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Biotest
From Nature for Life

ELPHA

(**E**nzyme **L**inked **P**robe **H**ybridization **A**ssay)

IVD

For In Vitro Diagnostic Use

ELPHA HLA-AB SL LowRes 48	REF 826 603
ELPHA HLA-AB DL LowRes 48	REF 826 613
ELPHA HLA-C SL LowRes 16	REF 826 621
ELPHA DRB SL LowRes 16	REF 826 641
ELPHA DRB1 DL All 48	REF 826 673
ELPHA DQB SL LowRes 16	REF 826 681
ELPHA DQB DL Extend 16	REF 826 691
ELPHA Primer HLA-AB LowRes	REF 826 604
ELPHA Primer HLA-C LowRes	REF 826 624
ELPHA Primer DRB SL LowRes	REF 826 644
ELPHA Primer DRB HiRes	REF 826 674
ELPHA Primer DRB1 DL HiRes	REF 826 675
ELPHA Primer DRB1 DL All	REF 826 677
ELPHA Primer DQB SL LowRes	REF 826 684
ELPHA Primer DQB DL Extend	REF 826 694
ELPHA Reagent 48	REF 826 503
ELPHA Reagent Supp 48	REF 826 513

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REF	GB	Article number	IVD	GB	For in vitro diagnostic use
	DE	Artikelnummer		DE	Nur zur in-vitro Diagnostik
	ES	Artículo número		ES	Sólo para el diagnóstico in vitro
	IT	Codice prodotto		IT	Solo per la diagnostica in vitro
	FR	Référence du produit		FR	Pour le diagnostic in vitro
	NL	Artikelnummer		NL	Voor in vitro gebruik
	DK	Artikelnummer		DK	Til diagnostik brug i glas
	CZ	Katalogové čislo		CZ	Použití pro in vitro diagnostiku

