Working Instruction

CTS-SEQUENCE HLA-B

For high-resolution typing of HLA-B

Product No. 334

Lot No. SB05-0

For research use only

University Clinic Heidelberg
Department of Transplantation Immunology
Im Neuenheimer Feld 305
69120 Heidelberg
Germany

Phone: +49-6221-564013
Fax: +49-6221-564200
www.ctstransplant.org

Content

Appendix

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-B with the CTS-SEQUENCE HLA-B 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- and locus-specific amplification of exon 2, exon 3 and exon 4 of HLA-B genes; second, the amplification products are sequenced in forward and reverse direction. Matching for exon 2 and 3 (antigen-recognition site) at allele-level is considered relevant in hematopoietic stem cell transplantation. Sequencing of exon 4 helps to reduce ambiguities. Furthermore, many null-alleles (not expressed alleles, e.g. B*51:11N which occur at relative high frequencies in specific haplotypes) can be detected by the sequence of exon 4.

The SEQUENCE HLA-B 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 I bases on amplification and sequencing primers which are located outside exon 2 and 3. For many HLA-class I variants only the sequence of the antigen recognition site (exon 2 and 3) 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-B Kit

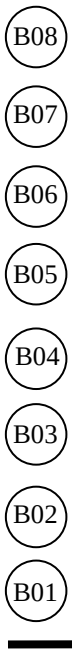
The SEQUENCE HLA-B Kit provides reagents sufficient for twenty four HLA-B high resolution typings and contains:

1) Twenty-four 8-well PCR stripes with prepipetted and dried primer mixes, each stripe for one HLA-B typing. Store at 4°C in pre-PCR area.
2) 2 tubes of CTS-SEQUENCE PCR Buffer (1400 µl). Store at -20°C in pre-PCR area.
3) Sequencing primers (500 µl each):
   B-E2F, B-E2R, B-E3F, B-E3R, B-E4F, B-E4R,
   Store at -20°C in post-PCR area.

a) PCR stripes and amplification mixes:

The amplification primers are prepipetted and dried in blue PCR stripes composed of 8 cavities. 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) amplified by each mix.
Mix B01 to B07 are group-specific, mix B08 is locus-specific.



Mix	Amplified Allels
B08	All HLA-B Alleles
B07	B*44 (ex. B*44:27)
B06	B*35, 51, 52, 53, 58, 78, 81:03
B05	B*18, 27, 37, 40:02, 40:06, 47, 82:02
B04	B*15, 46
B03	B*13, 57, 81:01/02
B02	B*08, 14, 38, 39, 42, 67, 73
B01	B*07, 40:01, 48, 81:01/02

Cave: Mix B06 amplifies B*35:43 weakly.

Figure 1: Mix position on CTS-SEQUENCE HLA-B stripe

b) Sequencing primers:

The tubes containing the sequencing primers (500 µl) have blue colored caps. Sequencing of exon 4 is only possible with the locus-specific mixes (mix B08).

Table 1: Labeling of the sequencing primers

HLA-Locus	Tube label	Sequenced Exon	Direction of sequencing
HLA-B	B-E2F	2	forward
	B-E2R	2	reverse
	B-E3F	3	forward
	B-E3R	3	reverse
	B-E4F	4	forward
	B-E4R	4	reverse

Special notes:

- In the following (rather uncommon) combinations of alleles/allele groups, ambiguities can be further resolved by using additional group-specific sequencing primers (not included in this kit) for sequencing reactions:

Combination	Positive amplification mix used for sequencing	Group-specific sequencing primers	Order number
B*35 + B*51	Mix B06 or mix B08	B-E2R35, B-E2R51, B-E3F35, B-E3F51	433-35+51

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/softwares	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 preperation	
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 measurememnt of DNA concentration	
50x TAE buffer	Inno-Train, Kronberg/Taunus, Germany Cat.No. GX12765
Analytical balance	

Table 3: Post-PCR area

Reagents/materials/softwares	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 elektophoresis 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/softwares	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 ddH_20.
- 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 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-B 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 Pilot™-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-B test (one stripe, 8 mixes), prepare a pre-mix for 10 mixes (108.5 µl of PCR Buffer + 40 µl of 25 ng/µl DNA + 1.5 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 65 °C	15 s 2 min	10
3	95 °C 61 °C 72 °C	15 s 50 s 1 min 30 s	22
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/cm^2).

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 1300-2000 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.

C) Interpretation hints:

- Some mixes may occasionally show faint, non-specific amplifications which do not affect the sequencing results.

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^{TM} (USB, Staufen, Germany). ExoSAP-IT^{TM} 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 I, exon 2 and exon 3 must be completely sequenced.
- If an allele is not separated by amplification (e. i. if only the locus-specific mix is positive or if only one of the group-specific mixes + the locus-specific mix are positive), we recommend to sequence the locus-specific mix (B08) in both directions (forward and reverse) to optimize base-calling and to reduce the risk of allelic drop out.
- 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).
- Sequencing of exon **4** (always use locus-specific amplicons for this step) should be performed in **forward and reverse** direction.

Table 5 exemplifies which sequencing primers should be used depending on positive amplification patterns.

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**.

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

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

Table 5: Sequencing strategy recommended for HLA-B (example)

Amplification mix	Exon to be sequenced	Only locus-specific mix (Mix B08) positive	One group-specific mix (e.g. mix B01) and locus-specific mix (mix B08) positive	Two group-specific mixes (e.g. mix B01+ mix B02) and locus-specific mix (mix B08) positive
Group-specific mix 1 (e. g. mix B01)	Exon 2		B-E2R	B-E2R
	Exon 3		B-E3R	B-E3R
Group-specific mix 2 (e. g. mix B02)	Exon 2			B-E2R
	Exon 3			B-E3R
Locus-specific Mix (mix B08)	Exon 2	B-E2F and B-E2R	B-E2F and B-E2R	B-E2R
	Exon 3	B-E3F and B-E3R	B-E3F and B-E3R	B-E3R
	Exon 4	B-E4F and B-E4R	B-E4F and B-E4R	B-E4F and B-E4R

Thermocycler program for sequencing reaction (**CTS-SEQ**):

Step	Temperature	Time	Numbers of cycles
1	96 °C	1 min	1
2	96 °C 60 °C	10 s 2 min	25
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 ddNPTs 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 2nd 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 then 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_B01_B-E2F) if amplification mix B01 was used in the sequencing reaction with the B-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-B

Name	B-E2F	B-E2R	B-E3F	B-E3R	B-E4F	B-E4R
Gene	B	B	B	B	B	B
Direction	fwd.	rev.	fwd.	rev.	fwd.	rev.
SeqPrimer gene parts	E2	E2	E3	E3	E4	E4
RFName	B-E2F	B-E2R	B-E3F	B-E3R	B-E4F	B-E4R
Sorting	0	0	0	0	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-Bllele.
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-B Amplification Protocol

For Lot SB05-0

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 Allels	Amplified Exon
	B01		1500 bp	B*07, 40:01, 48, 81:01/02	2-3
	B02		1600 bp	B*08, 14, 38, 39, 42, 67, 73	2-3
	B03		2100 bp	B*13, 57, 81:01/02	2-3
	B04		2000 bp	B*15, 46	2-3
	B05		2000 bp	B*18, 27, 37, 40:02, 40:06, 47, 82:02	2-3
	B06		1500 bp	B*35, 51, 52, 53, 58, 78, 81:03	2-3
	B07		1500 bp	B*44 (ex. B*44:27)	2-3
	B08		1800 bp	All HLA-B Alleles	2-4

Cave: Mix B06 amplifies B*35:43 weakly, B*44:27 is not amplified by Mix B07.

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_B01 _B-E2R)											
B	(Stan_B02 _B-E2R)											
C	(Stan_B01 _B-E3R)											
D	(Stan_B02 _B-E3R)											
E	(Stan_B08 _B-E2F)											
F	(Stan_B08 _B-E3F)											
G	(Stan_B08 _B-E4F)											
H	(Stan_B08 _B-E4R)											

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

Amplification pattern of the B-locus:

ⓧ	B08 positive
◯	
◯	
◯	
◯	
◯	
ⓧ	B02 positive
ⓧ	B01 positive

Position on plate	
A1	Mix B01 was sequenced with the B-E2R sequencing primer
A2	Mix B02 was sequenced with the B-E2R sequencing primer
A3	Mix B01 was sequenced with the B-E3R sequencing primer
A4	Mix B02 was sequenced with the B-E3R sequencing primer
A5	Mix B04 was sequenced with the B-E2F sequencing primer
A6	Mix B04 was sequenced with the B-E3F sequencing primer
A7	Mix B04 was sequenced with the B-E4F sequencing primer
A8	Mix B04 was sequenced with the B-E4R sequencing primer