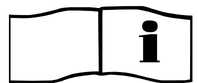

User Manual

Protrans HLA SSP Kits

Molecular genetic DNA Typing, SSP technique

PROTRANS Cyclerplate System		PROTRANS Domino System

REF	Article			REF	Article	
200 070	HLA- A*	IVD CE 0197		201 071	HLA- DRB1* 01	IVD CE 0197
200 080	HLA- B*			201 072	HLA- DRB1* 03	
200 030	HLA- DRB1*			201 070	HLA- DRB1* 04	
200 020	HLA- A*,-B*,-DRB1*			201 077	HLA- DRB1* 07/*09	
200 010	HLA- A*,- B*,- C*			201 073	HLA- DRB1* 08/*12	
200 011	HLA- A*,- B*			201 074	HLA- DRB1* 11	
200 050	HLA- DRB1*,- DQB1*			201 075	HLA- DRB1* 13	
200 090	HLA- C*	IVD CE		201 076	HLA- DRB1* 14	
200 040	HLA- DQB1* low			201 078	HLA- DRB1*15	
201 201	Negative Control HLA			201 079	HLA- DRB1*16	
200 048	HLA- DQB1* high			201 080	HLA- DRB1* 15/*16	
200 049	HLA- DQA1* high					

IVD	For In Vitro Diagnostic use	
	Buffer D, R and Y	- 20°C
	Cyclerplates and Domino Strips	2°- 8°C
	See label on the Testkit	
	User Manual	
	Warning and Precautions	

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Quality and Certification

We declare under our sole responsibility that the mentioned products are in compliance with the essential requirements of the Directive IVDD 98/79 EG and EN ISO 13485:2003 and EN ISO 9001:2000 on design, production and sales of HLA tissue typing products and are marked with the CE symbol.

License ARMS™ technology

With the Protrans SSP Typing Kits the **ARMS™ technology** is used. The Products are sold under license of BTG International Limited UK and DxS Ltd. UK. ARMS™ is the subject of worldwide patent property and is a trademark of the AstraZeneca group of companies.

Symbols

Country	REF	NC	IVD	🌡	⌛
DE	Artikelnummer	Negativkontrolle	in-vitro Diagnostik	Lagern bei	Haltbar bis
GB	Article number	negative controll	For in vitro diagnostic use	Store at	Expiration Date
ES	Artikulo número	control negativo	Sólo para el diagnóstico in vitro	Almacenar	Caducidad
IT	Codice prodotto	controllo negativo	Solo per la diagnostica in vitro	Conservare a	Scadenza
FR	Numéro de l´articl	contrôle negatif	Pour le diagnostic invitro	Conserver à	Péremption

1. Introduction

1.1 Intended Use

The Protrans HLA Sequence Specific Primer (SSP) Kits are intended for the determination of HLA-class I or -class II alleles based on the PCR-SSP method.

1.2 Summary and Explanation

The HLA-system is a complex, co-dominantly inherited system of antigens which plays an important role in the immune system by enabling it to distinguish „self“ from „non self“. In organ transplantation, HLA compatibility between donor and recipient is one of the major determinants of transplant outcome. For this reason, the determination of the individual combinations of HLA antigens is used as a basis for the selection of donors and recipients.
New dimensions have been opened up to modern diagnostics by the development of DNA-based test methods. HLA antigens partly differ from each other only by single amino acids within the polypeptide chain. Recognition of these largely identical structures by serological means is almost impossible; for this reason, the resolution capacity of the method is limited. As the DNA sequences of most important HLA alleles are now known, variations in sequences can be identified at the DNA level with the help of synthetic oligo nucleotides. Utilizing amplifications of genomic DNA (PCR, Polymerase Chain Reaction) together with specific primer pairs (SSP, Sequence Specific Primer) it is possible to identify a large number of HLA alleles by molecular test methods.

1.3 Principle of the Test

Protrans HLA SSP Kits are intend to determine HLA-class I or –class II alleles based on a PCR method using sequence specific primer (PCR-SSP). The principle of the SSP method is to generate an amplificate only when the sequence of a primer is perfectly complementary to the target sequence of a DNA sample. On the other hand, non-complementary primer do not bind to the DNA and for this reason no amplification takes place.
Using agarose gel electrophoresis, the amplified DNA can be determined. Successful amplification will generate a DNA fragment of defined length which appears as a band in the gel. If no amplification takes place, no specific band is seen.
The composition of the primer mixes in each well of the Cyclerplates and Domino Strips permits positive identification of different specific alleles of each HLA locus.
The Protrans SSP kits are in Vitro Diagnostic Test systems for the diagnosis of the immune system of organ donors and recipients and of patients receiving blood component substitution therapy.

2. Reagents

2.1 Contents of the Protrans SSP Kits

1	Component 1		
	Cyclerplates or Domino Strips	Oligonucleotides (dried)	2-8°C

2	Component 2		
	HLA class 1 Buffer D and Buffer Y	Master Mix for Amplification	-20°C
	HLA class 2 Buffer R and Buffer Y		
	HLA class 1 HLA class 2 in combination Buffer D and Buffer Y		

PROTRANS Cyclerplates and **PROTRANS Domino Strips**
contain pre-pipetted, dried, specific Primer Mixes (oligonucleotides).
The positions and specificities of the Primer Mixes are LOT – specific and described in the Kit Inserts
Reaction Protocol, Primer MixTable, Amplification Table and Typing Table.

Number of Primer Mixes of each Testkit in the PROTRANS Cyclerplate System refer to: **Table 7.7**
Number of Primer Mixes of each Testkit in the PROTRANS Domino System refer to: **Table 7.10**

The **PROTRANS Cyclerplates** are fit into each other and packed in zip-lock pouches. Each pouch contains 10 or 20 Cyclerplates. The pouch and the upper **Cyclerplate** are marked with a label.
Name of the product, specificities of the pre-pipetted Primer Mixes, LOT number, storage temperature and expiration date are written on the label.
Name of the product and LOT number are printed on each **PROTRANS Cyclerplate**.
Each well of the **PROTRANS Cyclerplate** is marked with a digit-letter combination from A1 to H12. Digits are visible on the top, letters on the left edge of the Cyclerplate.
Primer Mix 1 of each Testkit is located at position **H1**.
For better orientation the lower edge of the Cyclerplate has been marked with a black line.

96 wells											
8	16	24	32	40	48	56	64	72	80	88	96
7	15	23	31	39	47	55	63	71	79	87	95
6	14	22	30	38	46	54	62	70	78	86	94
5	13	21	29	37	45	53	61	69	77	85	93
4	12	20	28	36	44	52	60	68	76	84	92
3	11	19	27	35	43	51	59	67	75	83	91
2	10	18	26	34	42	50	58	66	74	82	90
1	9	17	25	33	41	49	57	65	73	81	89

The **PROTRANS Domino Strips** are fit into each other and packed in zip-lock pouches. Each pouch contains 12, 24 or 30 Domino Strips. The pouch is marked with a label.
On the label Name of the product, specificities of the pre-pipetted Primer Mixes, LOT number, storage temperature and expiration date are written.

PROTRANS Domino System
Position **Primer Mix 1** Domino Strip

8 wells
8
7
6
5
4
3
2
1

16 wells	
8	16
7	15
6	14
5	13
4	12
3	11
2	10
1	9

24 wells		
8	16	24
7	15	23
6	14	22
5	13	21
4	12	20
3	11	19
2	10	18
1	9	17

32 wells			
8	16	24	32
7	15	23	31
6	14	22	30
5	13	21	29
4	12	20	28
3	11	19	27
2	10	18	26
1	9	17	25

2.2 Warning and Precautions:

IVD Reagents only for In Vitro Diagnostic use

	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 and Domino Strips 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 Ethidium bromide and 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.

2.3 Storage and shelf life

Kit Components			**Ingredients**		
Cyclerplates 96-well PCR plates Domino-Strips 32-, 24-, 16-, or 8-well PCR-Strips			Primer (DNA Oligo-Nucleotides) Cresol Red	**2-8°C**	See label
Buffer	**D**	**R**	Ammonium Sulfate, Tris-Buffer, $MgCl_2$, Glycerol, Cresol Red, dNTPs (Deoxyribonucleotides)	**-20°C**	See label
Buffer	**Y**		Watery solution	**-20°C**	See label
Once the test kits have been opened, the remaining unused Cyclerplates or Domino Strips must be kept closed in their original package. Reseal the ziplock to prevent moisture accumulation during storage.					

3. Instrument requirements

3.1 Programming the Thermocycler

Thermal Cycling Program

For optimal results it is important to obtain **rapid ramp times (1° - 2,5°C/s)** and precise temperature control. The following thermal cycler profile is optimized and validated with the thermocyclers given in 5.2.3. for use with the PROTRANS SSP Cyclerplate System and Domino System.

The final volume of the amplification mix is **10 µl.**

<table>
<tr><td>Initial denaturation</td><td>94°C</td><td>2 min</td><td>Hold</td></tr>
<tr><td>Denaturation</td><td>94°C</td><td>15 sec</td><td rowspan="2">10 cycles</td></tr>
<tr><td>Annealing and Extension</td><td>65°C</td><td>60 sec</td></tr>
<tr><td>Denaturation</td><td>94°C</td><td>15 sec</td><td rowspan="3">20 cycles</td></tr>
<tr><td>Annealing</td><td>61°C</td><td>50 sec</td></tr>
<tr><td>Extension</td><td>72°C</td><td>30 sec</td></tr>
<tr><td>Hold</td><td>4°C</td><td>15 min</td><td>Hold</td></tr>
</table>

See **5.2.3**: list of validated Thermocyclers with 96-well block and heated lid.

4. Specimen collection and preparation

4.1 DNA Isolation

Genomic DNA may be obtained from all nucleated cells. Starting materials are EDTA- or citrate blood, buffy coat or cell suspensions. A vast range of various protocols exist for the isolation of DNA. For PCR-SSP testing only those methods which provide DNA of adequate quality and quantity should be considered.
The Protrans DNA Extraction Kit PROTRANS DNA Box 500/5000 provides DNA of high stability and quality. All DNA extraction methods must be validated before routine use.

The concentration of the DNA should be adjusted to **50 – 100 ng/µl**.
The **ratio** (purity) A_{260}/A_{280} should be **1.6 – 1.8**.
Concentration and purity of the DNA is of decisive importance for optimal test results.

DNA sample may be used immediately after isolation or stored at –20°C or below for extended period of time (over one year) with no adverse effects on the results.
For storage and stability information of isolated DNA please refer to the technical information provided by the DNA extraction test kit manufacturer.

Use either EDTA- or Citrate-blood samples for DNA extraction.

Do not use heparinized blood samples. Heparin inhibits the PCR.

4.2. Non-acceptable specimens

Contamination of the DNA by PCR inhibitors, such as haemoglobin, heparin, ethanol, ficoll-separated specimens (buffy coat, cell suspensions), etc. can result in serious interference with the PCR reaction.

For this reason, **heparin blood is not acceptable** as a starting material for DNA isolation.
If the patient is on heparin therapy, use an alternative source of DNA.

Avoid the use of lipemic or haemolysed specimens.

The use of specimens collected without anticoagulant or frozen/thawed multiple times is not recommended since these conditions may not provide sufficient quantity or quality of DNA for testing.

5. Materials

5.1 Materials Provided

See 2.1 contents of the PROTRANS SSP kits.

5.2 Additional materials, reagents and equipment required but not supplied

All reagents and equipment other than recommended requires validation by the user.

5.2.1 UV – spectrophotometer for photometric DNA measurement
e.g. Lambda Scan 200, MWG www.THE.MWG.com
PC photometer program: KC 4, Bio-Tek Instruments, Inc. www.biotek.com
Use of other UV-spectral photometers must be validated by the user.

5.2.2 DNA Taq Polymerase

The following enzymes are validated for use with the PROTRANS SSP Kits:

- AmpliTaq, Perkin Elmer, 5U/µl, Cat.No. N8010060
- MBI Fermentas DNA Taq Polymerase, 5U/µl, Cat.No. EP0402

Use of other DNA Taq Polymerase enzymes must be validated by the user.

5.2.3 Thermocycler

The following Thermocyclers with 96-well block and heated lid are validated for use with the PROTRANS SSP Kits:

- PE 2700, Applied Biosystems, www.appliedbiosystems.com
  (production PE 9600, Applied Biosystems, stopped)
- PTC-100, PTC-200 MJ Research, Inc., www.mjr.com

Thermocyclers other than the recommended have to be user-validated.
Thermocyclers which have no adjustable pressure plate require an adaptor mat in order to guarantee optimal heat transfer from the heat cover to the PCR tubes.

5.2.4 Pipettes

- Adjustable pipettes for volumes:
  - 1-10µl
  - 10-100µl
  - 100-1000µl
- Eppendorf Multipette Type 4720
- 8-channel pipette, e.g. 5-50µl adjustable pipetting volume
  (Finnpipette, ThermoLabsystems Cat.No. 4510020)

5.2.5 Disposables

- Suitable **Filtertips** specific for each pipettes
- 1,5 ml polypropylene reaction tubes (e.g. Eppendorf Safe-Lock, Cat.No. 0030 120.86)
- 0.5 ml Combitips for Eppendorf Multipette Type 4720

5.2.6 Gel - Electrophoresis

Agarose (for molecular biology)
TAE–Buffer (1x) – electrophoresis buffer

- TAE = Tris-buffer / conc. Acetic Acid (CH_3COOH) / 0,5 M NA_2-EDTA ph 8.0

Distilled water (dH_2O)
Ethidium bromide solution (10mg/ml); Caution: Ethidium bromide is mutagenic (see 2.2.)
Magnetic stirrer with hotplate or microwave oven
DNA molecular weight marker (50 – 1000 bp ladder)
Power Supply
PROTRANS Electrophoresis System (PROTRANS Electrophoresis Unit or Gel-Check REF 210 000, 210 001)
Gel documentation System

- Polaroid camera with UV Filter and Polaroid film type 667
- Gel documentation system
- Transilluminator (312nm) UV-light

6. PCR – Polymerase Chain Reaction

6.1 Precautionary measures

PCR is an extremely sensitive method which can efficiently amplify even the smallest amounts of DNA.
It follows from this that even traces of contaminating DNA in a sample can be amplified in the PCR reaction and falsifies the test results. 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 areas be strictly separated as follows:

6.2 Pre – PCR area:

All work carried out before PCR (DNA isolation and storage, preparations for the PCR, production and storage of reagents and solutions for DNA extraction and PCR).
When working in the pre-PCR area, pipettes with aerosol protection should be used (filtered tips).
It is recommended to use a negative control with every amplification to detect contaminations.

