HLA SBT Typing Kits:

SBT reagent kit for HLA sequence based typing

Product	REF	Package	
HLA-A SBT Typing Kit	800 001	50 Tests	CE 0197
HLA-A SBT Typing Kit	800 002	200 Tests	CE 0197
HLA-B SBT Typing Kit	800 003	50 Tests	CE 0197
HLA-B SBT Typing Kit	800 004	200 Tests	CE 0197
HLA-DRB1 SBT Typing Kit	800 005	50 Tests	CE 0197
HLA-DRB1 SBT Typing Kit	800 006	200 Tests	CE 0197
HLA-C SBT Typing Kit	800 007	50 Tests	CE
HLA-C SBT Typing Kit	800 008	200 Tests	CE
HLA-DQB1 SBT Typing Kit	800 009	50 Tests	CE
HLA-DQB1 SBT Typing Kit	800 010	200 Tests	CE
HLA-DPB1 SBT Typing Kit	800 011	50 Tests	CE
HLA-DPB1 SBT Typing Kit	800 012	200 Tests	CE

Revision Status: 2012-06

Contents

1. HLA-SBT Kit Systems 4

2. Components of the HLA-SBT Sequencing Kits 6

2.1. Ready to Use PCR Mix 6

2.2. Ready to Use Sequencing Mixes 6

2.3. Storage of Components of the HLA SBT Sequencing Kit 6

3. Additionally Required Reagents and Materials 7

4. Preparation of Samples Prior to Testing 9

5. PCR Amplification 9

5.1. Instruction for the Preparation of the Initial PCR: 9

5.2. Analysis of the PCR Amplifications 9

5.3. Purification of PCR Products before Sequencing 10

5.4. Dilution of PCR Products before Sequencing 10

6. Sequencing 10

6.1. Preparation of Cycle Sequencing Reactions 10

6.2. Purification of Cycle Sequencing Reactions 11

6.3. Sequencing Run 12

6.4. Sequence Analysis 13

7. Troubleshooting 14

7.1. PCR 14

7.2. Sequencing 15

8. Appendix: 15

8.1. Protocols (as an add on in A4 format) 15

8.1.1 PCR Protocol 15

8.1.2 Cycle Sequencing Protocol 15

8.2. Preparation of Additionally Required Solutions: 16

8.2.1 Gel Electrophoresis: 16

8.2.2 Purification after Cycle Sequencing 16

8.2.3 1x EDTA Running Buffer for the Sequencer 17

9. Performance Data: 17

10. References: 19

1. HLA-SBT Kit Systems

This R.O.S.E. Europe HLA sequence based typing (SBT) kit systems for 50/200 typings are in-vitro diagnostic (IVD) kits for high-resolution typing of the HLA loci A, B, C, DRB1, DQB1 and DPB1. In about 42% of all typings for HLA-A, 50% for HLA-B, 30 % for HLA C, 50% for HLA-DRB1, 70% for HLA-DQB1 and 52% of HLA-DPB1 the Core Kit Systems directly result in a 4-digit resolution which are in line with the EFI (European Federation of Immunogenetics) and the ASHI (American Society for Histocompatibility and Immunogenetics) standards available on: http://www.efiweb.org and http://www.ashi-hla.org. For all other cases R.O.S.E. Europe offers CE certified sequence specific sequencing primers (3SP) that help to resolve most of the common ambiguities according to EFI and ASHI standards.

Package sizes:

HLA-A SBT Kit System

Ref	Number of Tests	Ready to Use PCR Mix	Ready to Use Sequencing Mixes		
			Exon 2	Exon 3	Exon 4
800 001	50	1x (980µl)	SA2Fw (1x410µl) SA2Re (1x410µl)	SA3Fw (1x410µl) SA3Re (1x410µl)	SA4Fw (1x410µl) SA4Re (1x410µl)
800 002	200	4x (980µl)	SA2Fw (2x 825µl) SA2Re (2x 825µl)	SA3Fw (2x 825µl) SA3Re (2x 825µl)	SA4Fw (2x 825µl) SA4Re (2x 825µl)

HLA-B SBT Kit System

Ref	Number of tests	Ready to Use PCR Mix	Ready to Use Sequencing Mixes		
			Exon2	Exon3	Exon4
800 003	50	1x (980µl)	SB2Fw (1x410µl) SB2Re (1x410µl)	SB3Fw (1x410µl) SB3Re (1x410µl)	SB4Fw (1x410µl) SB4Re (1x410µl)
800 004	200	4x (980µl)	SB2Fw (2x 825µl) SB2Re (2x 825µl)	SB3Fw (2x 825µl) SB3Re (2x 825µl)	SB4Fw (2x 825µl) SB4Re (2x 825µl)

HLA-C SBT Kit System

Ref	Number of Tests	Ready to Use PCR Mix	Ready to Use Sequencing Mixes		
			Exon 2	Exon 3	Exon 4
800 001	50	1x (980µl)	SC2Fw (1x410µl) SC2Re (1x410µl)	SC3Fw (1x410µl) SC3Re (1x410µl)	SC4Fw (1x410µl) SC4Re (1x410µl)
800 002	200	4x (980µl)	SC2Fw (2x 825µl) SC2Re (2x 825µl)	SC3Fw (2x 825µl) SC3Re (2x 825µl)	SC4Fw (2x 825µl) SC4Re (2x 825µl)

The R.O.S.E. Europe HLA sequencing based typing (SBT) kits (Loci A, B and C) analysis sequences of the exons 2 and 3 (most relevant for clinical applications) as well as exon 4. The kit systems consist of initial PCR Ready to Use mix and Sequencing Ready to Use mixes. Each genomic DNA sample is amplified by one PCR for each HLA kit system with locus-specific primers resulting in two specific bands (approx. 1200bp and 1400bp). Before sequencing, PCRs are enzymatically purified, diluted with water and used as templates for direct sequencing according to the Sanger method. The reactions are carried out with Ready to Use Sequencing Mixes that include exon specific forward and reverse sequencing primers for the exons 2, 3 and 4. According to the histocompatibility test standards of EFI, sequencing in both directions is required especially if only one amplified product of a distinct gene region is tested. The analysis of the sequence data should be performed with HLA allele identification software.