MTP	GB	Microtest plate	PCR BUF ABC	GB	PCR buffer for HLA-ABC typing
	DE	Mikrotestplatte		DE	PCR Puffer zur HLA- ABC-Typisierung
	ES	Placa microtest		ES	PCR buffer para el tipaje de HLA-ABC
	IT	Micropiastra		IT	Tampone PCR per tipizzazione HLA-ABC
	FR	Test en microplaque		FR	Tampon de PCR pour le typage HLA-ABC
	NL	Microtiterplaat		NL	PCR buffer voor HLA-ABC typering
	DK	Mikrotestplade		DK	PCR buffer til HLA-ABC typning
	CZ	Mikrotestovací destička		CZ	PCR pufr pro HLA-ABC typizaci
PRIMER	GB	Primer mix	PCR BUF DR	GB	PCR buffer for HLA-DRB/DQB typing
	DE	Primermix		DE	PCR Puffer zur HLA-DRB/DQB-Typisierung
	ES	Mezcla cebadores		ES	PCR buffer para el tipaje de HLA-DRB/DQB
	IT	Miscela di primer		IT	Tampone PCR per tipizzazione HLA-DRB/DQB
	FR	Melange d'amorce		FR	Tampon de PCR pour le typage HLA-DRB/DQB
	NL	Primer mix		NL	PCR buffer voor HLA-DRB/DQB typering
	DK	Begyndelsesblanding		DK	PCR buffer til HLA-DRB/DQB typning
	CZ	Směs primerů		CZ	PCR pufr pro HLA-DRB/DQB typizaci
BUF A10x	GB	Buffer A 10x concentrated	NEUTRAL	GB	Solution for neutralization
	DE	Puffer A 10x konzentriert		DE	Neutralisierungslösung
	ES	Buffer A concentrado 10x		ES	Solución para la naturalizacion
	IT	Tampone A concentrato 10x		IT	Soluzione di neutralizzazione
	FR	Tampon A concentrèe 10x		FR	Solution de neutralisation
	NL	Buffer A 10x geconcentreerd		NL	Neutralisatie-oplossing
	DK	Buffer A 10x koncentreret		DK	Opløsning til neutralisering
	CZ	Pufr A 10x koncentrovaný		CZ	Roztok pro neutralizaci
SSC 20x	GB	20xSSC buffer	DENAT	GB	Solution for denaturation
	DE	20xSSC-Puffer		DE	Denaturierungslösung
	ES	Tampón 20xSSC		ES	Solución para la desnaturalizacion
	IT	Tampone SSC 20x		IT	Soluzione di denaturazione
	FR	Tampon SSC 20x		FR	Solution de dénaturation
	NL	Buffer SSC 20x		NL	Denaturatie-oplossing
	DK	Buffer SSC 20x		DK	Opløsning til denaturering
	CZ	Pufr pro SSC 20x		CZ	Roztok pro denaturaci
SDS 1%	GB	1% SDS solution	BUF A	GB	Buffer A, working solution
	DE	1%ige SDS-Lösung		DE	Puffer A, Gebrauchslösung
	ES	Solución 1% SDS		ES	Buffer A, solución de trabajo
	IT	Soluzione di SDS 1%		IT	Tampone A, soluzione di lavoro
	FR	Solution de SDS à 1%		FR	Tampon A, soluzion de travail
	NL	1% SDS oplossing		NL	Buffer A, werkoplossing
	DK	1% SDS opløsning		DK	Buffer A, arbejdsopløsning
	CZ	1% SDS roztok		CZ	Pufr A, pracovní roztok
CONJ FITC	GB	Conjugate (FITC)	CONJDIL FITC	GB	Diluted conjugate solution (FITC)
	DE	Konjugat (FITC)		DE	Verdünnte Konjugatlösung (FITC)
	ES	Conjugado (FITC)		ES	Solución diluída del conjugado (FITC)
	IT	Conjugato (FITC)		IT	Soluzione diluita di conjugato (FITC)
	FR	Conjugué (FITC)		FR	Solution diluée du conjugué (FITC)
	NL	Conjugaat (FITC)		NL	Verdunde conjugaat-olpossing (FITC)
	DK	Konjugat (FITC)		DK	Fortyndet konjugat opløsning (FITC)
	CZ	Konjugát (FITC)		CZ	Zředěný roztok konjugátu (FITC)
CONJ D	GB	Conjugate (D)	CONJDIL D	GB	Diluted conjugate solution (D)
	DE	Konjugat (D)		DE	Verdünnte Konjugatlösung (D)
	ES	Conjugado (D)		ES	Solución diluída del conjugado (D)
	IT	Conjugato (D)		IT	Soluzione diluita di conjugato (D)
	FR	Conjugué (D)		FR	Solution diluée du conjugué (D)
	NL	Conjugaat (D)		NL	Verdunde conjugaat-olpossing (D)
	DK	Konjugat (D)		DK	Fortyndet konjugat opløsning (D)
	CZ	Konjugát (D)		CZ	Zředěný roztok konjugátu (D)

BLOC REA	GB DE ES IT FR NL DK CZ	Blocking reagent Blockierungsreagenz Reactivo blocqueante Reagente bloccante Réactif d'arrêt Blocking reagens Blokerende reagens Blokující reagencie	BUF B	GB DE ES IT FR NL DK CZ	Conjugate dilution buffer Konjugatverdünnungspuffer Buffer de dilción del conjugado Tampone per la diluizione del conjugato Tampon de dilution du conjugué Conjugaat verdunningsbuffer Konjugat fortyndings buffer Pufr pro ředěni konjugátu
SUBS TMB	GB DE ES IT FR NL DK CZ	Substrate solution TMB Substratlösung TMB Solución substrato Soluzione substrato (TMB) Solution de substrat (TMB) TMB substraatoplossing Substrat opløsning TMB Roztok substrátu TMB	INHIB	GB DE ES IT FR NL DK CZ	POD inhibitor POD-Inhibitor POD inhibidor POD inhibitore POD inhibiteur POD inhibitor POD hæmmer POD inhibitor
			BUF SSC/SDS	GB DE ES IT FR NL DK CZ	Buffer 2xSSC/ 0.1% SDS Puffer 2xSSC/ 0.1% SDS Tampón 2xSSC/ 0,1%SDS Tampone di SSC 2x/ 0,1% SDS Tampon de SSC 2x/ 0,1% SDS Buffer 2xSSC/0,1% SDS Buffer 2x SSC/0,1% SDS Pufr pro SSC 2x/0,1% SDS

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1. Introduction

1.1 Intended Use

The Biotest ELPHA SL (**E**nzyme **L**inked **P**robe **H**ybridization **A**ssay **S**ingle **L**abelled) and the ELPHA-DL assays (**E**nzyme **L**inked **P**robe **H**ybridization **A**ssay **D**ouble **L**abelled), respectively, are DNA-hybridization tests for the determination of HLA class I or class II alleles.

