Genotyping of the DQA1 gene in the HLA Complex

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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 DQA1 gene. Previous studies have found that DQA1 alleles *0301and *0501 are predisposing for type 1 diabetes and the DQA1 allele *0102 has 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.

2. Safety Precautions

Standard safety precautions should be observed including wearing safety glasses, lab coats 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. The ABI 377, 310, and 3100 instruments contain lasers; during normal operation the laser is classified as Class I (not harmful).

a. *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 eyewear.

b. *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 fetal 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.

c. *Performance Optimized Polymer 4 (POP 4)*
This polymer is used within the automated sequencers and acts as medium through which DNA samples are transported through capillaries. This polymer contains 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 lab coat, fresh gloves, and protective eyewear.

d. *Acrylamide*
Acrylamide is used to pour the large sequencing gel used on the Applied Biosystems 377 Genetic analyzer. 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 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 ml 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 eighteen months. Extracted DNA can also be stored at –20°C 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. Procedures 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 the *Red Blood Cell Lysis Solution* (RBC Lysis Solution), *Cell Lysis Solution*, *RNase Solution*, *Proteins Precipitation Solution*, and *DNA Hybridization Solution*. All reagents except the RNase solution are stable at room temperature until the expiration date indicated by the manufacturer. The RNase Solution is stable at 4°C until the expiration date.

PCR primers are stable at –20°C indefinitely.

The Dye Terminator Cycle Sequencing Ready Reaction Kit contains three reagents. The *M13 Primer, the pGEM template, and the Terminator Ready Reaction Mix* may be stored at 4°C. Discard after expiration date. Be aware that the *Terminator Ready Reaction Mix* is light-sensitive and should be stored without exposure to light.

The sequencing reactions are re-suspended in Hi-DI Formamaide which may be stored until expiration date at-20°C.

The *Performance Optimized Polymer 6* (POP 6) as well as the *Performance Optimized Polymer 4* (POP 4) are stable at 4C until designated expiration date and is also light-sensitive.

The AmpF*l*STR Green I PCR Amplification consists of *AmpFISTR* PCR reaction Mix, AmpliTaq Gold DNA Polymerase, *AmpFISTR* Green I Primer, a tube of positive control DNA, and an appropriately sized DNA ladder which are all viable until expiration date marked on the kit box

The HotStarTaq Master Mix is stored at -20°C until the manufacture's expiration date.

b. Preparation of Reagents

See Appendix A, B and C.

c. Standards

This is a qualitative assay and calibration standards are not used. See Part 7.

d. Controls

HLA-DQA1 SSO-PCR SBT Method

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 ~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 –20°C 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 4°C in a "clean" laboratory and the Allelic Ladder is stored at 4°C 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 –20°C in a "clean" laboratory and the in house allelic ladder is stored at 4°C in a "dirty" laboratory where amplified DNA is in use.

e. Equipment and Materials

Puregene DNA Isolation Kit , catalog # D-50K (Gentra Systems, Minneapolis, MN)
70% ethanol
100% isopropanol
50 ml Falcon centrifuge tubes
Rainin pipet tips with filters (Rainin Instrument Co., Emeryville, CA)
Sterile, individually wrapped transfer pipets
Racks for centrifuge tubes and blood tubes (bleach after each use)
PipetAid, Drummond (Daigger, Lincolnshire, IL)
PipetAid, Drummond (Daigger, Lincolnshire, IL)
Qiagen Sigma centrifuge (Sigma Co., St. Louis, MO)
Dispensett III volume dispenser for reagents (Daigger, Lincolnshire, IL)
Nitrile Gloves

AmpF*l*STR 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)
MicroAmp Optical 96-well Reaction Plates #N8010560 (Applied Biosystems)
0.5ml Genetic Analyzer sample tubes and septum, catalog # 401957 and 401956 (Applied Biosystems)
1.5ml microfuge tubes (Marsh Biomedical Products, Rochester, NY)
Qiagen HotStarTaq MasterMix for PCR (Qiagen, Valencia, CA)
0.2 ml microAMP PCR tubes (Applied Biosystems)
Racks for microAMP tubes, reagent tubes, and microcentrifuge tubes (Applied Biosystems)
Appropriate primers for SSO-PCR
Applied Biosystems 9700 specific retainer tray (Applied Biosystems)
Deionized water
Ultra-pure agarose (Gibco BRL, Rockville, MD)
1X TBE buffer (Gibco BRL, Rockville, MD)
DNA marker/ladder (Gibco BRL, Rockville, MD)
Orange G Loading Dye (Sigma Co., St. Louis, MO)
Ethidium Bromide (Ameresco, Solon, OH)
Applied Biosystems 310 or 3100 Genetic Analyzer – automated sequencers (Applied Biosystems)
Appropriate primers for Cycle Sequencing Reactions
Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems)
Sequencing Capillaries (47cm x 50um) (Applied Biosystems)
POP 6 Sequencing Polymer (Applied Biosystems)
IEC benchtop Multi Centrifuge, ventilated (Forma Scientific Inc., Marietta, OH)
1.5ml microcentrifuge tubes (Marsh Biomedical Products, Rochester, NY)
0.5ml 310 Genetic Analyzer Sample Tubes (Applied Biosystems)
10x Genetic Analyzer Buffer with EDTA (Applied Biosystems)
Septae (Applied Biosystems)
Rainin pipets (P2, P10, P20, P100, P200, P1000) (Rainin Instrument Co. Emeryville, CA)
Applied Biosystems 9700 Thermocycler (Applied Biosystems)
Balance
Flasks
Electrophoresis unit (Owl Scientific, Portsmouth, NH)
PowerPac 300 power supply (BioRad, Herculues, CA)
AlphaImager Documentation System (Alpha Innotech Co., San Leandro, CA)
Heat Block
Ice Machine
Genie Vortex (Daigger, Lincolnshire, IL)
Mini-Centrifuge (Daigger, Lincolnshire, IL)
Speed-Vac (Forma Scientific, Marietta, OH)
Applied Biosystems 310/3100 or 377 Genetic Analyzer Automated Sequencers (Applied Biosystems)
Stratagene Stratalinker (Stratagene, Ceder Creek, TX)
Computer and software for analysis with GeneScan and Genotyper (Applied Biosystems)
Finnpette Biocontrol Pipettor with Multi-channel module (Lab Systems through Marsh Biomedical Products Inc.)
Electrofast (Advanced Biotechnologies through Marsh Biomedical Products Inc.)
IEC Multi Centrifuge with Double Deep Microplate Rotor (Forma Scientific Inc., Marietta, OH)
1XTBE (10x TBE from Gibco/BRL, Rockville, MD)
10X TBE (Trizma Base, Boric Acid, EDTA) for Sequencing
Long Ranger Singel Packs, catalog #50691 (FMC BioProducts, Rockland, ME)
Boekel Orbital Rocker (Boekel Scientific Inc., Feasterville, PA)

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 offers greater speed, oil free operation, lower reaction volumes, and cycle time reproducibility.

7. Calibration and Calibration Verification Procedures

The Applied Biosystems GeneAmp 9700 thermal cycler, as well as the Applied Biosystems 377/310/3100 Genetic analyzers are pre-calibrated by the respective manufacturer and annual preventive maintenance is preformed by the manufacturer's authorized service representative. This genotyping assay is a qualitative test as there are a specific number of possible genotypes. The SSO-PCR reactions are subsequently sequenced to obtain a more "finely tuned" genotype. If non of the four sequencing reactions performed for each DNA sample have positive sequence analysis, the PCR is repeated.

8. Procedure Operating Instructions; Calculations; Interpretation of Results

a. Procedure:

See Appendix A, B and C for DNA extraction, amplification and detection.

b. Calculations

All calculations are performed using the manufacturer's proprietary algorithms computed by the sequence detector software based on detection process described in 8c.

c. Interpretation of results

HLA DQA1 SSO-PCR SBT Method
Each of the DNA samples will undergo two PCR reactions: a "deletion" and an "03/01" reaction. The "del" and "03/01" reactions utilize SSO-PCR primers that specifically amplify the "deletion" subset of alleles and the "03/01" subset of alleles, respectively. The excess PCR primers and free nucleotides are removed from the reaction which is then used for sequence analysis. The electropherograms produced by the automated sequencer are qualitative and interpretations are based on the software programs MatchTools and MTNavigator which work together to assign alleles and allow manual review or editing of the sequencing data.

Human Identification. The AmpF*l*STR Green I PCR Amplification Kit amplifies the TH01, TPOX, CSF1PO short tandem repeat loci. In addition, the primers included in the AmpF*l*STR Green I Primer set amplify the Amelogenin locus which can be used for gender determination. The amplification products are 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 AmpF*l*STR 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 DQA1 genotyping method describes in this protocol has been well established in the Division of Environmental Health and Laboratory Sciences at the Centers for Disease Control and Prevention. Additionally, this method has been accepted for publication in Tissue Antigens [30]. This method has proven to be accurate, precise, and reliable.

The Human Identification with Short Tandem Repeat Loci using the AmpF*l*STR Green I PCR Amplification Kit, as described in Appendix B, is an internal quality control measure. In the case of the "trio" family samples we test, this assay ensures that the samples are appropriately related to each other to be eligible for statistical consideration with regards to any results generated by the tests. This assay also keeps samples organized as in the case of accidental mix up or mislabeling, the samples can be identified by this particular control measure.

Reliability of test results should be monitored by routine use of positive controls of known genotypes for both the SSO-PCR step as well as the sequencing step of this assay.

<u>HLA DQA1 SSO-PCR SBT Method</u>
A run is considered to be "out of control" if:
1. None of the sequencing reaction for a DNA sample produces any readable sequence.
2. If the positive controls (being the DNA of known genotype run with each set of samples produce false positives or negatives.
3. Reactions sequenced that produce un-readable electropherogtrams due to machine malfunction or operator error.
If a run is declared "out of control," both the SSO-PCR as well as the automated sequencing is repeated immediately.

<u>Human Idenfication</u>
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.

<u>Idenfication of Amelogenin and TH01 Markers</u>
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.

HLA DQA1 SSO-PCR SBT Method

DNA from Type I Diabetes samples whose genoptypes have been previously confirmed by DNA 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 ~25ng/ul. 3ul of control DNA is used in each PCR reaction. Control DNA is labeled with a unique identification number, and a number indicating the date and operator who prepared the sample. 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

HLA SSO-PCR Genotyping

There are several potential possibilities in a failed test. General precautions should be followed in order to avoid problematic results. 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.

a. If any of the control DNA samples give an unexpected genotype, repeat the experiment.
b. If a given sample fails to amplify, repeat the test on that sample in the next run. If again no amplification is seen, re-isolate the DNA using the stored cryopreserved cells or cell line and repeat the test.
c. If all samples including the control fails to amplify, it is likely due to one of the following reasons: (1) incorrect thermocycler 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

a. If less than 4 or more than 8 peaks appear on the electropherogram, repeat the fragment analysis.
b. If the fragment analysis appears to be the same, repeat the experiment from the beginning.
c. If the GeneScan-350 ROX Internal Lane standard does not show up on the electropherogram, repeat the fragment analysis.
d. 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

a. If less than 2 or more than 4 peaks appear on the electropherogram, repeat the fragment analysis.
b. If the fragment analysis appears to be the same, repeat the experiment from the beginning.
c. If the GeneScan-350 ROX Internal Lane standard does not show up on the electropherogram, repeat the fragment analysis.
d. 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 during PCR set-up and amplification/analysis. 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 has been shown to involve a genetic component as well as an environmental component [4]. Thus, an environmental trigger in a susceptible genetic background results in type 1 diabetes development. The DQA1 gene, located in the HLA complex, is known to play a role in the genetic protection and susceptibility for this detrimental disease.

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 DQA1 gene. Previous studies have found that DQA1 alleles *0301and *0501 are predisposing for type 1 diabetes and the DQA1 allele *0102 has a protective effect [12,27-29].

As of July 2000, there are 20 known alleles for the DQA1 gene [31]. The high risk alleles that contribute to susceptibility of the disease are found in 95% of Caucasian type I diabetics [32]. These alleles are also found in non-diabetic patients, so the search for the environmental trigger that initiates disease onset becomes increasingly more important. The frequencies of these alleles has yet to be determined for various populations. This project is not limited to a certain population and all ethnicities will be considered

14. Critical Call Results ("Panic Values")

Not applicable in this particular assay.

15. Specimen storage and Handling during testing

The blood specimens are received by the laboratory in 50 ml Falcon tubes partially processed up to the cell lysis stage (see Appendix A). At this stage, the specimens can be stored at room temperature for 18 months. Fully processed DNA can be stored at -20°C indefinitely. Prior to testing, DNA can be thawed at room temperature for 10-30 minutes.

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

When a run fails, it is usually due to one of the 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 (Anthony Nolan Web site www.anthonynolan.com). Results are proof read and entered into a common database, and given to the supervisor for review. After review of raw data, the supervisor forwards the final report to the Molecular Biology Branch Chief and EHLS division director for final approval. The approved 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 be kept in electronic or hard copy format. Only numerical identifiers should be used (e.g. Case ID numbers), all personal identifiers should be available only to the medical supervisor or project coordinator to safeguard confidentiality.

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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),
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.

a. **Cell Lysis**

1. Label 50 ml Falcon tubes appropriately and fill each with 30 ml of *Red Blood Cell Lysis* solution (RBC).
2. Add 10 ml of whole blood to the appropriately labeled Falcon tube containing the *RBC lysis solution.*
3. Invert the tubes to mix and incubate for 10 minutes at room temperature. Invert the tubes at least once during incubation.
4. Centrifuge at 2,000xg for 10 minutes.
5. Remove the supernatant, leaving behind the white pellet and approximately 200-400 ul of liquid.
6. Vortex each tube to resuspend cells.
7. Add 10 ml 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 Treatement

1. Add 50 ul *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 15-60 minutes.

C. Protein Precipitation
1. Cool samples at room temperature.
2. Add 3.33 ml of the *Protein Precipitation Solution* to the cell lysate solutions.
3. Vortex for 20 seconds.
4. Centrifuge at 2,000xg for 11 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 10 ml 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 2,000xg for 4 minutes.
5. Pour off supernatant and drain the tubes on a clean absorbent paper.
6. Add 10 ml of 70% ethanol and invert the tubes several times to wash the pellet.
7. Centrifuge at 2000xg for 2 minutes. Carefully pour off the ethanol.
8. Allow to air dry for 10-15 minutes.

E. DNA Hydration
1. Add 1 ml *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 a 1.5 ml tube.

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

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 2,000xg 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.

D. 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 2,000xg for 12 minutes. The precipitated proteins should form a tight, dark brown pellet. If the protein pellet is not tight, repeat step 3, followed by incubation on ice for 5 minutes and then repeat step 4.

E. 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 2,000xg 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 2,000xg 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 Short Tandem Repeat Loci using the AmpF*l*STR Green I PCR Amplification Kit.

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 pipette 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, catalog # 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

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, Daigger, 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

a. Record the ID of the samples to be typed and assign an internal ID number to each sample.
b. Label all processing MicroAmp 8-Strip Reaction Tubes (0.2 ml) with the internal ID number and place them into a MicroAmp 96-Well Tray/Retainer. There should be a tube for the samples, positive control and negative control.
c. Place the MicroAmp tubes/Tray and a 1.5 ml microcentrifuge tube into the Stratalinker (Stratagene) and UV crosslink twice at 120 joules (1200 on LED display) to sterilize the tubes. Remove the tubes from the Stratalinker.
d. Prepare a master mix of the following composition in a 1.5 ml microcentrifuge tube (all reagents are supplied in the AmpFlSTR Green I PCR Amplification Kit):
   number of samples x 10.5 µl of AmpF*l*STR PCR reaction Mix
   number of samples x 0.5 µl of AmpliTaq Gold DNA Polymerase
   number of samples x 5.5 µl of *AmpFlSTR* Green I Primer
   number of samples x 9.9 µl of deionized water
e. Mix by vortexing.
f. Dispense 24 µl of the master mix into each of the MicroAmp Reaction Tubes.
g. To each of the tubes containing the master mix, pipet 1 µl of genomic DNA(25 ng).
h. For the Positive Control tube add 1 µl (25ng) of Control DNA to the tube and for the Negative Control tube, add 1 µl of 1xTE buffer.
   NOTE: the final volume of the reaction is 25 µl.
i. Place the MicroAmp Caps on the tubes and seal tightly.

