User Manual

protrans
medizinische diagnostische produkte gmbh

HLA

SBT

protrans
medizinische diagnostische produkte gmbh

Protrans medizinische diagnostische Produkte GmbH
D- 68766 Hockenheim, Ketschau 2, Germany
Tel.: + 49-(0)6205-29299-0 Fax: + 49-(0)6205-29299-20
www.protrans.info - mail@protrans.info

Protrans Sequencing Testkits (SBT)

Protrans **S4**	**Single Allele and Locus Specific Sequencing System**	CE
REF	Article	
34 01	Protrans S4 HLA-A	CE 01 97
34 02	Protrans S4 HLA-B	CE 01 97
34 03	Protrans S4 HLA-C	CE
34 04	Protrans S4 HLA-DRB1	CE 01 97
24 1415	Protrans HLA-B*5701	CE 01 97

Protrans **S3**	**Allele and Locus Specific Sequencing System**	CE
REF	Article	
33 01	Protrans S3 HLA-A	CE 01 97
33 02	Protrans S3 HLA-B	CE 01 97
33 03	Protrans S3 HLA-C	CE
33 04	Protrans S3 HLA-DRB1	CE 01 97
33 09	Protrans S3 HLA-DQB1	CE

Protrans **Domino Stones**		**Haplotype-Specific Sequencing System**	CE
REF		Article	
541 01_13	641 01_13	Protrans Domino Stones HLA-A	CE 01 97
542 01_15	542 01_15	Protrans Domino Stones HLA-B	CE 01 97
543 01_13	543 01_13	Protrans Domino Stones HLA-C	CE
544 01_14	644 01_14	Protrans Domino Stones HLA-DRB1	CE 01 97
539 01_09	639 01_09	Protrans Domino Stones HLA-DQB1	CE

Protrans **S2**	**Allele-Group-Specific Sequencing**	CE
REF	Article	
32 04	Protrans S2 HLA-DRB1	CE 01 97
32 08	Protrans S2 HLA-DQA1	CE

Protrans **S1**	**Locus-Specific Sequencing System**	CE
REF	Article	
31 01	Protrans S1 HLA-A	CE 01 97
21 01	Protrans S1 HLA-A swift	CE 01 97
31 02	Protrans S1 HLA-B	CE 01 97
21 02	Protrans S1 HLA-B swift	CE 01 97
31 03	Protrans S1 HLA-C	CE
31 05	Protrans S1 HLA-DRB3	CE 01 97
31 06	Protrans S1 HLA-DRB4	CE 01 97
31 07	Protrans S1 HLA-DRB5	CE 01 97
31 09	Protrans S1 HLA-DQB1 Exon 2	CE
31 10	Protrans S1 HLA-DQB1 Exon 3	CE
31 11	Protrans S1 HLA-DPB1	CE

Σ	Protrans S4	24
	Protrans S3	24
	Protrans S2	24
	Protrans S1	24
	Protrans Domino Stones	24
		240
IVD	For In-Vitro-Diagnostic use	

	Amplification Unit 1 pre-pipetted PCR-Strips	4°-8°C
	Amplification Unit 2	-20°C
	Sequencing Unit	-20°C

1.0 PROTRANS Sequencing Strategy

The Protrans Kits should be used in accordance with the current guidelines for quality assurance established by the European Federation for Immunogenetics (EFI) or the American Society for Histocompatibility and Immunogenetics (Ashi).

Sequencing gives the most reliable and accurate information of the DNA sequence of a gene and is therefore of particular interest to fully characterize the genetic complexity and allelic diversity of the HLA genes in the human major histocompatibility complex (MHC). The full complexity of allelic diversity in HLA class I and class II makes PCR-SBT the method of choice for HLA typing. HLA typing by means of sequencing should be considered whenever the HLA type of an individual is needed. The recent developments have made sequencing equally simple and robust making it attractive for patient-related diagnostic and bone marrow registry typing. With the Protrans Sequencing System it is easy to type in low or high throughput formats at each level of automation and resolution desired. Sequencing can be carried out manually or fully automated depending on the laboratory's individual requirements.
Sequencing a gene will give the most reliable and accurate information of the DNA.
The quantity of known and permanently new identified HLA genes
in the human major histocompatibility complex (MHC) requires a high resolution, unambiguous and precise typing method for HLA-Alleles Class I and Class II.

The Protrans Sequencing System is a practical, reliable and adjustable typing method.
With the Protrans Sequencing System it is easy to type all HLA loci in the daily routine
low or high throughput formats manually or at each level of automation and resolution desired.

Protrans Sequencing System is easy to use not only for application in the bone marrow registry, but also as diagnostic tool for patient characterization in daily routine.

With the Software SEQUENCE PILOT© it is easy to do the analysis of the Sequencing Files
of the Protrans Sequencing System for HLA Class I und Class II.

Protrans Sequencing-Strategies

1	**PROTRANS S4**	Splitting the Haplotypes single-allele and Locus specific amplification and sequencing **No Ambiguities**
2	**PROTRANS S3**	Splitting the Haplotypes single-allele and locus specific amplification and sequencing **No Ambiguities**
3	**PROTRANS** **Domino Stones**	Haplotype specific sequencing. **No Ambiguities** **Domino Stones get your LCT, SSP, SSO results high**
4	**PROTRANS S2**	Splitting the Haplotypes HLA group specific amplification and sequencing
5	**PROTRANS S1**	HLA-locus specific amplification and sequencing (LSA)

2.0 PROTRANS HLA Sequencing System

The Protrans Sequencing System is designed to reach a maximum level of allele-specific sequencing and in turn the lowest number of ambiguities. With the separation of the DNA with specific primer Mixes in two separate Haplotypes ensures the recognition of both alleles in nearly all cases.

The Protrans Sequencing System allows
in HLA class I sequencing of Exon 1 to Exon 4.
in HLA class II sequencing of Exon 2 and codon 86TG
Special emphasis was put on the complete coverage of exons 1, 2, 3, and 4 to sort out nearly all ambiguities caused by variations outside Exons 2 and 3 as well as on the location of the sequencing primers to ensure complete Exon sequences in both orientations.

Intended use

Protrans Sequencing System is easy to use for the application of bone marrow registry typing as well as a diagnostic tool for patient characterization in daily routine.

Protrans S4 Sequencing Kits

The **Protrans S4** HLA SBT Typing kits are designed to reach a maximal level of allele-specific sequencing and in turn the lowest number of ambiguities.
This is achieved by splitting the Haplotypes before sequencing each Haplotype separately.

In **HLA class I** the DNA will be amplified with up to 14 Group-Specific PCR Amplifications (GSA) and in addition a locus specific Amplification (LSA) in parallel covering Exons 1, Exon 2, Exon 3 and Exon 4.
The Single Allele or Group-Specific Primer Mixes are pre-pipetted in 16-well PCR-Strips
In almost all cases sequence analysis of separately both alleles will be achieved.
In HLA class I special emphasis was put on the complete coverage of Exons 1, 2, 3, and 4 to sort out nearly all ambiguities caused by variations outside Exons 2 and 3.

In **HLA class II** the DNA will be amplified with up to 14 Group-Specific PCR Amplifications (GSA) in parallel covering the complete Exon 2 loci.
The Single Allele or Group-Specific Primer Mixes are pre-pipetted in 16-well PCR-Strips
In almost all cases sequence analysis of both alleles separately will be achieved.
In HLA-DRB1 a special emphasis was put on the separation of the DR52-associated HLA-DRB1 alleles to ensure unambiguous results in nearly all samples
Special emphasis was put on the location of the sequencing primers to ensure complete Exon sequences in both orientations.

For ease of use

- The Group-Specific Primer Mixes are pre-pipetted in 8-well PCR-Strips
- The Test procedure and the Thermocycler program for the Amplification, Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci identical
- The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
- It is very easy to type different HLA loci of several DNA Samples in parallel.
- It is always possible to combine the different Protrans Sequencing Kits in the different Protrans Sequencing Strategies.

Protrans S3 Sequencing Kits

The **Protrans S3** Sequencing Kits are designed to match the requirements of high sample throughput as well as allele-specific sequencing for less ambiguities.
This is achieved by splitting the Haplotypes before sequencing each Haplotype separately.

In the Protrans SBT Test kit HLA-A, B, C the DNA will be amplified with 7 Group-Specific PCR Amplification Mixes (GSA) and in addition a Locus specific Amplification Mix (LSA).

In the Protrans SBT Test kit HLA-DQB1 the DNA will be amplified with 6 Group-Specific PCR Amplifications Mixes (GSA) and in addition 2 Locus specific Amplification Mixes (LSA).

In the Protrans SBT Test kit HLA-DRB1 the DNA will be amplified with 8 Group-Specific PCR Amplifications (GSA).

If the GSA reactions do not indicate two separate alleles the LSA reaction must be sequenced. This ensures in all cases the recognition of both alleles.
In the Protrans SBT Test kit HLA-class I Exons 1, Exon 2, Exon 3 and Exon 4 and in the Protrans SBT Test kit HLA-class II Exon 2 (DRB1, DQB1) and Exon 3 (DQB1)are covered.

The Amplification Primer Mixes are pre-pipetted in 8-well PCR-Strips and each HLA-locus in a different colour.
In most cases sequence analysis of separately both alleles will be achieved.
In HLA class I special emphasis was put on the complete coverage of Exons 1, 2, 3, and 4 to sort out nearly all ambiguities caused by variations outside Exons 2 and 3.
Special emphasis was put on the location of the sequencing primers to ensure complete Exon sequences in both orientations.

For ease of use

- The Group-Specific Primer Mixes are pre-pipetted in 8-well PCR-Strips
- The Test procedure and the Thermocycler program for the Amplification, Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci identical.
- The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
- It is very easy to type different HLA loci of several DNA Samples in parallel.
- It is always possible to combine the different Protrans Sequencing Kits in the different Protrans Sequencing Strategies.

Protrans Domino Stones HLA SBT

are designed for a maximal flexibility to match individual requirements of the HLA laboratory.
The Domino Stones Locus- and different Group-Specific PCR Amplification Mixes (Domino Stones) are supplied separately to allow an individual set up according to the individual requirements.
Using the Protrans Domino Stones it is for all HLA loci possible to change the low resolution typing result from other techniques (SSP or SSO) in a 4 digit high resolution result.
The Locus- and Group-Specific PCR Amplifications are covering at least Exons 2 and 3 in HLA class I loci and Exon 2 in HLA class II loci.

Protrans S2 Sequencing Kits

The Protrans S2 sequencing kits are designed to match the requirements of very high sample throughput as well as allele-specific sequencing for less ambiguities.
This is achieved by applying 4 Group-Specific PCR Amplifications (GSA) in parallel covering at least exons 2 and 3 in HLA class I loci and exon 2 in HLA class II loci allowing in many cases sequence analysis of both alleles separately.

For ease of use

- The Group-Specific Primer Mixes are pre-pipetted in 8-well PCR-Strips
- The Test procedure and the Thermocycler program for the Amplification, Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci identical.
- The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
- It is very easy to type different HLA loci of several DNA Samples in parallel.
- It is always possible to combine the different Protrans Sequencing Kits in the different Protrans Sequencing Strategies.

Protrans S1 Sequencing Kits

The Protrans S1 sequencing kits are designed to match the requirements of very high sample throughput.
This is achieved by Locus-Specific PCR Amplification (LSA) covering in
HLA class I Exon 1, Exon 2, Exon 3 and Exon 4, and in
HLA class II Exon 2, allowing sequence analysis of both alleles simultaneously.
A special emphasis was put on the location of the sequencing primers to ensure complete Exon sequences in both orientations.
Protrans Sequencing Kits Protrans S1 swift REF 2101 and 2102 are a variant of Protrans S1 Kits.
These Kits are designed to match the requirements of very high sample throughput. This is achieved by Locus-Specific PCR Amplification (LSA) covering Exons 2 and 3 in HLA class I loci leaving ambiguities due to variations outside Exon 2 and 3 unsolved. A special emphasis was put on the generation of short PCR Products to increase robustness in case of DNA of lower quality.

For ease of use

- The Group-Specific Primer Mix is in a single Tube.
- The Test procedure and the Thermocycler program for the Amplification, Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci identical.
- The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
- It is very easy to type different HLA loci of several DNA Samples in parallel.
- It is always possible to combine the different Protrans Sequencing Kits in the different Protrans Sequencing Strategies.

Primer Mix Specificities and Specification Tables

The Protrans HLA Sequencing Kits are continuously updated. In order to provide the user with the most recent version of the kit, the Primer Mix Specifications are listed as an Attachment in the Primer Mix Specification Tables. Please make sure that the version of the kit is identical with the version of the Primer Mix Specification Table.

PCR Amplification

The method is based on PCR amplifications starting with genomic DNA. The PCR amplification reactions are covering at least Exons 2 and 3 in HLA class I loci and Exon 2 in HLA class II loci and have been designed to be specific for a single group of alleles only. Each of the PCR formulations has been validated against a panel of well characterized cell lines to ensure against non-specific amplification and preferential amplification of one allele over another in heterozygous combinations.

Sequencing

After PCR clean up the positive Group-Specific Amplification reactions are used as templates for direct sequencing at least Exons 2 and 3 in HLA class I loci and Exon 2 in HLA class II loci. The PCR templates have been optimized for the use of dye terminator cycle sequencing chemistries to generate the allele typing information.

Allele Assignment

The final step in sequence analysis consists out of the allele assignment using the SEQUENCE PILOT© allele identification Software. This program performs allele identification, allows manual reviewing or editing of the sequencing data as well as reporting, exporting and archiving of sequences or results SEQUENCE PILOT© allele identification software enables quick and easy allele assignment.
The software allows manual reviewing or editing of the sequencing data of Exon 1 to Exon 8. Heterozygous positions as well as mismatches are detected and reported. Typing results and sequence electropherograms can be printed, exported and archived.
The software is available as a Windows™ single-user-version or as a Windows™ or Linux™ server-client-version for use in a network with several workstations.
The HLA database is continuously updated in line with the latest scientific research of the official IMGT/HLA database.

Instrument Platforms

The PCR and sequencing cycle profiles provided in this manual have been used with the Thermal Cyclers from Applied Biosystems GeneAmp PCR Systems 2700, 9600 and 9700. They should also work with compatible instruments but may require adjustments of the cycling profile or emulation of the above instruments.
The Sequencing Kits as well as the SEQUENCE PILOT© Allele Identification Software are compatible with all four-dye capillary-sequencing instruments available.

Applied Biosystems	Capillary: 310, 3100, 3130A, 3130xl ,3500, 3500xl, 3730, 3730xl
GE Healthcare	Capillary: MegaBACE 500, 1000, 4000
Beckman	Capillary: CEQ8000; GeXP

3.0 Procedure Protrans Sequencing System

Pre-PCR	
Separation of Haplotypes	**DNA Amplification PCR SSP**

Post PCR	
Purification	**of the separated Haplotypes**
Sequencing the single Haplotypes	**HLA Class I** EXON 1-2-3-4 forward and reverse
	HLA Class II EXON 2 forward and reverse and codon 86TG
Purification	**of the Sequencing Products**
Running Sequencing Products	**on all Sequencers**
Automated Analysis of the Sequencing results Protrans Software	**Sequence Pilot©**

4.0 Materials supplied with the HLA Sequencing Kits

Amplification Unit 1 Pre-PCR					
Protrans S4 HLA-A Single Allele and Locus-specific Sequencing	**Primer Mixes**			**Typings**	
16 well PCR Strips (yellow) precoated with Primer Mixes	12 x GSA	1 x LSA	1 x neg.ctrl.	24	4-8℃
Protrans S3 HLA-A Single Allele and Locus-specific Sequencing					
8 well PCR Strips (yellow) precoated with Primer Mixes	7 x GSA	1 x LSA		24	4-8℃

Protrans S4 HLA-B Single Allele and Locus-specific Sequencing	**Primer Mixes**			**Typings**	
16 well PCR Strips (blue) precoated with Primer Mixes	12 x GSA	1 x LSA	1 x neg.ctrl.	24	4-8℃
Protrans S3 HLA-B Single Allele and Locus-specific Sequencing					
8 well PCR Strips (blue) precoated with Primer Mixes	7 x GSA	1 x LSA		24	4-8℃

Protrans S4 HLA-C Single Allele and Locus-specific Sequencing	**Primer Mixes**			**Typings**	
16 well PCR Strips (green) precoated with Primer Mixes	12 x GSA	1 x LSA	1 x neg.ctrl.	24	4-8℃
Protrans S3 HLA-C Single Allele and Locus-specific Sequencing					
8 well PCR Strips (green) precoated with Primer Mixes	7 x GSA	1 x LSA		24	4-8℃

Protrans S4 HLA-DRB1 Single Allele and Allele-Group specific Sequencing	**Primer Mixes**			**Typings**	
16 well PCR Strips (natural) precoated with Primer Mixes	14 x GSA	1 x pos.ctrl.	1 x neg.ctrl.	24	4-8℃
Protrans S3 HLA-DRB1 Single Allele and Allele-Group specific Sequencing					
8 well PCR Strips (natural) precoated with Primer Mixes	8 x GSA			24	4-8℃
Protrans S2 HLA-DRB1 Allele Group specific Sequencing	**Primer Mixes**			**Typings**	
4 well PCR Strips (natural) precoated with Primer Mixes	4 x GSA			24	4-8℃

Protrans S2 HLA-DQA1 Allele Group specific Sequencing	**Primer Mixes**			**Typings**	
4 well PCR Strips precoated with Primer Mixes	4 x GSA			24	4-8℃

Protrans S3 HLA-DQB1 Single Allele and Locus-Specific Sequencing					
8 well PCR Strips (pink) precoated with Primer Mixes	6 x GSA	2 x LSA		24	4-8℃

Amplification Unit 2

Protrans S4 HLA-A Single Allele and Locus-specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
PCR Solution D	PSD	4 x	1.750	-20℃

Protrans S3 HLA-A Single Allele and Locus-specific Sequencing				
PCR Solution D	PSD	2 x	1.750	-20℃
Negative control	NC-ABC	1 x	140	-20℃

Protrans Domino Stone HLA-A Haplotype-specific Sequencing				
Allele- and Allele-Group specific Amplification	GSA	1 x	1.400	-20℃
PCR Solution L	PSL	3 x	1.000	-20℃

Protrans S1 HLA-A Locus-specific Sequencing (LSA) **EXON 1-4**				
Locus-specific Amplification Primer	LSA	1 x	360	-20℃

Protrans S1 HLA-A swift Locus-specific Sequencing (LSA) **EXON 2-3**				
Locus-specific Amplification Primer	LSA	1 x	360	-20℃

Amplification Unit 2

Protrans S4 HLA-B Single Allele and Locus-specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
PCR Solution D	PSD	4 x	1.750	-20℃

Protrans S3 HLA-B Single Allele and Locus-specific Sequencing				
PCR Solution D	PSD	2 x	1.750	-20℃
Negative control	NC-ABC	1 x	140	-20℃

Protrans Domino Stone HLA-B Haplotype-specific Sequencing				
Allele- and Allele-Group specific Amplification	GSA	1 x	1.400	-20℃
PCR Solution L	PSL	3 x	1.000	-20℃

Protrans S1 HLA-B Locus-specific Sequencing (LSA) **EXON 1-4**				
Locus-specific Amplification Primer	LSA	1 x	360	-20℃

Protrans S1 HLA-B swift Locus-specific Sequencing **EXON 2-3**				
Locus-specific Amplification Primer	LSA	1 x	360	-20℃

Amplification Unit 2

Protrans S4 HLA-C Single Allele and Locus-specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
PCR Solution D	PSD	4 x	1.750	-20℃

Protrans S3 HLA-C Single Allele and Locus-specific Sequencing				
PCR Solution D	PSD	2 x	1.750	-20℃
Negative control	NC-ABC	1 x	140	-20℃

Protrans Domino Stone HLA-C Haplotype-specific Sequencing				
Allele- and Allele-Group specific Amplification	GSA	1 x	1.400	-20℃
PCR Solution L	PSL	3 x	1.000	-20℃

Protrans S1 HLA-C Locus-specific Sequencing **EXON 2-4**				
Locus-specific Amplification Primer	LSA	1 x	360	-20℃

Amplification Unit 2

Protrans S4 DRB1 Allele- and Allele-Group specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
PCR Solution D	PSD	4 x	1.750	-20℃

Protrans S3 DRB1 Allele- and Allele-Group specific Sequencing				
PCR Solution D	PSD	2 x	1.750	-20℃
Negative control	NC-ABC	1 x	140	-20℃

Protrans Domino Stone DRB1 Haplotype-specific Sequencing				
Allele- and Allele-Group specific Amplification	GSA	1 x	1.400	-20℃
PCR Solution L	PSL	3 x	1.000	-20℃

Protrans S2 DRB1 Allele-and Allele-Group-specific Sequencing				
PCR Solution D	PSD	1 x	1.750	-20℃

Protrans S1 HLA-DRB3 Locus-specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
Locus-specific Amplification	LSA	1 x	360	-20℃

Protrans S1 HLA-DRB4 Locus-specific Sequencing	**Solution**	**Quantity**	**Volume/µl**	
Locus-specific Amplification	LSA	1 x	360	-20℃

Protrans S1 HLA-DRB5 Locus-specific Sequencing	**Solution**	**Quantity**	**Volume/µl**	
Locus-specific Amplification	LSA	1 x	360	-20℃

Protrans S3 HLA-DQB1 Single Allele and Locus-specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
PCR Solution D	PSD	2 x	1.750	-20℃
Negative control	NC-DQ	1 x	140	-20℃

Protrans Domino Stone HLA-DQB1 Haplotype-specific Sequencing				
Allele- and Allele-Group specific Amplification	GSA	1 x	1.400	-20℃
PCR Solution L	PSL	3 x	1.000	-20℃

Protrans S1 HLA-DQB1 EXON 2 Locus-specific Sequencing				
Locus-specific Amplification	LSA	1 x	360	-20℃

Protrans S1 HLA-DQB1 EXON 3 Locus-specific Sequencing				
Locus-specific Amplification	LSA	1 x	360	-20℃

Protrans S2 HLA-DQA1 Allele Group specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
PCR Solution D	PSD	1 x	1.750	-20℃

Protrans S1 HLA-DPB1 Locus-specific Sequencing	**Solution**	**Tubes**	**Volume/µl**	
Locus-specific Amplification	LSA	1 x	360	-20℃

Content of the Amplification Unit components

Protrans S4 precoated Strips	5’ and 3’-primers
	Cresol red in the negative control well (last position)
Protrans S3 precoated Strips	5’ and 3’-primers
Protrans S2 precoated Strips	5’ and 3’-primers
Protrans Domino Stone Primer Mix	5’ and 3’-primers
Protrans S1 LSA Mix	5’ and 3’-primers
	Buffer
	Nucleotides
PCR-Solution D (PSD)	Buffer
	Nucleotides
PCR-Solution L (PSL)	Buffer
	Nucleotides
Negative Control (NC)	5’ and 3’-primers

Sequencing Unit
Post PCR Area

Protrans HLA-A

Testkit							Tubes			
Sequencing Primers	**S4**	**S3**	**S1**	**S1 sw**	**Do. St.**	**Tube**	**S4, S3, S2**	**Do. St**	**µl**	
Exon 1 forward (natural)	**x**	**x**	**x**			A-E1F	**1 x**		360	-20℃
Exon 1 reverse (natural)	**x**	**x**	**x**			A-E1R	**1 x**		360	-20℃
Exon 2 forward (red)	**x**	**x**	**x**	x	x	A-E2F	**1 x**	**5 x**	360	-20℃
Exon 2 reverse (red)	**x**	**x**	**x**	x	x	A-E2R	**1 x**	**5 x**	360	-20℃
Exon 3 forward (blue)	**x**	**x**	**x**	x	x	A-E3F	**1 x**	**5 x**	360	-20℃
Exon 3 reverse (blue)	**x**	**x**	**x**	x	x	A-E3R	**1 x**	**5 x**	360	-20℃
Exon 4 forward (yellow)	**x**	**x**	**x**			A-E4F	**1 x**		360	-20℃
Exon 4 reverse (yellow)	**x**	**x**	**x**			A-E4R	**1 x**		360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x;S4 2 x**		400	4-8℃

Protrans HLA-B

Testkit							Tubes			
Sequencing Primers	**S4**	**S3**	**S1**	**S1 sw**	**Do. St.**	**Tube**	**S4, S3, S2**	**Do.St**	**µl**	
Exon 1 forward (natural)	**x**	**x**	**x**			B-E1F	**1 x**		360	-20℃
Exon 1 reverse (natural)	**x**	**x**	**x**			B-E1R	**1 x**		360	-20℃
Exon 2 forward (red)	**x**	**x**	**x**	x	x	B-E2F	**1 x**	**5 x**	360	-20℃
Exon 2 reverse (red)	**x**	**x**	**x**	x	x	B-E2R	**1 x**	**5 x**	360	-20℃
Exon 3 forward (blue)	**x**	**x**	**x**	x	x	B-E3F	**1 x**	**5 x**	360	-20℃
Exon 3 reverse (blue)	**x**	**x**	**x**	x	x	B-E3R	**1 x**	**5 x**	360	-20℃
Exon 4 forward (yellow)	**x**	**x**	**x**			B-E4F	**1 x**		360	-20℃
Exon 4 reverse (yellow)	**x**	**x**	**x**			B-E4R	**1 x**		360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x;S4 2 x**		400	4-8℃

Protrans HLA-C

Testkit							Tubes			
Sequencing Primers	**S4**	**S3**	**S1**		**Do. St.**	**Tube**	**S4, S3, S2**	**Do. St**	**µl**	
Exon 1 forward (natural)	in preparation					C-E1F				
Exon 1 reverse (natural)	in preparation					C-E1R				
Exon 2 forward (red)	**x**	**x**	**x**		x	C-E2F	**1 x**	**5 x**	360	-20℃
Exon 2 reverse (red)	**x**	**x**	**x**		x	C-E2R	**1 x**	**5 x**	360	-20℃
Exon 3 forward (blue)	**x**	**x**	**x**		x	C-E3F	**1 x**	**5 x**	360	-20℃
Exon 3 reverse (blue)	**x**	**x**	**x**		x	C-E3R	**1 x**	**5 x**	360	-20℃
Exon 4 forward (yellow)	**x**	**x**	**x**			C-E4F	**1 x**		360	-20℃
Exon 4 reverse (yellow)	**x**	**x**	**x**			C-E4R	**1 x**		360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x;S4 2 x**	400	4-8℃	

Protrans HLA-DRB1

Testkit							Tubes			
Sequencing Primers	**S4**	**S3**	**S2**		**Do. St.**	**Tube**	**S4, S3, S2**	**Do. St**	**µl**	
Exon 2 forward (blue)	**x**	**x**	**x**		**x**	DR-E2F	**1 x**	**5 x**	360	-20℃
Exon 2 reverse (red)	**x**	**x**	**x**		**x**	DR-E2R	**1 x**	**5 x**	360	-20℃
Exon 2 Codon 86TG	**x**	**x**	**x**		x	DR-codon 86TG	**1 x**	**1 x**	360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x;S4 2 x**		400	4-8℃

Protrans HLA-DRB 3; 4; 5

Testkit							Tubes		
Sequencing Primers				**S1**		**Tube**	**S1**	**µl**	
Exon 2 forward (blue)				**x**		DR-E2A	**1 x**	360	-20℃
Exon 2 reverse (red)				**x**		DR-E2B	**1 x**	360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x**	400	4-8℃

Protrans HLA-DQB1 Exon 2

Testkit							Tubes			
Sequencing Primers				**S1**	**Do. St.**	**Tube**	**S1**	**Do. St**	**µl**	
Exon 2 forward (blue)				**x**	**x**	DQB-2F	**1 x**	**5 x**	360	-20℃
Exon 2 reverse (red)				**x**	**x**	DQB-2R	**1 x**	**5 x**	360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x**		400	4-8℃

Protrans HLA-DQB1 Exon 3

Testkit							Tubes			
Sequencing Primers				**S1**	**Do. St.**	**Tube**	**S1**	**Do. St**	**µl**	
Exon 3 forward (blue)				**x**	**x**	DQB-3F	**1 x**	**5 x**	360	-20℃
Exon 3 reverse (red)				**x**	**x**	DQB-3R	**1 x**	**5 x**	360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x**		400	4-8℃

Protrans HLA-DQB1 Exon 2 + 3

Testkit							Tubes			
Sequencing Primers		**S3**			**Do. St.**	**Tube**	**S3**	**Do. St**	**µl**	
Exon 3 forward (blue)		**x**			**x**	DQB-F	**2 x**	**5 x**	360	-20℃
Exon 3 reverse (red)		**x**			**x**	DQB-R	**2 x**	**5 x**	360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x**		400	4-8℃

Protrans HLA-DQA1

Testkit							Tubes			
Sequencing Primers			**S2**			**Tube**	**S2**		**µl**	
Exon 2 forward (blue)			**x**			DQA-E2F	**1 x**		360	-20℃
Exon 2 reverse (red)			**x**			DQA-E2R	**1 x**		360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x**		400	4-8℃

Protrans HLA-DPB1										
Testkit							Tubes			
Sequencing Primers				**S1**		**Tube**	**S1**		**µl**	
Exon 2 forward (blue)				**x**		DPB-E2F	**1 x**		360	-20℃
Exon 2 reverse (red)				**x**		DPB-E2R	**1 x**		360	-20℃
Agarose-Gel Loading Buffer (colourless)						LB	**1 x**		400	4-8℃

Content of the Sequencing Unit components

Sequencing Primers	5’ and 3’-primers
Loading Buffer	Gel Loading Solution

Note Refer to the Primer Mix Specificity Tables **(Attachment)** delivered with the kits to select the appropriate PCR products and Sequencing Primers. Make sure that the version of the kit and the version of the Primer Mix Specificity Tables are identical

Note All reagents supplied with the Protrans HLA Sequencing Kits should be used
Amplification Primer **Version specific** and
Sequencing Primer **LOT** specific
Amplification Primer can not be mixed between different **Versions** as indicated on the Attachment of each Kit
Sequencing Primer can not be mixed between different LOT´s as indicated as indicated on the Attachment of each Kit

Note In case of damaged tubes or boxes malfunctions cannot be excluded.
Those Kits must not be used.

5.0 Storage and Shelf Life

Pre PCR Area		
Amplification Unit 1	**Precoated Strips**	**4 – 8 ℃**
Amplification Unit 2	**Buffers**	**-20℃**

Post PCR Area		
Sequencing Unit	**Sequencing Primers**	**-20℃**

When stored under appropriate conditions the kit components can be used until the expiry date indicated on the Pre PCR Box and Post PCR Box kit.
Open packages and Tubes must be closed and stored under the same conditions as closed packages and Tubes and can be used until their expiry date.
Only components of the **same kit Version** can be used with each other and only until the marked expiry date

6.0 Precautions and Warning:

IVD Reagents only for In Vitro Diagnostic use
In the US only for research (RUO)

	The PROTRANS Testkits must be performed by well-trained and authorised laboratory technicians.
	All reagents should be handled in accordance to good laboratory practice using appropriate precautions.
	In addition , handle all patient samples as potentially infectious. Do not pipette by mouth.
	All used PCR-Cyclerplates should be treated as potentially infectious and should be destroyed according to the valid national guidelines.
	Do not use reagents which are expired. See expiration date printed on the label.
	Pre-PCR and Post-PCR rooms must be strictly separated.
	Use separate pipettes in the Pre-PCR area and in the Post-PCR area
	Ethidium bromide used for staining of DNA is a potential carcinogen. Always wear protective gloves when handling stained gels. Waste management according to national guidelines.
	Wear UV-blocking eye protection and avoid direct UV light when viewing or photographing gels.
	See Material Safety Data Sheet (**MSDS**) for detailed information. Available from Protrans.

6.1 Materials and Equipment not supplied with the Kit

Pre PCR Area				
[1] SEQUENCE PILOT© Software for automated allele assignment				PROTRANS
PROTRANS Pipetting Assistant (PPA) Software for documentation of DNA-samples (pipetting regime). Transfer of generated information to the Platerecord of the sequencer				PROTRANS
DNA Extraction PROTRANS DNA Box 500				PROTRANS
Photometer for adjusting DNA concentration				multiple suppliers
Protrans PCR Workstation deep frozen block for pipetting PCR				PROTRANS
Vortexer				multiple suppliers
Mini Centrifuge				multiple suppliers
AmpliTaq Gold™ polymerase (5U/µl)				Applied Biosystems
Eppendorf Tubes 1,5ml				
Rainin EDP3-plus electronic Multistepper	20-200µl	Dispensing Mastermix		PROTRANS
Rainin L-20 Pipet-Lite	2-20µl	Taq-polymerase		PROTRANS
Rainin L-1000 Pipet-Lite	100-1000µl	PSD		PROTRANS
Pipettes filter tips	2-20µl	GP-L10F	red	PROTRANS
	20-200µl	GP-L 200F	green	PROTRANS
	100-1000µl	GP-L 1000F	blue	PROTRANS
Closing roller PCR-caps				multiple suppliers
Thermal cycler with heated lid **Note** The PCR and sequencing cycle profiles provided in this manual have been used with the Thermal Cyclers from Applied Biosystems GeneAmp PCR Systems 2700, 9600 and 9700. They should also work with compatible instruments but may require adjustments of the cycling profile or emulation of the above instruments. See Appendix A, troubleshooting guide.				multiple suppliers

6.2 Materials and Equipment not supplied with the Kit

Post PCR Area				
Rainin L-20 Pipet-Lite	2-20µl	ExoSAP–IT® ; Set-up sequencing reaction		PROTRANS
Rainin EDP3 E3-20 Electronic Multistepper	2-20µl	Big-Dyes		PROTRANS
Rainin EDP3 E3-100 Electronic Multistepper	10-100µl	Sequencing primers		PROTRANS
Rainin EDP3-Plus E8-20 Electronic Multistepper 8 channel Multistepper	2-20µl	PCR products		PROTRANS
Rainin EDP3-Plus E8-300 Electronic Multistepper 8 channel Multistepper	20-300µl	Ethanol precipitation		PROTRANS
Pipettes filter tips	2-20µl	GP-L10F	red	PROTRANS
	20-200µl	GP-L 200F	green	PROTRANS
	200-300µl	RT-L300F	blue	PROTRANS
Elektrophorese Unit PROTRANS Gel Check or Protrans Quattro-Gel Check 4 x 96 Amplificats in 4 Gels in 1 chamber				PROTRANS
Agarose, molecular biology grade				PROTRANS
1x TAE/TBE-Buffer				PROTRANS
Heating block				multiple suppliers
Microwave oven				multiple suppliers
Ethidium bromide solution (10mg/ml) ⚠ **WARNING CHEMICAL HAZARD.** Ethidium bromide is a known mutagen. It can change genetic material in a living cell. Before using ethidium bromide, read the manufacturer's MSDS, which gives information on physical characteristics, hazards, precautions, first-aid, spill clean up, and disposal procedures. Always wear appropriate protective eyewear, clothing, and gloves.				multiple suppliers
Photograph unit				multiple suppliers
Power supply				multiple suppliers
Transilluminator				PROTRANS
PCR 8-strips for Loading Buffer				multiple suppliers
PCR reaction plate 96 well and cap-strips				multiple suppliers
8 channel pipette transferring the Loading Buffer into a MicroAmp 96 –well reaction plate or MicroAmp strips for mixing the PCR Amplificats with Loading Buffer for loading into the Gel				multiple suppliers
PROTRANS AmpliPUR Fast for purification of PCR products				PROTRANS
[1] ExoSAP-IT® Exonuclease I/Shrimp Alkaline Phosphatase for purification of PCR products				USB Europe GmbH www.usbweb.de Art-Nr.: 78201, 78202
MicroAmp optical 96-well reaction Plate Part Number N801-0560				ABI
Automated DNA sequencing instrument and consumables				[1] Applied Biosystems [2] Beckman Coulter [3] GE Healthcare
ABI Sequencer [1] Big Dye™ Terminator Cycle Sequencing Kit version 1.1 (BDT v 1.1)				Applied Biosystems
[2] **CEQ™ 8000** QuickStart Kit Beckmann Coulter				Beckman Coulter
[1] **MegaBACE** DYEnamic™ ET Dye Terminator Kit (MegaBACE™)				GE Healthcare

Note The Protrans HLA Sequencing Kits have been validated for the Applied Biosystems DNA Sequencers 3100 Genetic Analyzer and 3730 DNA Analyzer. They should also work with all other four-dye sequencing instruments available	[1] Applied Biosystems [2] Beckman Coulter [1] MegaBACE™ GE Healthcare
PROTRANS DyePUR for purification of sequencing products	PROTRANS
[1] Sephadex G-50 Fine DNA Grade for purification of sequencing products	GE Healthcare
96er plate centrifuge for purification of sequencing products on 96-well plates. (Ethanol or PROTRANS PlatePUR)	multiple suppliers
EDTA-di Sodium 0,5M	Merck and other
Na Acetate 3M solution	Merck and other
Ethanol absolute pro analysis 100% for ethanol precipitation method	Merck
HPLC water	Merck
Aluminium cover foil for ethanol purification	PROTRANS

[1] **Note** The reagents and instruments listed with [1] have been validated for Protrans HLA Sequencing Kits. Protrans does not take any responsibility when other materials or equipment are used. The user must validate reagent and instruments other than those listed with [1]

[2] **Note** The reagents and instruments listed with [2] have not been validated for Protrans HLA Sequencing Kits. Protrans does not take any responsibility when these materials or equipment are used. The Reagents and instruments listed with [2] must be validated by the user.

7.0 Preparation and Processing of Samples

7.1 DNA Extraction and DNA concentration

Genomic DNA can be obtained from all nucleated cells. The simplest method is to isolate DNA from cell suspensions (blood, buffy coat or cultured cells) for this multiple protocols and kits are available. For DNA sequencing only those methods should be considered which provide DNA of high quality and quantity.

PROTRANS DNA Box 500

The PROTRANS DNA extraction-kit PROTRANS DNA Box 500 provides high quality DNA, optimized for PROTRANS HLA sequencing kits. Other extraction methods must be validated before used in routine sequencing.

Note Use either EDTA or ACD- blood. Heparin anticoagulated blood should be avoided as heparin has been shown to seriously affect PCR yield.

The DNA concentration must be 50 – 100 ng/µl

Working with human blood samples has the potential to transmit infectious diseases and should be handled with precaution. Always wear appropriate protective eyewear, clothing, and gloves

8.0 Program Thermocycler Protrans SBT

All Protrans HLA Sequencing Kits (Protrans S4, S3, S2, S1, and Domino Stones) are running with the same Cycling Profiles for PCR Amplification and Cycle Sequencing
The program is standardised for all HLA Loci **(Important Note: Ramp Rate 1℃/s)**

8.1 PCR Amplification

CR - Step	Temperature	Time	Cycles
Initial Denaturation	95℃	2 min	Hold
Denaturation	96℃	40 sec	15 Cycles
Annealing	64℃	1 min	
Extension	72℃	2 min	
Denaturation	96℃	20 sec	15 Cycles
Annealing	60℃	1 min	
Extension	72℃	2 min	
Denaturation	96℃	20 sec	10 Cycles
Annealing	56℃	1 min	
Extension	72℃	2 min	
Terminal cooling	4℃	∞	Hold
Volume	**15 µl**		

8.2 Purification of PCR Products with ExoSAP-IT®

Step	Temperature	Time	Process
1	37℃	15 min	Degradation of primers and dNTPs
2	80℃	15 min	Degradation of enzymes
3	4℃	After incubation and cooling to room temperature the PCR products are ready for sequencing set-up	

8.3.1 Cycle Sequencing ABI

Step	Temperature	Time	Cycles
Initial Denaturation	96℃	1 min	Hold
Denaturation	96℃	10 sec	25 Cycles
Annealing	50℃	5 sec	
Extension	60℃	4 min	
Terminal-Cooling	4℃	∞	Hold
Volume		**10 µl**	

8.3.2 Cycle Sequencing Beckman

Step	Temperature	Time	Cycles
Denaturation	96℃	20 sec	30 Cycles
Annealing	50℃	20 sec	
Extension	60℃	3 min	
Terminal-Cooling	4℃	∞	Hold
Volume		**10 µl**	

Note The PCR and sequencing cycle profiles provided in this manual have been used with the Thermal Cyclers from Applied Biosystems GeneAmp PCR Systems 2700, 9600 and 9700. They should also work with compatible instruments but may require adjustments of the cycling profile or emulation of the above instruments. See Appendix A, troubleshooting guide.

9.0 Specificities of the Amplification Primers

The method is based on PCR amplifications starting with genomic DNA. The PCR amplification reactions are covering at least exons 2 and 3 in HLA class I loci and exon 2 in HLA class II loci and have been designed to be specific for a single group of HLA alleles only and a single HLA locus. Each of the PCR formulations has been validated against a panel of well characterized cell lines to ensure against non-specific amplification and preferential amplification of one allele over another in heterozygote combinations.
The Protrans SBT Kits are continuously updated
The Attachment for each kit is LOT- Version and Database-specific. For interpretation of the amplification-results and selection of the sequencing primers the Attachment delivered with the kit Version and Database-specific must be used exclusively.

The Protrans HLA Sequencing Kits are continuously updated.
In order to provide the user with the most recent version of the kit, the Primer Mix Specifications are listed as an Attachment in the Primer Mix Specification Tables delivered with each kit.
Please make sure that the **Version** of the kit is identical with the **Version** of the ATTACHMENT the Primer Mix Specification Table in the Testkit.

10.0 PROTRANS S4 Sequencing Kits

The **Protrans S4 HLA SBT Typing kits** are designed to reach a maximal level of allele-specific sequencing and in turn the lowest number of ambiguities.
This is achieved by splitting the Haplotypes before sequencing each Haplotype separately.

In **HLA class I** the DNA will be amplified with up to 14 Group-Specific PCR Amplifications (GSA) and in addition a locus specific Amplification (LSA) in parallel covering Exons 1, Exon 2, Exon 3 and Exon 4.
The Single Allele or Group-Specific Primer Mixes are pre-pipetted in 16-well PCR-Strips
In almost all cases sequence analysis of separately both alleles will be achieved.
In HLA class I special emphasis was put on the complete coverage of Exons 1, 2, 3, and 4 to sort out nearly all ambiguities caused by variations outside Exons 2 and 3.

In **HLA class II** the DNA will be amplified with up to 14 Group-Specific PCR Amplifications (GSA) in parallel covering the complete Exon 2. loci
The Single Allele or Group-Specific Primer Mixes are pre-pipetted in 16-well PCR-Strips
In almost all cases sequence analysis of separately both alleles will be achieved.
In HLA-DRB1 a special emphasis was put on the separation of the DR52-associated HLA-DRB1 alleles to ensure unambiguous results in nearly all samples
Special emphasis was put on the location of the sequencing primers to ensure complete Exon sequences in both orientations.

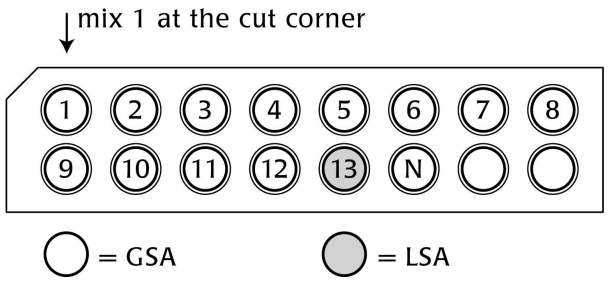
For ease of use
The Allele- and Allele-Group-Specific Primer Mixes are pre-pipetted in 16-well PCR-Strips. The colour of the strips are for each HLA-locus different.

The Test procedure and the Thermocycler-program for the Amplification, for Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci and identical.

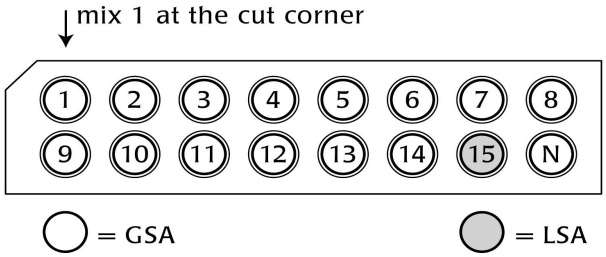
The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
It is very easy to type different HLA loci of several DNA Samples in parallel.
It is always possible to combine the different Protrans Sequencing Kits with the different Protrans Sequencing Strategies.

10.1. Protrans S4 Sequencing Kits pre-coated PCR Strips

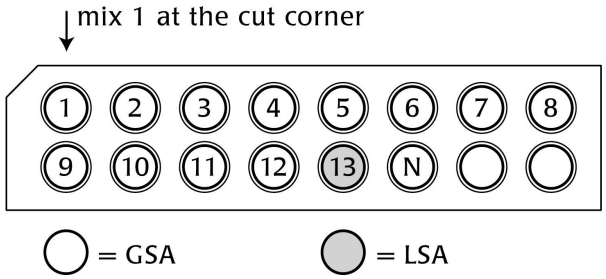
HLA-A



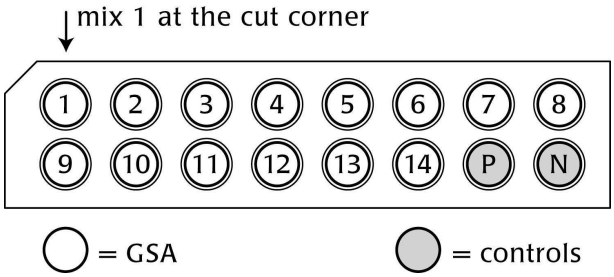
HLA-B



HLA-C



HLA-DRB1



10.2. Set-up Amplification Protrans S4 Sequencing Kits

Documentation of DNA-samples with Protrans Pipetting Assistant or pipetting scheme form
Place reagents and strips in PROTRANS PCR Workstation –20℃

1	16-well PCR Strip
2	PROTRANS PCR Solution D (PSD)
3	AmpliTaq Gold™ DNA Polymerase
4	DNA sample
Vortex all reagents and spin down with mini-table centrifuge	

Reaction tube 1,5ml		**Master Mix** for each DNA sample
1	**280 µl**	**PCR Solution (PSD)**
2	**3,0 µl**	**AmpliTaq Gold™ DNA polymerase**
Vortex master mix and spin down with mini-table centrifuge		

3	**15µl**	**Master Mix** in **Position 14 Negative Control (N)** in Protrans 16-well PCR Strip
4	**20µl**	**DNA sample** (50-100ng/µl) in the **Master Mix**
Vortex master mix and spin down with mini-table centrifuge		

5	**15µ**	**Master Mix** in Protrans 16-well PCR Strip with electronic Multistepper (RAININ)

Close 16-PCR strip. Place the strips in the thermocycler and start the PCR amplification program

10.3. Protrans S3 Sequencing Kits

The **Protrans S3** Sequencing Kits are designed to match the requirements of high sample throughput as well as allele-specific sequencing for less ambiguities.
This is achieved by splitting the Haplotypes before sequencing each Haplotype separately.

In the Protrans SBT Test kit HLA-A, B, C the DNA will be amplified with 7 Group-Specific PCR Amplification Mixes (GSA) and in addition a Locus specific Amplification Mix (LSA).

In the Protrans SBT Test kit HLA-DQB1 the DNA will be amplified with 6 Group-Specific PCR Amplifications Mixes (GSA) and in addition 2 Locus specific Amplification Mixes (LSA).

In the Protrans SBT Test kit HLA-DRB1 the DNA will be amplified with 8 Group-Specific PCR Amplifications (GSA).

If the GSA reactions do not indicate two separate alleles the LSA reaction must be sequenced. This ensures in all cases the recognition of both alleles.
In the Protrans SBT Test kit HLA-class I Exons 1, Exon 2, Exon 3 and Exon 4 and in the Protrans SBT Test kit HLA-class II Exon 2 (DRB1, DQB1) and Exon 3 (DQB1)are covered.

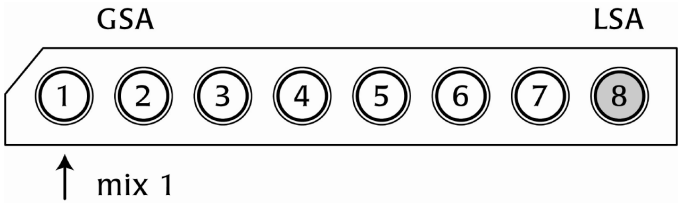
The Amplification Primer Mixes are pre-pipetted in 8-well PCR-Strips and each HLA-locus in a different color.
In most cases sequence analysis of separately both alleles will be achieved.
In HLA class I special emphasis was put on the complete coverage of Exons 1, 2, 3, and 4 to sort out nearly all ambiguities caused by variations outside Exons 2 and 3.
Special emphasis was put on the location of the sequencing primers to ensure complete Exon sequences in both orientations.

For ease of use

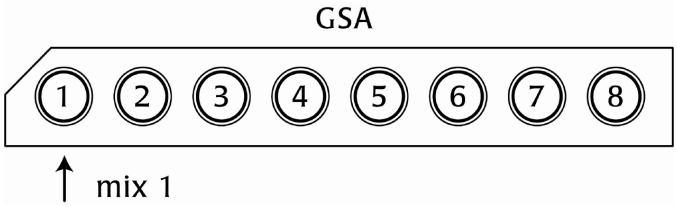
- The Group-Specific Primer Mixes are pre-pipetted in 8-well PCR-Strips
- The Test procedure and the Thermocyclerprogram for the Amplification, Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci identical.
- The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
- It is very easy to type different HLA loci of several DNA Samples in parallel.
- It is always possible to combine the different Protrans Sequencing Kits in the different Protrans Sequencing Strategies.

10.4 Protrans S3 Sequencing Kits pre-coated PCR Strips

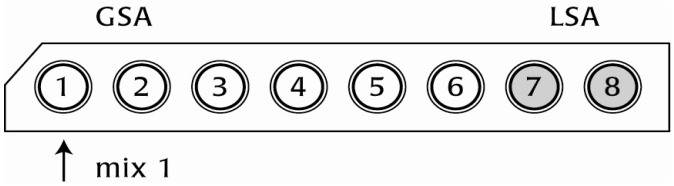
HLA-A, B, C



HLA-DRB1



HLA-DQB1



10.5 Set-up Amplification Protrans S3 Sequencing Kits

Documentation of DNA-samples with Protrans Pipetting Assistant or pipetting scheme form
Place reagents and strips in PROTRANS PCR Workstation –20℃

1	8-well PCR Strip
2	PROTRANS PCR Solution D (PSD)
3	AmpliTaq Gold™ DNA Polymerase
4	DNA sample
Vortex all reagents and spin down with mini-table centrifuge	

Reaction tube 1,5ml		**Master Mix** for each DNA sample
1	**140 µl**	**PCR Solution (PSD)**
2	**1,5 µl**	**AmpliTaq Gold™ DNA polymerase**
Vortex master mix and spin down with mini-table centrifuge		

3	**10µl**	**DNA sample** (50-100ng/µl) in the **Master Mix**
Vortex master mix and spin down with mini-table centrifuge		

4	**15µ**	**Master Mix** in Protrans 8-well PCR Strip with electronic Multistepper (RAININ)

Close 16-PCR strip. Place the strips in the thermocycler and start the PCR amplification program

Protrans S3 HLA Sequencing Kit negative control should be performed for each series of DNAs tested to exclude contamination of the PCR solution and AmpliTaq Gold™ DNA Polymerase

10.6. PROTRANS Domino Stones HLA SBT Sequencing Kits

The **Protrans Domino Stones** are designed for a maximal flexibility to match individual requirements of the HLA laboratory.
The Domino Stones Locus- and different Group-Specific PCR Amplification Mixes (Domino Stones) are supplied separately to allow an individual set up according to the individual requirements.
Using the Protrans Domino Stones it is for all HLA loci possible to change the low resolution typing result from other techniques (SSP or SSO) in a 4-digit high resolution result.
The Locus- and Group-Specific PCR Amplifications are covering at least Exons 2 and 3 in HLA class I loci and Exon 2 in HLA class II loci.

For ease of use

- The Test procedure and the Thermocycler-program for the Amplification, Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci identical.
- The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
- It is very easy to type different HLA loci of several DNA Samples in parallel.
- It is always possible to combine the different Protrans Sequencing Kits in the different Protrans Sequencing Strategies.

10.7 Set-up Amplification Protrans Domino Stone Sequencing Kits

Documentation of DNA-samples with Protrans Pipetting Assistant or pipetting scheme form
Place reagents and PCR-strips in PROTRANS PCR Workstation –20℃

1	Single Tube, 8-well PCR Strip or 96 well PCR plate
2	Domino Stones (Amplification Primer Mixes) for different HLA loci Allele or Allele-Group specific
3	PROTRANS PCR Solution L (PSL)
4	AmpliTaq Gold™ DNA Polymerase
5	DNA sample
Vortex all reagents and spin down with mini-table centrifuge	

Reaction tube 1,5ml		**Master Mix for 1 DNA sample**
1	**8,85 µl**	**PCR Solution (PSL)**
2	**0,15 µl**	**AmpliTaq Gold™ DNA polymerase**
Vortex Master Mix and spin down with mini-table centrifuge		

2	**9 µl**	**Master Mix in 1 -well of a PCR Strip with electronic Multistepper (RAININ)**

3	**5 µl**	**Domino Stone Amplification Primer Mix in 1 -well of a PCR Strip**
Vortex DNA sample and spin down with mini-table centrifuge		

3	**1 µl**	**DNA sample** (50-100ng/µl) in the specific well of the strip to the Domino Stone.

Close PCR strip. Place the strips in the thermocycler and start the PCR amplification program

Protrans Domino Stone negative control should be performed for each series of DNAs tested to exclude contamination of the PCR solution and AmpliTaq Gold™ DNA Polymerase

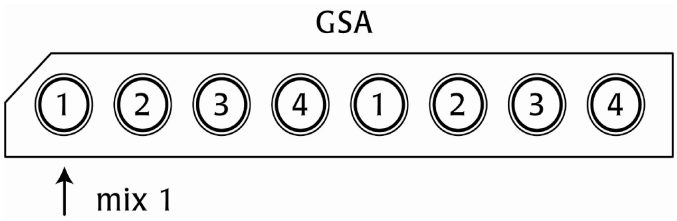
10.8. PROTRANS S2 Sequencing Kits

The **Protrans S2 sequencing kits** are designed to match the requirements of very high sample throughput as well as allele-specific sequencing for less ambiguities.
This is achieved by applying 4 Group-Specific PCR Amplifications (GSA) in parallel covering at least exons 2 and 3 in HLA class I loci and Exon 2 in HLA class II loci allowing in many cases sequence analysis of both alleles separately.

For ease of use

- The 4 Group-Specific Primer Mixes are pre-pipetted twice in a 8-well PCR-Strips
- The Test procedure and the Thermocycler-program for the Amplification, Cycle Sequencing and the Settings of the Sequencer are for all Protrans Sequencing Kits and for all HLA loci identical.
- The Purification of the PCR Products and Sequencing Products are identically for all HLA loci.
- It is very easy to type different HLA loci of several DNA Samples in parallel.
- It is always possible to combine the different Protrans Sequencing Kits in the different Protrans Sequencing Strategies.

10.9 Protrans S2 Sequencing Kits pre-coated PCR Strips



10.10 Set-up Amplification Protrans S2 Sequencing Kits

Documentation of DNA-samples with Protrans Pipetting Assistant or pipetting scheme form
Place reagents and strips in PROTRANS PCR Workstation –20℃

1	8-well PCR Strip
2	PROTRANS PCR Solution D (PSD)
3	AmpliTaq Gold™ DNA Polymerase
4	DNA sample
Reaction tube 1,5ml	**Master Mix** for each DNA sample

1	**70 µl**	**PCR Solution (PSD)**
2	**0,75 µl**	**AmpliTaq Gold™ DNA polymerase**
Vortex master mix and spin down with mini-table centrifuge		

3	**5 µl**	**DNA sample** (50-100ng/µl) in the **Master Mix**
Vortex master mix and spin down with mini-table centrifuge		

4	**15µ**	**Master Mix** in Protrans 8-well PCR Strip with electronic Multistepper (RAININ)

Close 16-PCR strip. Place the strips in the thermocycler and start the PCR amplification program

Protrans S2 HLA Sequencing Kit negative control should be performed for each series of DNAs tested to exclude contamination of the PCR solution and AmpliTaq Gold™ DNA Polymerase

10.11. Protrans S1 Sequencing Kits

The **Protrans S1 Sequencing Kits** are designed to match the requirements of very high sample throughput as well as extended sequencing in HLA class I to sort out all ambiguities caused by variations outside exons 2 and 3. This is achieved by Locus-Specific PCR Amplification (LSA) covering exons 1, 2, 3 and 4 in HLA class I Loci and exon 2 in HLA class II loci allowing sequence analysis of both alleles simultaneously. A special emphasis was put on the location of the sequencing primers to ensure complete exon sequences in both orientations. For ease of use the Locus-Specific Amplification Mix is ready for use.
A variant of the **Protrans S1** kit design is the **Protrans S1 swift** Sequencing Kit. The **Protrans $S1_{swift}$** Sequencing Kit is designed to match the requirements of very high sample throughput. This is achieved by Locus-Specific PCR Amplification (LSA) covering exons 2 and 3 in HLA class I loci leaving ambiguities due to variations outside Exon 2 and 3 unsolved. A special emphasis was put on the generation of short PCR products to increase robustness in case of DNA of lower quality. For ease of use the Locus-Specific Amplification Mix is ready for use.
The PCR Amplification set up for **Protrans S1** and **Protrans $S1_{swift}$** Sequencing Kits is identical.

10.12 Set-up Amplification Protrans S1 Sequencing Kits

Documentation of DNA-samples with Protrans Pipetting Assistant or pipetting scheme form
Place reagents and PCR-strips in PROTRANS PCR Workstation –20℃

1	Single Tube, 8-well PCR Strip or 96 well PCR plate
2	Domino Stones (Amplification Primer Mixes) for different HLA loci Allele or Allele-Group specific
3	PROTRANS PCR Solution D (PSL)
4	AmpliTaq Gold™ DNA Polymerase
5	DNA sample
Vortex all reagents and spin down with mini-table centrifuge	

Reaction tube 1,5ml		**Master Mix for 1 DNA sample**
1	**12 µl**	**S1 LSA Mix**
2	**0,15 µl**	**AmpliTaq Gold™ DNA polymerase**
Vortex Master Mix and spin down with mini-table centrifuge		

3	**12 µl**	**Master Mix in 1 -well** of a PCR Strip with electronic Multistepper (RAININ)

Vortex DNA sample and spin down with mini-table centrifuge		
4	**3 µl**	**DNA sample** (50-100ng/µl) in the specific well of the strip
Close PCR strip. Place the strips in the thermocycler and start the PCR amplification program		

Protrans S1 HLA Sequencing Kit negative control should be performed for each series of DNAs tested to exclude contamination of the PCR solution and AmpliTaq Gold™ DNA Polymerase

11.0 Analysis of PCR Amplification results. Determing the Haplotypes

The PCR products are identified by fluorescent agarose gel electrophoresis followed by UV detection of the PCR bands.

1	Preparation of 2% Agarose gel.		
2	1x TBE oder 1x TAE buffer		0.2 µg/ml ethidium bromide.
3	**2 µl**	**Loading Buffer** dispense with Multi-Stepper in a PCR strip or 96-well PCR plate.	
4	**5 µl**	**PCR amplification reaction** mix each PCR amplification reaction with 2µl loading buffer. (Use a 8-channel pipette compatible with microplate formats)	
5	**5 µl**	of this **Mixture** load directly on the gel with the same tips (e.g. use PROTRANS Quattro-Gel-Check system 1 chamber, 4 Gels, 96 slots).	
6		Run the gel in 1X TBE at 20 V/cm for 5 - 20 min. For example, with the PROTRANS Gel-Check electrophoresis chamber a run at 200 V for 5 min.	

12.0 Documentation of positive Reactions Assignment of Haplotypes

Photograph the PCR amplification pattern and record the results on a copy of the Protrans Attachment, the Primer Mix Specification Tables provided with each kit and determine the PCR products to be sequenced. An amplification reaction is considered positive if an intensive PCR fragment occurs.
The Protrans S1 and Protrans Domino Stones PCR product can directly be purified and sequenced without Agarose gel analysis.
In the Protrans S4, S3 and S2 HLA Sequencing Kits depending on the alleles present in the DNA sample to be investigated one, two, three or four PCRs can be positive. The amplification pattern should be consistent with the specificities listed in the Protrans Attachment (Primer Mix Specification Tables).

Note It is recommended to select those PCR products for sequencing that have not been generated by overlapping primer mixes. If a PCR-based separation of alleles is not achieved, it is strongly recommended to sequence in both orientations.

Note The PCR amplification pattern should be in concordance with the specificity table and give a conclusive result. If the PCR amplification pattern is inconclusive, e.g. by indicating the presence of more than two alleles, then contamination or a variant allele may exist. If the negative control excludes contamination sequencing of two PCR products can be performed. If the sequencing results indicate identical sequences the other group-specific PCR products should be sequenced.

Note The related positive amplification control should be positive and the related negative amplification control must be negative to exclude contamination.

2 PCR are positive (2 Haplotypes)

If two PCRs are positive, both PCR products must be sequenced, each in a single orientation, forward **or** reverse.
If more than two PCRs are positive, those PCR products with the least specificity overlap must be sequenced, each in a single orientation, forward **or** reverse.

If the PCR amplification pattern is not consistent with the Primer Mix Specification Tables, e.g. by indicating the presence of more than two alleles, a contamination or a variant allele may exist. If the negative control excludes contamination single orientation sequencing of the PCR products with the least specificity overlap must be performed. If the sequencing results give identical sequences of these PCR products the other Group-Specific PCR products have to be sequenced to exclude homozygosity.

Only one PCR is positive

If only one PCR is positive, the LSA PCR product or, in HLA-DRB1, this GSA PCR product must be sequenced in both orientations, forward **and** reverse.

Note The HLA-DRB1 sequencing kits are based on group-specific sequence diversities outside exon 2. For several HLA-DRB1 alleles these sequences are not available yet. It has been shown that outside exon 2 HLA-DRB1 alleles are conserved within allelic groups and are thus amplified by the dedicated primer mix. However, it cannot be completely excluded that some alleles with unknown motifs outside exon 2 are not covered by the HLA-DRB1 primer mixes. It is important to be aware of this possible limitation and it is strongly recommended to perform low-resolution typing by PCR-based methods if only a single allele has been identified.

Mark the positive PCR primer mixes in the Protrans Attachment
Select Haplotypes that will be sequenced
Type result in Protrans Pipetting Assistant or pipetting scheme form
Generate purification plate and sequencing plate with PROTRANS Pipetting Assistant

13.0 Protrans Attachment

LOT			Version		
PCR MIX		**S4 HLA -*A Specificity**	**PCR MIX**		**S4 HLA -*B Specificity**
01		A*01, *36	**01**		B*07, *48, *8101, w.o. B*4802
02		A*02	**02**		B*08,*42
03		A*03	**03**		B* 13
04		A*11	**04**		B*15, *45, *46, *49, *50, *4026 *4427
05		A*23, *24, *0103, w.o.A *2433	**05**		B*18, *37
06		A*25, *26, *34, *43, *66	**06**		B*27, *3542, *4002 group, *47, *82, w.o. B*270504
07		A*68, *69, *3401, *3405, *6602, *6603	**07**		B*35, *4802, *51, *52, *53, *5606, *58, *78
08		A*29, *31, *32, *33, *74, w.o A*3204	**08**		B*14, *38, *39, *6701
09		A*31, *33	**09**		B*4001 group, *41
10		A*29, *32, *74, w.o.A*3204	**10**		B*41
11		A*30, *0102	**11**		B*42, w.o. B*4202
12		A*8001	**12**		B*44, *8301?, w.o.B*4415, *4418
13		**All HLA-A Alleles**	**13**		B*54, *55, *56, *5901, w.o. B*5606
14		Negative Amplification control	**14**		B*57
15			**15**		**All HLA-B Alleles**
16			**16**		Neg. Amplification Control

LOT			Version		
PCR MIX		**S4 HLA -*C Specificity**	**PCR MIX**		**S4 HLA -*DRB1 Specificity**
01		Cw*01	01		DRB1*01
02		Cw*02 , *1511	02		DRB1*15, *16
03		Cw*03	03		DRB1*03, *1315, *1402, *1406, *1413, w.o. DRB1*0317,*1417
04		Cw*04 , *14	04		DRB1*04
05		Cw*05, *08	05		DRB1*03, *11, *13, *14, *0806, w.o. DRB1*1317
06		Cw*06, *18, w.o. Cw *06020102	06		DRB1*08, *11, *13, *14, w.o. DRB1*1402, *1406, *1413
07		Cw*05, *07, *08	07		DRB1*03, *11, *13, *1402, *1403, *1406, *1413, *1417, *1421, DRB1*0806, w.o. DRB1 *0317, *1313
08		Cw*14	08		DRB1*12
09		Cw*16	09		DRB1*1301, *1302, *1334, *1417, *1421
10		Cw*18	10		DRB1*14, *1117, w.o. DRB1*1402, *1403, *1406,*1413, *1417
11		Cw*01, *03, *04, *14, *18	11		DRB1*07
12		Cw*02, *05, *06, *08, *12, *15, *16, *17	12		DRB1*08, *1317, w.o. DRB1 *0806
13		**All HLA-C Alleles**	13		DRB1*09
14		Negative Amplification Control	14		DRB1*10
15			15		**Positive Amplification Control**
16			16		Negative Amplifikation Control

LOT			Version	
PCR MIX		**S3 HLA -*DQB1 Specificity**	**Foto**	
01		DQB1*02		
02		DQ3 – Group 1 (DQB1 *0301, *0304, *0309, *0310, *0312,*0313)		
03		DQ3 – Group 2 (DQB1 *0302,*0303, *0305 - *03080, *0311)		
04		DQB1*04		
05		DQB1*05		
06		DQB1*06		
07		**All HLA – DQB1 Alleles Exon 3**		
08		**All HLA – DQB1 Alleles Exon 2**		

14.0 Purification of the Haplotypes
Purification of the positive PCR-Products

The PCR products have to be purified before using them as sequencing templates because residual PCR primers and nucleotide triphosphates (dNTPs) can interfere with the sequencing chemistry resulting in lower data quality.

14.1 PROTRANS AmpliPur-Fast

The **PROTRANS AmpliPUR-Fast** PCR purification kit provides a convenient tool for fast and efficient direct purification of PCR products.

Instruction

1	**150µl**	**Binding Buffer** vortex and pipet into the PCR products which have to be purified	
2		place **Protrans Spin filter** in the marked **Protrans Receiver Tubes 2,0ml**	
3		PCR product with Binding Buffer pipet in the center of the spin column	
4		**Protrans Receiver Tubes** centrifuge at 10.000 rpm	**3 Min**
5		place **Protrans Spin filter** in new marked **Protrans Receiver Tubes 1,5ml**	
6	**30µl**	**Elution Buffer (EB)** pipet in the center of the Protrans spin column	
7		Incubation at RT	**1 Min**
8		centrifuge at 6.000 rpm	**1 Min**

14.2 ExoSAP-IT® Purification

Purification of the PCR-products with Exonuclease I and shrimp alkaline phosphatase enzyme mix **ExoSAP-IT®**. Degradation of unbound primers and dNTPs.

Instruction

1	**3µl**	**ExoSAP-IT®** Vortex before use. Pre-pipet in PCR strips or PCR plate
2	**10µl**	**PCR Product (Haplotype)** which has to be purified pipet to defined position on PCR plate
3		PCR strips or PCR plate seal, spin down and place in thermocycler

Thermocyclerprogram			
	37℃	15 min	Degradation of primers and dNTPs
	80℃	15 min	Degradation of enzymes
	4℃	After incubation and cooling to room temperatur e the PCR products are ready for sequencing set-up	

14.3 Beads fishing Method

An alternative to the ExoSAP-IT® purification is the use of Agencourt AMPure beads-based technology (Beckman Coulter, 2.3) which fishes the PCR products by attaching them to the beads and allowing to wash away the residual primers and dNTPs. This technique does not need a thermal cycler and can easily be automated. The beads purification is compatible with low to high throughput formats using single tubes, 96 or 384 well microplates.

15.0 Selection of Sequencing Primers

The **Protrans S4, S3, S2** and
Protrans Domino Stones HLA Sequencing Kits
are designed to amplify and sequence both alleles separately.

The **Protrans S1 HLA Sequencing Kits**
are designed to amplify and sequence both alleles simultaneously.

Note Refer **Protrans Attachment** (Primer Mix Specification Tables) for sequencing primer selection.

In HLA class I
at least Exons 2 and 3 should be sequenced to get 4-digit HLA typing results.

In HLA class II
at least Exon 2 should be sequenced to get 4-digit HLA typing results.

For lower resolution HLA typing results less sequence information may be sufficient.
If separation of the alleles is achieved, (2 Haplotypes)
it is sufficient to sequence each of the two selected PCR products in a single orientation.

In HLA class I
getting complete Exon sequences it is recommended to use the
Reverse Sequencing Primers Exon 2 (E2R) and Exon 3 (E3R)

In HLA class II
getting complete Exon sequences it is recommended to use the
Forward sequencing primers Exon 2 (E2F)

If a PCR-based separation of the alleles is not archieved,
sequencing in both orientations is required. In order to avoid ambiguities even in heterozygous sequencing due to undefined cis linkages of sequence motifs the use of Sequence-Specific Sequencing Primers allows a selective sequencing of only one of the two alleles. Together with the result of the heterozygous sequencing an unambiguous cis linkage definition of both alleles can then be obtained.

Sequence-Specific Sequencing Primer HLA-DRB1 (DR-86TG)
is presently available for codon 86-selective sequencing

15.1 Set up Sequencing Reaction
The DNA is splitted into 2 Haplotypes

HLA class I			
Haplotype 1	**Exon 2**	**Reverse -E2R**	forward -E2F
	Exon 3	**Reverse -E3R**	forward -E3F

Haplotype 2	**Exon 2**	**Reverse -E2R**	forward -E2F
	Exon 3	**Reverse -E3R**	forward -E3F

HLA class II			
Haplotype 1	**Exon 2**	**Forward -E2F**	reverse -E2R

Haplotype 1	**Exon 2**	**Forward -E2F**	reverse -E2R

It is possible to sequence each Haplotype only in one direction

15.2 Set up Sequencing Reaction
Only 1 Allele or only the Locus specific Amplification Product (LSA)

HLA class I			
Haplotype	**Exon 2**	**forward -E2F**	**reverse -E2R**
	Exon 3	**forward -E3F**	**reverse -E3R**

Locus specific Amplification (LSA)	**Exon 2**	**forward -E2F**	**reverse -E2R**
	Exon 3	**forward -E3F**	**reverse -E3R**

HLA class II				
Haplotype	Exon 2	**forward -E2F**	**reverse -E2R**	**Codon 86TG**

Locus specific Amplification (LSA)	Exon 2	**forward -E2F**	**reverse -E2R**	**Codon 86TG**

In most cases sequencing of exons 2 and 3 may be sufficient even for 4-digit typing and is always sufficient if only a 2-digit typing information is required
The sequences of exons 1 and 4 may be required to sort out certain ambiguities as well as non-expressed (null) alleles.

Attachment (see Kit insert):

shows Specificities of the Amplification Primer and additional possibilities to use Sequencing-Primers for Exon 1 or Exon 4.

Exon 1 reverse sequencing may also be possible by extending the sequencing run of Exon 2 reverse.
Exon 2 forward sequencing may also be possible by extending the sequencing run of Exon 1 forward.
However, this requires longer high quality reads and specific settings of the sequencing instrument. Moreover, in certain allele-combinations a deletion in intron 1 causes a chromatogram shift inhibiting sequence analysis of Exons 1 and 2, respectively.

16.0 Instruction Set up Sequencing reaction (3-dot-Method)

1	Generate sequencing plate with Protrans Pipetting Assistant (Protrans SEQ 1) or Pipetting Scheme form

2	In Protrans PCR Workstation -20℃	PCR plate or MicroAmp optical plate
		Big DyeTerminators
		Sequencing primers

3	Vortex Big DyeTerminators

4	**2µl**	**Big Dyes** dispense to the left side of the wells in the MicroAmp optical plate. (RAININ Multistepper)

5	Spin down sequencing primers with mini-table centrifuge

6	**6µl**	**Sequencing Primers** dispense to the left side of the wells in the MicroAmp optical Plate. (RAININ Multistepper)

7	**2µl**	**Purified PCR Product** pipet in the middle of the wells in the MicroAmp optical plate (RAININ Multistepper)

8	Seal the plate carefully and place the plate in the thermocycler. Start the cycle sequencing program

	Note If sequencing reactions give to strong signals a dilution of the BDT RR Mix might be appropriate, beginning with a 1:2 dilution. Dilute Big Dye Terminators 1.1 with 5x Sequencing Buffer 1:2,
	Note If sequencing reactions give rise to background signals or a to strong signal decline a dilution of the PCR product may be appropriate, beginning with a 1:2 dilution.

17.0 Purifying Sequencing Reactions

17.1 PROTRANS DyePUR

The sequencing products have to be purified to remove unincorporated dye terminators.
The **PROTRANS DyePUR** purification kit for purifying sequencing reactions is based on spin filtration using Sephadex G-50 and PROTRANS DyePUR columns. The method provides high quality sequence data.

Preparation of the Sephadex gel for 20 columns

1	50ml Falcon tube		
2	**1g**	**Sephadex™ G-50 DNA Grade F**	
3	**12ml**	**HPLC Water (Merck)**	
4	Incubation at RT mix or vortex several time during incubation		**30 min.**

	Mark the Protrans Receiver Tubes (2ml)		
5	**A few minutes before** thermocycler program for sequencing reaction ends		
	Fill the columns with Sephadex gel suspension about ¾ of the tube. Use a plastic Pasteur pipette		
	Centrifuge the Protrans Receiver Tubes with the Filter Columns at Spinning time and g force must be kept exactly	**2000x g**	**90 sec.**
8	Mark Protrans Receiver Tubes 1,5 ml reaction tubes according to the number of sequencing reactions which have to be purified, and place the PROTRANS Filter columns with Sephadex gel suspension in Protrans Receiver Tubes 1,5 ml.		

Purification of Sequencing Products

1		Take one spin column out of Protrans Receiver Tubes 1,5 ml and keep in hand		
2	**10µl**	**Sequencing Product** pipet carefully to the center of the angled surface of the pre-spinned Sephadex column. Pay attention that the tip is not touching the Sephadex gel. Careful application of the sample to the center of the bed is essential for good separation. Do not allow any of the sample to flow around the sides of the Sephadex column		
3		Place spin filter back in marked 1,5ml tube		
4		Spin Protrans column down at exactly	**2000xg**	**1 Min**.
		Spinning time and g force must be kept exactly		
5		Until loading the marked sequencing products should be stored at 4-8℃ or for longer periods at -20° **protected from light**.		

Sequencing set-up

1	**18µl**	**HPLC water** pipet in a MicroAmp Optical plate.
2	**2µl**	**Purified Sequencing Product** pipet in a MicroAmp Optical plate, always use new filtertips
3	The sequencing products are ready for loading into sequencing instruments Until loading the plates should be stored at 4-8℃ **protected from light**	

17.2 Ethanol Precipitation

1		**80% Ethanol**: 8 parts **Ethanol 100%** and 2 parts **HPLC water**.	

2		**Spin** briefly the 96-well MicroAmp Optical plate with the Sequencing products		
3		Take the **EDTA/NaOAc buffer** vortex/spin down gently before opening vial and keep on ice (Protrans PCR-Workstation)		
4	**2µl**	**NaOAc/EDTA buffer** (Teknova, cat.-no. S2080) pipet to each sequencing reaction. Add the drop to the wall of each well using an 8-channel-multi dispensing pipette. **Note**: The static can keep the drop at the tip, assure dispensing.		
5		**Spin** MicroAmp Optical plate briefly		
6	**25µl**	**100% Ethanol** pipet to each well using an 8-channel-multi dispensing pipette		
7		**Vortex the plate thoroughly.** Using a flat support over the tray to prevent any splash (e.g. aluminium plate) **Note**: Incomplete mixing will result in poor quality data.		**30 sec**
8		**Spin** MicroAmp Optical plate at for	**2.000x g**	**30 min**
9		**Immediately** remove supernatant by inverting and gently tapping tray on paper towels Place the inverted tray with the paper towels in the centrifuge.		
10		**Spin** MicroAmp Optical plate at to remove the supernatant.	**180x g**	**20 sec**
		Note: Centrifugation is important to ensure complete removal of the degraded products		
11	**50µl**	**80% Ethanol** pipet to each well using an 8-channel-multi dispensing pipette.		
12		**Spin** MicroAmp Optical plate at for	**2.000 x g**	**5 min**
13		Immediately remove supernatant by inverting and gently tapping tray on paper towels. Place the inverted MicroAmp Optical plate with the paper towels in the centrifuge		
14		**Spin** MicroAmp Optical plate to remove the supernatant at	**180x g**.	**20 sec**
		Note: Centrifugation is important to ensure complete removal of the degraded products		
15		Dry the tray for **in dark** ambient (reactions are light sensitive)		**15 min**
16	**10µl**	**HPLC water** to each well, using an 8-channel pipette. Resuspend the sequencing Products by pipetting 3 x up and down		
17	**90µl**	**HPLC water** to each well, using an 8-channel pipette. Close the plate and vortex!		
18		**Spin** MicroAmp Optical plate briefly and place on the ABI Support Base®,		
19		Place a Full Plate Septa® mat on the tray, followed by the 96 Well Plate Retainer and place the complete tray onto the ABI Sequencer		

Alternative Steps		
Preparing **125mM Sodium EDTA (pH 7.0): 1 part** 0,5M EDTA and **3 parts** HPLC-H_2O		
4	**2µl**	**125 mM EDTA (pH 7.0)** add to the sequencing reactions (Single or 8 channel Multistepper). Use filter tip.
	2µl	**3 M Sodium Acetate (pH 5.2)** add to the sequencing reactions (Single or 8 channel Multistepper). Use filter tip.

16	**15µl of Hi Di Formamide** to each well, using an 8-channel-multi dispensing pipette. Close the plate	
17.1	Incubate at 95℃ (Thermocycler)	**2 min**
17.2	Keep on ice (Protrans PCR-Workstation)	**2 min**

17.3 Alternative Purification methods

comprise amongst others Agencourt CleanSEQ beads purification technology, Ethanol precipitation or Millipore Montage filter technology which have all been shown to yield high-quality data. These techniques can also be applied in a 96-well format for high throughput requirements. Moreover, the Agencourt and Millipore technologies have a good potential for automation. Multiple other methods are available for purifying sequencing reactions and may be used if validated in the laboratory.

18.0 Preparing Sequencing Reactions for Electrophoresis

The different sequencing instruments may require different preparations of the samples prior to loading them on the gel or the capillary. The following chapters describe preparing samples for the capillary sequencers 310, 3100, 3130, 3700 and 3730 and the slab gel sequencer 377 (Applied Biosystems). For other DNA Sequencers the preparations described may be suitable as well. For detailed information refer to the manufacturer's recommendations.

To prepare purified sequencing reactions for capillary electrophoresis resuspension in formamide or water is possible. For ease of use, higher reproducibility and higher data quality the water protocol is recommended for the Protrans HLA Sequencing Kits.

Preparing Sequencing Reactions for Capillary Electrophoresis

As already desribed in 22.1.and 22.2 dilute the purfied Sequencing Reaction	
10 µl	Purified Sequencing Reaction with
90 µl	HPLC water
Seal the plate or tubes appropriately and place them on the autosampler.	

Note Different sequencers may have different fluorescence detection sensitivities and may therefore require different dilutions.
Note If less or more DyeTerminators are used in the sequencing reaction as specified in 17 the dilution of the purfied Sequencing Reaction must be adapted.
Note The remaining Sequencing Reaction may be stored as a back-up at 4℃ for several days in the dark.

Preparing Sequencing Reactions for Slab Gel Electrophoresis

Dry the purified sequencing samples in a vacuum centrifuge or in a heating block at 70℃	45 min.
4 µl	**Loading Buffer** (recrystallized formamide and blue dextran/EDTA solution 5:1; prepare fresh for each use).resuspend the dried Sequencing Sample.
If spin filtration was used (18.1 skip 5) don´t dilute add **4µl Loading Buffer**	
If Etanol precipitation was used (18.2 step 15) don´t dilute add **4µl Loading Buffer**	
Note Different sequencers may have different fluorescence detection sensitivities and may therefore require different dilutions.	
Note If less or more DyeTerminators are used in the sequencing reaction as specified in 3.4.1 the dilution of the purfied sequencing reaction must be adapted.	
Denature resuspended samples at 90℃ for 2 minutes and keep on ice until loading on the gel.	

19.0 Running the Instruments

Instrument platforms

The Protrans HLA Sequencing Kits are compatible with all four-dye sequencing instruments available. Follow the manufacturer's instructions for standard runs. A read length of 350 bases is sufficient.

Applied Biosystems	Capillary: 310, 3100, 3130, 3700 and 3730 Slab Gel: 377
GE Healthcare	Capillary: MegaBACE 500, 1000, 4000
Beckman	Capillary: CEQ8
MJ Research	Slab Gel: BaseStation, BaseStation51

For comprehensive information on these instruments and for running other four-dye DNA Sequencers refer to the user's manual of the manufacturers.

Note The Protrans HLA Sequencing Kits have been validated for the Applied Biosystems DNA Sequencers 3100 Genetic Analyzer and 3730 DNA Analyzer. Other four-dye sequencing instruments should be validated before routine use

20.0 Identifying HLA alleles Protrans Allele Identification Software

The final step in sequence analysis consists in the allele assignment using the Protrans Software Sequence Pilot© Allele Identification Software (JSI medical systems, www.jsi-medisys.de). This program performs allele identification based on the cDNA sequence database of all HLA class I and class II exon sequences, detects heterozygous positions as well as mismatches with the sequence database, allows manual review or editing of the sequencing data as well as reporting, exporting, printing and archiving of sequences and results. The HLA cDNA sequence library is updated with each new Sequence Database release of the HLA informatics group (www.anthonynolan.com/HIG) and can be downloaded from the www.jsi-medisys.de website.
The software is availabel as a Windows™ single-user version or as a Windows™ or Linux™ server-client version.
This chapter is designed to be a quick reference to help the user through the basic steps involved in performing Sequence Pilot© software analysis. For more details, refer to the Sequence Pilot© User's Manual.
The Sequence Pilot© Allele Identification Software is compatible or adaptable to all four-dye sequencing instruments available

Applied Biosystems	Capillary: 310, 3100, 3130, 3700 and 3730 Slab Gel: 377
GE Healthcare	Capillary: MegaBACE 500, 1000, 4000
Beckman	Capillary: CEQ8
MJ Research	Slab Gel: BaseStation, BaseStation51

21.0 Sample Naming Conventions

Guideline

The Sequence Pilot© Software automatically recognizes the locus and exon sequenced as well as the direction of sequencing. In order to allow the software for automated joining or pairing of sequencing results that belong together (e.g. forward and reverse sequencing direction of the same PCR product) certain rules of sample naming conventions must be followed. Even if you are not using the automated joining features of the Sequence Pilot© software, sample naming conventions should be followed to simplify clarity for manual processes.

Enter the following types of information in brackets:

(SampleID_Amplification Mix_Sequencing Primer)_any other information

Alleles Separated

The sequencing reactions of a DNA sample that was amplified by the Group-Specific Mixes S4R2 (DRB1*15, *16) and S4R4 (DRB1*04) have the following file names

Example

(SampleID_S4R2_DR-E2F)_...	corresponds to Haplotype 1 forward
(SampleID_S4R4_DR-E2F)_...	corresponds to Haplotype 2 forward

Alleles not separated

The sequencing reactions of a DNA sample that was amplified by the single Group-Specific Mix S4R2 (DRB1*15, *16) have the following file names:

Example

(SampleID_S4R2_DR-E2F)_...	corresponds to Haplotype 1+2 forward
(SampleID_S4R2_DR-E2R)_...	corresponds to Haplotype 1+2 reverse

Due to these sample naming conventions the Sequence Pilot© Software automatically recognizes that these sequencing results belong together and performs allele assignment based on both result files.

22.0 Performing Allele Identification

Result Files Menu

1	Mark the result file(s) belonging to the same Haplotype(s) of the DNA sample. Select 'save' to transfer them as joined or paired result files in the 'joined result files' table
Process	Selects, pairs (forward and reverse) or joins (different exons) the result files that belong together and have to be analyzed together.
	Note This is done automatically if the Sample Naming Conventions have been followed
	Generates a combined sequence for paired forward and reverse sequences. Starts allele identification for paired or joined result files by comparing the result sequences with the HLA sequence library

Analysis Menu

2	Mark the joined result files in the 'Worklist' and select 'Sequence' in the analysis window.
Process	Displays the electropherograms and result of allele assignment in the 'Data Analyzer' window
3	Check the results using the electro-pherogram options.
Process	Edits and re-analyzes the result sequences if required.
4	Approve final analysis.
Process	Validates allele identification, saves data in the result database, transfers the result into the local LIS (if desired), and prints the final report for documentation.

23.0 Trouble shooting Guide

PCR Troubleshooting

PCR is an extremely sensitive method, which can efficiently amplify even the smallest amount of DNA. It follows from this that even traces of contaminating DNA in a sample can be amplified in the PCR reaction and falsify the test result. One particular source of contamination is amplified DNA coming into contact with samples, which are still to be amplified. To avoid contamination with amplified material, it is recommended that the work area is strictly partioned as follows:

Precautionary measures

Pre-PCR area:

All work carried out before PCR (preparing and storing sample DNA, preparing PCR amplification reactions, setting up and storing reagents and solutions for DNA isolation and PCR).

Post-PCR area:

All work carried out after PCR (running thermal cyclers and DNA sequencers, performing agarose gel electrophoresis, preparing and purifying sequencing reactions, storing amplified DNA or sequencing reactions).

Materials and Equipment from the post-PCR area must not be taken into the pre-PCR area. When pipetting in the pre-PCR area and for setting up the sequencing reactions, tips with aerosol protection (filtered tips) should be used. It is recommended that for each amplified DNA sample a related negative control is performed as an indication of contamination with foreign DNA.

The following table lists possible causes and solutions for PCR problems.

PCR Troubleshooting Table

Problem	Possible Cause	Solution
No PCR product or weak PCR product	No ethidium bromide in gel	Secondary staining of the gel in a staining bath (1X TBE with 0.5 µg/ml ethidium bromide); remember to add ethidium bromide prior to pouring the gel
	Incomplete mixing of AmpliTaq Gold™ and PCR reaction mix	Repeat PCR with attention to mixing
	Degraded DNA	Evaluate on agarose gel and re-extract the DNA if necessary
	DNA not measured correctly	Re-measure DNA and adjust to 50 - 100 ng/µl
	PCR inhibitors in the genomic DNA	Re-extract genomic DNA using one of the recommended methods (see “Preparing Sample DNA”); avoid using blood treated with heparin
	Inappropriate cycling parameters	Check the cycling profile and correct it if necessary
	Thermal cycler problems	The cycling profiles provided in this manual are optimized for the GeneAmp PCR Systems 2400, 2700, 9600 and 9700 (Applied Biosystems) as well as the PTC-100, PTC-200, Dyad and Tetrad (MJ Research). The use of other thermal cyclers may require adjustment of the cycling profile.
2 PCR bands	Inappropriate agarose gel conditions	Run agarose gel according to appropriate conditions
Non-specific PCR bands	Inappropriate cycling parameters	Check the cycling profile and correct it if necessary
	Thermal cycler problems	The cycling profiles provided in this manual are optimized for the GeneAmp PCR Systems 2400, 2700, 9600 and 9700 (Applied Biosystems) as well as the PTC-100, PTC-200, Dyad and Tetrad (MJ Research). The use of other thermal cyclers may require adjustment of the cycling profile.
	Inappropriate DNA Taq polymerase	Repeat PCR with AmpliTaq Gold™ DNA Taq polymerase
	Contamination of the PCR reagents	Check the related PCR negative control to exclude contamination of the PCR reagents. Use filter tips.
	Contamination of the DNA sample	To exclude contamination of the DNA sample re-extract DNA from a new source sample. Use filter tips.

Sequencing Troubleshooting

Sequencing problems may be related to sequences in general or only to heterozygous sequences. Problems with heterozygous sequences may be peak shifts or weak heterozygous signals. The majority of these anomalies occur in only one sequencing orientation for a certain base position and can be resolved by reviewing data from the other orientation.
The following table lists possible causes and solutions for general sequencing problems.

Sequencing Troubleshooting Table

Problem	Possible Cause	Solution
Weak signal strength	Inappropriate injection time or injection voltage	Because of variations between instruments, adjustments of the injection time and/or the injection voltage may be needed to get a signal range from 100 – 2000 relative fluorescent units
	Too little sequencing reaction applied	Capillary DNA sequencer: Increase injection time, injection voltage or concentration of sequencing reaction. Slab gel DNA sequencer: Increase loading volume or concentration of sequencing reaction.
Too strong signal strength	Inappropriate Injection time or injection voltage	Because of variations between instruments, adjustments of the injection time and/or the injection voltage may be needed to get a signal range from 100 – 2000 relative fluorescent units.
	The finally prepared sequencing reaction was too concentrated	Increase dilution in the final step of preparing sequencing reactions for electrophoresis (36 µl instead of 18 µl HPLC water).
Noisy baseline	Inappropriate PCR product purification or purification omitted before sequencing	See “Purifying PCR products for Sequencing”.
	Inappropriate sequencing reaction purification	Re-purify the sequencing reaction (see “Purifying Sequencing Reactions”). Remember to pipet the complete sequencing reaction carefully on the center of the sloping Sephadex surface without touching it. Pipetting on the center is essential for high-quality purification. Do not allow the sample to flow beside the resin.

Sequencing Trouble shooting Table (continued)

Noisy baseline	Contamination of the PCR product	Check the related PCR negative control to exclude contamination of the PCR reagents. To exclude contamination of the DNA sample re-extract DNA from a new source sample. Use filter tips.
	Contamination of the sequencing primer	Repeat sequencing with a fresh tube of sequencing primer. Use filter tips.
	Contamination of the dye terminator reagents	Repeat sequencing with a fresh tube of dye terminators. Use filter tips.
Broad fluorescent terminator artifacts (dye blobs)	Inappropriate sequencing reaction purification	Re-purify the sequencing reaction. Remember to pipet the complete sequencing reaction carefully on the center of the sloping Sephadex surface without touching it. Pipetting on the center is essential for high-quality purification. Do not allow the sample to flow beside the resin.
High fluorescent artifact peaks	Air bubbles in the capillary or polyacrylamide gel	Capillary DNA sequencer: Refill the capillaries. Consider possibly to change the capillary array or to contact the manufacturer's service for your sequencing instrument. Slab gel DNA sequencer: Pour the gels carefully and appropriately treat the gel sides of the glass plates to avoid air bubbles. Contact the manufacturer's service for your sequencing instrument.

Protrans medizinische diagnostische Produkte GmbH
Ketschau 2 68766 Hockenheim, Germany

Telephone ++49 (6205) 29299-0
Telefax ++49 (6205) 29299-20

www.protrans.info
mail@protrans.info