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", thus allowing the triggering of a targeted immune response to foreign antigens. In organ transplantation, HLA compatibility between the donor and the 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.

In the past determination of HLA antigens by serology, although subject to certain restrictions, has brought about great improvement and contributed to our understanding in the areas mentioned.

New dimensions have been opened up to modern diagnostics by the development of DNA-based test methods. HLA antigens may differ from each other by single amino acids within the polypeptide chain. Recognition of these largely identical structures by serological means has proved almost impossible; for this reason, the resolution of this method is limited. As the DNA sequences of nearly all important HLA alleles are now known, variations in sequences can be identified at the DNA level with the help of synthetic oligonucleotides. Utilizing amplification of genomic DNA (PCR, **P**olymerase **C**hain **R**eaction) together with suitable probes (SSO, **S**equence **S**pecific **O**ligonucleotides) it is possible to identify a large number of HLA alleles.

1.3 Principle of the Test

Sequence-specific oligonucleotide (SSO-) probes are used as diagnostic tools to identify polymorphic sequence motifs. The hybridization between probe and target DNA is detected by a method adapted from the protein ELISA technique. The microtest plate format allows the use of standardized apparatus for automation of the test procedure and data evaluation. The test, however, also can be easily performed manually.

Starting from genomic DNA which can be readily obtained from peripheral blood, the polymorphic region in the 2nd exon of the class II genes and the 2nd Exon and the 3rd Exon of the class I genes are amplified by means of the **P**olymerase **C**hain **R**eaction (PCR) using Biotin-labelled primers. After amplification and denaturation of the PCR products the resulting biotinylated single strand molecules are bound to Streptavidin-coated wells of a microtest plate. The labelled SSO-probes are in a dried form which redissolves when the diluted solution of the PCR products is transferred to the wells. Fixation of the PCR products to the wells through the Biotin-Streptavidin bond and hybridization with the SSO probes thus occur in one step. The specificity of the assay is dependent on the subsequent stringent washing step in which probes of insufficient sequence homology to the PCR products are removed. Hybridised probes are visualized by means of a colour reaction involving antigen-specific antibodies coupled to Horseradish peroxidase (POD). The results are quantified photometrically in an ELISA reader. The combination of HLA alleles in the test material is determined from the pattern of positive reactions by means of the reaction pattern schemes or by means of the Biotest HLA Typing Software (REF 847 070) and the lot update available on the Biotest homepage.
In the case of the Biotest ELPHA DRB HiRes and Biotest ELPHA DQB-Typing two separate amplification reactions are performed to increase resolution.
Using Biotest ELPHA DQB-Typing all alleles serologically belonging to the DQ1 group will be separated from the alleles of group DQ2-4 with the help of the PCR reaction.
In the Biotest ELPHA DRB HiRes test the polymorphism at amino acid 86 in the second DRB exon is used to divide the alleles into two amplification groups of roughly equal size. The products of the two amplifications are separated and analyzed with two equal sets of oligonucleotide probes.
In ELPHA-DL every well contains two different labelled probes (digoxigenin and fluorescein) both binding their specific sequence motifs. These can be detected with antibody conjugates of the respective specificity (DIG or FITC) in two subsequent POD reactions. To determine the results of the second run independently of the first one an inhibitor inactivates the first bound POD conjugate after result reading and reaction products are then washed off before the second conjugated antibody is added. In ELPHA-

DL the complete pattern of positive reactions results from the combination of both colour reactions. The combination of HLA alleles in the test material is determined from the pattern of positive reactions by means of the reaction pattern schemes or by use of the Biotest HLA Typing software (REF 847 070) and the lot update you can download from the Biotest homepage.

