Sequence-Based Typing of the HLA-DRB 1 gene

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1. Summary of Test Principle and Clinical Relevance

Type 1 diabetes mellitus is a chronic autoimmune disease that involves a T cell-mediated destruction of the pancreatic beta cells, the body's sole source for insulin (1). This disorder is the most common chronic disease among children and young adults (2). Complications include kidney failure, blindness, amputations, nerve damage as well as an increased risk for heart attacks and strokes (3).

Type 1 diabetes has been shown to involve a genetic component and an environmental component (4). Thus, an environmental trigger in a susceptible genetic background results in type 1 diabetes development. This genetic component is the earliest predictor of type 1 diabetes and may eventually allow prediction in the prenatal phase leading to early prevention and/or treatment. The genes that are known to play a role in the genetic susceptibility include those in the Human Leukocyte Antigen (HLA) complex on chromosome 6p21, and the insulin gene on chromosome 11p15(5).

The role of the HLA region in type 1 diabetes was discovered in the 1970s by both association studies and affected-sib-pair studies (6-8). The DR3 and DR4 haplotypes, which consist of specific combinations of the class II genes DQA1, DQB1 and DRB1, have been implicated in disease susceptibility, and the DR2 haplotype has been associated with disease protection as determined by serotyping (9-12). These class II HLA genes are highly polymorphic and molecular genetic analysis has identified certain alleles with protection or susceptibility to type 1 diabetes (12-26). The assay described below involves genotyping the HLA DRB1 gene. Previous studies have found that DRB1 alleles *0301, *0401, *0402, *0404, and *0405 are predisposing for type 1 diabetes and the DRB1 alleles *1501and *0701have a protective effect (12,27-29). This study will allow confirmation of these results as well as allow the minor genetic risk factors to be identified by controlling for the major genetic risk.

The genetic complexity of the DRB region of the human major histocompatibility complex(MHC) has required development of molecular typing techniques with increasing levels of resolution. Methods such as Sequence Specific Primer-Polymerase Chain Reaction(SSP-PCR) are not always able to discriminate among the 288 alleles recognized alleles at DRB1. To address this need, a system that combines a low-resolution SSP-PCR followed by a high resolution allele typing using automated DNA sequencing was developed. Since the low resolution SSP-PCRs are based on allele group-specific motifs in the first hypervariable region of exon 2, the following allele groups and genes are amplified with the eleven specific PCR mixes provided: DR1, DR2, DR3/11/6, DR4, DR7 &, DR8/12, DR9, DR10, DRB3, DRB4, and DRB5. The positive amplification reactions from the SSP-PCR are used as sequencing templates to generate the high resolution allele typing information. Although the PCR and sequencing protocols enable direct sequencing with no PCR purification steps, this method is time consuming and has the possibility of sample mix-ups. To address this need, a single-tube PCR that amplifies exon 2 of all the allele groups was developed by Forensic Analytical. The AlleleSEQR DRB1 PCR is a single-tube reaction that amplifies exon 2 of all allele groups which makes it possible to eliminate the agarose gel step common to most other DRB assays based on a preliminary SSP-PCR typing. This design greatly enhances throughput and efficiency as well as eliminating the possibility of sample mix-ups. Three custom sequencing mixes(Forward, Reverse, and a primer specific for the GTG motif of Codon 86)are provided. The Codon 86 sequencing data minimizes the number of ambiguities that are encountered in DRB typing. The AllelSEQR DRB1 assay will be used as the primary assay for DRB1 genotyping, however the Applied Biosystems assay will be used in cases where it can resolve ambiguities.

The final step in the analysis procedure is to perform the allele assignment using MatchTools Software and MTNavigator Software. These programs work together to assign alleles and to allow manual review or editing of the sequence data.

2. Safety Precautions

Standard safety precautions should be observed including wearing safety glasses, a lab coat, and gloves during the preparation of blood specimens. Follow Universal Precautions when handling all blood and blood products. Vaccination for hepatitis B is strongly encouraged. Laboratory items exposed to blood or blood products should be disposed of or decontaminated in compliance with guidelines from the Office of Health and Safety, CDC. Any mutagen or toxigenic organic solution used in this genotyping process is listed below:

1. *Ethidium Bromide (EtBr)*
   Ethidium Bromide is used to visualize double-stranded DNA that has been separated by size on an agarose/acrylamide gel matrix. The EtBr intercolates into double-stranded DNA, and will fluoresce when visualized on a UV transilluminator. EtBr is a potential carcinogen, and extreme caution should be taken when working with this chemical. Observe all safety precautions such as wearing a lab coat, fresh gloves, and protective eye-wear.

2. *HiDi Formamide*
   HiDi Formamide is used in small amounts within the laboratory to resuspend DNA for automated sequencing (also found in *Template Suppression Reagent*). Formamide is a teratogen which can affect development. Always exhibit extreme caution while in contact with formamide, and observe all safety precautions such as wearing a lab coat, fresh gloves, and protective eyewear. *Note: women who are or plan to become pregnant should not work with formamide due to its adverse effects on fetal development.

3. *Performance Optimized Polymer 4 and 6 (POP 4) / (POP 6)*
   These polymers are used within the automated sequencers and acts as a medium through which the DNA samples are transported through capillaries. Both of these polymers contain high amounts of urea, which is a potential mutagen and has been shown to have reproductive and tumorigenic effects. Observe all safety precautions such as wearing a lab coat, fresh gloves, and protective eyewear.

4. *Acrylamide*
   Acrylamide is used to pour the large sequencing gel used on the Applied Biosystems 377 DNA Sequencer. Acrylamide is a poison, neurotoxin, irritant, carcinogen, and possible teratogen.
   The effects of this chemical are cumulative, so always use it with the upmost caution. Observe all safety precautions such as wearing a lab coat, fresh gloves, and protective eyewear.

3. Computerization; Data System Management

Integrity of specimen data generated by this method is maintained by proofreading all transcribed data by the analyst. All data is copied to a CD-R for transfer to a Microsoft Access database created to store all raw data generated in the GoKinD study. Only authorized personnel from the Molecular Biology Branch (as determined by the supervisor) have access to this database. Analyzed genotype results are recorded by the analyst in a Microsoft Access database located on CDC's LAN, and only authorized personnel from the Molecular Biology Branch (as determined by the supervisor) have access to the data.

4. Specimen Collection, Storage, and Handling Procedures; Criteria for Specimen Rejection

a. Specimen collection: Whole blood obtained with EDTA as an anticoagulant may be used. All 10 mls of the venous blood collected will be processed for DNA.

b. Specimen storage: Blood samples which have been processed by the Puregene method through the cell lysis step (see appendix A) can be stored at room temperature for up to 18 months. Extracted DNA can be stored at -20C indefinitely until assayed.

c. Freeze-Thaw effect: Repeated freeze-thaws may cause slight fragmentation of DNA. However, the size DNA targeted for amplification is very small (<400bp) and there is no documented deleterious effect of freeze-thaw on this test.

5. Procedure For Microscopic Examination; Criteria for Rejection of Inadequately prepared slides

Not applicable for this procedure

6. Preparation of Reagents, Calibrators (standards), Controls, and All Other Materials

a. Reagents

The Puregene DNA Isolation Kit (Gentra systems) contains *Red Blood Cell Lysis Solution* (RBC Lysis Solution), *Cell Lysis Solution*, *RNase solution*, *Protein Precipitation Solution*, and *DNA Hydration Solution.* All reagents except for the *RNase solution* are stable at room temperature until the manufacture's specified expiration date. The *RNase solution* is stable at 4C until the manufacture's specified expiration date.

The AlleleSEQR HLA-DRB1 SBT Kit is stored at -20C until the manufacture's specified expiration date. Check the expiration date before each use and discard the kit after the expiration date.

The HLA-DRB kit is stored at 4C until the manufacture's specified expiration date. Check the expiration date before each use and discard the kit after the expiration date.

The HotStarTaq Master Mix is stored at -20C until the manufature's specified expiration date. Check the expiration date before each use and discard the kit after the expiration date.

The Dye Terminator Cycle Sequencing Ready Reaction Kit, which contains the reagents for the control reaction for cycle sequencing and sequencing, may be stored at -20C until the manufacture's specified expiration date. Discard after expiration date. The *Terminator Ready Reaction Mix* is light-sensitive and should be stored without exposure to light.

The AmpFlSTR Green I PCR Amplification Kit is stored at 4C until the manufacture's specified expiration date. Check the expiration date before each use and discard the kit after the expiration date.

The HiDi Formamide is aliquoted into separate tubes and stored at -20C.

b. Preparation of Reagents

See Appendix A ,B, C, D and E.

c. Standards

These are qualitative assays and calibration standards are not used. See part 7.

d. Controls

AlleleSEQR HLA-DRB1 SBT
In each set of PCR and subsequent sequencing reactions, an in-house control DNA as well as control DNA supplied in the kit is tested. These control DNAs have been previously genotyped by sequencing analysis. The positive DNA control supplied in the kit is used as a control to test the chemistries for PCR as well as for sequencing.

SSP-PCR and Sequencing
In each set of PCR reactions and subsequent sequencing reaction, an in-house control DNA is tested. This control DNA has been previously genotyped by sequencing analysis. Other internal controls for the SSP-PCR and sequencing are the ALL tube for the SSP-PCR and pGEM for sequencing. This ALL reaction tube should always have a positive PCR band and pGEM has been previously sequenced and is used as a control for sequencing chemistries.

