfaced with a data handling computer program capable of tracking each sample, sorting the data by various parameters and assisting with analysis and reporting. Remaining PCR products are blotted "as is", without re-arraying, for SSOP typing. Using this strategy enables the detection of any human error introduced during the re-arraying step prior to sequencing.

The software filters the scanned images after hybridization specifically for HLA-C. Each amplification is analyzed separately and, upon completion of the group-specific analysis, results are combined to yield the final typing for the HLA-C locus. Scoring for positive group-specific amplifications is entered into HistoLab software where instructions for the

"cherry-picking" application are generated. Aliquots of the sequencing reactions are re-arrayed and a new work list is generated for the technician.

Currently, exonuclease (Exo) I and shrimp alkaline phosphatase (SAP) are used as the treatment method for PCR clean-up prior to sequencing. The actual sequencing is performed using BigDye terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA). Four sequencing primers, which are derived from sequences flanking the respective exons, are used for exons 2 and 3. All sequencing extension products are purified by EtOH + NaOAc precipitation in a 96 well format using 8 or 96-channel automatic pipetters. Histogenetics is equipped with two 3730 ABI sequencers (Applied Biosystems). One instrument is capable of running, unattended, 3000 sequencing reactions in a 24 hour period. Sequencing templates for the 3730 sequencers are automatically generated. Sequence results files are also automatically sorted according to the sample id number following data extraction. From there, results are compared to the allele database exon by exon. After combining and filtering results from the two exons, the software automatically makes a typing assignment.

In addition to SBT, SSOP is performed as a confirmatory test for HLA-C. Histogenetics has developed the sequence-specific oligo-probes used in this testing. These probes are conjugated with alkaline-phosphatase (AP) and have the same washing conditions suitable for multiplex hybridization. These probes are also thoroughly tested for allele and locus cross reactivity (vital information for multiplexing hybridization). A total of 15 HLA-A, B and C group-specific amplifications performed for SBT are tested by SSOP on two sets of membranes. Prior to blotting onto the membrane, an aliquot of each PCR amplification product is transferred to a denaturing solution using a robotic liquid handler. All HLA-A, B and C groups are then blotted onto the C1-set membranes in a specific and fixed location. These membranes contain amplification groups from HLA-A and HLA-B as well. The membranes are co-hybridized with probes for all loci. As a result, each hybridization chamber may contain up to three non-cross reacting probes. The signals captured on films are scanned and processed by software specifically developed for this project. This software captures, sorts and analyzes the SSOP signals to make a final allele assignment.

The current probe sets developed for this SSOP procedure do not produce allele level resolution for class I loci. As long as the primary methodology of SBT produces a class I allele level result for a given sample, no further groups are introduced to obtain this same level of class I typing via SSOP.