6.3 Post – PCR area:

Thermocycler, gel electrophoresis, evaluation, storage of amplified DNA. Equipment and consumables from the post-PCR area **must not** be taken into the pre-PCR area.

7 Instruction for PROTRANS SSP DNA Typing Kits

7.1 Preparation of the PCR

7.1.1	Out of the refrigerator collect the desired PCR-Cyclerplates or PCR-Domino-Strips.
7.1.2	Out of the freezer (-20°C) collect the DNA Taq Polymerase and the corresponding Protrans Buffers. **See Tables 7.5 and 7.8**.
7.1.3	Place the PCR-Cyclerplates or the PCR-Domino-Strips and the DNA Taq Polymerase into the cooled (-20°C) PROTRANS „PCR Workstation“ or into a 96-well PCR-rack.
7.1.4	With the Protrans Cutter (scissors) it is possible to cut the PCR-Cyclerplates into single tests.
7.1.5	Thaw the Protrans **Buffers D** or **R** and **Buffer Y.**

7.2 Preparation of Master Mix for the Protrans PCR-SSP System

7.2.1	For each DNA sample to be tested pipette the appropriate volume of **Buffer D** or **R** and **Buffer Y** and DNA Taq Polymerase into a 1,5ml reaction tube. **See Tables 7.6 and Table 7.9.**
7.2.2	Vortex Mastermix well
7.2.3	When using a negative control pipette 10 µl of this Mastermix without the DNA into the appropriate well. **See Tables 7.6, 7.7 and 7.9, 7.10.**
7.2.4	Add to each Mastermix the appropriate volume of DNA recommended for the test **See Tables 7.6 and 7.9.**
7.2.5	Vortex Mastermix well

7.3 Dispensing the Master Mix in the Protrans PCR-Cyclerplates or PCR-Domino-Strips

7.3.1	Dispense 10 µl of the Mastermix into each well of the PCR-Cyclerplate or PCR-Domino-Strip but not into the negative control. When pipetting at the upper edge of each well the drop will run down to the bottom. The bottom of the wells must not be touched with the tip.

7.4 Capping the Protrans PCR-Cyclerplates or PCR-Domino-Strips

7.4.1	Ensure that the Mastermix is at the bottom of all wells by either centrifugation or gentle tapping the Cyclerplate or Domino-Strip onto the work bench. Visual control ensures that the Mastermix is at the bottom of each well.
7.4.2	Seal Cylerplates or Domino-Strips carefully using the caps provided or with re-usable PROTRANS 24-/96-well Coverplates. Ensure caps completely seal the wells to prevent evaporation.
7.4.3	Place the Cyclerplate or Domino-Strips into Thermocycler and start amplification. **Final volume in each well is 10 µl.** If unable to start amplification immediately after PCR set-up, the PCR-Cyclerplates or -Domino-Strips must be stored at 2°-8° C. Start amplification within 2 hours.

7.5	PROTRANS Cyclerplate System		
REF	**Article**	**Buffer**	**Buffer**
200 070	**HLA- A***	**D**	**Y**
200 080	**HLA- B***	**D**	**Y**
200 090	**HLA- C***	**D**	**Y**
200 030	**HLA- DRB1***	**R**	**Y**
200 040	**HLA- DQB1* low**	**R**	**Y**
200 020	**HLA- A*,- B*,- DRB1***	**D**	**Y**
200 010	**HLA- A*,- B*,- C***	**D**	**Y**
200 011	**HLA- A*,- B***	**D**	**Y**
201 201	**Negative Control HLA**	**D or R**	**Y**
200 050	**HLA- DRB1*- DQB1***	**R**	**Y**
200 048	**HLA- DQB1* high**	**R**	**Y**
200 049	**HLA- DQA1* high**	**R**	**Y**

7.6	Volumes of PROTRANS Buffers and Taq Polymerase for the Master Mix							
REF	**Protrans SSP Cyclerplate System**	**Buffer/µl**		**Buffer/µl**		**Taq**	**NC in Position**	**DNA 50-100 ng/µl**
200 070	**HLA- A***	**D**	70	**Y**	140	1,6 µl	-	50 µl
200 080	**HLA- B***	**D**	140	**Y**	280	3,3 µl	-	100 µl
200 090	**HLA- C***	**D**	70	**Y**	140	1,6 µl	A3, 6, 9, 12	50 µl
200 030	**HLA- DRB1***	**R**	70	**Y**	140	1,6 µl	A3, 6, 9, 12	50 µl
200 040	**HLA- DQB1* low**	**R**	46	**Y**	94	1,1 µl	C2, 4, 6, 8, 10, 12	34 µl
200 020	**HLA- A*,- B*,- DRB1***	**D**	280	**Y**	560	6,5 µl	A12	200 µl
200 010	**HLA- A*,- B*,- C***	**D**	280	**Y**	560	6,5 µl	A12	200 µl
200 011	**HLA- A*,- B***	**D**	210	**Y**	420	5,0 µl	H10	150 µl
201 201	**Negative Control HLA**	**D or R**		**Y**	Volume as indicated for each REF			
200 050	**HLA- DRB1*- DQB1***	**R**	115	**Y**	230	2,8 µl	A 3,9 C 5,11	85 µl
200 048	**HLA- DQB1* high**	**R**	140	**Y**	280	3,3 µl	B 6,12	100 µl
200 049	**HLA- DQA1* high**	**R**	70	**Y**	140	1,6 µl	A 3,6,9,12	50 µl

7.7	Number and Positions of Primer Mixes of PROTRANS Cyclerplate System			
REF	**Protrans Cyclerplate System**	**Number of Primer Mixes**	**Position of 1. Primer Mix**	**Position NC**
200 070	**HLA- A***	4x 24	H1, 4, 7, 10	-
200 080	**HLA- B***	2x 48	H1, 7	-
200 090	**HLA- C***	4x 24	H1, 4, 7, 10	A 3, 6, 9, 12
200 030	**HLA- DRB1***	4x 24	H1, 4, 7, 10	A 3, 6, 9, 12
200 040	**HLA- DQB1* low**	6x 14	H1, 3, 5, 7, 9, 11	C 2, 4, 6, 8, 10, 12
200 020	**HLA- A*,- B*,- DRB1***	96	A*: H1; B*: H4; DRB1*: H10	A12
200 010	**HLA- A*,- B*,- C***	96	A*: H1; B*: H4; Cw*: H10	A12
200 011	**HLA- A*,- B***	73	A*: H1; B*: H4	H10
200 050	**HLA- DRB1*- DQB1***	2x 38	DRB1* H1, H7; DQB1* H4, H10	A3, C5, A9, C11
200 048	**HLA- DQB1* high**	2x 47	H1, H7	B6, B12
200 049	**HLA- DQA1* high**	4x 24	H1, H4, H7, H10	A3, A6, A9, A12

7.8	PROTRANS Domino System		
REF	Article	Buffer	Buffer
201 071	HLA- DRB1* 01	R	Y
201 072	HLA- DRB1* 03	R	Y
201 070	HLA- DRB1* 04	R	Y
201 077	HLA- DRB1* 07/*09	R	Y
201 073	HLA- DRB1* 08/*12	R	Y
201 074	HLA- DRB1* 11	R	Y
201 075	HLA- DRB1* 13	R	Y
201 076	HLA- DRB1* 14	R	Y
201 078	HLA-DRB1*15	R	Y
201 079	HLA-DRB1*16	R	Y
201 080	HLA- DRB1* 15/*16	R	Y

7.9	Volumes of PROTRANS Buffers, Taq Polymerase and DNA for the Master Mix							
REF	Protrans SSP Domino System	Buffer/µl		Buffer/µl		Taq Polymerase	NC in Position	DNA 50-100 ng/µl
201 071	HLA- DRB1* 01	R	46	Y	94	1,1 µl	13	34 µl
201 072	HLA- DRB1* 03	R	70	Y	140	1,6 µl	20	50 µl
201 070	HLA- DRB1* 04	R	95	Y	190	2,2 µl	32	70 µl
201 077	HLA- DRB1* 07/*09	R	40	Y	80	1,0 µl	12	30 µl
201 073	HLA- DRB1* 08/*12	R	70	Y	140	1,6 µl	24	50 µl
201 074	HLA- DRB1* 11	R	95	Y	190	2,2 µl	30	70 µl
201 075	HLA- DRB1* 13	R	70	Y	140	1,6 µl	24	50 µl
201 076	HLA- DRB1* 14	R	75	Y	145	1,7 µl	27	55 µl
201 078	HLA-DRB1*15	R	70	Y	140	1,6 µl	24	50 µl
201 079	HLA-DRB1*16	R	40	Y	80	1,0 µl	-	30 µl
201 080	HLA- DRB1* 15/*16	R	46	Y	94	1,1 µl	16	34 µl

7.10	Number and Positions of Primer Mixes of PROTRANS Domino System			
REF	Protrans Domino System	Number Primer Mixe	Position 1.Primer Mix	Position NC
201 071	HLA- DRB1* 01	13	1	13
201 072	HLA- DRB1* 03	20	1	20
201 070	HLA- DRB1* 04	32	1	32
201 077	HLA- DRB1* 07/*09	12	1	12
201 073	HLA- DRB1* 08/*12	24	1	24
201 074	HLA- DRB1* 11	30	1	30
201 075	HLA- DRB1* 13	24	1	24
201 076	HLA- DRB1* 14	27	1	27
201 078	HLA-DRB1*15	24	1	24
201 079	HLA-DRB1*16	12	1	-
201 080	HLA- DRB1* 15/*16	16	1	16

8. Post PCR

After thermal cycling, remove the PCR-Cyclerplate or -Domino-Strips and proceed to gel electrophoresis. If not performing electrophoresis immediately, store the Cyclerplate at 4°C for up to two days; or at –20°C for longer.

8.1 Gel electrophoresis

The PCR products are identified using agarose gel electrophoresis followed by the detection of the DNA bands in UV light.

8.2 Performing gel electrophoresis

A 2% solution of agarose is prepared by boiling 4g of agarose in 200ml of 1x TAE using a magnetic stirring hotplate or microwave oven until the solution becomes clear. Allow the solution to cool to below 60°C before adding 10µl of ethidium bromide solution.

Ethidium bromide – solution (10mg/ml)
Dissolve 100mg of ethidium bromide in 10ml of distilled water. Store at 2-8°C protected from light.
Caution: Ethidium bromide is mutagenic and toxic. Always wear protective gloves when working with ethidium bomide (also in diluted form). In case of contact with the skin, wash off immediately with copious amounts of water.

Place the PROTRANS UV Gel Tray on a horizontal surface. Fill the agarose solution into the PROTRANS UV Gel Tray avoiding air bubbles, and place the PROTRANS Gel Combs (4 combs with x 4x25 slots, each containing 10µl) into the gel. The distance of the combs' teeth correspond to that of a standard 8-channel pipette which allows rapid load of the samples onto the gel.
After polymerization (about 30-60 minutes at room temperature) remove the combs and place the gel into the gel chamber that has been filled with **1x TAE buffer.** The level of electrophoresis buffer should be 2-3 mm above the gel surface and completely cover the gel.
Carefully remove the caps or Protrans Coverplates from the Cyclerplates or Domino strips and pipette all PCR products with a 8 channel pipette into the slots of the gel. Make sure that the order in which you load the samples is standardized. The PROTRANS PCR Primer Mixes are containing Loading Buffer (glycerol and cresol-red) don´t use an additional loading buffer.
Caution: Sudden movement of the Cyclerplate or Domino Strip can disperse amplified product, contaminating the laboratory and may require repetition of the test.
To measure the size of the PCR Products a molecular weight marker standard (50–1000 bp ladder) can be included in each row.
Run the **PROTRANS Electrophoresis** for about **20 min at 150 V**
the **PROTRANS Gel Check** for about **5 min at 200 V**

8.3 Result Interpretation and result documentation

Place the gel on a UV Transilluminator or on the PROTRANS UV Transilluminator
(suitable face protection against UV radiation should be worn). For interpretation and documentation of the results place the **PROTRANS Number-Plate** on the Gel and take a Polaroid picture

8.4 Result Interpretation

Each of the PROTRANS primer mixes contains a non-allelic amplification control primer pair which amplifies either a part (89 bp) of the β-globin gene (HLA class I) or a part (440 bp) of the C-reactive-protein gene (HLA class II). The concentration of these primer pairs is lower than the allele specific primer pairs and their purpose is to provide an internal control of successful PCR amplification. This amplification generally always occurs, i.e. both in presence or in absence of an allele-or group-specific PCR fragment. The control band can therefore generally be seen in all PCR reactions. In the presence of an allele specific PCR product the control band can appear weaker or is completely missing. This is not a limitation of the method, as the specific band provides a check on the success of the PCR amplification.
The composition of the primer mixes permits positive identification of the HLA characteristics. The interpretation is based on whether a specific band is present or not. The size of the amplified DNA fragment does not need to be taken into consideration when evaluating the test, nevertheless it might be helpful for the test interpretation. For evaluation the pattern of the specific bands is transferred to the LOT specific Protrans Reaction Protocol supplied and the typing result read off with the aid of the reaction pattern. For the interpretation the Primer Mix tables and the Amplification tables are very helpful. Additionally the Score Program (www.ihwg.org) can be used for detailed result interpretation.
If the testkit contains a negative control, any band in this amplification is evidence of contamination. The results of the test would be invalid and the test must be repeated.

8.5 Gel Interpretation

HLA class I	PROTRANS Positive reaction	HLA class II
Gel pocket		Gel pocket
Specific band >100-900bp		Control band 440bp
Control band 89 bp		Specific band <260bp
Primer cloud		Primer cloud

1. If weak bands of incorrect size are present, disregard them if the overall strength and clarity of the amplification is good.

2. Unused primer will form a diffuse band below 50 base pair.

3. Occasionally the formation of primer-dimers (< 80 bp) can be observed.
   The primer-dimers do not invalidate the test. A wrong interpretation of the result can be avoided by checking the approximate product size against the correct product size of the PROTRANS Primer Mix Table (see package insert).

4. Some of the primer mixes of HLA-A (mixes 5, 16, 19 and 24) and of HLA-B (mixes 5, 9, 43 and 47) will give short PCR products (< 200 bp).
   These PCR products might be difficult to distinguish from the 89 bp control PCR product.
   In general, these specific products will give a much stronger signal than that of the controls and will not have migrated as far into the gel as the control bands. If you are not sure whether the strong signal is due to a specific or a generic PCR product, you might let the gel run for an additional 15 minutes at a lower voltage. This way the specific band will be separated from the control band and you will be able to clearly see a double signal at this position: a very strong, specific, and a weaker, shorter, control band.

5. Some lanes have two or more different sizes of PCR products. These wells have multiplexed primer pairs which give rise to different amplicons depending upon the allele present. Refer to the locus specific tables in the LOT specific kit inserts

6. False negative reactions can be caused by
   - inefficient amplification
   - poor quality of DNA
   - uneven placement of the PCR plate in the block of the thermocycler
   - temperature variations across the wells of the thermal cycler itself
   - inadequate cycler calibration

7. It is possible, in rare instances, that the false negatives are due to a new or yet uncharacterized allele.
   In such cases it is recommended to repeat the test using another technique (SBT)

8. The test must be repeated if
   - absent control bands with no specific amplification are indicating failed reactions
   - there is an apparent homozygous result, or the missed reaction could change an allele assignment
   - the reaction pattern gives no clear result
   - the reaction pattern shows the prescence of 3 alleles
   - the negative control is not negative

9. If alleles can be determined in the presence of a failed PCR reaction, and that failed reaction does not change the allele assignment, the test does not need to be repeated.

10. For detailled informations refer to the HLA locus- and LOT specific kit inserts.

9. Limitation of the Procedure

1. Intensity of positive bands will vary due to the quality and amount of the PCR product.
   Quality of the PCR product will directly affect the intensity of the specific and the control bands visualized through the UV Transilluminator.
   In case of missing internal control bands and no accounted alleles the test should be repeated.

2. DNA samples may be used immediately after isolation or stored at –20°C or below for extended periods of time (over 1 year) with no adverse effects on test results.

3. Performance of the test can only be guaranteed if enclosed instructions are strictly adhered to.

4. The SSP HLA Typing tests should only be used for initial HLA typing.
   Other clinical and diagnostics findings should be used in addition,
   when determining suitability for transplant.

5. Use of the PROTRANS SSP HLA typing kits cannot resolve all combinations.

10. Quality Control

Each manufactured LOT is checked against a panel of DNA samples representative of specific detection by the primer**.** See certificates of Analysis for each LOT.
All **PROTRANS SSP kits**
are produced on the basis of the quality standards
EN ISO 9001:2008,
EN ISO 13485:2003 + AC:2007
and attachment IV of the IVDD 9879/EG.

11. Troubleshooting

Problem	Possible Cause	Solution
No PCR products.	Multiple thawing and freezing of the Mastermix (Buffer D and R).	Aliquot the Mastermix and freeze aliquots: For the PROTRANS Cyclerplate System make aliquots of 280µl and freeze them. For the PROTRANS Domino System make aliquots of 23, 46, 69 and 92µl and freeze them.
Dry PCR tubes after amplification.	Wells not sealed properly.	Repeat test and seal wells properly.
No visible bands in gel.	No Ethidium bromide in the gel.	Re-stain gel
	Inefficient Thermocycler, e.g. block defect.	Control of Thermocycler by Protrans Cycler Check, REF 200 100
		Repeat test with validated Thermocycler (see 5.2.3). The described program applies to the recom-mended thermal cyclers. Other thermal cycler as recommended have to be user-validated.
	Incorrect Thermocycler program used.	Repeat test with correct amplification program
	Wrong Taq Polymerase used.	Repeat test with validated DNA Taq Polymerase (see 5.2.2).
	DNA concentration used was too high/low.	Repeat test with correct DNA concentration.
	Degraded DNA (smears in gel lanes).	Repeat test with a new, re-extracted DNA sample.
	PCR inhibitors in the genomic DNA or impure DNA (see 4.2).	Repeat test with a new, re-extracted DNA sample out of EDTA-/Citrate-blood.
	Lack of DNA Taq Polymerase activity.	Repeat test with a known reference DNA to verify activity of Taq Polymerase.
Weak specific bands. No control bands in the gel.	Inefficient Thermocycler, e.g. block defect.	Control of thermocycler by Protrans Cycler Check, REF 200 100
		Repeat test with validated Thermocycler (see 5.2.3). The described program applies to the recom-mended thermal cyclers. Other thermal cycler as recommended have to be user-validated.
	Contact of the PCR plate to the block of the thermocycler is not correct.	The provided rack from PE (sample holder) must not be used in combination with the 96-well PCR Cyclerplates.
	PCR inhibitors (heparin, ficoll, ethanol) in the genomic DNA. Impure DNA (see 4.2).	Repeat test with re-extracted DNA, from appropriate sample (EDTA or citrate blood). Use approx. 100 ng/µl DNA. After washing the DNA pellet with wash buffer including ethanol, allow sample to dry thoroughly
	DNA concentration used was too high/low.	Repeat test with correct DNA concentration.
	Degraded DNA (smears in gel lanes).	Repeat test with a new, re-extracted DNA sample.
	Lack of DNA Taq Polymerase activity.	Repeat test with a known reference DNA to verify activity of Taq Polymerase.
Non-specific bands.	Inefficient Thermocycler, e.g. block defect.	Control of Thermocycler by Protrans Cycler Check, REF 200 100.
		Repeat test with validated Thermocycler (see 5.2.3). The described program applies to the recom-mended thermal cyclers. Other thermal cycler as recommended have to be user-validated.
	Incorrect thermocycler program used.	Repeat test with correct amplification program.
	DNA concentration used was too high.	Repeat test with correct DNA concentration.
	Wrong Taq Polymerase used.	Repeat test with validated DNA Taq Polymerase (see 5.2.2).
No visible control bands	Inefficient thermocycler, e.g. block defect.	Control of thermocycler by Protrans Cycler Check REF 200 100.
		Repeat test with validated Thermocycler (see 5.2.3). The described program applies to the recom-mended thermal cyclers. Other thermal cycler as recommended have to be user-validated.

Problem	Possible Cause	Solution
No visible control bands.	Contact of the PCR plate to the block of the thermocycler is not correct.	The provided rack from PE (sample holder) must not be used in combination with the 96-well PCR Cyclerplates.
	Incorrect Thermocycler program used.	Repeat test with correct amplification program.
No visible control bands (continued).	Wrong Taq Polymerase used.	Repeat test with validated DNA Taq Polymerase (see 5.2.2).
	In case of a homozygous typing result.	Repeat test.
	DNA not evenly re-suspended in original diluent or in Mastermix.	Vortex DNA sample and Mastermix thoroughly before PCR set-up and repeat test .
		If two alleles can be positively identified, no action need to be taken. In all other cases it is recommended to repeat the test.
Specific bands occasionally missing.	Inefficient Thermocycler, e.g. block defect.	Control of Thermocycler by Protrans Cycler Check REF 200 100.
		Repeat test with validated Thermocycler (see 5.2.3). The described program applies to the recom-mended thermal cyclers. Other thermal cycler as recommended have to be user-validated.
	Contact of the PCR plate to the block of the Thermocycler is not correct.	The provided rack from PE (sample holder) must not be used in combination with the 96-well PCR Cyclerplates.
	DNA not evenly re-suspended in original diluent or in Mastermix.	Vortex DNA sample and Mastermix thoroughly before PCR set-up and repeat test .
		If two alleles can be positively identified, no action need to be taken. In all other cases it is recommended to repeat the test.
	Wrong loading of the agarose gel.	Control of gel and gel lanes concerning the loaded primer mixes.
False positive bands.	Inefficient Thermocycler, e.g. block defect.	Control of Thermocycler by Protrans Cycler Check REF 200 100.
		Repeat test with validated Thermocycler (see 5.2.3). The described program applies to the recom-mended thermal cyclers. Other thermal cycler as recommended have to be user-validated.
	Excess of DNA.	Repeat test with adequate amount of DNA Concentration ~ 100ng/µl.
	Excess of DNA Taq Polymerase.	Repeat test with adequate amount of Taq. Validate each LOT of DNA Taq Polymerase before using it routinely.
	Extensive delay between PCR set-up and start of thermal cycling.	Start amplification directly after PCR set-up or store pipetted Cyclerplate at 2°-8°C until start of amplification (max. for 2 hours).
	Mis-interpretation of primer-dimer as specific amplification.	Check correct band size.
	DNA contaminated with other DNA or PCR product.	Separate strictly pre-PCR area from post-PCR area. Perform wipe test.
Overall fuzzy bands, smeared lanes.	Gel is too thin due to excess evaporation while heating.	Compensate for lost volume by adding water.
	Agarose not completely dissolved.	Boil for an additional 30 seconds after melting.
	Overheating gel, too high voltage .	Lower voltage.
	Heavy streaking in random wells can be caused by uneven suspensions of DNA .	Using an 8 channel pipettor, mix the PCR product up and down twice before loading.
	Rapid release of amplified product during gel loading can cause product to float out of well.	Use slow, steady pipetting when loading gel.
Occasional faint lanes.	Product floated out of gel slot.	Pipette tip needs to be properly aligned with the slots of the agarose gel.
Gel picture too dark.	Wrong amount of Ethidium bromide used.	Use 5µl Ethidium bromide (10mg/ml) for each 100ml gel solution.
	Gel tray not UV-transparent.	Remove gel from tray before viewing. Use PROTRANS Electrophoresis Equipment (REF 210 000).
	Incorrect camera setting.	Increase exposure time or aperture setting.

Problem	Possible Cause	Solution
Gel picture too bright.	Excessive amount of Ethidium bromide used.	Use 5µl ethidium bromide (10mg/ml) for each 100ml gel solution.
	Incorrect camera setting	Increase exposure time or aperture setting.

11. Literatur / References / Références Bibliographiques/ Bibliografia

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