Package size:

HLA-DRB1 SBT Kit System

Ref	Number of tests	Ready to use PCR Mix	Ready to Use Sequencing Mixes
			Exon 2
800 005	50	1x (980µl)	SR2Fw (1x410µl); SR2Re (1x410µl)
800 006	200	4x (980µl)	SR2Fw (2x 825µl); SR2Re (2x 825µl)

The R.O.S.E. Europe GmbH HLA-DRB1 SBT kit analyze sequences of exon 2. The kit consists of the Initial PCR Ready to Use mix and the Sequencing Ready to Use mixes. For HLA typing with the kit each genomic DNA sample is amplified by PCR with locus-specific primers resulting in a specific band. The size of the specific band varies according to different allele groups of DR (400-800bp). Before sequencing, samples are enzymatically purified, diluted and used as templates for direct sequencing according to the Sanger method, carried out with exon 2 specific forward and reverse sequencing primers. According to the histocompatibility test standards of EFI sequencing in both directions is required, especially if only one amplified product of a distinct gene region is tested. The analysis of sequence data should be performed with HLA-allele identification software.

Package size:

HLA-DQB1 SBT Kit System

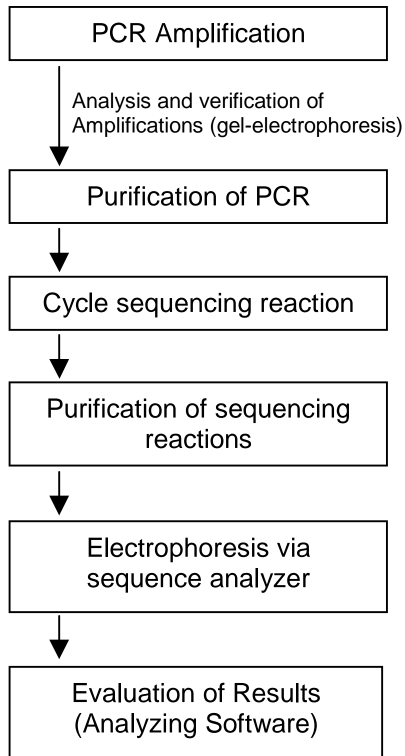
Ref	Number of tests	Ready to use PCR Mix	Ready to Use Sequencing Mixes	
			Exon 2	Exon 3
800 009	50	1x (980µl)	SQ2Fw (1x410µl) SQ2Re (1x410µl)	SQ3Fw (1x410µl) SQ3Re (1x410µl)
800 010	200	8x (980µl)	SQ2Fw (2x 825µl) SQ2Re (2x 825µl)	SQ3Fw (2x825µl) SQ3Re (2x825µl)

The R.O.S.E. Europe GmbH HLA-DQB1 SBT kit analyses sequences of exon 2 and 3. The kit consists of the Initial PCR Ready to Use mix and the Sequencing Ready to Use mixes. For HLA typing with the kit each genomic DNA sample is amplified by PCR with locus-specific primers resulting in two specific bands (400bp and 860bp). Before sequencing, samples are enzymatically purified, diluted and used as templates for direct sequencing according to the Sanger method, carried out with exon 2 and 3 specific forward and reverse sequencing primers. According to the histocompatibility test standards of EFI sequencing in both directions is required, especially if only one amplified product of a distinct gene region is tested. The analysis of sequence data should be performed with HLA-allele identification software.

Ref	Number of tests	Ready to use PCR Mix	Ready to Use Sequencing Mixes	
			Exon 2	Exon 3
800 011	50	1x (980µl)	SP2Fw (1x410µl) SP2Re (1x410µl)	SP3Re (1x410µl)
800 012	200	8x (980µl)	SP2Fw (2x 825µl) SP2Re (2x 825µl)	SP3Re (2x825µl)

The R.O.S.E. Europe GmbH HLA-DPB1 SBT kit analyses sequences of exon 2 and 3. The kit consists of the Initial PCR Ready to Use mix and the Sequencing Ready to Use mixes. For HLA typing with the kit each genomic DNA sample is amplified by PCR with locus-specific primers resulting in two specific bands (325bp and 1150bp). Before sequencing, samples are enzymatically purified, diluted and used as templates for direct sequencing according to the Sanger method, carried out with exon 2 specific forward and reverse sequencing primers and 3 with a specific reverse sequencing primer. According to the histocompatibility test standards of EFI sequencing in both directions of exon 2 is required, especially if only one amplified product of a distinct gene region is tested. Exon 3 of this class II gene helps to resolve ambiguities, therefore the sequencing in only one direction is sufficient. The analysis of sequence data should be performed with HLA-allele identification software.

Test principle:



Key issues of the software are: Analysis of the relevant exons; report of heterozygous positions and mismatches; the possibility to modify sequences; print out and archiving results; continuously updated HLA-database according to the latest official information. The manufacturer does NOT issue any guarantee or responsibility for the use of the analyzing software.
HLA-typing with the SBT kits requires also components, which are not part of the kit. The user can purchase these directly from the respective manufacturer or from R.O.S.E. Europe GmbH.
Enzymes and components additionally needed are Taq DNA Polymerase, ExoSAP-IT® as well as BigDye™ Terminator Mix v3.1 and Formamide.
R.O.S.E. Europe GmbH explicitly excludes liability for enzymes produced by “non-original parts manufacturers”. We point out that R.O.S.E. Europe GmbH is only taking responsibility for the products produced under its premises.