10. Place the tubes into the thermal cycler, GeneAmp PCR System 9700,and program the following conditions into the machine and start the run under the reaction volume of 25µl (refer to the Geneamp PCR System 9700 Users Manual for details):

1 Cycle @	11 min at 95°C
27 Cycles @	1 min at 94°C
	1 min at 59°C
	1 min at 72°C
1 Cycle @	45 min at 60°C
Hold @	at 25°C

(Store amplified products away from light at 2-6°C for short periods and at -20°C for longer periods.)

Preparing samples for Genescan using the 310 Genetic Analyzer

a. 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.
b. 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.
c. 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.
d. Calculate and combine the necessary amounts of Hi Di formamide and GeneScan-350 [ROX] Internal Lane Standard in a 1.5ml 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 µl of Hi Di formamide
   (Number of samples + 2) x 1.0 µl of genescan-350[ROX] size standard
e. Aliquot 25 µl of the Hi Di formamide/genescan-350[ROX] mixture into 0.5ml Genetic Analyzer tubes.
f. Add 1.0 µl of *AmpFl* STR Green I PCR Product or 1.0 µl of AmpF*l* STR Green I Allelic Ladder per tube and mix by pipetting up and down.
g. Seal each tube with a septum.
h. Denature each sample at 95°C for 3 minutes and chill the tubes for 3 minutes in an ice water bath.
i. Place the tubes in the sampler tray of the 310 Genetic Analyzer and start the Genescan run.
j. Analyze the data using the PE Biosystem's Genotyper software.
k. Input all data into the database..

Appendix C

Identification of the Amelogenin (sex) and TH01 Markers with the Short Tandem Repeat Loci (This method will be used as Quality Control for indivduals who are not part of a Trio)

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, Foster City, CA
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,

LabelingLabeling
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 $\leq$ 0.45μm 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 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.
   - #of samples x 12.5 µl of HotStarTaq Master Mix
   - # of samples x 1.0 µl of FAM-AmeloF primer (3.6 pM/µl)
   - # of samples x 1.0 µl of AmeloR primer (3.6 pM/µl)
   - # of samples x 1.0 µl of TH01F primer (8 pmol/µl)
   - # of samples x 1.0 µl of FAM-TH01R primer (8 pM/µl)
   - # of samples x 7.5 µl of water
5. Mix by Vortexing.
6. Dispence 24 µl of the master mix into each of the MicroAmp Reaction Tubes.
7. To each of the tubes containing master mix, pipet 1ul of Genomic DNA at a concentration of 25 ng/ml.
8. For the Positive Control tube, add 1 µl of the selected control to the tube and for the Negative control tube, add 1 µl of water. Note: The final volume for the PCR is 25 µl.
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 at	95°C for 10min,
27 Cycles at	94°C for 45sec, 60°C for 45sec, 72°C for 1min,
1 Cycle at	60°C -45min
Hold at	4°C

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

GeneScan using the 377 DNA Sequencer

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

i. <u>Gel Preparation</u>

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 the polymerization of the gel.

ii. <u>Gel Casting</u>

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 µl of the PCR product and 1.0 µl of GeneScan Rox[350] with 5 µl of a 5:1 ratio of Hi Di formamide and blue dextran/EDTA loading dye (for example 5ml of the Hi Di formamide combined with 1ml of the blue dextran/EDTA loading dye).
9. Vortex the samples and centrifuge briefly.
10. Denature the samples by heating the samples at 95 $\pm$ 5°C 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 50°C and rinse out the top of the gel with 1XTBE buffer.
13. Load 1.8 ml 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