2. Materials

2.1 ELPHA Sets of microtestplates

Following test sets are necessary to perform HLA typings:

ELPHA Set of microtestplates	REF	Reagent set	REF	Set	Number of typings
1 ELPHA HLA-AB SL LowRes 48	826 603	1 ELPHA Reagent 48	826 503	826 603 900	48 HLA-AB low resolution
1 ELPHA HLA-AB DL LowRes 48	826 613	1 ELPHA Reagent 48 + 1 ELPHA Reagent Supp 48	826 503 826 513	826 613 900	96 HLA-AB low resolution
1 ELPHA HLA-C SL LowRes 16	826 621	1/3 ELPHA Reagent 48	826 503	--	48 HLA-C low resolution
1 ELPHA DRB SL LowRes 16	826 641	1/3 ELPHA Reagent 48	826 503	--	64 HLA-DRB low resolution
1 ELPHA DRB1 DL All 48	826 673	1 ELPHA Reagent 48 + 1 ELPHA Reagent Supp 48	826 503 826 513	826 673 900	192 HLA-DRB1 medium resolution
1 ELPHA DQB SL LowRes 16	826 681	1/3 ELPHA Reagent 48	826 503	--	64 HLA-DQB
1 ELPHA DQB DL Extend 16	826 691	1/3 ELPHA Reagent 48	826 503	--	96 HLA-DQB medium resolution

2.2 Contents of the Biotest ELPHA Microtest Plate Sets

In the sets the ELPHA microtestplate set and the reagent sets needed are included.

For manually processing the Biotest ELPHA microtest plates, the cover foils (Cover MTP 96, REF 826 550) are needed additionally.

- Microtest plates MTP, each with 12 strips of 8 wells, marked with black numbers. The wells are individually coated with dried SSO probes.
- PCR buffer for HLA-ABC typing PCR BUF ABC contains 500 mM Tris/HCl, 150 mM $(NH_4)_2SO_4$, 15 mM $MgCl_2$.
- PCR buffer for HLA-DRB/DQB typing PCR BUF DR contains 100 mM Tris/HCl, 500 mM KCl, 15 mM $MgCl_2$.
- Quick reference guide (ELPHA Microtestplate sets and ELPHA reagent sets)
- The lot information containing lot-specific software updates, reaction pattern chart and an information sheet to the positioning of probes as well as the currently valid package insert, is available from the internet (http://www.biotest.com).

	Contents of packages				
Test	MTP	PCR BUF ABC	PCR BUF DR	PRIMER	PRIMER
HLA-AB SL LowRes 48	48	3x1,0 ml		HLA-A (red) 3x180 µl	HLA-B (blue) 3x180 µl
HLA-AB DL LowRes 48	48	3x1,0 ml		HLA-A (red) 3x240 µl	HLA-B (blue) 3x240 µl
HLA-C SL LowRes 16	16	1x1,0 ml		HLA-C (green) 1x300 µl	
DRB SL LowRes 16	16		1x1,0 ml	C (green) 1x640 µl	
DRB1 DL All 48	48		3x1,0 ml	DRB1 All (orange) 3x640 µl	
DQB SL LowRes 16	16		1x1,0 ml	A (blue) 1x160 µl	B (red) 1x160 µl
DQB DL Extend 16	16		1x1,0 ml	DQB DL (red) 1x480 µl	

2.3 ELPHA Primersets

ELPHA Primerset	REF	ELPHA Set of microtestplates	REF	Number of Typings
1 ELPHA Primer HLA-AB LowRes	826 604	1 ELPHA HLA-AB SL LowRes 48 or 1 ELPHA HLA-AB DL LowRes 48	826 603 826 613	48 HLA-AB low resolution
1 ELPHA Primer HLA-C LowRes	826 624	1 ELPHA HLA-C SL LowRes 16	826 621	48 HLA-C low resolution
1 ELPHA Primer DRB SL Low Res	826 644	1 ELPHA DRB SL LowRes 16	826 641	64 DRB low resolution
1 ELPHA Primer DRB HiRes	826 674	1 ELPHA DRB1 DL All 48	826 673	96 DRB high resolution
ELPHA Primer DRB1 DL HiRes	826 675	1 ELPHA DRB1 DL All 48	826 673	96 DRB1 high resolution
1 ELPHA Primer DRB1 DL All	826 677	ELPHA DRB1 DL All 48	826 673	192 DRB1 medium resolution
1 ELPHA Primer DQB SL LowRes	826 684	ELPHA DQB SL LowRes 16	826 681	64 DQB low resolution
1 ELPHA Primer DQB DL Extend	826 694	ELPHA DQB DL Extend 16	826 691 826 693	96 HLA-DQB medium resolution

The reagent sets mentioned in chapter 2.2 are needed additionally.