Human Identification
In each set of PCR reactions, a positive control of previously genotyped DNA as well as a no DNA negative control is included. For the fragment analysis using the Green I kit, a Green I Allelic Ladder is always run with the samples. For fragment analysis of Amelogenin and TH01, an inhouse internal allelic ladder is always run with the samples.

e. Equipment and Materials

Puregene DNA Isolation Kit , catalog # D-50K (Gentra Systems, Minneapolis, MN)
70% ethanol % ethanol
100% isopropanol % isopropanol
glycogen(Gentra Systems)
50 ml Falcon centrifuge tubes
Rainin pipet tips with filters (Rainin Instrument Co., Emeryville, CA)
Sterile, individually wrapped transfer pipets
Racks for 50ml centrifuge tubes (bleach after each use)
PipetAid, Drummond (Daigger, Lincolnshire, IL)
Qiagen Sigma centrifuge (Qiagen Inc, Chatsworth, CA)
Dispensett III volume dispenser for reagents (Daigger, Lincolnshire, IL)
Nitrile Gloves
HLA-DRB BigDye Terminator Sequencing-Based Typing Kit(catalog # 4305213, Applied Biosystems)
1.5 and 0.5 ml microfuge tubes (Marsh Biomedical Products Inc., Rochester, NY)
MicroAmp 8-Strip Reaction Tubes[0.2ml](catalog # N801-0580, Applied Biosystems)
MicroAmp Caps[8 caps/strip](catalog # N801-0535, Applied Biosystems)
MicroAmp 96-Well Tray/Retainer Sets(catalog # 403081, Applied Biosystems)
Microtiter plate
Finnpette Biocontrol Pipettor with Multi-channel module (Lab Systems/Marsh Biomedical Products Inc.)
Geneamp PCR System 9700(Applied Biosystems)
ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA)
MicroAmp 96-well reaction plate(catalog # N8010560, Applied Biosystems)
96-well plate septa (catalog # 4315933, Applied Biosystems)
3100 POP-6 polymer(catalog # 4316357, Applied Biosystems)
Performance Optimized Polymer 6 (POP-6) (Applied Biosystems)
Stratalinker 2400 UV Crosslinker (Stratagene, La Jolla, CA)
Rainin pipettors, Rainin (Emeryville, CA)
Electrofast (Advanced Biotechnologies through Marsh Biomedical Products Inc.)
Boekel Orbital Rocker, Boekel Scientific, Inc. (Feasterville, PA)
Balance
Incubator
Power Pak 300 Power Supply (BioRad, Hercules, CA)
Computer software for analysis with Sequencing Analysis, Match Tools, MTNavigator, GeneScan and Genotyper (Applied Biosystems)
Heating block
IEC Multi Centrifuge with Double Deep Microplate Rotor (Forma Scientific Inc., Marietta, OH)
Savant Vacuum centrifuge (Forma Scientific Inc., Marietta, OH)
Vortexer Genie (Diagger, Licolnshire, IL)
Alpha Imager documentation System (Alpha Innotech, San Leandro, CA)
1XTBE(10x TBE from Gibco/BRL, Rockville, MD)
10X TBE(Trizma Base, Boric Acid, EDTA) for Sequencing
deionized water
microwave
flasks and beakers
plastic wrap
Orange G gel loading dye(Orange G, Ficoll 400, EDTA)
Hi Di Formamide (catalog # 4311320, Applied Biosystems)
blue dextran/EDTA loading dye (Applied Biosystems)
Ultra- pure agarose (Gibco/BRL, Rockville, MD)
Long Ranger Singel Packs (catalog #50691, FMC BioProducts, Rockland, ME)
Ethidium Bromide (Ameresco, Solon, OH)
Low DNA Mass Ladder (cat # 10068-013, Gibco/BRL, Rockville, MD)

Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA)
Sequencing Capillaries (47cm x 50um) (Applied Biosystems, Foster City, CA)
AmpF*l* STR Green I PCR Amplification Kit (catalog # 402902, Applied Biosystems)
AmpF*l* STR Green I PCR Amplification Kit User's Manual (catalog # 402944, Applied Biosystems)
Performance Optimized Polymer 4 (POP-4) (catalog # 402838, Applied Biosystems)
0.5ml Genetic Analyzer sample tubes and septum(catalog # 401957 and 401956, Applied Biosystems)
ABI PRISM 310 Genetic Analyzer (Applied Biosystems)
ABI PRISM 3100 Genetic Analyzer(Applied Biosystems)
1xTE(10mM Tris-HCl, 0.1mM EDTA, pH 8.0)
GeneScan-350[ROX] Internal Lane Size Standard (catalog # 401735, Applied Biosystems)
10x Genetic Analyzer Buffer with EDTA(catalog # 402824, Applied Biosystems)

f. Instrumentation

The ABI PRISM 377 DNA Sequencer, ABI PRISM 310 Genetic Analyzer, and ABI PRISM 3100 Genetic Analyzer can be used for both fragment analysis as well as for sequencing applications. All instruments utilize electrophoresis, laser excitation and detection via a charged-coupled device (CCD) camera which provides simultaneous detection of all four colors from a single sample run.

The GeneAmp PCR System 9700 is an automated thermal cycler with interchangeable sample blocks, used to carry out PCR amplification reactions. Methods, instructions that specify how the instrument should heat or cool samples in a PCR thermal profile, are programmed and stored in the instrument software. The Geneamp PCR System 9700 combines integrated instrument design and thin walled 0.2ml MicroAmp Reaction Tubes to offer greater speed, oil free operation, lower reaction volumes, and unsurpassed cycle time reproducibility.

7. Calibration and Calibration Verification Procedures

The ABI PRISM 377 DNA Sequencer, ABI PRISM 310 Genetic Analyzer, and ABI PRISM 3100 Genetic Analyzer are calibrated by the manufacturer and annual preventative maintenance is performed by the manufacturer's authorized service representative. The genotyping assays are qualitative tests. There are a number of possible genotypes in the test population. If the in-house sequence does not give the correct genotype, the test is repeated.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

a. Procedure:
see Appendix A, B, C, and D for DNA extraction, Human Identification, AlleleSEQR HLA-DRB SBT and SSP-PCR and Sequencing.

b. Calculations:
Not Applicable.

c. Interpretation of results:
AlleleSEQR HLA-DRB1 SBT
The AlleleSEQR DRB1 PCR is a single-tube reaction that amplifies exon 2 of all allele groups except DRB1*0814. This design makes it possible to eliminate the agarose gel step common to most other DRB assays based on a preliminary SSP-PCR typing. The results obtained are qualitative and interpretations are based on the software programs MatchTools and MTNavigator which work together to assign alleles and to allow manual review or editing of the sequencing data. Genotypes are entered into the database according to standard HLA nomenclature.
SSP-PCR and Sequencing
The positive amplification reactions from the SSP-PCR are used as sequencing templates to generate the high resolution allele typing information. The PCR and sequencing protocols enable direct sequencing with no purification steps. The design of the amplification primers allows sequencing of both strands from

a single amplification fragment. The BigDye terminator cycle sequencing chemistry has been optimized for ease of use and high throughput. The results obtained are qualitative and interpretations are based on the software programs MatchTools and MTNavigator which work together to assign alleles and to allow manual review or editing of the sequencing data. Genotypes are entered into the database according to standard HLA nomenclature.

Human Identification

The AmpF*l* STR Green I PCR Amplification Kit amplifies the TH01, TPOX, CSF1PO short tandem repeat loci. In addition, primers included in the AmpF*l* STR Green I Primer set amplify the Amelogenin locus which can be used for gender determination. The amplified product is run on an ABI PRISM instrument and the collected multicolor fluorescent data is analyzed using GeneScan Analysis Software. The Genotyper Software converts fragment sizes to genotypes which can then be uploaded into a database. The AmpFlSTR Green I Kit is used for quality control for the trio samples

Identification of Amelogenin and TH01 Markers

This PCR based method amplifies the Amelogenin locus and the TH01 short tandem repeat loci. These markers are used for quality control purposes for samples that are not trios.

9. Reportable Range of Results

Not applicable. See item 7 for details

10. Quality Control(QC) Procedures

a. Quality Control Principles

The type I diabetes genotyping method described in this protocol has been well established in the Division of Environmental Health and Laboratory Sciences. These methods have proven to be accurate, precise, and reliable.

Reliability of test results should be monitored by routine use of positive controls of known genotypes for both the AlleleSEQR HLA-DRB1 sequence-based typing, SSP-PCR and sequencing step for the DRB SSP-PCR/sequencing assay as well as for the microsatellites assay.

AlleleSEQR HLA-DRB1 SBT

A run is considered to be "out of control" if:

1. The reactions sequenced produces an un-readable electropherogram for the Forward or Reverse sequence reaction due to machine malfunction or operator error.

HLA-DRB SSP-PCR and sequencing

A run is considered to be "out of control" if:

1. There are more than 2 positive PCR products for DRB1.
2. The band patterns do not match one of the possible combinations in the HLA-DRB Comparison Table(Table 2).
3. There is no positive band for the ALL tube or no bands at all.
4. The positive control(the in-house control DNA sample) produces a false positive or false negative band.
5. The reactions sequenced produces an un-readable electropherogram due to machine malfunction or operator error.

If the run is declared "out of control," both the SSP-PCR as well as the automated sequencing is repeated immediately.

Human Idenfication

A run is considered to be "out of control" if:

1. The electropherogram for the fragment analysis contains less than 4 or greater than 8 peaks,
2. The GeneScan-350 ROX Internal Lane standard does not show up on the electropherogram, or
3. The signal is too weak on the electropherogram.

If the run is declared "out of control," the fragment analysis should be repeated. If the run is "out of control" again, the PCR as well as the fragment analysis is repeated.

Idenfication of Amelogenin and TH01 Markers

A run is considered to be "out of control" if:

1. The electropherogram for the fragment analysis contains less than 2 or greater than 4 peaks,
2. The GeneScan-350 ROX Internal Lane standard does not show up on the electropherogram, or
3. The signal is too weak on the electropherogram.

If the run is declared "out of control," the fragment analysis should be repeated. If the run is "out of control" again, the PCR as well as the fragment analysis is repeated.

b. Preparation of controls

AlleleSEQR HLA-DRB1 SBT

DNA from samples whose genoptypes have been previously confirmed by DNA sequence analysis are used as positive controls in this assay. The control DNA is stored in a 1.5ml microcentrifuge tube and stored at a concentration of 20ng/µl Two µl of control DNA is used in each PCR reaction. The control DNA is labeled with a unique identification number, and a number indicating the date and operator who prepared the sample. The control DNA is stored at -20C in a "clean" laboratory in which no amplified DNA is present.