Kit components of each Kit System are tested individually and can be combined with any other LOT of the same Kit System.

The kit is shipped on dry ice and should be stored immediately at –20°C until use. In case of damage, the components of the HLA SBT kits should be checked carefully. If the components are undamaged the system may be used.
None of the components in the HLA SBT kits carries any health risks and no specific security precautions are required.

2. Components of the HLA-SBT Sequencing Kits

2.1. Ready to Use PCR Mix

The Ready to Use PCR Mixes are shipped in 1.5ml tubes and contain PCR buffer, $MgCl_2$, dNTPs; primers, and water. To separate ROSE GmbH Kit Systems easily from each other the following color code for lids is used: Locus A: green; Locus B: red; Locus C: yellow; Locus DRB1: blue and Locus DQB1: white.

2.2. Ready to Use Sequencing Mixes

Ready to Use Sequencing Mixes are shipped in 1.5ml tubes and contain sequencing buffer, sequencing primers and water. The Ready to Use Sequencing Mixes for the different exons can be easily distinguished by different colored labels: blue for exon 2; yellow for exon 3 and pink for exon 4.
In addition each of the SBT Kit systems has different colored lids as mentioned above.

2.3. Storage of Components of the HLA SBT Sequencing Kit

All components of the HLA SBT Sequencing kit must be stored at –20°C in the pre-PCR area of the laboratory upon arrival.
The Kit system should be used up in 8 months after the first opening.
After thawing the Kit System, desired amounts of Ready to Use PCR-Mix / Ready to Use Sequencing Mixes should be taken from the Kit System. Afterwards all components should be stored at –20°C again.
Thawing and re-freezing cycles should be kept as low as possible, since frequent thawing and re-freezing might influence the quality of the reagents. Users may prepare smaller aliquots in order to avoid repeated freezing and thawing processes. Freezing and thawing up to 35 cycles has no influence on function and quality of the HLA SBT Kit Systems.

3. Additionally Required Reagents and Materials

To perform typings with the HLA SBT Sequencing Kits additional materials and reagents are required which are not part of the kit:

Reagents	Provider/Manufacturer	Storage and application
Platinum Taq DNA Polymerase (5U/µl) or Go Taq Hot Start Polymerase (5U/µl)	Invitrogen GmbH Promega	Pre-PCR Area
Big Dye™ Terminator Cycle Sequencing Kit Version 3.1	Applied Biosystems	Pre-PCR Area
ExoSAP-IT®	USB Corp.	Post-PCR Area
High purified water, e.g. HPLC grade (LiChrosolv Water)	e.g. Merck KGaA	Pre-PCR Area

All these reagents might be purchased from R.O.S.E. Europe GmbH in sufficient amounts for 50/200 typings.

R.O.S.E. Europe GmbH Kit Systems are optimized with the following equipment and reagents:

Thermocycler:	GeneAmp PCR Systems 9700 (Applied Biosystems)
Sequence analyzer:	3130*xl* / 3730xl (Applied Biosystems)
Taq DNA Polymerase	Invitrogen (Platinum Hot start Taq) or Promega (Go Taq Hot start Polymerase)
Labeling Mix:	BigDye™ Terminator Mix v3.1 (Applied Biosystems)
Initial PCR Purification:	ExoSAP-IT® (USB Corp)
Cycle Sequencing Purification	Ethanol / EDTA Method or Sephadex Method

If other equipment / reagents / protocols are used validation experiments are strongly recommended. Using other Taq Polymerase might lead to drop out of alleles and therefore mistakes in typing results.

Additional reagents needed are:

Reagents		Provider / Manufacturer	Storage / application
Agarose gel electrophoresis:			Post-PCR Area
10x Borate buffer (running buffer)	Boric acid p.a.	e.g. Merck KGaA	
	EDTA p.a.	e.g. Merck KGaA	
	Tris(hydroxymethyl)aminomethane p.a.	e.g. Merck KGaA	
Agarose		e.g. Biozym Diagnostik GmbH	
Ethidium bromide (10mg/ml) **Ethidium bromide is potentially carcinogen. Wear protective gloves when handling ethidium bromide!**		e.g. AppliChem GmbH	
Loading dye 5-6x		e.g. Fermentas GmbH	
Purification after cycle sequencing: e.g. Ethanol method			
EDTA		e.g. AppliChem GmbH	
Ethanol		e.g. AppliChem GmbH	
70% Ethanol			
Purification after cycle sequencing: e.g. Sephadex method:			
Sephadex G50 Superfine (e.g. G-5050)		Sigma-Aldrich Co.	
Multiscreen HV plates (e.g. MAHVN4510)		Millipore GmbH	
Multiscreen 45µl Column Loader (MACL09645)		Millipore GmbH	
Multiscreen Centrifuge Alignment Frame (MACF09604)		Millipore GmbH	
ABI Micro Amp Splash free 96-well Base (P/Nr. 4312063)		Applied Biosystems GmbH	
Denaturing DNA			
HiDi Formamide		Applied Biosystems GmbH	

Additional reagents needed (continued):

Running buffer and polymer for Sequencer		
Various reagents for the ABI Sequencing device like: - 1x EDTA Running buffer (preparation see 8.2.3) - POP 7	Applied Biosystems GmbH	

Equipment, consumables, and software additionally required:

Equipment, consumables and software	Provider/ Manufacturer	Application
Spectral photometer (for adjusting DNA concentration)	e.g. Beckmann Coulter	Post-PCR Area
Micro wave oven(preparation of Agarose gels)	e.g. AEG Elektrolux	Post-PCR Area
8- or 12- channel pipette 5–50µl, and standard filter tips 2-200µl	e.g. Socorex Isba S.A., Eppendorf AG	
Multipette with corresponding adapters 0.1; 0.2; 0.5; 1.25 ml	e.g. Eppendorf AG, Gilson Inc.	
Equipment, consumables and software	Provider/ Manufacturer	Application
Pipettes and filter tips for volumes of: 0.5–10µl, 10–100µl, 100–1000µl	e.g. Eppendorf AG, Gilson Inc.	
Analytical balance	e.g. Sartorius AG	
Vortex	e.g. VWR International GmbH	
Consumables for initial PCR: 96-well plates: AB-0600 48-well plates: AB-0916 16/24-well plates: SP-0151	ABgene Ltd.	
96-well plates for Sequencer: AB-1100	ABgene Ltd.	
Reaction tubes 1.5 ml	e.g. Eppendorf AG	
Sequencer e.g. 3130*xl*	Applied Biosystems	Post-PCR Area
Thermocycler e.g. 9700, PTC-200	Applied Biosystems,	
Plate centrifuge (900-2500xg)	e.g Heraeus Instruments GmbH	
Gel electrophoresis tank	e.g. GE Healthcare Life Sciences	
Voltage supplier	e.g GE Healthcare Life Sciences	
UV Tran illuminator	e.g. MWG Biotech AG	
Photo documentation	e.g. Mitsubishi, UVItec Ltd.	
8-channel pipette and tips 2-200µl, corresponding filter tips	e.g. Eppendorf AG, Gilson Inc.	
Multipette with corresponding adapter, see above	e.g. Eppendorf AG, Gilson Inc.	
Pipettes and filter tips (Safe seal tips) for : 0.5–10 µl	e.g. Eppendorf AG, Gilson Inc.	
0.2 ml 96-well PCR plates (see above)	ABgene Ltd.	
Adhesive foil for 96-well plates	e.g. ABgene Ltd., Nunc	
Allele identification software: e.g. Sequence Pilot ™-HLA SBT; ATF	e.g. JSI Medical Systems GmbH; Conexio GmbH	

4. Preparation of Samples Prior to Testing

DNA isolation and measurement of the concentration:
In general, genomic DNA can be extracted from any nucleated cell. DNA sources are Buffy Coat, cell suspensions or EDTA-/Citrate- blood. Heparin-blood should not be used as heparin may inhibit PCR. It is recommended to use only extraction systems for genomic DNA which guarantee a good quality and high amount of DNA, e.g. Chemagen, Magnetic Separation, QIAamp Blood Kit (QIAGEN), and others. The concentration of the extracted DNA should be adjusted with HPLC-water between 5-50ng/µl.
Human material is considered as potentially infectious and must be handled with extra care. Always wear protective clothes!

5. PCR Amplification

5.1. Instruction for the Preparation of the Initial PCR:

<table>
<tr><td>1.</td><td colspan="3">Fill in the PCR documentation protocol (e.g. see 8.1.1).</td></tr>
<tr><td>2.</td><td colspan="3">Thaw Ready to Use PCR Mix.</td></tr>
<tr><td>3.</td><td colspan="3">Prepare and label plate for initial PCR.</td></tr>
<tr><td rowspan="3">4.</td><td colspan="3">Prepare amplification mix: for DNA samples + one negative control per PCR run.</td></tr>
<tr><td>Ready to Use PCR Mix</td><td>Use 17.85µl for each sample + 5% as reserve volume: n*17.85+(0.05*(n*17.85))</td><td rowspan="2">Pipette Ready to Use PCR Mix and Taq DNA polymerase together.</td></tr>
<tr><td>Taq DNA polymerase</td><td>Use 0.15µl for each sample + 5% as reserve volume: n*0.15+(0.05*(n*0.15))</td></tr>
<tr><td>5.</td><td colspan="3">Vortex Amplification Mix containing Taq DNA polymerase, centrifuge briefly to collect the solution on the bottom of the tube and pipette 18µl into each well of the plate.</td></tr>
<tr><td>6.</td><td colspan="3">Change to the post–PCR room.</td></tr>
<tr><td>7.</td><td colspan="3">Pipette 2.0µl DNA sample (5-50ng/µl) in each well of the plate, except for the negative control. (Use instead 2µl H_2O for the negative control)</td></tr>
<tr><td>8.</td><td colspan="3">Seal plate with adhesive foil, vortex and spin down briefly the liquid to the bottoms of the wells.</td></tr>
<tr><td>9.</td><td colspan="3">Place plate into the thermocycler and start the PCR program.</td></tr>
</table>

Thermocycler program for the initial PCR:

1 Cycle: 96°C: 120 sec	5 Cycles: 96°C: 30 sec. 65°C: 30 sec. 72°C: 120 sec.	35 Cycles: 96°C: 30 sec. 62°C: 30 sec. 72°C: 120 sec.	10°C: ∞

5.2. Analysis of the PCR Amplifications

Following PCR the amplification products are evaluated on a 1.6%-agarose gel (see 8.2.1).

1.	For each amplification pipette 2µl loading dye (5-6x) into one well of an empty PCR plate.
2.	Add 5µl of each amplification reaction with an 8-channel pipette and mix carefully.
3.	Pipette this mixture into the gel slots.
4.	Perform the gel run according to the following conditions: 150 V approx. 45-60 min.
5.	Evaluate the results: each amplification should display two visible bands of approx. 1200bp and 1400bp (Loci A, B, C); one or two bands between 400-800bp (Locus DRB1); two bands of approx 400 and 860bp (Locus DQB1) and two bands approx 325bp and 1150bp (Locus DPB1).
6.	Document the gel result with a photo documentation system and add the picture to the PCR documentation protocol.