Genotyping the DQA1 gene in the HLA Complex with the HLA-DQA1 SSO-PCR SBT Method

Materials

Nitrile Gloves
Qiagen HotStarTaq MasterMix for PCR (Qiagen, Valencia, CA)
Optical 96-well Reaction Plates (Applied Biosystems, Foster City, CA)
MicroAmp Optical 96-well Reaction Plates (Applied Biosystems, Foster City, CA)
MicroAmp Full Plate Cover (Applied Biosystems, Foster City, CA)
Rainin pipet tips with fibers (Rainin Instrument Co., Emeryville, CA)
Racks for microAMP tubes, reagent tubes, and microcentrifuge tubes (Applied Biosystems)
Appropriate primers for SSO-PCR (obtained from the CDC Biotechnology Core Facility)
Appropriate primers for Cycle Sequencing Reactions (obtained from the CDC Biotechnology Core Facility)

Primer Name	Orientation	Nucleotide Sequence	Genotype
Intron IA/M13F	Forward	GTA AAA CGA CGG CCA GTC ATC TTC ACT CAT CAG CTG ACC	
DelB/M13R	Reverse	CAG GAA ACA GCT ATG ACG TAG AGT TG(G) AGC GTT TAA TCA **G**	*01 subset Exon 2
03B/M13R	Reverse	CAG GAA ACA GCT ATG ACG TAG AGT TG(G) AGC GTT TAA TCA **C**	*03 subset Exon 2
01B/M13R	Reverse	CAG GAA ACA GCT ATG ACG TAG AGT TG(T) AGC GTT TAA TCA **T**	*del subset Exon 2
SeqA/M13F	Forward	GTA AAA CGA CGG CCA GT	All Exon 2
SeqB/M13R	Reverse	CAG GAA ACA GCT ATG AC	All Exon 2
DQA1Ex3/M13F	Forward	GTA AAA CGA CGG CCA GTA GGT TCC TGA GGT CAC AGT GTT T	All Exon 3
DQA1Ex3/M13R.v2b	Reverse	CAG GAA ACA GCT ATG ACC TTG ACA GAC AAG AAA GCA TC	All Exon 3

Deionized water
Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA)
Sequencing Capillaries (36cm x 50um) (Applied Biosystems, Foster City, CA)
10x Genetic Analyzer Buffer (Applied Biosystems, Foster City, CA)
POP 6 Sequencing Polymer (Applied Biosystems, Foster City, CA)
Hi-Di Formamide (Applied Biosystems, Foster City, CA)
Exonuclease I (USB Corporation, Cleveland, OH)
Shrimp Alkaline Phosphaase (USB Corporation, Cleveland, OH)
1.5ml microcentrifuge tubes
Plate Septae (Applied Biosystems, Foster City, CA)
Sodium Acetate (NaOAc)
Ethylene Diamine Tetracetic Acid (EDTA)
Ethanol, 100% and 80% (ETOH)
Standard paper towels

Equipment
Rainin pipets (P2, P10, P20, P100, P200, P1000) (Rainin Instrument Co., Emeryville, CA)
Applied Biosystems 9700 Thermocycler (Applied Biosystems, Foster City, CA)
Heat Block
Ice Machine
Genie Vortex (Daigger, Lincolnshire, IL)
Mini-Centrifuge (Daigger, Lincolnshire, IL)
Applied Biosystems 310/3100 or 377 Genetic Analyzer – automated sequencers (Applied Biosystems, Foster City, CA)
Stratagene Stratalinker (Stratagene, Ceder Creek, TX)
Finnpette Biocontrol Pipettor with Multi-channel module (Lab Systems via Marsh Biomedical Products Inc.)
IEC Multi Centrifuge with Double Deep Microplate Rotor (Forma Scientific Inc., Marietta, OH)

Labeling
Label all reagents and aliquots of reagents with the reagent name, concentration, date prepared, operator name, and appropriate expiration date. Labels are either hand-written or created with the Eltron label printer and label making system (LabelView).

Preparation of Reagents

a. Sequence Specific Oligonucleotide Polymerase Chain Reaction (SSO-PCR) is performed by utilizing unique PCR primers that must be diluted to 15 pmol/µl in concentration and stored at 4°C. PCR primers used for the amplification of HLA-DQA1 exon 3 must be diluted to 30 pmol/µl in concentration.
b. Primers used for Cycle Sequencing Reactions must be diluted to 3.2 pmol/µl in concentration and stored at 4°C. An appropriate amount of concentrated primer is diluted in deionized water using sterile techniques.
c. 1x Genetic Analyzer Buffer for use on the 3100 Sequencer is prepared by adding 2.5ml of 10x Genetic Analyzer Buffer to 22.5ml of de-ionized water.
d. Preparation of the stock solutions: N/A
e. 3M Sodium Acetate(pH 4.6)

Final concentration	*grams/500 mL*
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 0.45 :m membrane

Procedures:

A. General PCR practices: Although SSO-PCR is carried out in a closed tube system, and the chance of carry-over contamination rare, we still adhere to the precautions below.

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 set-up, and amplification/analysis.
4. Open and close all sample tubes carefully to avoid reagent or samples splashes.
5. Use positive displacement pipettes or air-displacement pipettors with filter-plugged tips. Change tips after each use.
6. Clean the general area using 5% bleach solution and rinse with de-ionized water. Cover lab benches with clean sheet or KayDry or disposable absorbent pad and remove at the end of each day.

7. Work in a "clean room" in which no amplified DNA has been introduced.
8. UV irradiate racks and tubes in Stratalinker at 120,000 uJoules before using.

B. Polymerase Chain Reaction (PCR) Protocol

1. Label all MicroAmp PCR tubes appropriately and UV cross-link in the Stratagene Stratalinker to ensure no DNA contamination.
2. Add 12.5 µl of MasterMix, 7.5 µl-8.5 µl of de-ionized water, 1 µl of each appropriate SSO-PCR primers at 15 pmol/µl (Table 1), and 50ng DNA to each tube. (See table 1)
3. Each of the DNA samples will undergo two PCR reactions: a "deletion" and an "03/01" reaction. The "del" and "03/01" reactions utilize SSO-PCR primers that specifically amplify either the "deletion" subset of alleles, the "03/01" subset of alleles, or both
4. Create a tube for the negative control sample, which contains all of the reagents but no DNA.
5. Vortex and spin well.
6. Place into Applied Biosystems GeneAmp 9700 thermal cycler specific retainer tray and place in machine using appropriate cycling method: 95°C for 10 minutes, followed by 30 cycles of 95°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute, and then ending with a 7 minute hold at 72°C.

Table 1 – HLA-DQA1 Exon 2 PCR Protocol for each DNA sample (2 reactions per sample)

Reaction #1	**DEL**	reaction #2	**03 / 01**
Master Mix	**12.5 µl**	**Master Mix**	**12.5 µl**
DQA1-Intron1A	**1 µl-15pmol**	**DQA1-Intron1A**	**1 µl-15pmol**
DQA1-delB	**1 µl-15pmol**	**DQA1-03B**	**1 µl-15pmol**
X	**x**	**DQA1 -01b**	**1 µl-15pmol**
DNA (25 ng/ul)	**2 µl**	**DNA (25 ng/µl)**	**2 µl**
Water	**8.5 µl**	**water**	**7.5 µl**
TOTAL:	**25 µl**	**TOTAL:**	**25 ul µl**

1. Label all MicroAmp PCR tubes appropriately and UV cross-link in the Stratagene Stratalinker to ensure no DNA contamination.
2. Add 12.5 µl of MasterMix, 8.5 µl of de-ionized water, 1 µl of each appropriate SSO-PCR primers at 30 pmol/µl (Table 2), and 50ng DNA to each tube. (See table 1)
3. Each of the DNA samples will undergo one PCR reactions for the amplification of HLA-DQA1 Exon 3.
4. Create a tube for the negative control sample, which contains all of the reagents but no DNA.
5. Vortex and spin well.
6. Place into Applied Biosystems GeneAmp 9700 thermal cycler specific retainer tray and place in machine using appropriate cycling method: 95°C for 10 minutes, followed by 30 cycles of 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute, and then ending with a 7 minute hold at 72°C.

Table 2 – HLA-DQA1 Exon 3 PCR Protocol for each DNA sample (1 reaction per sample)

Master Mix	**12.5 µl**
DQA1 Ex3/M13F	**1 µl-30pmol**
DQA1 Ex3/M13R.v2b	**1 µl-30pmol**
DNA (25 ng/ul)	**2 µl**

Water	**8.5 µl**
TOTAL:	**25 µl**

C. Exo-Sap (Exonuclease I and Shrimp Alkaline Phosphatase) PCR Purification Kit Protocol

1. Add 1.5 µl Exonuclease I and 1.5 µl Shrimp Alkaline Phosphatase to each PCR reaction to remove excess PCR primers and dNTP's.
2. Vortex samples.
3. Heat for 37C for 30 minutes and then at 80C for 15 minutes.

D. Cycle Sequencing Reaction Protocol

1. Label MicroAmp 96-well optical reaction plate appropriately.
2. Add 4 µl Ready Reaction Mix, 13 µl de-ionized water, 1 µl sequencing primers (Seq A or Seq B), and 2 µl DNA to each tube (See table 3). Prepare a forward (SeqA/M13F primer) and reverse (SeqB/M13R primer) reaction for each of the three PCR reactions for each DNA sample. There should be four sequencing reactions per DNA sample for exon 2, and two sequencing reactions per DNA samples for exon 3.
3. Vortex and spin well.
4. Place into Applied Biosystems 9700 specific retainer tray and place in machine using appropriate cycling method: 96°C for 30 seconds, 50°C for 15 seconds, followed by 60°C for 4 minutes. These cycling conditions are then repeated 25 times.

Table 3 - Cycle Sequencing Reactions: Forward and Reverse reactions for each PCR reaction

DNA Extraction	PUREGENE
PCR clean-up	Exo-Sap
Big Dye Terminator clean-up	ETOH
Terminator Ready Reaction Mix	**4 µl**
DNA 10-30ng	**2 µl**
Primer @3.2pmol	**1 µl (SeqA or SeqB)**
De-ionized water	**13 µl**
Final Volume	**20 µl**

E. Ethanol Precipitation Protocol

1. Add 2µl of NaOAc/EDTA buffer to each sequencing reaction.
2. Prepare a mixture of EtOH/NaOAc by adding 20 µl of 3M NaOAc(pH4.6) per mL of absolute ethanol (1mL of this mixture is sufficient for 20 reactions).
3. Add 50 µl 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 100 µl 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.

F. Preparing and Loading Samples on the ABI 3100 Genetic Analyzer

1.Resuspend the cycle sequencing reactions in 20ul of Hi-Di 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 or heat block at 95°C for 3 minutes then remove and chill on ice for 3 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 Users Manual. Use the filter set that corresponds to the Big Dye 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 samples can also be run on the ABI 310 Genetic Analyzer or the ABI 377 DNA Sequencer.

G. 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 DT3100POP6(BD)v2.mob mobility file and the BC-3100RRv2_SeqOffFtOff.saz analysis file.
2. Determine the genotype from the sequence data using the MatchTools and MTNavigator Software, using the L022 library which includes 22 DQA1 alleles.
(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.)

H. Genotype Determinations

1. Save the plate record, run log, and electropherograms to a common place on the hard drive. Every week these files will be saved to a CD with the use of a CD burner. This sufficiently backs up all computer files needed to genotype samples run.
2. Analyze each of the hard copy electropherograms with the MatchTools software program to genotype the samples.
3. It is important to first analyze the control DNA that is tested with each run to make sure the expected genotypes are determined. If the control samples work, it can be assumed that all other genotypes are accurate
4. Genotypes are lastly entered into a database designated for the collection of sample genotypes from all analysts working on the samples in question.