HLA-DRB SSP-PCR/Sequencing

DNA from samples whose genoptypes have been previously confirmed by DNA sequencing and sequencing analysis are used as positive controls in this assay. The control DNA is stored in a 1.5ml microcentrifuge tube and stored at a concentration of ~200ng/µl. Two µl of control DNA is used in each set of PCR reactions. The control DNA is labeled with a unique identification number, and a number indicating the date and operator who prepared the sample. The control DNA is stored at -20C in a "clean" laboratory in which no amplified DNA is present.

Human Identification

Control DNA of a known Genotype and a Green I Allelic Ladder are supplied in the AmpF*l* STR Green I PCR Amplification Kit at a set concentration. The control DNA is stored at 4C in a "clean" laboratory and the Allelic Ladder is stored at 4C in a "dirty" laboratory where amplified DNA is in use.

Identification of Amelogenin and TH01 Markers

An in house control DNA of a known genotype and an in house allelic ladder are prepared at a set concentration. The in house control DNA is stored at -20C in a "clean" laboratory and the in house allelic ladder is stored at 4C in a "dirty" laboratory where amplified DNA is in use.

11. Remedial Action if calibration or QC systems fail to meet acceptable criteria.

There are several potential possibilities in a failed test. To determine the cause of failure the analyst and the supervisor must use their scientific knowledge in solving the problem. All the pre-PCR reagents should be kept in small aliquots, and the preparatory area should be kept clean at all times. Positive displacement pipettes or pipette tips that contain a fiber plug are used to decrease risk of contamination. Gloves are changed frequently, and analysts never work with amplified DNA before working with genomic DNA samples.

AlleleSEQR HLA-DRB1 SBT

1. If any of the control DNA samples give an unexpected genotype, repeat the experiment.
2. If a given sample fails to sequence, repeat the test on that sample in the next run. If again no sequence is seen, re-isolate the DNA from either cryopreserved cells or the immortalized cell line.
3. If a sample fails to amplify, it is likely due to one of the following reasons: (1) incorrect thermal cycler program, (2) interruption during the PCR run, (3) an error in the PCR reaction mixture (i.e., failure to add key component to tubes).

HLA-DRB SSP-PCR/Sequencing

1. If more than 2 bands in the SSP-PCR reactions for DRB1 turns out to be positive, the results of all reactions are disregarded due to possible contamination. Carefully clean the PCR preparatory space and repeat the experiment.
2. If any of the control DNA samples give an unexpected genotype, repeat the experiment.
3. If a given sample fails to amplify, repeat the test on that sample in the next run. If again no amplification is seen, recheck/reread the DNA concentration and repeat the test. If the sample still fails, re-isolate the DNA from the immortalized cell line.
4. If all samples including the ALL control tube fails to amplify, it is likely due to one of the following reasons: (1) incorrect thermal cycler program, (2) interruption during the PCR run, (3) an error in the PCR reaction mixture (i.e., failure to add key component to tubes).

Human Identification

1. If less than 4 or more than 8 peaks appear on the electropherogram, repeat the fragment analysis. If the fragment analysis appears to be the same, repeat the experiment from the beginning.
2. If the GeneScan-350 ROX Internal Lane standard does not show up on the electropherogram, repeat the fragment analysis.
3. If there is no/low green signal compared to the internal lane standard on the electropherogram, check the concentration of the DNA again and repeat the experiment.

Identification of the Amelogenin and TH01 Markers

1. If less than 2 or more than 4 peaks appear on the electropherogram, repeat the fragment analysis. If the fragment analysis appears to be the same, repeat the experiment from the beginning.
2. If the GeneScan-350 ROX Internal Lane standard does not show up on the electropherogram, repeat the fragment analysis.
3. If there is no/low blue signal compared to the internal lane standard on the electropherogram, check the concentration of the DNA again and repeat the experiment.

12. Limitations of Method; interfering substances and conditions

This method is not labor-intensive as compared to other non-automated methods using manual sequencing and analysis. However, it requires expensive instrumentation and thus is not widely used. Adequate precautions must be taken to prevent the introduction of foreign DNA into the PCR reactions.
The following guidelines should be followed:

Wear a clean laboratory coat and fresh gloves when preparing samples or reagents for PCR amplification. Changing gloves often is instrumental in preventing cross contamination and should be done often. Open and close all sample tubes carefully top avoid reagent or sample splashes. Use positive displacement or air-displacement pipettors with filter-plugged tips. Change tips after each use.

13. Reference Ranges (Normal Values)

Type 1 diabetes mellitus is a chronic autoimmune disease that involves a T cell-mediated destruction of the pancreatic beta cells, the body's sole source for insulin (1). Type 1 diabetes has been shown to involve a genetic component and an environmental component (4). The genes that are known to play a role in the genetic susceptibility include those in the Human Leukocyte Antigen (HLA) complex on chromosome 6p21, and the insulin gene on chromosome 11p15(5).

The role of the HLA region in type 1 diabetes was discovered in the 1970s by both association studies and affect-sib-pair studies (6-8). The DR3 and DR4 haplotypes, which consist of specific combinations of the class II genes DQA1, DQB1 and DRB1, have been implicated in disease susceptibility, and the DR2 haplotype has been

associated with disease protection as determined by serotyping (9-12). These class II HLA genes are highly polymorphic and molecular genetic analysis has identified certain alleles with protection or susceptibility to type 1 diabetes (12-26). The assay described below involves genotyping the HLA DRB1 gene. Various genotypes of the DRB1 gene are present within the population. There are 304 known alleles for the DRB1 gene as of January 2001(30). The frequencies for a number of these 304 alleles within various populations can be found in the HLA Facts Book (31).

14. Critical Call Results ("Panic Values")

Not applicable in this particular assay.

15. Specimen storage and Handling during testing

The blood specimens will arrive at the laboratory, partially processed, in 50ml Falcon tubes. The partially processed blood is at the cell lysis stage of the Puregene protocol and are stable for 18 months at room temperature(see appendix A). Fully precessed DNA can be stored at -20C indefinitely. Prior to testing, thaw DNA completely to room temperature for 10-30 minutes.

16. Alternative methods for performing test or storing specimens if test system fails

When a test fails, it is usually due to one reasons mentioned previously in item 10. If the automated sequencer fails prior to a run, use another instrument that is located in Biotechnology Core Facility, Scientific Resource Program, or CDC, and have the failed one repaired immediately by the manufacturer. If the instrument fails during a run, the entire test must be repeated promptly.

17. Test Result Reporting Systems; Protocol for reporting critical calls (if applicable)

Each allele is reported according to standard HLA nomenclature (30). Results are proofread and entered into a common database by the analyst, and given to the supervisor to review. After review of run data, supervisor forwards the final report to the Molecular Biology Branch Chief and DLS division director for final approval. After approval, report is forwarded to requestor. Critical calls are not applicable.

18. Transfer or Referral of Specimens; Procedures for Specimen Accountability and Tracking

Standard record keeping means (including the use of Excel and/or Access database software) should be used to track specimens. It is recommended that records be maintained for 2 years, including related QC data and that duplicate records should be kept in electronic or hard copy format. Only numerical identifiers (patient/participant ID#) will be available.

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Appendix A

Puregene Method for DNA Isolation from Whole Blood for PCR Using the Puregene Genomic DNA Isolation Kit

Materials

Puregene DNA Extraction Kit, catalog # D-50K, Gentra Systems, Minneapolis, MN
70% ethanol
100% isopropanol
glycogen, Gentra Systems
50 ml Falcon centrifuge tubes
Rainin pipet tips with filters, Rainin Instrument Co., Emeryville, CA
Sterile, individually wrapped transfer pipets
Racks for 50ml centrifuge tubes (bleach after each use)

Equipment

PipetAid, Drummond, Daigger, Lincolnshire, IL
Qiagen Sigma centrifuge, Qiagen Inc, Chatsworth, CA
Dispensett III volume dispenser for reagents, Daigger, Lincolnshire, IL
Incubator
Boekel Orbital Rocker, Boekel Scientific Inc., Feasterville, PA

Labeling

Label all reagents and aliquots of reagents with the reagent name, concentration, date prepared and appropriate expiration date. Labels are created using computer label making system Label View Pro and Eltron printer.

Preparation of reagents:

NOTE: prepare all reagents and aliquots in clean lab, also be sure to record date reagents opened.

Purification Protocol

Record ID of samples to be extracted and assign a temporary ID number to each (for example 1-10). Label all processing tubes and columns with the temporary ID number.

NOTE: use universal precautions when working with blood, and perform all steps in a biological safety cabinet to avoid contamination or exposure to biological agents within the blood.

A. Cell Lysis

1. Label 50mL falcon tubes appropriately and fill each with 30mL *Red Blood Cell Lysis* solution(RBC).
2. Add 10mL whole blood to appropriately labeled falcon tube containing the *RBC lysis solution.*
3. Invert to mix, and incubate 10 minutes at room temperature. Invert at least once during incubation.
4. Centrifuge at 2,000 x g for 10 minutes.
5. Remove supernatant leaving behind white pellet and about 200-400 ml of liquid.
6. Vortex tube to resuspend cells.
7. Add 10mL *Cell Lysis Solution* to cells and pipet up and down to lyse cells. Usually no incubation is required, however, if cell clumps are visible, incubate at 37°C until solution is homogenous and no clumps are detected.

Note: samples are stable in cell lysis solution for at least 18 months at room temperature.

B. RNase Treatment

1. Add 50 ul *RNase A* Solution to cell lysate solution in falcon tube.
2. Mix by inverting the tube 25 times and incubate at 37°C for 15-60 minutes.

C. Protein Precipitation

1. Cool sample to room temperature.
2. Add 3.33mL *Protein Precipitation Solution* to cell lysate solution.
3. Vortex for 20 seconds.
4. Centrifuge at 2,000 x g for 11 minutes. The precipitated proteins should form a tight, dark brown pellet. If the protein pellet is not tight, repeat step 3, followed by incubating on ice for 5 minutes and then repeat step

D. DNA Precipitation

1. Pour the supernatant containing the DNA (leaving the protein pellet behind) into a clean 50mL tube containing 10mL 100% isopropanol.
2. Add 16.7 ul glycogen solution per 10mL isopropanol to increase DNA yield.
3. Mix the sample by inverting gently 50 times until the white threads of DNA form a visible clump.
4. Centrifuge at 2,000 x g for 4 minutes.
5. Pour off supernatant and drain tube on a clean absorbent paper.
6. Add 10ml 70% ethanol and invert tube several times to wash the DNA pellet.
7. Centrifuge at 2,000 x g for 2 min. Carefully pour off ethanol.
8. Allow to air dry 10-15 minutes.

E. DNA Hydration

1. Add 1mL DNA *Hydration Solution.*
2. Rehydrate DNA by incubating at 65°C for 1 hour and place on Boekle orbital rocker at room temperatutre for seven days. Tap tube periodically to aid in dispersing DNA.
3. For storage, sample may be centrifuged briefly and then transferred to a 1.5ml tube.

Store at 4°C, or at –20°C for long-term storage of DNA

Puregene Method for DNA Isolation from Cell Culture for PCR
Using the Puregene Genomic DNA Isolation Kit

Materials
Puregene DNA Extraction Kit , catalog # D-50K, (Gentra Systems, Minneapolis),
70% ethanol
100% isopropanol
50 ml Falcon centrifuge tubes
Ranin pipette tips with filters, (Rainin Instrument Co., Emeryville, CA)
Sterile, individually wrapped transfer pipettes
Racks for centrifuge tubes and blood tubes (bleach after each use)

Equipment
PipetAid, Drummond, Daigger, Lincolnshire,
Qiagen Sigma centrifuge, Sigma Co., St. Louis,
Dispensett III volume dispenser for reagents, Daigger, Lincolnshire, IL
Boekel Orbital Rocker (Boekel Scientific, Inc.)

Labeling
Label all reagents and aliquots of reagents with the reagent name, concentration, date prepared and appropriate expiration date. Labels are created using a computer label making system Label View Pro and an Eltron printer.

Preparation of reagents:
NOTE: prepare all reagents and aliquots in clean lab and record the date reagents were opened.

Purification Protocol

Record the ID of samples to be extracted and assign a temporary ID number to each (for example 1-10). Label all processing tubes and columns with the temporary ID number.

NOTE: use universal precautions when working with blood, and perform all steps in a biological safety cabinet to avoid contamination or exposure to biological agents within the blood.

NOTE: for appropriate amounts of reagents see table after section E.

A. **Cell Lysis**

1. Add appropriate cell volume to a 50 ml Falcon tube.
2. Spin in a centrifuge at 2,000 x g for 5 minutes.
3. Remove the supernatant, leaving behind the white pellet and a small volume of liquid.
4. Vortex each tube to resuspend cells.
5. Add appropriate volume of the *Cell Lysis Solution* to the cells and pipette up and down to lyse the cells. Incubation is usually not required, however, if cell clumps are visible, incubate at 37°C until the solution is homogenous and no clumps are detected.

NOTE: The samples are stable in the cell lysis solution for at least 18 months at room temperature.

B. **RNase Treatment**

1. Add appropriate volume of RNase*A Solution* to the cell lysate solutions in the Falcon tubes.
2. Mix by inverting the tube 25 times and then incubate at 37°C for 60 minutes.

C. Protein Precipitation

1. Cool samples at room temperature.
2. Add appropriate volume of the *Protein Precipitation Solution* to the cell lysate solutions.
3. Vortex for 20 seconds.
4. Centrifuge at 2,000 x g for 12 minutes. The precipitated proteins should form a tight, dark brown pellet. If the protein pellet is diffuse, repeat step 3, followed by incubation on ice for 5 minutes and then repeat step 4.

D. DNA Precipitation

1. Pour the supernatant containing the DNA (leaving the protein pellet behind) into a clean 50 ml tube containing appropriate volume of 100% isopropanol.
2. Add 16.7 ul of glycogen solution per 10 ml of isopropanol to increase the DNA yield.
3. Mix the sample by inverting the tubes gently 50 times until the white threads of DNA form a visible clump.
4. Centrifuge at 2,000 x g for 20 minutes.
5. Pour off supernatant and drain the tubes on a clean absorbent paper.
6. Add appropriate volume of 70% ethanol and invert the tubes several times to wash the pellet.
7. Centrifuge at 2,000 x g for 12 minutes. Carefully pour off the ethanol.
8. Allow to air dry for 10-15 minutes.

E. **DNA Hydration**

1. Add appropriate volume of *DNA Hydration Solution*.
2. Rehydrate DNA by incubating at 65°C for 1 hour and place on Boekel orbital rocker for 7 days at room temperature.
3. For storage, samples may be centrifuged briefly and then transferred to an appropriate tube.

Store the DNA samples at 4°C, or at -20°C for long-term storage.

Number Cells	100-10,000	0.5-1.0 Million	3-5 Million	30-50 Million	60-90 Million	100 Million
Cell Lysis (ml)	0.06	0.15	0.6	6.0	10	15
Rnase A (ul)	0.50	0.75	3.0	30	50	70
Protein Precipitation (ml)	0.02	0.033	0.20	2.0	3.3	5.0
100% Isopropanol (ml)	0.06	0.15	0.6	6.0	10	15
70% Ethanol (ml)	0.06	0.15	0.6	6.0	10	15
DNA Hydration (ul)	10	10	60	500	750	1000

Appendix B

Human Identification with the Short Tandem Repeat Loci using the AmpF*l*STR Green I PCR Amplification Kit.
(This method will be used for Quality Control for Trio samples)

Materials
AmpFlSTR Green I PCR Amplification Kit, catalog # 402902, Applied Biosystems, Foster City, CA
AmpF*l* STR Green I PCR Amplification Kit User's Manual, catalog # 402944, Applied Biosystems
Performance Optimized Polymer 4 (POP-4), catalog # 402838, Applied Biosystems
MicroAmp 8-Strip Reaction Tubes(0.2ml), catalog #N801-0580, Applied Biosystems
MicroAmp Caps(8 caps/strip), catalog # N801-0535, Applied Biosystems
MicroAMp 96-Well Tray/Retainer, catalog # 403081, Applied Biosystems
0.5ml Genetic Analyzer sample tubes and septum, catalog # 401957 and 401956, Applied Biosystems
Rainin pipet tips, Rainin, Emeryville, CA
1.5ml microfuge tubes, Marsh Biomedical Products, Rochester, NY
1xTE(10mM Tris-HCl, 0.1mM EDTA, pH 8.0)
Hi Di formamide, catalolg # 4311320, Applied Biosystems
GeneScan-350[ROX] Internal Lane Size Standard, catalog # 401735, Applied Biosystems, Foster City, CA
10x Genetic Analyzer Buffer with EDTA, catalog # 402824, Applied Biosystems
Performance Optimized Polymer-4 (POP-4), Applied Biosystems

Equipment
Geneamp PCR System 9700, Applied Biosystems, Foster City, CA
ABI PRISM 310 DNA sequencer, Applied Biosystems
Rainin pipettors, Rainin, Emeryville, CA
Stratalinker 2400 UV Crosslinker, Stratagene, La Jolla, CA
Vortex Genie, Diagger, Lincolnshire, IL
Heating block
Computer and software for analysis with GeneScan and Genotyper Software, Applied Biosystems,

Labeling

Label all reagents and aliquots of reagents with the reagent name, concentration, date prepared and appropriate expiration date. Labels are created using computer label making system Label View Pro and Eltron printer.

PCR Amplification

1. Record ID of samples to be typed and assign an internal ID number to each sample.
2. Label all processing MicroAmp 8-Strip Reaction Tubes (0.2ml tubes) with the internal ID number and place them into a MicroAmp 96-Well Tray/Retainer. There will be a positive and negative control tube as well.
3. Place the MicroAmp tubes/Tray and a 1.5ml microcentrifuge tube into the Stratalinker (Stratagene) and UV crosslink twice at 120 joules(1200 on LED display) to sterilize the tubes prior to use to avoid contamination.
4. Make a master mix of the following contents in a 1.5ml microcentrifuge tube. All reagents are supplied in the AmpFlSTR Green I PCR Amplification Kit
   number of samples x 10.5 ul of AmpF*l*STR PCR reaction Mix
   number of samples x 0.5 ul of AmpliTaq Gold DNA Polymerase
   number of samples x 5.5 ul of *AmpFlSTR* Green I Primer.
   number of samples x 9.9 ul of deionized water
5. Mix by vortexing.
6. Dispense 24 ul of the master mix into each of the MicroAmp Reaction Tubes.

7. To each of the tubes containing master mix, pipet 1 ul of Genomic DNA at a concentration of 25 ng/ul. For the Positive Control tube, add 1 ul(25ng) of Control DNA to the tube and for the Negative control tube, add 1 ul of 1xTE buffer. Note: The final volume for the PCR is 25ul.
8. Place the MicroAmp Caps on the tubes and seal tightly.
9. Place the Tubes in the thermal cycler, Geneamp PCR System 9700, and program the following conditions into the machine and start the run under the reaction volume of 25ul(refer to the Geneamp PCR System 9700 Users Manual for details).

   1 cycle @ 95C-11min,
   27 cycles @ 94C-1min, 59C-1min, 72C-1min,
   1 cycle @ 60C-45min

   hold at 25C (store the amplified products protected from light at 2-6C for short periods and at –15 to –25C for longer periods.)

Preparing samples for Genescan using the 310 Genetic Analyzer

1. Clean the machine out and prepare the 310 Genetic Analyzer for running GeneScan using Performance Optimized Polymer 4 (POP-4). Follow instructions described in the ABI PRISM 310 Genetic Analyzer User's Manual.
2. Open a new GeneScan sample sheet in the 310 Data Collection Software. Fill in the sample names and mark the red box as the standard. Save the sample sheet.
3. Open up a new injection list and open the sample sheet that was just created and select Genescan-350-ROX as the internal lane standard.
4. Calculate and combine the necessary amounts of Hi Di formamide and GeneScan-350 [ROX] Internal Lane Standard in a 1.5 ml microcentrifuge tube. (Remember to include in the sample number the positive control and a Green I Allelic Ladder that is supplied in the kit)
   (Number of samples + 2) x 24 ul of Hi Di formamide
   (Number of samples + 2) x 1.0 ul of genescan-350[ROX] size standard
5. Aliquot 25 ul of the Hi Di formamide/genescan-350[ROX] mixture into 0.5ml Genetic Analyzer tubes.
6. Add 1.0ul of *AmpFl* STR Green I PCR Product or 1.0 ul of AmpF*l* STR Green I Allelic Ladder per tube and mix by pipetting up and down.
7. Seal each tube with a septum.
8. Denature each sample at 95C for 3 minutes and chill the tubes for 3 minutes in an ice water bath.
9. Place the tubes in the sampler tray of the 310 Genetic Analyzer and start the GeneScan run.
10. The GeneScan data is analyzed by the Genotyper Software and can be incorporated directly into a database.

Appendix C

Identification of the Amelogenin(sex) and TH01 Markers
(This method will be used as Quality Control for samples other than Trio samples)

Materials

HotStarTaq Master Mix Kit, catalog # 203443, Qiagen, Valencia, CA
MicroAmp 8-Strip Reaction Tubes(0.2ml), catalog #N801-0580, Applied Biosystems
MicroAmp Caps(8 caps/strip), catalog # N801-0535, Applied Biosystems
MicroAMp 96-Well Tray/Retainer, catalog # 403081, Applied Biosystems
Rainin pipet tips, Rainin, Emeryville, CA
1.5ml microfuge tubes, Marsh Biomedical Products, Rochester, NY
10X TBE(Trizma Base, Boric Acid, EDTA) for Sequencing
GeneScan-350[ROX] Internal Lane Size Standard, catalog # 401735, Applied Biosystems
Hi Di Formamide, catalog # 4311320, Applied Biosystems
blue dextran/EDTA loading dye, Appled Biosystems
Long Ranger Singel Packs, catalog #50691, FMC BioProducts, Rockland, ME
beakers
deionized water
The following oligonucleotides (Obtained from the CDC Biotechnology Core Facility):
    FAM-AmeloF primer (5'-FAM-CCCTGGGCTCTGTAAAGAATAGTG-3')
    AmeloR primer (5'-ATCAGAGCTTAAACTGGGAAGCTG-3')
    TH01F primer (5'-ATTCAAAGGGTATCTGGGCTCTGG-3')
    FAM-TH01R primer (5'-FAM-GTGGGCTGAAAAGCTCCCGATTAT-3')

Equipment
Geneamp PCR System 9700, Applied Biosystems, Foster City, CA
ABI PRISM 377 DNA sequencer, Applied Biosystems
Rainin pipettors, Rainin, Emeryville, CA
Stratalinker 2400 UV Crosslinker, Stratagene, La Jolla, CA
Vortex Genie, Diagger, Lincolnshire, IL
Heating block
Computer and software for analysis with GeneScan and Genotyper Software, Applied Biosystems,

Labeling
Label all reagents and aliquots of reagents with the reagent name, concentration, date prepared and appropriate expiration date. Labels are created using computer label making system Label View Pro and Eltron printer.

Reagent Preparation
10X TBE for sequencing:

Final concentration	grams/L
890mM Tris Base (Trizma Base)	108g
890mM Boric Acid	55g
20mM Disodium EDTA	7.44g

Add deionized water to a final volume of 1000mL, mix thoroughly and filter through $\leq$ 0.45mm membrane.
Store at room temperature and do not use if precipitate forms.
For 1X TBE for sequencing, dilute 150mL of the
10X TBE stock and bring the volume up to 1.5L.

PCR Amplification

1. Record ID of samples to be typed and assign an internal ID number to each sample.
2. Label all processing MicroAmp 8-Strip Reaction Tubes(0.2ml tubes) with the internal ID number and place them into a MicroAmp 96-Well Tray/Retainer. There will be a positive and negative control tubes as well.

3. Place the MicroAmp tubes/Tray and a 1.5ml microcentrifuge tube into the Stratalinker (Stratagene) and UV crosslink twice at 120 joules (1200 on LED display) to sterilize the tubes prior to use to avoid contamination.
4. Make a master mix of the following content in a 1.5ml microcentrifuge tube.
   # of samples x 12.5 ul of HotStarTaq Master Mix
   # of samples x 1.0 ul of FAM-AmeloF primer(3.6 pmol/ul)
   # of samples x 1.0 ul of AmeloR primer(3.6 pmol/ul)
   # of samples x 1.0 ul of TH01F primer(8 pmol/ul)
   # of samples x 1.0 ul of FAM-TH01R primer(8 pmol/ul)
   # of samples x 7.5 ul of water
5. Mix by Vortexing
6. Dispense 24 ul of the master mix into each of the MicroAmp Reaction Tubes.
7. To each of the tubes containing master mix, pipet 1 ul of Genomic DNA of a 25ng/ul concentration.
8. To the Positive Control tube, add 1 ul of the selected control and to the Negative control tube, add 1 ul of water. Note: The final volume for the PCR is 25 ul.
9. Place the MicroAmp Caps on the tubes and seal tightly.
10. Place the Tubes in the thermal cycler, Geneamp PCR System 9700, and program the following conditions into the machine and start the run under the reaction volume of 25ul (refer to the Geneamp PCR System 9700 Users Manual for details).
    1 cycle @ 95C-10min,
    27 cycles @ 94C-45sec, 60C-45sec, 72C-1min,
    1 cycle @ 60C-45min
    hold at 4C (store the amplified products protected from light at 2-6C for short periods and at –15 to –25C for longer periods.)

GeneScan using the 377 DNA Sequencer

A. Gel Preparation and casting using the Long Ranger Singel Pack.
i. Gel Preparation
1. Assemble glass plates and spacers in the cassette following the method described in the ABI PRISM 377 DNA Sequencer Users Manual.
2. Have the Long Ranger Singel pack at room temperature.
3. Remove the BLACK clip and mix the contents of the compartments by hand thoroughly but gently for 1 minute.
4. Place the pack on an orbital shaker for 5 minutes at medium speed.
5. Mix by hand thoroughly but gently for 1 minute
6. Place the pack on an orbital shaker for 5 minutes at medium speed.

NOTE: Do not over mix. This may interfere with gel polymerization.

ii. Gel Casting
NOTE: The following steps must be completed without delay.
1. Remove only the RED clip and mix the contents of the compartment well by hand for 1 minute.
2. Remove the WHITE clip to expose the filter to gel solution.
3. Hold the pack so the contents drain into the filter end. Fold the pack in half at the indicated line.
4. Hold the pack with the cut mark at the top and cut the corner within the space marked CUT. To avoid introducing bubbles cut a large enough hole in the pouch to allow steady flow of the solution through the filter into a beaker.
5. Avoid introducing air into solution after mixing. Cast gel and insert comb according to your standard procedure.
6. Once the gel is polymerized (30 minutes), place paper towels soaked in electrophoresis buffer over the ends of the plates and then cover with plastic wrap. This will prevent moisture loss as the polymerization process continues.
7. Allow 2 hours for complete gel polymerization.

B. Preparing for Electrophoresis

1. Remove the comb, wash the plates and load the comb as described in the ABI PRISM 377 DNA Sequencer Users Manual.
2. Prepare a sufficient quantity of electrophoresis buffer to fill both anodal and cathodal chambers by diluting 10X TBE stock for sequencing with deionizd water to 1X.
3. Mount the gel cassette onto the sequencing apparatus and prepare the gel for the sequence run according to the 377 DNA Sequencers Users Manuals instructions.
4. Open a new GeneScan sample sheet in the 377-96 Data Collection Software, and input sample names to be run on the gel.
5. Save the sample sheet and open a new GeneScan sample run in the 377-96 Data Collection Software and open the new sample sheet that was just created in the previous step.
6. To assure plates and gel are clean, perform a plate check using the Plate Check module.
7. Pre-warm the acrylamide gel by running the GS PR 36A-2400 module.
8. Prepare the samples for the GeneScan run by combining 1.5 ul of PCR product and 1.0 ul of GeneScan Rox[350] with 5 ul of a 5:1 ratio of Hi Di formamide and blue dextran/EDTA loading dye(for example 5ul of the Hi Di formamide combined with 1ul of the blue dextran/EDTA loading dye).
9. Vortex the samples and centrifuge briefly.
10. Denature the samples by heating the samples at 95± 5C for 2 minutes.
11. Ice the samples immediately for 2 minutes and keep on ice until ready to use.
12. Stop the PRE-RUN when the temperature reaches 50C and rinse out the top of the gel with 1XTBE buffer.
13. Load 1.8ul of the denatured samples on the gel. The odd lanes should be loaded first then run in for 1 minute before the even lanes are loaded.
14. Cancel the PRE-RUN and change the module to the GS Run 36A-2400 module and start the run. The run will take 2.5 hours.
15. Analyze the Results using the GeneScan Analysis and Genotyper Software.

Appendix D:
Sequence-Based typing of HLA-DRB1 using the AlleleSEQR HLA-DRB1 Sequence-Based Typing(SBT) Kit

<u>Materials</u>
AlleleSEQR HLA-DRB1 Sequence-Based Typing Kit,catalog #R7000, Forensic Analytical, Hayward, CA
Group Specific Sequencing Primers(Gr3 and G4), Forensic Analytical, Hayward, CA
1.5 and 0.5 ml microfuge tubes, Marsh Biomedical Products Inc., Rochester, NY
MicroAmp 8-Strip Reaction Tubes(0.2ml), catalog # N801-0580, Applied Biosystems
MicroAmp Caps(8 caps/strip), catalog # N801-0535, Applied Biosystems
MicroAmp 96-Well Tray/Retainer Sets, catalog # 403081, Applied Biosystems
MicroAmp Full Plate Cover, catalog # N801-0500, Applied Biosystems
Rainin pipett tips, Rainin, Emeryville, CA
MicroAmp 96-well reaction plate, catalog # N8010560, Applied Biosystems
96-well plate septa, catalog # 4315933, Applied Biosystems
deionized water
Hi Di Formamide, catalog # 4311320, Applied Biosystems
10x Genetic Analyzer Buffer with EDTA,catalog # 402824, Applied Biosystems
3100 POP-6 polymer, catalog # 4316357, Applied Biosystems
Absolute ethanol
80% ethanol

<u>Equipment</u>
Finnpette Biocontrol Pipettor with Multi-channel module, Lab Systems through Marsh Biomedical Products Inc.
Geneamp PCR System 9700, Applied Biosystems
ABI PRISM 3100 Genetic Analyzer, Applied Biosystems
Stratalinker 2400 UV Crosslinker, Stratagene, La Jolla, CA
Rainin pipettors, Rainin, Emeryville, CA
Balance
Computer software for analysis with Sequencing Analysis, MatchTools, and MTNavigator, Applied Biosystems
IEC Multi Centrifuge with Double Deep Microplate Rotor, Forma Scientific Inc., Marietta, OH
Vortexer Genie, Diagger, Licolnshire, IL

<u>Labeling</u>
Label all reagents and aliquots of reagents with the reagent name, concentration, date prepared and appropriate expiration date. Labels are created using computer label making system Label View Pro and Eltron printer.

Procedures
A. General PCR practices:
1. Wear a new disposable laboratory coat and new gloves when preparing samples or reagents for PCR amplification.
2. Change gloves frequently.
3. Maintain separate areas and dedicated equipment and supplies for sample preparation, PCR setup and amplification/analysis.
4. Open and close all sample tubes carefully to avoid reagent or sample splashes.
5. Use air-displacement pipettors with filter-plugged tips. Change tips after each use.
6. Clean the general area using 10% bleach solution and rinse with deionized water. Cover lab benches with clean sheet of disposable absorbent pad and remove at the end of each day.

B. Reagent Preparation

3M Sodium Acetate(pH 4.6)

Final concentration	grams/500mL
3M Sodium Acetate	204.12 grams

Adjust the pH of the solution to pH4.6 then bring up the volume to 500mL and filter through a < 0.45um membrane.

C. PCR Amplification

1. Record ID of samples to be typed and assign an internal ID number to each sample (there will be one reaction per sample being typed).
2. Label all processing MicroAmp 8-Strip Reaction Tubes (0.2ml tubes) with the internal ID number and place them into a MicroAmp 96-Well Tray/Retainer. Place the MicroAmp tubes/tray and 0.5ml microcentrifuge tubes to be used into the
3. Stratalinker (Stratagene) and UV crosslink twice at 120 joules (1200 on LED display) to sterilize the tubes prior to use to avoid contamination.
4. Calculate the number of reactions to be typed including the controls and prepare the PCR/Taq Mix according to the table below.

Number of Reactions	Volume of PCR Pre-Mix	Volume of AmpliTaq Gold
10	80 ul	1 ul
20	160 ul	2 ul
50	400 ul	5 ul

5. To each of the strip-reaction tubes, add 8 ul of PCR/Taq Mix.
6. Add 2 ul of sterile water to the negative control tube and to the remaining reactions, add 2 ul of Genomic DNA(20 ng/ul) to each reaction tube.
7. Place the MicroAmp Caps on the tubes and seal tightly.
8. Place the tubes in the thermal cycler, Geneamp PCR System 9700, and program the following conditions into the machine and start the run. (refer to the Geneamp PCR System 9700 Users Manual for details).

   1 cycle @ 95C for 10 min
   36 cycles @ 96C for 20 sec
   60C for 30 sec
   72C for 3 min
   Hold at 4C (after amplification store PCR products at 2-6C until needed)

D. Purifying the PCR Amplicons(Exo/Sap)

1. Prepare a fresh 1:1 mixture of Exonuclease I(Exo) and Shrimp Alkaline Phosphatase(Sap). You will need 0.5ul of each enzyme per reaction(Exo and Sap are provided in the AlleleSEQR HLA-DRB1 SBT kit)
2. Add 1 ul of the Exo/Sap mixture to each of the reaction tubes.
3. Cover the tubes with strip-caps and place them in the thermal cycler, Geneamp PCR system 9700, and program the following conditions into the machine and start the run.

   37C for 15 min
   80C for 15min
   Hold at 4C until ready to use

E. Cycle Sequencing

1. Dilute each PCR amplicon by adding 2 volumes of sterile water to 1 volume PCR product in a separate tube.
2. Label the MicroAmp 96-well reaction plate with internal ID numbers. There will be a Forward (F), Reverse (R), and Codon 86 reaction tube per PCR amplicon.
3. To the F tube for each sample, add 8ul of the Forward sequencing mix.
   To the R tube for each sample, add 8ul of the Reverse sequencing mix
   To the Codon 86 tube for each sample, add 8 ul of the Codon 86 sequencing mix
   (All mixes are supplied in the AlleleSEQR HLA-DRB1 SBT kit).
4. To each of the forward, reverse, and codon 86 tubes per sample, add 2ul of the appropriate diluted Exo/Sap treated PCR product and mix well.

5. Seal the tubes with the MicroAmp Strip caps tightly.
6. Place the tubes/tray in the thermal cycler, GeneAmp PCR system 9700, and run under the following conditions: (refer to the GeneAmp PCR system 9700 Users Manual for details)
   25 cycles @ 96C for 20 sec
   60C for 2 min
   hold at 4C until ready to proceed.

F. Clean up of Cycle Sequencing Reaction
1. Add 1ul of NaOAc/EDTA buffer included in the AlleleSEQR HLA-DRB SBT kit to each sequencing reaction.
2. Prepare a mixture of EtOH/NaOAc by adding 20 ul of 3M NaOAc (pH4.6) per mL of absolute ethanol (1 mL of this mixture is sufficient for 40 reactions).
3. Add 25 ul of EtOH/NaOAc prepared in step 2 to each sequencing reaction.
4. Vortex the reaction tubes well. Incomplete mixing will result in poor quality sequence data.
5. Centrifuge at 2000 x g for 30 minutes.
6. Remove the supernatant by inverting the tray onto a paper towel and centrifuging at 500 x g for 30 seconds.
7. Add 50 ul of 80% EtOH to each of the sequencing reactions..
8. Centrifuge at 2000 x g for 5 minutes.
9. Remove the supernatant by inverting the tray onto a paper towel and centrifuging at 500 x g for 30 seconds.
10. Store the reactions in the freezer if you are not going to proceed with sequencing at this time.

Sequencing using the 3100 Genetic Analyzer

Preparing and Loading Samples on the 3100 Genetic Analyzer
1. Resuspend the cycle sequencing reactions in 20ul of HiDi Formamide in the MicroAmp 96-well reaction plate and cover the plate with a MicroAmp Full Plate Cover.
2. Heat samples in the Geneamp PCR System 9700 thermal cycler at 95°C for 3 minutes then remove and chill on ice for 2 minutes.
3. Replace the Plate Cover with the 96-well plate septa.
4. Create a new “sample sheet” in the ABI PRISM 3100 sequencing software and appropriately label all samples according to the method described in the ABI PRISM 3100 Genetic Analyzer ‘s Users Manual. Use the filter set that corresponds to the ET terminators for the run.
5. Place the sample tray into the 3100 Genetic Analyzer.
6. Link the sample sheet to the corresponding plate.
7. Run the 3100 Genetic Analyzer according to manufacture’s instructions.
8. Analyze.
   *The sequence can also be run on the ABI 310 Genetic Analyzer or the ABI 377 DNA Sequencer.

Analysis of data from the 3100 Genetic Analyzer
1. Once the run is complete, analyze the sequence data using the Sequence Analysis software with the DT3700POP6{ET}mob file
2. Determine the genotype from the sequence data using the MatchTools and MTNavigator Software.
3. Input all data into the database.
   (See the user’s manual for the 3100 Genetic Analyzer, 3100 Collection Software, Sequence Analysis Software, MatchTools and MTNavigator software for operation and usage of the 3100 Genetic Analyzer and the software.)

Group Specific Sequencing (GSS) following Sequence Based Typing of HLA-DRB1
(The Group Specific Sequencing protocol is used to clarify a subset of genotypic ambiguities that remain following analysis of the Allele SEQR HLA-DRB1 Sequence Based Typing kit)

Note: In the Group Specific Sequencing Protocol the GSS Gr3 group will sequence most of the *03, *11, *13, and *14 alleles and the GSS Gr4 group will sequence most of the *04 alleles. If there is a combination of 2 alleles in the same subgroup, this protocol will not clarify either of those ambiguities.

Cycle Sequencing

1. Dilute the PCR amplicon to be sequenced by adding 2 volumes of sterile water to 1 volume PCR product in a separate tube.
2. Label the MicroAmp 96-well reaction plate with internal ID numbers. Add 8ul of the appropriate GSS mix to the wells.
3. Add 2 ul of the appropriate diluted Exo/Sap treated PCR product and mix well.
4. Seal the tubes with the MicroAmp Strip caps tightly.
   Place the tubes/tray in the thermal cycler, GeneAmp PCR system 9700, and run under the following conditions: (refer to the GeneAmp PCR system 9700 Users Manual for details)
   25 cycles @ 96C for 20 sec
   60C for 2 min
   hold at 4C until ready to proceed

Clean up of Cycle Sequencing Reaction

1. Add 1ul of NaOAc/EDTA buffer included in the AlleleSEQR HLA-DRB SBT kit to each sequencing reaction.
2. Prepare a mixture of EtOH/NaOAc by adding 20 ul of 3M NaOAc (pH4.6) per mL of absolute ethanol (1 mL of this mixture is sufficient for 40 reactions).
3. Add 25 ul of EtOH/NaOAc prepared in step 2 to each sequencing reaction.
4. Vortex the reaction tubes well. Incomplete mixing will result in poor quality sequence data.
5. Centrifuge at 2000 x g for 30 minutes.
6. Remove the supernatant by inverting the tray onto a paper towel and centrifuging at 500 x g for 30 seconds.
7. Add 50 ul of 80% EtOH to each of the sequencing reactions..
8. Centrifuge at 2000 x g for 5 minutes.
9. Remove the supernatant by inverting the tray onto a paper towel and centrifuging at 500 x g for 30 seconds.
10. Store the reactions in the freezer if you are not going to proceed with sequencing at this time.