2.4 Contents of the ELPHA Primersets

	Contents of package			
Primerset	PCR BUF ABC	PCR BUF DR	PRIMER	PRIMER
ELPHA Primer HLA-AB LowRes	1x1,0 ml		HLA-A (red) 1x180 µl	HLA-B (blue) 1x180 µl
ELPHA Primer HLA-C LowRes	1x1,0ml		HLA-C (green) 1x300 µl	
ELPHA Primer DRB SL LowRes		1x1,0 ml	C (green) 1x640 µl	
ELPHA Primer DRB HiRes		1x1,0 ml	G (blue) 1x320 µl	T (red) 1x320 µl
ELPHA Primer DRB1 DL HiRes		1x1,0 ml	DRB1 G (yellow) 1x320 µl	DRB1 T (black) 1x320 µl
ELPHA Primer DRB1 DL All		1x1,0 ml	DRB1 All (orange) 1x640 µl	
ELPHA Primer DQB SL LowRes		1x1,0 ml	A (blue) 1x160 µl	B (red) 1x160 µl
ELPHA Primer DQB DL Extend		1x1,0 ml	DQB DL (red) 1x480 µl	

- Primer mixes for generic amplification:
  PRIMER HLA-A contains two HLA-A generic primer pairs with which the complete exon 2 and the complete exon 3 of the HLA-A gene are amplified.
  PRIMER HLA-B contains two HLA-B generic primer pairs with which the complete exon 2 and the complete exon 3 of the HLA-B gene are amplified.
  PRIMER HLA-C contains two HLA-C generic primer pairs with which the complete exon 2 and the complete exon 3 of the HLA-C gene are amplified.
  PRIMER C contains one HLA-DRB generic primer pair with which the complete exon 2 is amplified and also a DR2 specific primer pair with which a 339 bp fragment of the first DRB intron is amplified. Within that intron DR15 and DR16 specific sequences are located that will give in case of DR2 positive specimen with the specific probes E3 and D3 a positive signal and thus allow the typing of DR15 and DR16.
  PRIMER A contains a specific primer pair with which the complete exon 2 of the HLA-DQB gene are amplified for the detection of DQB1*05 and *06 alleles.
  PRIMER B contains a specific primer pair with which the complete exon 2 of the HLA-DQB gene are amplified for the detection of DQB1*02, *03 and *04 alleles.
  PRIMER DQB DL contains a specific primer pair which amplifies all alleles of the HLA-DQB1 gene.
  PRIMER G contains a specific primer pair which amplifies alleles coding for glycine in position 86 of the exon 2 of the HLA-DRB gene.
  PRIMER T contains a specific primer pair which amplifies alleles coding for valine in position 86 of the exon 2 of the HLA-DRB gene.
  The primer mixes PRIMER A, B, G and T contain not only the specific primers but also a control primer pair with which a 422 bp fragment of human growth hormone (hGH) is amplified. This amplicon serves as a control of the PCR and should be detectable in the samples in which no specific PCR product is present.
  PRIMER DRB1G contains a specific primer pair which amplifies alleles coding for glycine in position 86 of exon 2 of the HLA-DRB1 gene.
  PRIMER DRB1T contains a specific primer pair which amplifies alleles coding for valine in position 86 of the exon 2 of the HLA-DRB1 gene.
  PRIMER DRB1All contains a specific primer pair which amplifies all alleles of the HLA-DRB1 gene.
  The primer mixes PRIMER A, PRIMER B, PRIMER G, PRIMER T, PRIMER DRB1G and PRIMER DRB1T contain not only the specific primers but also a control primer pair with which a 422 bp fragment of human growth hormone (HGH) is amplified. This amplicon serves as a control of the PCR and should be detectable in the samples in which no specific PCR product is present.