5.3. Purification of PCR Products before Sequencing

Before the performance of the cycle sequencing reaction the PCR products must be purified to remove free primers and dNTPs, which might influence the sequencing reaction.
For the enzymatic purification ExoSAP-IT® is recommended. This enzyme combination ensures that the free primers and dNTPs are digested.

Protocol for the purification with ExoSAP-IT®:

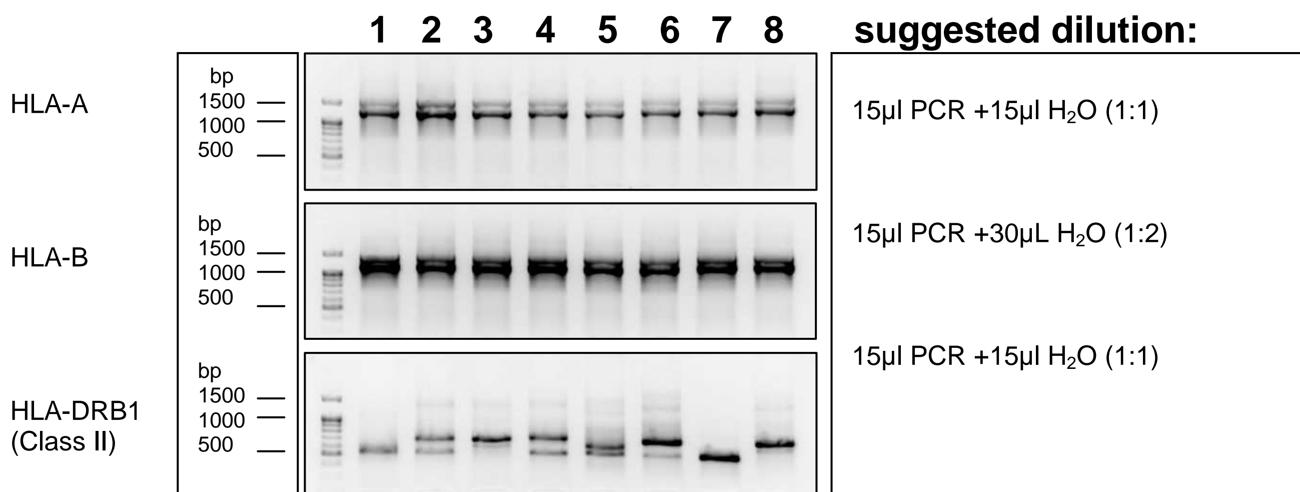
1.	Remove lids of the plates.
2.	Use a multipipette to add 2µl of ExoSAP-IT® to each sample. To ensure that every sample contains ExoSAP-IT® pipette the enzyme just below the upper rim of the well. Check carefully for completeness after pipetting.
3.	Close plate, and spin down briefly to ensure that the enzyme mix is resolved in the sample.
4.	Afterwards mix (vortex) carefully and spin down briefly again.
5.	Incubate samples in a thermocycler with the program given below.
6.	After the incubation step dilute samples 1:1 or up to 1:3 with highly purified water.
7.	Document purification step in the PCR Protocol

Thermocycler incubation program for ExoSAP-IT®:

Incubation:	Enzyme inactivation	
1 Cycle: 37°C: 15 min.	1 Cycle: 85°C: 15 min.	10°C ∞

5.4. Dilution of PCR Products before Sequencing

Depending on the band intensity in the gel electrophoresis it is necessary to dilute the samples after the enzymatic purification for the cycle sequencing reaction. A standard dilution of the purified PCR product of 1:1 or 1:2 is recommended. There might be cases where a higher dilution is necessary. The following picture helps to evaluate the dilution ratio of the purified PCR products:



After purification and dilution the samples are ready for cycle sequencing.

6. Sequencing

6.1. Preparation of Cycle Sequencing Reactions

After purification and dilution of the PCR products the cycle sequencing reactions are performed. High-resolution typing requires six (Loci A, B, C), two (Locus DRB1) three (Locus DPB1) or four (Locus DQB1) sequencing reactions to obtain the complete sequences from exons 2, 3 and 4 (Loci A, B, C) exon 2

(Locus DRB1) and exon 2 and 3 (Locus DQB1 and DPB1). Each exon is sequenced in forward and reverse direction, except exon 3 in DPB1 where only the reverse primer is used.

The cycle sequencing reaction for each sequencing primer is prepared separately:

1.	Provide and fill in cycle sequencing protocol(s).	
2.	Provide and thaw BigDye™ Terminator Mix (Cycle Sequencing Kit Version 3.1).	Pre-PCR Area
3.	Prepare 96-well reaction plate(s) (ABgene: AB-1100) with barcode (if needed) and a corresponding adhesive foil. Provide, label and note barcode-No. into the cycle sequencing protocol.	
4.	Use 7.75µl of the Ready to Use Sequencing Mix and 0.25µl of BigDye™ Terminator Mix Version 3.1 per sample; calculate a 5% reserve volume according to the following formula: for Ready to Use Sequencing Mix: n*7.75µl Sequencing Mix + (0.05*n*7.75µl Sequencing Mix) for BigDye™ Terminator Mix Version 3.1: n*0.25µl BigDye + (0.05*n*0.25µl BigDye)	
5.	Prepare a mixture of Ready to Use Sequencing Mix and BigDye™ Terminator Mix Version 3.1 and pipette 8µl in each well of the 96-well plate.	
6.	Change to the post-PCR Area.	**Post-PCR Area**
7.	Add 2µl of each purified and diluted PCR sample per well.	
8.	Close the 96-well plate(s) with a corresponding adhesive foil.	
9.	Vortex the plate(s) and spin contents down briefly.	
10.	Place the 96-plate(s) into the thermocycler(s) and run the cycle sequencing program as given below.	

Thermocycler Cycle Sequencing Program:

1 Cycle: 96°C: 1 min.	40 Cycles: 96°C: 10 sec. 60°C: 2 min.	10°C: ∞

6.2. Purification of Cycle Sequencing Reactions

Following cycle sequencing the sequencing products are purified to remove non-inserted dNTPs and ddNTPs (stop-nucleotides) to avoid sequencing artifacts. For the HLA SBT Sequencing Kits the Sephadex purification method and the Ethanol precipitation method have been tested and these methods are recommended for the purification of the sequencing products (please see 8.2.2 for details).
All purification steps must be performed in the post-PCR area.

6.3. Sequencing Run

The purified and denatured sequencing products can be put directly into the sequencing apparatus 3130xl or 3730xl, see instruction manual 3130xl / 3730xl DNA Analyzer (Applied Biosystems).

Running conditions for the 3130xl / 3730xl DNA Analyzer (Applied Biosystems):
Calibrations for 3130xl / 3730xl Capillary sequencer:

	3130xl	3730xl
POP-Polymer	POP-7	POP-7
Capillary	36 cm array	36 cm array
Electrophoresis Buffer	1x Buffer with EDTA	1x Buffer with EDTA
Instrument Protocol Type: RunModule: Dye Set:	 Regular UltraSeq36_POP7_IT20_Z Z-Big-DyeV3	 Regular StdSeq36_1200s_POP7_IT10 Z-Big-DyeV3
Sequencing Analyses Software	3130xl Gene Analyzer Data Collection Software v.3.0	3730/3730xl Gene Analyzer Data Collection Software v.3.0
Run Module (Protocol) Oven Temperature : Poly fill volume: Current stability Pre Run Voltage Pre Run Time Injection Voltage Injection Time Voltage_Number_Of_Steps Voltage_Step_Interval Data Delay time Run Voltage Run Time First_ReadOut_Time Second_ReadOut_Time Ramp_Delay	UltraSeq36_POP7_IT20_Z 60°C 6500 steps 5.0 uAMPS 15 kV 180 sec 1.2 kV 20 sec 20 nk 15 sec 80 sec 13.2 kV 1200 sec	StdSeq36_1200s_POP7_IT10_Z 60°C 30.0 uA 15 kV 180 sec 1.2 kV 10 sec 30 nk 15 sec 120 sec 8.5 kV 1200 sec 250 msec 250 msec 450 sec
Analyze Module **General:** Sequence File Format: **Base Calling**: Basecaller: Dye set/primer file: Processed data: Ending base Quality threshold	(KB_POP7_Z) ABI KB.bcp KB_3130_POP7_BDTv3.mob True Profile not set Do not assign N's to Basecalls	(STD_POP7_Z) ABI KB.bcp KB_3730_POP7_BDTv3.mob True Profile not set Do not assign N's to Basecalls
Mixed Base: **Clear range methods:**	Use mix base identification not set (default Call IUB if 2nd highest peak is 25% of the highest peak) Use quality values; Remove bases from ends until fewer than 4 bases out of 20 have QVs less than 20	
Settings Plate Record RunModule (Protocol):	Dye Set: Z UltraSeq36_POP7_IT20_Z	Dye Set: Z StdSeq36_1200s_POP7_IT10_Z

Define the sample set up (cycle sequencing protocol) in a personal computer connected to the DNA Analyzer. Assigned names of the sequence preparations need to be coordinated with the HLA allele identification software.

The communication between sequence interpretation software and HLA allele analyzing software should be organized in a standardized way with a pre-defined naming system containing sample or DNA name, amplification product name and sequence primer name.

For example:

Serial Number:	Internal number:	Sample amount [µl]	Ready to Use Sequencing Mix	BigDye [µl]	Sequencing Mix [µl]	Exon
1	01(XY_TTTT_TTTT)	2	[name]	0.25	7.75	2
2	02(XY_TTTT_TTTT)		[name]			
3	03(XY_TTTT_TTTT)		[name]			
4	04(XY_TTTT_TTTT)		[name]			

XY= sample identification; TTTT = amplification product; TTTT = **Ready to Use Sequencing Mix** name.

Load samples into the 3130xl DNA Analyzer and start the run.

6.4. Sequence Analysis

Sequence based allele identification software is mandatory for the analysis of the sequencing data. Key requirements are: single sequences of the relevant exons can be analyzed, heterozygous situations and mismatches are indicated and the sequences can be edited. Results can be printed out and archived. The HLA database is continuously updated to the newest official data of the IMGT allele database.

Follow the manual of the used allele identification software for result evaluation. Before using the allele identification software it is obligatory to set up the software for the sequence based typing kit system used by following the manual of your allele identification software. It is also recommended to establish a pre-defined way of how to handle and analyze incoming data from the sequencer.

We recommend the Software package form Conexio Genomics. This Software was tested and evaluated positively on the Kit System. Other Software packages should be validated before use.

Ambiguities that might occur with this Kit System can be resolved with our sequence specific sequencing Primers (3SP). These Primers are suitable to resolve the most common ambiguities. For detailed information please contact R.O.S.E. Europe. Our Technical Support is pleased to support you with any kind of information.

Please notice that the reading length of exon specific primers might be shorter when ethanol precipitation is used. This might lead to the coverage of the exon ends by only one sequencing primer. In the specific case of **HLA SBT Typing Kit Locus: DQB1** it is highly advised to check out the3´-end of exon 2 in order to avoid mistyping.

7. Troubleshooting

7.1. PCR

Pre-PCR room:
All test steps that are carried out prior to amplification (preparation and storage of reagents, buffers and solutions for DNA isolation and PCR preparation, storage of prepared DNA) must be performed in the pre-PCR area. PCR is an extremely sensitive method even slightest amounts of foreign DNA can lead to the falsification of the results. A negative control (water) can exclude contamination by foreign DNA. Furthermore, filter tips should be used in the pre-PCR area. No material or equipment must be transferred from the post-PCR room to the pre-PCR room.

Post-PCR room:
All working steps after the PCR (incl. PCR run) must take place in the post-PCR room.

PCR Troubleshooting

Problem	Cause	Troubleshooting
No or only weak PCR product present.	Ethidium bromide is missing in the Gel.	Prepare fresh agarose gel with ethidium bromide.
	DNA degraded.	Check DNA by gel electrophoresis. Re-extract DNA.
	No or not enough DNA used.	Use higher concentrated sample for PCR template. Re-extract DNA.
	Genomic DNA contains PCR inhibiting factors.	Check DNA extraction method: Do not use heparin containing blood for DNA extraction. Make sure the protocol of DNA extraction is followed in a correct way.
	Forgot to mix the PCR.	Repeat amplification.
	Use of wrong thermocycler or protocol.	Repeat amplification. Notice: This method was optimized for the 9700 (Applied Biosystems). If another thermocycler is used, the PCR program must be adjusted and validated.
	Thermocycler malfunction.	Check thermocycler.
Unspecific PCR bands	Wrong Taq DNA polymerase.	This method is adjusted to the Platinum Taq DNA Polymerase (Invitrogen). If other enzymes are used, the PCR reaction must be adjusted and validated.
	Thermocycler.	Check the program used for amplification. Check the function of the thermocycler. Check the calibration status of the thermocycler.
	Contamination of PCR reagents or DNA sample.	Use negative control to exclude contaminations. Carefully check or exchange reagents. Extract new DNA.

7.2. Sequencing

Problem	Cause	Troubleshooting
No signals	No Sample.	Repeat sequencing reaction.
	Filling level of 96-well plate too low; no formamide.	Add the adequate amount of formamide.
	Air bubbles in the wells.	Remove bubbles by centrifugation of the 96-well plate.
Signals too weak	Wrong “Injection Time“.	Differences between single sequencers can occur, “Injection Time“ must be adjusted in a way to reach a curve-signal strength between 400 and 3000 for the raw data. Average should be 500-2000 and should not exceed 8000 (maximum).
	Not enough sequencing product used.	Increase “Injection Time“, “Injection Voltage“ or concentration of the sequencing reaction.
Signals too strong.	Wrong “Injection Time“.	Differences between single sequencers can occur, “Injection Time“ must be adjusted in a way to reach a curve-signal strength between 400 and 3000 for the raw data. Average should be 500-2000 and should not exceed 8000 (maximum).
	Concentration of the sequencing product too high.	Dilute PCR product with water prior to the sequencing reaction, e.g. 1:1 - 1:3.
Sequence overlaps, background too high.	Poorly purified PCR product.	Repeat PCR reaction and purification, see 5.1 ff.
	Poorly purified sequencing product.	Repeat cycle sequencing reaction and purification, see 6.1 ff.
	Contamination of PCR product.	Extract new DNA.
	Contamination of sequencing primers.	Use sequencing primers from a new tube.
	Contamination of the sequencing chemistry.	Use sequencing chemistry from a new tube.
DyeBlobs, artifacts of the sequencing chemistry.	Inadequate purification of the sequencing products.	Repeat sequencing reaction and purification, see 6.1ff.
Late initiation of Sequence reading	Precipitation used as a purification method.	Shorter fragments (50b or less) of labeled product might be lost during precipitation. In the case this would affect the analysis use a more conservative Purification method e.g. sephadex purification or re-check your centrifugation parameters.

8. Appendix:

8.1. Protocols (as an add on in A4 format)

8.1.1 PCR Protocol

8.1.2 Cycle Sequencing Protocol

8.2. Preparation of Additionally Required Solutions:

8.2.1 Gel Electrophoresis:

8.2.1.1 10x Borate Buffer

Add 108.92g Tris, 55.6g boric acid and 9.3g EDTA to an adequate container and fill up to 1000ml with Aqua dest. Stir the mixture on a magnetic stirrer until the ingredients are completely dissolved and adjust the volume to 1000ml. Store the buffer at room temperature.

8.2.1.2 1x Borate Buffer:

Add Aqua dest to 100ml 10x Borate buffer to the volume of 1000ml. Store the buffer at room temperature.

8.2.1.3 Preparation of Agarose Gel (1.6%):

Weigh 1.6g agarose and add 1x Borat buffer to 100g. Boil the solution in a microwave oven until it is clear. Replace the missing fluid from the boiling process with Aqua dest. Cool the agarose solution down to approx. 60°C and add 1µl of ethidium bromide (10mg/ml). Mix well and pour the agarose solution into a prepared gel chamber or slide (with combs). Remove combs after polymerization. Fill the gel chamber with 1x Borate buffer. Run the gel at 150V; 45-60min.

8.2.2 Purification after Cycle Sequencing

The purification step after cycle sequencing can be performed in many ways. We recommend either an Ethanol precipitation step or purification via Sephadex columns e.g. G-5050 (Sigma-Aldrich Co.). Other methods may work as well but must be evaluated in the customer's laboratory.

8.2.2.1 EDTA/Ethanol Method

According to the ABI BigDye Terminator protocol (10µl):

Preparation of 125mM EDTA Solution (pH 8.0):
Add 200ml purified H_2O (e.g. Merck HPLC water) to 11.63g of EDTA (372.24 g/mol), adjust pH to 8.0 with HCl (Notice: EDTA dissolves in water at pH 8.0) and fill up with H_2O to 250ml. Filter the solution (filter size approx. 0.2µM).

70% Ethanol:
Prepare a 70% Ethanol solution with purified water (e.g. Merck HPLC water).

Procedure:

1.	Remove the 96-well reaction plate from the thermocycler and briefly spin.
2.	Add 2.5µl of 125mM EDTA solution (pH 8.0) to each well. **Make sure the EDTA reaches the bottom of the wells.**
3.	Add 30µl of 100% ethanol to each well.
4.	Seal the plate with adhesive foil and mix by inverting 4 times.
5.	Incubate at room temperature for 15 min.
6.	*Use a centrifuge with a plate adapter and spin the plate at the maximum speed as follows:* • 1400–2000×*g* for 45 min or • 2000–3000×*g* for 30 min **Proceed to the next step immediately. If this is not possible, then spin the plate for an additional 2 min before performing the next step.**
7.	Remove the liquid by inverting the plate, place it upside-down on a paper tissue and spin again at 185×g for 1min.
8.	Add 50µl of 70% ethanol to each well.

9.	With the centrifuge set to 4 °C, spin at 2500×*g* for 15 min.
10.	Invert the plate, place it upside-down on a paper tissue and spin at 185×*g* for 1 min, and then remove from the centrifuge. **Start timing when the rotor starts moving.**
11.	Dry samples in the dark for 5-10min.
12.	Resuspend each sample in 10-20µl of HIDI formamide.

8.2.2.2 Sephadex Method:

Procedure:

Loading of dry Sephadex into all 96 wells of a Multi-Screen HV plate using the 45µl column loader:	
1.	Add Sephadex to the column loader.
2.	Remove residual resin off the top of the column loader with the scraper supplied.
3.	Place Multi-Screen HV plate upside-down on top of the column loader.
4.	Invert both Multi-Screen HV plate and column loader.
5.	Tap on top or side of the column loader to release the resin.
6.	Using a multi channel pipette, add 300µl H_2O to each well to swell the resin.
7.	Incubate at room temperature for 3h.
Once the mini columns are swollen in the Multi-Screen plates, they can be stored in the refrigerator at 4°C for up to two weeks, either by tightly sealing the plates individually with parafilm, or storing them in a sealed plastic container with a moist, lint-free cloth (to assure humidity).	
Sephadex purification:	
1.	Place a centrifuge alignment frame on the top of a standard 96-well plate, and then place the Multi-Screen HV plate on the assembly and centrifuge at 800xg for 5min. to pack the mini columns.
2.	Carefully add the sequencing reactions to the center of each well.
4.	Using the centrifuge alignment frame place the Multi-Screen HV plate on top of a 96-well AB1100 plate containing 10µl of HIDI formamide/per well and centrifuge the samples at 800xg for 5min. into wells.

8.2.3 1x EDTA Running Buffer for the Sequencer

Mix 90ml LiChrosolv Water with 10ml 10x EDTA buffer from Applied Biosystems.
Store at RT.

9. Performance Data:

Sensitivity: Based on Sensitivity Tests 10-100ng DNA / PCR can be used; we recommend using 40ng DNA / PCR (2µl of 20ng/µl DNA).
Specificity: Specificity Tests were performed with 400 samples for each Locus A, B, DRB1; more than 100 samples for Locus C and 50 samples for Locus DQB1 and Locus DPB1.
Variability: Inter Assay Variability; Intra Assay Variability and Lot to Lot Variability Testing were performed positively with Ready to Use PCR Mixes, as well as all Ready to Use Sequencing Mixes. Based on these results each Lot of Ready to Use PCR Mix might be combined with each Lot of Ready to Use Sequencing Mixes. Freezing and thawing cycles were tested to confirm quality and function of the Kit Systems. 35 freezing and thawing cycles did not influence the quality and function of the Kit Systems.

10. References:

Voorter CE, Mulkers E, Liebelt P, Sleyster E, van den Berg-Loonen EM. Reanalysis of sequence-based HLA-A, -B and -Cw typings: how ambiguous is today's SBT typing tomorrow. Tissue Antigens. 2007;70:383-9

Sayer DC, Whidborne R, De Santis D, Rozemuller EH, Christiansen FT, Tilanus MG. A multicenter international evaluation of single-tube amplification protocols for sequencing-based typing of HLA-DRB1 and HLA-DRB3,4,5. Tissue Antigens. 2004 May;63(5):412-23.

Dormoy A, Froelich N, Leisenbach R, Weschler B, Cazenave JP, Tongio MM. Mono-allelic amplification of exons 2-4 using allele group-specific primers for sequence-based typing (SBT) of the HLA-A, -B and -C genes: preparation and validation of ready-to-use pre-SBT mini-kits. Tissue Antigens. 2003 Sep;62(3):201-16.

Tiercy JM. Molecular basis of HLA polymorphism: implications in clinical transplantation. Transpl Immunol. 2002 May;9(2-4):173-80.

Kurz B, Steiert I, Heuchert G, Müller CA. New high resolution typing strategy for HLA-A locus alleles based on dye terminator sequencing of haplotypic group-specific PCR-amplicons of exon 2 and exon 3. Tissue Antigens. 1999;53:81-96

Kotsch K, Wehling J, Blasczyk R. Sequencing of HLA class II genes based on the conserved diversity of the non-coding regions: sequencing based typing of HLA-DRB genes.Tissue Antigens. 1999 May;53(5):486-97.

Kotsch K, Wehling J, Köhler S, Blasczyk R. Sequencing of HLA class I genes based on the conserved diversity of the noncoding regions: sequencing-based typing of the HLA-A gene. Tissue Antigens. 1997 Aug;50(2):178-91.

R.O.S.E. Europe GmbH
Daimler Str. 6,
61449 Steinbach / Taunus
phone: +49-(0)6171 2868298
fax: +49-(0)6171 2688299
Homepage: www.rose-europe.de