Sequencing using the 3100 Genetic Analyzer

See the previous page for instructions on preparing and loading samples on the 3100 Genetic Analyzer

Analysis of data from the 3100 Genetic Analyzer

See the previous page for instructions on analyzing data obtained from the 3100 Genetic Analyzer

Appendix E:

Genotyping of HLA-DRB1 using the HLA-DRB BigDye Terminator Sequencing-Based Typing Kit

Materials

HLA-DRB BigDye Terminator Sequencing-Based Typing Kit, catalog # 4305213, Applied Biosystems
1.5 and 0.5 ml microfuge tubes, Marsh Biomedical Products Inc., Rochester, NY
MicroAmp 8-Strip Reaction Tubes(0.2ml), catalog # N801-0580, Applied Biosystems
MicroAmp Caps(8 caps/strip), catalog # N801-0535, Applied Biosystems
MicroAmp 96-Well Tray/Retainer Sets, catalog # 403081, Applied Biosystems
MicroAmp Full Plate Cover, catalog# N801-0500, Applied Biosystems
Rainin pipett tips, Rainin, Emeryville, CA
Dye Ex 96 kit, Qiagen Inc, Chatsworth, CA
Microtiter plate
MicroAmp 96-well reaction plate, catalog # N8010560, Applied Biosystems
96-well plate septa, catalog # 4315933, Applied Biosystems
1XTBE(10x TBE from Gibco/BRL, Rockville, MD)
10X TBE(Trizma Base, Boric Acid, EDTA) for Sequencing
deionized water
Orange G gel loading dye(Orange G, Ficoll 400, EDTA)
Hi Di Formamide, catalog # 4311320, Applied Biosystems
blue dextran/EDTA loading dye, Appled Biosystems
Dye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems
Ultra- pure agarose, Gibco/BRL, Rockville, MD
Long Ranger Singel Packs, catalog #50691, FMC BioProducts, Rockland, ME
Ethidium Bromide, Ameresco, Solon, OH
Low DNA Mass Ladder, cat # 10068-013, Gibco/BRL, Rockville, MD
10x Genetic Analyzer Buffer with EDTA,catalog # 402824, Applied Biosystems
3100 POP-6 polymer, catalog # 4316357, AppliedBiosystems
Performance Optimized Polymer 6 (POP-6), Applied Biosystems
flasks
beakers
microwave
plastic wrap

Equipment

Finnpette Biocontrol Pipettor with Multi-channel module, Lab Systems through Marsh Biomedical Products Inc.
Geneamp PCR System 9700, Applied Biosystems
ABI PRISM 377 DNA sequencer, Applied Biosystems
ABI PRISM 3100 Genetic Analyzer, Applied Biosystems
ABI PRISM 310 Genetic Analyzer, Applied Biosystems
Stratalinker 2400 UV Crosslinker, Stratagene, La Jolla, CA
Rainin pipettors, Rainin, Emeryville, CA
Electrofast, Advanced Biotechnologies through Marsh Biomedical Products Inc.
Balance
Power Pak 300 Power Supply, BioRad, Hercules, CA
Computer software for analysis with Sequencing Analysis, MatchTools, and MTNavigator, Applied Biosystems
Heating block
IEC Multi Centrifuge with Double Deep Microplate Rotor, Forma Scientific Inc., Marietta, OH
Savant Vacuum centrifuge, Forma Scientific Inc., Marietta, OH
Vortexer Genie, Diagger, Licolnshire, IL
Alpha Imager documentation System, Alpha Innotech, San Leandro, CA

Labeling

Label all reagents and aliquots of reagents with the reagent name, concentration, date prepared and appropriate expiration date. Labels are created using computer label making system Label View Pro and Eltron printer.

Procedures:

A. General PCR practices:

1. Wear a new disposable laboratory coat and new gloves when preparing samples or reagents for PCR amplification.
2. Change gloves frequently.
3. Maintain separate areas and dedicated equipment and supplies for sample preparation, PCR setup and amplification/analysis.
4. Open and close all sample tubes carefully to avoid reagent or sample splashes.
5. Use air-displacement pipettors with filter-plugged tips. Change tips after each use.
6. Clean the general area using 10% bleach solution and rinse with deionized water. Cover lab benches with clean sheet of disposable absorbent pad and remove at the end of each day.

B. Reagent Preparation

10X TBE for sequencing:

Final concentration	grams/L
890mM Tris Base (Trizma Base)	108g
890mM Boric Acid	55g
20mM Disodium EDTA	7.44g

Add deionized water to a final volume of 1000mL, mix thoroughly and filter through a $\leq$ 0.45µm membrane.
Store at room temperature.
Do not use if precipitate forms.

For 1X TBE for sequencing, dilute 150mL of the 10X TBE stock and bring the volume up to 1.5L.

Orange G Loading Dye

Final concentration	grams/100mL
15% Ficoll 400	15g/100mL
25mM EDTA pH 8	5µl of 0.5M EDTA pH 8
0.25% Orange G	0.25g/100mL

Add deionized water to a final volume of 100mL, mix thoroughly with a little heat and filter through a 0.45mm membrane.
Store at room temperature.

C. PCR Amplification (SSP-PCR)

1. Record ID of samples to be typed and assign an internal ID numbers to each sample(for example A1-12, B1-12 where A refers to the sample ID and 1-12 refers to the tube number- there will be 12 reaction tubes per individual being typed).
2. Label all processing MicroAmp 8-Strip Reaction Tubes(0.2ml tubes) with the internal ID number and place them into a MicroAmp 96-Well Tray/Retainer.
3. Place the MicroAmp tubes/Tray and 0.5ml microcentrifuge tubes to be used into the Stratalinker (Stratagene) and UV crosslink twice at 120 joules(1200 on LED display) to sterilize the tubes prior to use to avoid contamination.
4. Pipet 40µl of Genomic DNA at a concentration of 10ng/µl into the 0.5ml microcentrifuge tubes that have been labeled appropriately.
5. Add 1.0µl of AmpliTaq Gold DNA polymerase(5U/µl) to the 40ul genomic DNA template(10ng/µl) and mix well by vortexing.
6. To each of the strip-reaction tubes labeled 1-12, add 10µl of the allele group specific PCR mix(supplied in the HLA-DRB BigDye Terminator Sequencing-Based Typing Kit). For example:

Tube number	PCR mix		Tube number	PCR mix		Tube number	PCR mix
1	DRB1G1		5	DRB1G7		9	DRB3
2	DRB1G2		6	DRB1G8/12		10	DRB4
3	DRB1G3/11/6		7	DRB1G9		11	DRB5
4	DRB1G4		8	DRB1G10		12	All

7. To each of the 12 tubes with the allele specific PCR mix, add 2.5µl of the genomic DNA template/AmpliTaq Gold DNA Polymerase mixture and mix up and down with a pipettor. (For example, add 2.5µl of mixture A to each of the 12 tubes 1-12 to create A1-A12.)
8. Place the MicroAmp Caps on the tubes and seal tightly.
9. Place the tubes in the thermal cycler, Geneamp PCR System 9700, and program the following conditions into the machine and start the run under the reaction volume of 12µl (refer to the Geneamp PCR System 9700 Users Manual for details).
   1 cycle @ 95C for 10 min
   36 cycles @ 96C for 20 sec
   65C for 30 sec
   72C for 30 sec
   1 cycle @ 99C for 10 min
   Hold at 4C (after amplification store PCR products at 2-6C until needed)

D. Gel Electrophoresis
1. Prepare a 2% agarose gel containing 1XTBE buffer.
   Measure 1.6 grams of ultra-pure grade agarose and place it in a 250ml flask with 80mls of 1xTBE buffer. Cover the flask with plastic wrap and place into the microwave to melt the agarose to go into solution. Once the solution is cool enough to touch (around 60c) pour the solution into the electrofast gel apparatus and wait for at least 30 minutes to cool.
2. Combine 7.5µl of each PCR amplification mixture with 3µl of the Orange G gel loading dye in a microtiter plate.
3. Combine 48µl of the Low DNA Mass Ladder and 12µl of the Orange G gel loading dye in a microcentrifuge tube.
4. Load 10µl of the PCR/loading dye mixture on the 2% agarose gel as well as 5µl of the Low DNA Mass Ladder/loading dye mixture.
5. Run the gel in 1XTBE at 80 volts for 30-45 minutes.
6. Stain the gel in an Ethidium Bromide/1XTBE solution for 5 minutes then de-stain the gel in 1XTBE for 10 minutes.
7. Photograph the gel and record the results. Record the samples that have a positive band on the sample worksheet provided(Table 1) and attach the picture of the gel.

E. Cycle Sequencing
1. Figure out how many sequencing reactions are going to be run. There will be a forward and reverse sequencing reaction for each band that was recorded from the gel for DRB1. DRB3, DRB4, and DRB5 do no need to be sequenced if there are 2 positive bands for DRB1. If there is only 1 positive band for DRB1 and DRB3 is has a positive band, DRB3 should be sequenced since the DRB1*1130 allele amplifies in the DRB3 tube.
2. Record ID of samples to be sequenced and assign another internal ID number to each sample. For example 1-10 for the sample number and F or R for forward and reverse reactions so there will be tubes labeled 1F, 1R, 2F, 2R, etc.
3. Label all MicroAmp 8-Strip Reaction Tubes with the internal ID number and place them into a MicroAmp 96-Well Tray/Retainer.
4. In the F tubes, add 8µl of the forward ready reaction mix and to the R tubes, add 8µl of the reverse ready reaction mix(both mixes are supplied in the HLA-DRB BigDye Terminator Sequencing-Based Typing kit).
5. In a separate 0.5ml microcentrifuge tube, dilute the PCR product 1:3 in DNA Diluent Buffer supplied in the kit (for example, 2µl of the PCR product in 6µl of the diluent buffer, mix well).
6. To each of the forward and reverse tubes per sample, add 2µl of the diluted PCR product and mix well.

7. Seal the tubes with the MicroAmp Strip caps tightly.
8. Set up a control reaction for sequencing of pGEM DNA(all components are supplied in the Dye Terminator Cycle Sequencing Ready Reaction Kit).

   Combine pGEM control reaction as the following:

1μl of pGEM(10-30ng/μl)	4μl of M13 primer(3.2pmol/μl)
8μl of terminator ready mix	7μl of water

9. Place the tubes/tray in the thermal cycler, Geneamp PCR system 9700, and run under the following conditions for the reaction volume of 10μl (refer to the Geneamp PCR system 9700 Users Manual for details):

1 cycle @	96C for 10 sec
20 cycles @	96C for 10 sec
	50C for 10 sec
	60C for 2 min

   hold at 4C until ready to proceed with the cleanup of the sequencing reactions.

F. Clean up of Cycle Sequencing Reaction

1. Add 10μl of water to each of the cycle sequencing reaction tubes. This is to bring the sample volume up so that it is easer to work with for the clean up procedure.
2. Take the DyeEx 96 plate out of the bag and remove the tape sheets from the top and bottom of the DyeEx 96 plate. When handling the DyeEx 96 plate ensure that it remains horizontal.
3. Place the DyeEx 96 plate on the top of the collection plate(provided in the kit) and centrifuge for 3 minutes at 1000 x g.
4. Discard the flow-through.
5. Carefully place the DyeEx 96 plate on an appropriate elution plate(MicroAmp 96-well reaction plate) with a suitable adaptor.
6. Slowly apply the cycle sequencing samples(20μl) to the gel bed of each well.
7. Centrifuge for 3 minutes at 1000 x g.
8. Dry the samples in a vacuum centrifuge until dry(about 20 minutes). Make sure that you do not over dry the samples.
9. Store the dried down samples in the freezer(-20c) until ready to proceed to the sequencing run.

**Note: Automated Sequencing can be performed on the Applied Biosystems 310 Genetic Analyzer, 377 DNA Sequencer, or 3100 Genetic Analyzer.

Sequencing using the 377 DNA Sequencer

A. Gel Preparation and casting using the Long Ranger Singel Pack.

i. Gel Preparation

1. Assemble glass plates and spacers in the cassette following the method described in the ABI PRISM 377 DNA Sequencer Users Manual.
2. Have the Long Ranger Singel pack at room temperature.
3. Remove the BLACK clip and mix the contents of the compartments by hand thoroughly but gently for 1 minute.
4. Place the pack on an orbital shaker for 5 minutes at medium speed.
5. Mix by hand thoroughly but gently for 1 minute
6. Place the pack on an orbital shaker for 5 minutes at medium speed.

NOTE: Do not over mix. This may interfere with gel polymerization.

ii. Gel Casting

NOTE: The following steps must be completed without delay.

1. Remove only the RED clip and mix the contents of the compartment well by hand for 1 minute.
2. Remove the WHITE clip to expose the filter to gel solution.
3. Hold the pack so the contents drain into the filter end. Fold the pack in half at the indicated line.

4. Hold the pack with the cut mark at the top and cut the corner within the space marked CUT. To avoid introducing bubbles cut a large enough hole in the pouch to allow steady flow of the solution through the filter into a beaker.
5. Avoid introducing air into solution after mixing. Cast gel and insert comb according to your standard procedure.
6. Once the gel is polymerized(30 minutes), place paper towels soaked in electrophoresis buffer over the ends of the plates and then cover with plastic wrap. This will prevent moisture loss as the polymerization process continues.
7. Allow 2 hours for complete gel polymerization.

B. Preparing for Electrophoresis

1. Remove the comb, wash the plates and load the comb as described in the ABI PRISM 377 DNA Sequencer Users Manual.
2. Prepare a sufficient quantity of electrophoresis buffer to fill both anodal and cathodal chambers by diluting 10X TBE stock for sequencing with deionizd water to 1X.
3. Mount the gel cassette onto the sequencing apparatus and prepare the gel for the sequence run according to the 377 DNA Sequencers Users Manuals instructions.
4. Open a new sample sheet in the 377-96 Data Collection Software, and input sample names to be run on the gel.
5. Save the sample sheet and open a new sample run in the 377-96 Data Collection Software and open the new sample sheet that was just created in the previous step.
6. To assure plates and gel are clean, perform a plate check using the Plate Check module.
7. Pre-warm the acrylamide gel by running the PRE-RUN module.
8. Prepare the samples for the sequence run by re-suspending the dried down samples in 4µl of a 5:1 ratio of Hi Di formamide and blue dextran/EDTA loading dye(for example 5µl of the Hi Di formamide combined with 1µl of the blue dextran/EDTA loading dye).
9. Vortex the samples and centrifuge briefly.
10. Denature the samples by heating the samples at 95 ± 5C for 2 minutes.
11. Ice the samples immediately for 2 minutes and keep on ice until ready to use.
12. Stop the PRE-RUN when the temperature reaches 50C and rinse out the top of the gel with 1XTBE buffer.
13. Load 1.5µl of the denatured samples on the gel. The odd lanes should be loaded first then run in for 1 minute before the even lanes are loaded.
14. Cancel the PRE-RUN and change the module to the 377 HLA-DRB DBT module and start the run. The run will take 4 hours.

Sequencing using the 3100 Genetic Analyzer

Preparing and Loading Samples on the 3100 Genetic Analyzer

1. Resuspend the dried down sample in 20ul of HiDi Formamide in the MicroAmp 96-well reaction plate and cover the plate with a MicroAmp .
2. Heat samples in the Geneamp PCR System 9700 thermal cycler at 95°C for 2 minutes then remove and chill on ice for 2 minutes.
3. Replace the Plate Cover with a 96-well plate septae.
4. Create a new "sample sheet" in the ABI PRISM 3100 sequencing software and appropriately label all samples according to the method described in the ABI PRISM 3100 Genetic Analyzer 's Users Manual.
5. Place the sample tray into the 3100 Genetic Analyzer.
6. Link the sample sheet to the corresponding plate.
7. Run the 3100 Genetic Analyzer according to manufacture's instructions.
8. Analyze.

Sequencing using the 310 Genetic Analyzer

Preparing and loading samples on the 310 Genetic Analyzer

1. Resuspend the dried down sample in 25 µl of TSR (Template Suppression Reagent).
2. Vortex and spin the sample plate in a micro-centrifuge.
3. Heat samples at 95°C for 2 minutes.
4. Chill on ice for 2 minutes.
5. Vortex and spin the samples again in a micro-centrifuge and place back on ice.
6. Transfer the sample resuspended in TSR to an appropriately labeled 0.5ml Genetic Analyzer tube and place a septum on the tube.
7. Create a new "sample sheet" in the ABI PRISM 310 sequencing software and appropriately label all samples according to the method described in the ABI PRISM 310 Genetic Analyzer's Users Manual.
8. Import the sample sheet into the "injection list" and make appropriate adjustments.
9. Place the tubes/septum into the 310 loading tray.
10. Run the 310 Genetic Analyzer using the POP-6 Rapid E(1ml) module.
11. Analyze.

Genotype Determination

For the 377 DNA Sequencer

1. Once the run is completed, track the lanes on the gel using the 377 Collection Software.
2. Once the tracking is complete, analyze the raw sequencing data using the Sequencing Analysis Software.
3. Once the raw data is extracted, determine the genotype from the sequence data using the MatchTools and MT Navigator Software.
4. Input all data into the database.
   (See the user's manual for the 377 DNA Sequencer, 377 Collection Software, Sequence Analysis Software, MatchTools and MTNavigator software for operation and usage of the 377 DNA Sequencer and the software.)

For the 310 and 3100 Genetic Analyzer

1. Once the run is complete, analyze the sequence data using the Sequence Analysis software with the dye/primer set 310 HLA-DRB DBT and 350 basepair caller for the 310 Genetic Analyzer and DT3700POP6{ET}mob file for the 3100 Genetic Analyzer.
2. Determine the genotype from the sequence data using the MatchTools and MTNavigator Software.
3. Input all data into the database.
   (See the user's manual for the 310/3100 Genetic Analyzer, 310/3100 Collection Software, Sequence Analysis Software, MatchTools and MTNavigator software for operation and usage of the 310/3100 Genetic Analyzer and the software.)

Table 1: HLA-DRB sample worksheet
HLA-DRB Typing Results using the BigDye Terminator Sequencing-Based Typing Kit
(Applied Biosystems)
Instructions:
*Enter the ID number of the sample tested under the column "Sample name"
*Place a positive mark"+" in the appropriate boxes if there is a band present on the agarose gel.

Sample Name	DRB1 G1	DRB1 G2	DRB1 G3/11/6	DRB1 G4	DRB1 G7	DRB1 G8/12	DRB1 G9	DRB1 G10	DRB3	DRB4	DRB5	ALL

Date of PCR Reaction:
Date of agarose gel run: ____________________________
Date of Cycle Sequencing Reaction: _________________
Date of DyeEx clean up: ___________________________
Date Analyzed: __________________________________

Thermal Cycler: 9700
Thermal Cycler #(circle): 1 2 3 4 5 6 7 8
Instrument used to Sequence(circle): 377 3100 310
Date of Sequence Run: ___________________________
Date entered into Access database: ________________

Attach photographs of the agarose gels below:

Table 2: HLA-DRB Comparison Table

Possible DRB Combinations

	G1	G2	G3/11/6	G4	G7	G8/12	G9	G10	DRB3	DRB4	DRB5
1	+										
2		+									+
3			+						+		
4				+						+	
5					+					+	
6						+					
7						+			+		
8							+			+	
9								+			
10	+	+									+
11	+		+						+		
12	+			+						+	
13	+				+					+	
14	+					+					
15	+					+			+		
16	+						+			+	
17	+							+			
18		+	+						+		+
19		+		+						+	+
20		+			+					+	+
21		+				+					+
22		+				+			+		+
23		+					+			+	+
24		+						+			+
25			+	+					+	+	
26			+		+				+	+	
27			+			+			+		
28			+				+		+	+	
29			+					+	+		
30				+	+					+	
31				+		+				+	
32				+		+			+	+	
33				+			+			+	
34				+				+		+	
35					+	+				+	
36					+	+			+	+	
37					+		+			+	
38					+			+		+	
39						+	+			+	
40						+		+			
41						+	+		+	+	
42							+	+		+	