2.5 Contents of the Biotest ELPHA Reagent 48

1.	DENAT, ready to use Solution for Denaturation 0.1 M NaOH	120 ml
2.	NEUTRAL, ready to use Solution for Neutralization 0.1 M HCl	120 ml
3.	SSC 20x 20xSSC Buffer 10fold concentrated stock solution (3 M NaCl; 0.3 M Sodium Citrate)	2 x 500 ml
4.	SDS 1% 1% SDS Solution 10fold concentrated stock solution (1% Sodium Dodecyl Sulfate)	2 x 500 ml
5.	BUF A10x buffer A 10fold concentrated stock solution (1 M Tris/HCl pH 7.5; 1.5 M NaCl; 0.5% Tween 20)	3 x 500 ml
6.	BLOC REA blocking reagent dry reagent	7 x 500 mg
7.	CONJ FITC conjugate Affinity purified sheep IgG, specific for Fluorescein, conjugated to Peroxidase	400 µl
8.	SUBS TMB, ready to use Substrate solution TMB 3,3´,5,5´Tetramethylbenzidine	27 x 13 ml

Caution: TMB is toxic. Avoid inhaling the vapour and avoid contact of the solution with the skin and eyes. Store in the dark! Waste management by burning.

2.6 Contents of the Biotest ELPHA Reagent DL Supp 48

1.	BUF A10x buffer A 10fold concentrated stock solution (1 M Tris/HCl pH 7.5; 1.5 M NaCl; 0.5% Tween 20)	3 x 500 ml
2.	BLOC REA blocking reagent dry reagent	7 x 500 mg
3.	SUBS TMB, ready to use Substrate solution TMB 3,3´,5,5´Tetramethylbenzidine	27 x 13 ml
4.	CONJ D conjugate Affinity purified sheep IgG, specific for Digoxygenin, conjugated to Peroxidase	400 µl
5.	INHIB, ready to use 2 M ammonium thiocyanate	3 x 125 ml

Caution: TMB is toxic. Avoid inhaling the vapour and avoid contact of the solution with the skin and eyes. Store in the dark! Waste management by burning.

Caution: Ammonium thiocyanate is unhealthy. Avoid inhaling the vapour and avoid contact of the solution with the skin and eyes. Contact with acids liberates very toxic gases!

Safety data sheets are available for all reagents.

2.7 Storage and shelf life

All reagents and kit components should be stored at 2...8ºC. The shelf life is printed in the outer label. After first opening the pouch, the microtest plates should be used within 6 weeks.

2.8 Warning and Precautions

For In Vitro Diagnostic Use IVD

Caution: The test must be performed by fully-trained and authorised laboratory technicians.

Caution: All reagents should be handled in accordance with good laboratory practice using appropriate precautions. In addition, handle all patient samples with appropriate precautions. Do not pipette by mouth.

Caution: Do not use reagents past the expiry date printed on the label.

Caution: Do not use reagents with any evidence microbial contamination.

Caution: Pipettes used for **Post**-PCR manipulations should **not** be used for **Pre**-PCR manipulations.

Caution: Biohazard Warning: **Ethidium bromide is mutagenic, poisonous and a potential carcinogen. Wear protective gloves when working with ethidium bromide (even in diluted form). If it touches the skin, wash off immediately with copious water. Waste management by burning.**

Caution: Biohazard Warning: All blood products should be treated as potentially infectious.

Caution: Wear UV-blocking eye protection, and do not view UV light source directly when viewing or photographing gels.

Caution: All used microtest plates MTP should be treated as potentially infectious and should be destroyed according to the valid national guidelines.

Caution: Do not discard the desiccant contained in ELISA plate pouches. Once opened, the plates should be kept in their original pouch along with desiccant, resealed with tape to prevent moisture accumulation during storage. Do not use plates with evidence of excessive moisture accumulation.

2.9 Additional materials required

2.9.1 PCR

- Taq DNA polymerase (5 U/µl, e.g. