

Working Instruction

CTS-SEQUENCE HLA-DRB1

For high-resolution typing of HLA-DRB1

Product No. 237

Lot No. SDRB04-0

For research use only

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The CTS-SEQUENCE HLA-B Kit is delivered at room temperature. Immediately upon receipt, store PCR Buffer & sequencing primers at -20°C and PCR minitrays at 4°C.

1 Introduction

This working instruction describes the procedure for high-resolution genotyping of the human leukocyte antigens HLA-DRB1 with the CTS-SEQUENCE HLA-DRB1 Kit. PCR-sequencing based typing (PCR-SBT) is an accurate and reliable method, allowing high resolution of HLA alleles at least 4-digit level.

The strategy is based on two consecutive steps: first, group-specific amplification of exon 2 of HLA-DRB1; second, the amplification products are sequenced in forward and reverse direction. Matching for exon 2 (antigen-recognition site) at allele-level is considered relevant in hematopoietic stem cell transplantation.

The SEQUENCE HLA-DRB Kit is validated and optimized with following reagents, instruments, softwares and methods:

- GeneAmp® PCR System 2700 Thermocycler (Applied Biosystems, Darmstadt, Germany).
- Amplification with the MBI Taq polymerase (Fermentas, St. Leon-Rot, Germany).
- Purification of amplification products with EXO-SAP-IT (USB, Staufen, Germany).
- Sequencing reaction with BigDye terminator v1.1 Kits (Applied Biosystems, Darmstadt, Germany).
- Purification of the sequencing products using ethanol precipitation.
- Resuspension of sequencing products with HiDi formamide (Applied Biosystems, Darmstadt, Germany).
- Separation of sequencing products with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany).
- Sequence analysis and HLA allele assignment with Sequence Pilot™-HLA SBT (JSI Medical Systems, Kippenheim, Germany).

Other reagents, instruments etc. may be used, but should be validated by the user. The CTS-SEQUENCE kits have been validated to be performed with the GeneAmp® PCR System 2700 thermocycler. If other cyclers are used, the ramp rate has to be set at 1°C/sec.

According to EFI standards for histocompatibility testing (Version 5.6.1; L3.2520) PCR-SBT typing of HLA-class II bases on amplification and sequencing primers which are located outside of exon 2. For many HLA-class II variants only the sequence of the antigen recognition site (exon 2) are reported. Even though the PCR-SBT HLA-SEQUENCING Kits have been extensively tested and validated, an allelic drop out of a rare or new allele due to mutations in the priming sites cannot be categorically ruled out.

2 Materials and Equipment

2.1 Materials included in the CTS-SEQUENCE HLA-DRB1 Kit

The SEQUENCE HLA-DRB1 Kit provides reagents sufficient for twenty four HLA-DRB1 high resolution typings and contains:

- 1) Twenty-four 16-well PCR minitrays. 12 wells contain dried primer mixes, each tray for one HLA-DRB1 typing. Store at 4°C in pre-PCR area.
- 2) 3 tubes of CTS-SEQUENCE PCR Buffer (1400 µl). Store at -20°C in pre-PCR area.
- 3) Sequencing primers (500 µl each):
DRB-E2F, DRB-E2R
Store at -20°C in post-PCR area.

a) PCR stripes and amplification mixes:

The amplification primers are prepipetted and dried in purple PCR stripes (Note: only 12 of the 16 cavities contain primers). For quality reasons, we recommend to use only the caps included in the package.

Figure 1 shows the positions of the PCR mixes on the stripe and the allele group(s) and the exons amplified by these mixes.

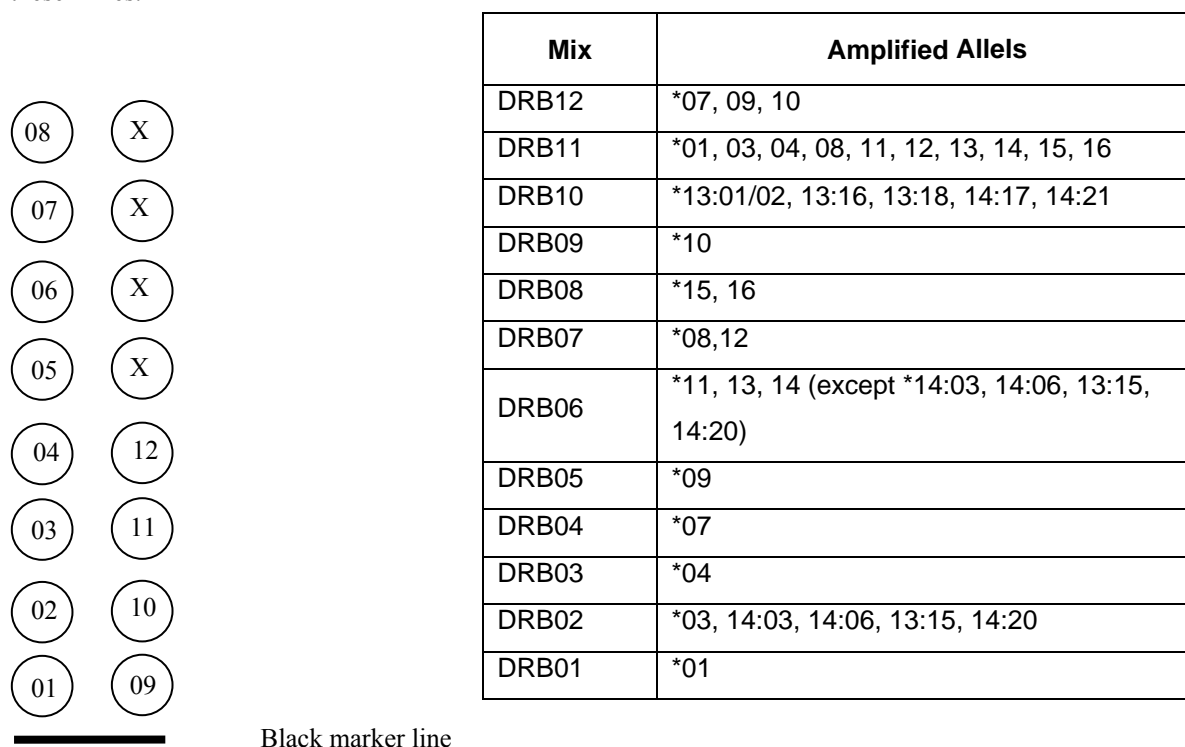


Figure 1: Mix position on CTS-SEQUENCE HLA-DRB1 tray. The X marked wells contain no primer mixes.

b) Sequencing primers:

The tubes containing the sequencing primers (500 µl) have purple colored caps. Mix DRB11 can only be sequenced in reverse direction (DRB-E2R) and mix DRB12 can only be sequenced in forward direction (DRB-E2F).

Table 1: Labeling of the sequencing primers

| HLA-Locus | Tube label | Sequenced Exon | Not Applicable for Mix | Direction of sequencing |
|-----------|------------|----------------|------------------------|-------------------------|
| HLA-DRB1 | DRB-E2F | 2 | DRB11 | forward |
| | DRB-E2R | 2 | DRB12 | reverse |

2.2 Storage and expiration

All kit components are labeled with storage condition and date of expiration.

Frequent thawing and freezing can reduce the quality of the reagents and should be avoided. It is recommended to make aliquots of appropriate volumes and store them as indicated.

2.3 Materials and equipment not included

Table 2: Pre-PCR area

| Reagents/materials/software | Company/Catalogue number |
|--|--|
| Taq DNA Polymerase (5 U/μl) | Fermentas, St. Leon-Rot, Germany Cat.No EP0401/ EP0402 |
| Ultra Pure Agarose | Inno-Train, Kronberg/Taunus, Germany Cat. No. GX04090 |
| Ethidium bromide (10 mg/ml) Cave: potentially carcinogenic! | Sigma-Aldrich GmbH, Steinheim, Germany Cat.No. E1510-10ML |
| Magnetic stirring hotplate or a microwave oven for gel preparation | |
| Pipettes and filter tips for 0.5-10 μl, 10- 200 μl and 200-1000 μl volumes | Eppendorf, Wessing-Berzdorf, Germany |
| Sequence Pilot™-HLA SBT | JSI Medical Systems GmbH, Kippenheim, Germany |
| Photometer for spectral measurement of DNA concentration | |
| 50x TAE buffer | Inno-Train, Kronberg/Taunus, Germany Cat.No. GX12765 |
| Analytical balance | |

Table 3: Post-PCR area

| Reagents/materials/software | Company/Catalogue number |
|--|--|
| ExoSAP-IT™ | USB, Staufen, Germany Cat.No. 78202 |
| BigDye™ Terminator Cycle Sequencing Kit v1.1 (Sequencing buffer (5x) included) | Applied Biosystems, Darmstadt, Germany Cat.No.4336791 |
| 1x TAE electrophoresis buffer | See section 3 below for instruction |
| HiDi Formamide | Applied Biosystems, Darmstadt, Germany Cat.No. 4311320 |
| Loading buffer (bromophenol blue) | Fermentas, St. Leon-Rot, Germany |
| Sodium-Acetat 3M pH 5.2 for precipitation | Sigma Aldrich, Germany Cat.No. S7899 |
| Ethanol absolute GR for analysis | Merck, Darmstadt, Germany Cat.No. 1.00983.1000 |
| Ethanol 70% | See section 3 below for instruction |
| 10x EDTA running buffer for the sequencer | Applied Biosystems, Darmstadt, Germany Cat.No. 402824 |
| 1x EDTA running buffer for the sequencer | |
| Centrifuge for PCR plates | |
| GeneAmp® PCR System 2700 thermocycler | Applied Biosystems, Darmstadt, Germany |
| Power supplier for electrophoresis | |
| Gel Documentation System | |
| Gel electrophoresis chamber | |
| Capillary sequencer: ABI PRISM 3100 Genetic Analyzer | Applied Biosystems, Darmstadt, Germany |
| 8-channel pipette and filter tips 0.5-10 µl | Eppendorf, Wessing-Berzdorf, Germany Cat.No. 0030.077.040 |
| Pipettes and filter tips for 0.5-10 µl volume | Eppendorf, Wessing-Berzdorf, Germany Cat.No. 0030.077.040 |
| Multipette and combitips (0.1, 0.2 ,0.5, 1.0, 2.5ml) Not mandatory | Eppendorf, Wessing-Berzdorf, Germany |
| Adhesive aluminium foils for 96-well PCR plate | Kisker, Steinfurt, Germany Cat.No. GO71 |
| Optical 96-well reaction plate and optical caps | Applied Biosystems, Darmstadt, Germany Cat.No. N801-0560, N801-0535 |

Table 4: Pre-PCR and post-PCR area (two sets are needed!)

| Reagents/materials/software | Company/Catalogue number |
|--------------------------------|--|
| HPLC water (LiChrosolv® water) | Merck, Darmstadt, Germany Cat.No. 1.15333.1000 |
| Vortexer | |
| Reaction tubes 1.5 ml | Eppendorf, Wessing-Berzdorf, Germany Cat.No. 0030 120.086 |
| Examination gloves | |
| Nitril gloves | |

3 Preparation of buffers and agarose gel

1x TAE electrophoresis buffer:

49 volume parts of deionised water + 1 volume part of 50x TAE electrophoresis buffer

Ethanol 70%:

7 volume parts of absolute ethanol + 3 volume parts of HPLC water

2% agarose gel:

If you use CTS electrophoresis chamber and CTS combs (see www.ctstransplant.org for order information) proceed as follows:

- Add 7 g of agarose and 7 ml of 50x TAE buffer to 350 ml of ddH₂O.
- Boil to dissolve the agarose, using a magnetic stirring hot plate or a microwave oven.
- Cool down to 60 °C, add 17 µl of ethidium bromide (10 mg/ml), mix and pour the gel. Allow the gel to set for 1 hour at room temperature. Cave: Ethidium bromide is potentially carcinogenic! Wear appropriate protection, e.g. nitril gloves.
- On a 20x25 cm gel, you can place up to six CTS combs. These combs have a tooth distance corresponding to that of the channels of a standard 8-channel pipette. This allows the use of such a pipette for rapid loading of the samples onto the gel.

4 Isolation and concentration measurement of DNA

Genomic DNA can be isolated from all nucleated cells. Starting material can be EDTA or citrate blood, buffy coats, cell suspensions etc. Heparinized blood should not be used. DNA can be isolated by the salting out method (Miller SA et al., Nucleic Acid Research 1999) or magnetic particle technology (e.g. GenoM-6/Qiagen EZ1 robot, Qiagen, Vienna, Austria). Magnetic beads should be separated from the DNA (e.g. by centrifugation). It is likely that other commercial kits or automats for DNA isolation will also work, but they should be validated by the users.

For optimal reaction, adjust the DNA concentration to approximately 25 ng/µl with HPLC water.

Cave: Human material should always be considered to be potentially infectious and be handled with care. See your own standard laboratory safety guidelines.

5 Test procedure

High resolution HLA-typing with the CTS-SEQUENCE HLA-DRB1 Kit is performed in 7 steps:

- Amplification of the HLA locus by PCR (setup in pre-PCR area; thermal cycling in post-PCR area)
- Electrophoresis to check for positive amplifications (“gel control”) (post-PCR area)
- Purification of the (positive) amplification products for sequencing (post-PCR area)
- Sequencing reaction (post-PCR area)
- Purification of the sequencing products (post-PCR area)
- Separation of the sequencing products in the capillary sequencer (post-PCR area)
- Sequence analysis and allele assignment with the Sequence PilotTM-HLA SBT software

5.1 Amplification

Prepare PCR on ice.

- Fill in your PCR protocol.
- Label your PCR-minitray.
- Thaw PCR Buffer.
- Pre-mix 10.85 µl of PCR Buffer with 4 µl of 25 ng/µl genomic DNA and 0.15 µl of Taq polymerase for each mix (each PCR). An excess volume to compensate loss during pipetting is recommended. For example, if you want to perform one CTS-SEQUENCE HLA-DRB1 test (12 mixes), prepare a pre-mix for 14 mixes (151.9 µl of PCR Buffer + 56 µl of 25 ng/µl DNA + 2.1 µl of Taq).
- Vortex the pre-mix.
- Pipette 15 µl of the pre-mix into each well of the minitray.
- Close the tubes and spin them down.
- Put the minitray into the thermocycler and start the amplification program **CTS-AMP** (see below).

| | |
|-----------|-----------------|
| 10.85 µl | PCR Buffer |
| + 4 µl | DNA (25 ng/µl) |
| + 0.15 µl | Taq Polymerase |
| 15 µl | reaction volume |

Cave: DNA resolved in buffers should always be diluted at least 1:1 with HPLC water prior to use in the amplification (buffers often contain PCR inhibitors e.g. EDTA).

Cave: Do not use hot start polymerase (e.g. AmpliTaq Gold, Applied Biosystems) or a proofreading polymerase!

Thermocycler program for amplification (CTS-AMP):

| Step | Temperature | Time | Numbers of cycles |
|------|-------------|------------|-------------------|
| 1 | 95 °C | 2 min | 1 |
| 2 | 95 °C | 15 s | 10 |
| | 65 °C | 2 min | |
| 3 | 95 °C | 15 s | 22 |
| | 61 °C | 50 s | |
| | 72 °C | 1 min 30 s | |
| 4 | 4 °C | ∞ | |

Cave: Do not forget to enter the reaction volume of 15 µl!

5.2 Gel control

The amplification products are separated on a 2% agarose gel by electrophoresis. This step is to check for success of the amplification step and to identify the amplification mix(es) which will be subjected to sequencing.

A) Electrophoresis

- Pre-pipette 5 µl of loading buffer for each amplification product into a PCR plate.
- Add 5 µl of your amplification product. Use filter tips to avoid contamination.
- Load the gel with 10 µl of the amplification/loading buffer mixture.
- If you use CTS electrophoresis chamber, run the electrophoresis for 20 min at 170 Volts (approx. 0.4 V/cm²).

Cave: Ethidium bromide is potentially carcinogenic! Wear appropriate protection, e.g. nitril gloves!

B) Documentation and interpretation

Place the gel on a UV light transilluminator (312 nm) and take a polaroid picture for interpretation and documentation. Wear UV-protection goggles!

You can proceed with an amplification product if a band representing the specific amplicon is visible in the gel picture. The length of the specific amplification products range from 610 to 1030 bp.

Cave: Do not mistake primer dimers or primer clouds for specific amplification products! Primer dimers are very small (15-50 bp). Use a size marker if you are not confident.

5.3 Purification of the amplification products

Before an amplification product is subjected to sequencing, it has to be purified e. g. with ExoSAP-IT™ (USB, Staufien, Germany). ExoSAP-IT™ contains an exonuclease digesting single-stranded DNA (e.g. primers) and a phosphatase inactivating the nucleotides. This enzymatic purification method is simple and appropriate to perform large-scale testing. A further advantage compared with other methods is that the enzymatic digest is performed in the same tube that will subsequently be used for the amplification step. This avoids contaminations and a mix-up of samples.

- Add 4 µl of ExoSAP-IT™ (2µl ExoSAP-IT™ per 5µl PCR products) to each well with a positive PCR reaction (based on the gel control). For large-scale performances, a Multipette can be used.
- Close the reaction tubes (avoid contaminations!).
- Spin down the ExoSAP-IT™ in the reaction tubes.
- Put the PCR reaction wells into the thermocycler and start the purification program **CTS-PUR** (see below).

Cave: ExoSAP-IT™ is a viscous fluid, vortex well before use and get rid of excessive enzyme hanging at the tip of your pipette.

Thermocycler program for purification with ExoSAP-IT™ (CTS-PUR):

| Step | Temperature | Time | Numbers of cycles |
|------|-------------|--------|-------------------|
| 1 | 37 °C | 15 min | 1 |
| 2 | 80 °C | 15 min | 1 |
| 3 | 4 °C | ∞ | |

Cave: Do not forget to enter the reaction volume of 14 µl.

5.4 Sequencing reaction

General strategy

- For high resolution typing of HLA class II, exon 2 must be completely sequenced.
- If an allele is not separated by amplification, we recommend to sequence in both directions (forward and reverse) to optimize base-calling
- Mix DRB11 and DRB12 are especially designed to cover all HLA-DRB1 allele groups in minimum by two amplification mixes (on of the mixes DRB01 to DRB10 plus mix DRB11 or DRB12). This strategy is used to reduce the risk of an allelic drop out due to a failed amplification. In case of homozygous results (single allele), in addition to the one positive mix of the mixes DRB01 to DRB10, mix DRB11 or DRB12 (if positive) should be sequenced with DRB-E2R or DRB-E2F, respectively.
- If the alleles are separated by amplification (e. i. if two group-specific mixes are positive), it is sufficient to sequence the positive amplicons in only one direction (we recommend to use the reverse primers).

Setting-up a sequencing reaction

- Create a pipetting scheme determining which amplicon(s) and which sequencing primer(s) are pipetted into which position(s) of the optical 96-well reaction plate. An example of a pipetting scheme can be seen in the appendix.
- Place an optical 96-well reaction plate on ice.
- Mix one volume of BigDye terminators (BDT) with one volume of 5x BigDye sequencing buffer (always prepare freshly). Keep an excess volume to compensate loss during pipetting. Pipette 2 µl of the mixture into the optical 96-well reaction plate.
Alternatively, pipette 1 µl of BigDye terminators + 1 µl of 5x BigDye sequencing buffer directly into the optical 96-well reaction plate.
Close the wells with caps and spin down.
- Add 6 µl of sequencing primer.
- Add 2 µl of purified amplification product (DNA template).
- Spin down, close the plate with caps and place it into the thermocycler.
- Start the thermocycler program **CTS-SEQ**.

| |
|------------------|
| 1 µl BDT |
| + 1 µl 5x buffer |
| + 6 µl Primer |
| + 2 µl Template |
| 10 µl |

Cave: Keep the BigDye terminators cool and minimize their exposure to light.

Thermocycler program for sequencing reaction (CTS-SEQ):

| Step | Temperature | Time | Numbers of cycles |
|------|-------------|-------|-------------------|
| 1 | 96 °C | 1 min | 1 |
| 2 | 96 °C | 10 s | 25 |
| | 60 °C | 2 min | |
| 3 | 4 °C | ∞ | |

Cave: Do not forget to enter the reaction volume of 10 µl. Proceed with the purification of the sequencing products immediately when the sequencing reaction has finished.

5.5 Purification of the sequencing products

Residual ddNTPs must be removed to avoid sequencing artifacts (e.g. dye blobs). This can be done e. g. by ethanol precipitation which is a cheap method and can be used for high-throughput.

- Pre-mix 1 µl of 3 M Sodium-Acetate (pH 5.2) with 25 µl of absolute ethanol for each sequencing reaction to be purified. An excess volume to compensate loss during pipetting is recommended.
- Add 25 µl of the pre-mix to each sequencing reaction.
- Close the optical 96-well reaction plate with an adhesive aluminium foil and vortex well (30 sec). Vortexing is crucial for a good precipitation!
- Incubate the optical 96-well reaction plate at room temperature in a dark place for 15 min (keep light exposure of ddNTPs low).
- Centrifuge the optical 96-well reaction plate for 30 min at 2000 x g. Proceed immediately with the next step. If you can not proceed immediately, centrifuge again for 3min at 2000 x g before the next step.
- Remove the adhesive aluminium foil, flip the optical 96-well reaction plate and remove the supernatant.
- Place the optical 96-well reaction plate upside down on paper towel into the centrifuge. Spin the plate for a few seconds at 180 x g to dry.
- Add 75 µl of 70% ethanol to the precipitated sequencing products and vortex briefly.
- Centrifuge the optical 96-well reaction plate for 10 min at 2000 x g. Proceed immediately with the next step. If you can not proceed immediately, centrifuge again for 3min at 2000 x g before the next step.
- Remove the adhesive aluminium foil, flip the optical 96-well reaction plate and remove the supernatant.
- Place the optical 96-well reaction plate upside down on paper towel into the centrifuge. Spin the plate for a few seconds at 180 x g to dry.
- Keep the plate in a dark place until all ethanol has evaporated (~ 20 min).

In dried form, the sequencing products are quite stable when kept in the dark.

5.6 Sample preparation for sequencing runs

- Add 15µl of HiDi Formamide onto the dried sequencing products, close the wells with caps and spin down.
- Put the plate into a thermocycler and denature for 2 min at 95 °C.
IMPORTANT: Vapours at high temperatures. Cool down the HiDi Formamide at 4 °C before opening the caps.

6 Start of a sequencing run on the sequencer

6.1 Instrument protocol for ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany)

| | | |
|------------------------|--|-------------|
| POP medium | 3100 POP-6 | |
| Capillary | 36 cm array | |
| Electrophoreses buffer | 1x buffer with EDTA | |
| Instrument Protocol | Type | Regular |
| | Run Module | CTS2600 |
| | Dye Set | E-Big-DyeV1 |
| Sequence File Format | True Profile | |
| Ending Base | At PCR Stop Do not assign N's to Basecalls | |
| Mixed Base | Use Mixed Base Identification Call IUB if 2 nd highest Peak is 25% of the highest peak | |
| Clear Range Method | Use quality values, Remove bases from ends until viewer | |

| | | |
|------------------------------|---|------------|
| | then 10 bases out of 20 have QVs less than 15 | |
| Mobility file | 3100_POP6_BDTv1 | |
| Sequencing Analysis Software | Vers. 5.1.1 | |
| Run Module (CTS2600) | Run Temperature | 55 °C |
| | Leak Threshold | 25 steps |
| | Current tolerance | 100 uAmps |
| | Run current | 100 uAmps |
| | Voltage tolerance | 0.6 kVolts |
| | Pre Run Voltage | 15 KVolts |
| | Pre Run Time | 180 sec |
| | Injection Voltage | 1,2 kVolts |
| | Injection Time | 10 sec |
| | Run Voltage | 15 kVolts |
| | Number of Steps | 10 steps |
| | Voltage Step Interval | 60 sec |
| | Data delay Time | 240 sec |
| Run Time | 2600 sec | |
| Basecaller | KB.bcp | |
| Settings Sample Manager | Basecaller:KB.bcp Dye set/primer file: KB 3100 POP6 BDTv1.mob | |
| Settings Plate Record | Dye Set: E Mobility File: 3100_POP6_BDTv1.mob Run Module: CTS2600 | |

6.2 Run Sequencing

- 1) Transfer your sequencing pipetting scheme into the “Plate Record” of the ABI PRISM 3100 Genetic Analyzer.
If the sequences should be later analyzed with the software Sequence Pilot™ (JSI Medical Systems GmbH, Kippenheim, Germany) (see section 7), the sample naming conventions are:
(Sample name_Amplification mix_Sequencing primer)
Example: (Sample_DRB01_DRB-E2F) if amplification mix DRB01 was used in the sequencing reaction with the DRB-E2F sequencing primer.
- 2) Place samples into the ABI PRISM 3100 Genetic Analyzer and run the instrument.

For details, refer to the User Guides of ABI PRISM 3100 Genetic Analyzer and its softwares.

7 **Result evaluation**

For allele assignment, the sequences are loaded into the Sequence Pilot™-HLA SBT Allele Identification Software (JSI Medical Systems GmbH, Kippenheim, Germany). This software shows the electropherograms and aligns them with HLA alleles as listed in the IMGT/HLA Sequence Database (<http://www.ebi.ac.uk/imgt/hla/>). Mismatches to the proposed HLA alleles, if shown, can be edited. The sequencing results can be printed and archived. For details, see User Manual of the Sequence Pilot™-HLA SBT Allele Identification Software.

Add the sequencing primers with following names and parameters in the “Seq. Primer master file”:

HLA-DRB1

| | | |
|-----------------------------|---------|---------|
| Name | DRB-E2F | DRB-E2R |
| Gene | DRB1 | DRB1 |
| Direction | fwd. | rev. |
| SeqPrimer gene parts | E2 | E2 |
| RFName | DRB-E2F | DRB-E2R |
| Sorting | 0 | 0 |

Adding the sequencing primer to the “Seq. Primer master file” is not mandatory. However, by doing so, one can avoid a situation in which a forward sequence of exon 3 is shown, which has been sequenced by the forward sequencing primer of exon 2; such a sequence will have bad quality and can be omitted.

8 Troubleshooting

8.1 Amplification

| Observation | Possible Cause(s) | Solution |
|--|---|--|
| No, weak or non-specific PCR-product(s). → Some primary checks: Did you follow the amplification protocol? Did you vortex the solution well? Was the correct cycler program used? Was ethidium bromide included in the gel? | Degraded DNA | New extraction of DNA |
| | DNA concentration to low | New extraction of DNA |
| | DNA contains PCR inhibitors | Heparinized blood? New extraction of DNA |
| | Thermocycler is defect. | Check cycler (e.g. with the CTS Cycler Control Kit) |
| | Incorrect thermocycler program | Correct programm and repeat PCR |
| | Thermocycler program needs to be adapted. | Our method was optimized for the GeneAmp® PCR System 2700 Thermocycler. For other thermocyclers, the cycling program may have to be adjusted and validated. |
| | Taq Polymerase needs to be adapted. | Our method was optimized for the Taq DNA Polymerase purchased from Fermentas, St. Leon-Rot, Germany, Cat.No EP0401/ EP0402. Repeat PCR with this polymerase. |

8.2 Sequencing

| Observation | Possible Cause(s) | Solution |
|------------------------|---|---|
| No signal | No sample was in sequencing reaction. | Repeat sequencing reaction. |
| | Not enough formamide or air bubble at the bottom of the well. | Pipette enough formamide and spin down well. |
| Weak signals | Wrong “injection time” or “injection voltage”. | Differences between capillary sequencer can occur. Adapt “injection time” or “injection voltage” to get fluorescent intensities between 400 and 9000 in raw data. |
| | Not enough sequencing products after purification. | Cleaning-up by ethanol precipitation requires very precise ethanol concentrations. Ethanol concentration can vary when tubes are frequently opened. Aliquot ethanol solutions for single use. |
| | Not enough sequencing products were loaded. | Increase “injection time” or “injection voltage”. Salt can reduce the amount of loaded sequencing products. Reduce salt contamination during ethanol precipitation. |
| Signals are too strong | Wrong “injection time” or “injection voltage”. | Differences between capillary sequencer can occur. Adapt “injection time” or “injection voltage” to reach fluorescent intensities between 400 to 9000 in raw data. |
| | High concentration of sequencing products. | Reduce the amount of PCR product used in the sequencing reaction. The |

| | | |
|---|---|---|
| | | reduced amount should be substituted with HPLC water (e.g. dilute amplicon with HPLC water) |
| Electropherogram has high background. | Purification of PCR amplification products did not work well (primer contamination). | Repeat PCR and purification of amplification products. |
| | Contamination with a second sequencing primer. | Avoid contamination during pipetting sequencing primers. |
| | Double sequence which starts in the forward and reverse sequencing reaction at the same base (in different directions). | Double sequence due to inserts or deletions within an HLA-B allele. |
| DyeBlobs | Purification of sequencing products did not work well (leftover of dye). | Ethanol concentration during precipitation to high. |
| Very high, randomly occurring peaks (spikes) | Air bubbles or polymer crystals in capillaries. | Refill capillaries with new polymer. |
| Two different peaks run at nearly the same position in the electropherogram | Secondary structures of sequencing products (gel compression) | This phenomenon is sequence-dependent and occurs only in one sequencing direction of a limited region. Analyze this region with the sequencing primer for the other direction. The sequences obtained with the forward primers tend to show gel compressions more often than reverse primers. |

CTS-SEQUENCE HLA-DRB1 Amplification Protocol

For Lot DRB04-0

DNA-No.: _____ Date: _____

Thermocycler: _____

| | Lot | Volume |
|---------------|-----|----------|
| PCR Buffer | | 10,85 µl |
| TAQ | | 0,15 µl* |
| DNA (25ng/µl) | | 4 µl |

*The exact amount of Taq-Polymerase needed may vary depending on brand and lot; it should therefore be established through your own validation.

| Photo | Mix | Positive/ purified | Length of Amplificat | Amplified Alleles | Amplified Exon |
|-------|-------|-----------------------|-------------------------|--|-------------------|
| | DRB01 | | 840 | *01 | 2 |
| | DRB02 | | 990 | *03, 14:03, 14:06, 13:15, 14:20 | 2 |
| | DRB03 | | 760 | *04 | 2 |
| | DRB04 | | 740 | *07 | 2 |
| | DRB05 | | 850 | *09 | 2 |
| | DRB06 | | 886 | *11, 13, 14 (except *14:03, 14:06, 13:15, 14:20) | 2 |
| | DRB07 | | 1030 | *08, 12 | 2 |
| | DRB08 | | 850 | *15, 16 | 2 |
| | DRB09 | | 710 | *10 | 2 |
| | DRB10 | | 730 | *13:01/02, 13:16, 13:18, 14:17, 14:21 | 2 |
| | DRB11 | | 850 | *01, 03, 04, 08, 11, 12, 13, 14, 15, 16 | 2 |
| | DRB12 | | 610 | *07, 09, 10 | 2 |

Cave: Mix DRB11 can only be sequenced with the sequencing primer DRB-E2R and Mix DRB12 only with DRB-E2F.

Comment:

Date, Signature Operator: _____

Date, Signature Reviewer: _____

Pipetting scheme

(Example)

Optical 96-well reaction plate

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|--------------------------|---|---|---|---|---|---|---|---|----|----|----|
| A | (Stan_DRB02 _DRB-E2F) | | | | | | | | | | | |
| B | (Stan_DRB02 _DRB-E2R) | | | | | | | | | | | |
| C | (Stan_DRB05 _DRB-E2F) | | | | | | | | | | | |
| D | (Stan_DRB05 _DRB-E2R) | | | | | | | | | | | |
| E | (Stan_DRB11 _DRB-E2R) | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

DNA sample ID: Name (e.g. Stan)

Amplification pattern of the B-locus:

-
-
-
- DRB05 positive
-
- DRB11 positive
- DRB02 positive
-

Position on
plate

- A1 Mix DRB02 was sequenced with the DRB-E2F sequencing primer
- A2 Mix DRB02 was sequenced with the DRB-E2R sequencing primer
- A3 Mix DRB05 was sequenced with the DRB-E2F sequencing primer
- A4 Mix DRB05 was sequenced with the DRB-E2R sequencing primer
- A5 Mix DRB11 was sequenced with the DRB-E2R sequencing primer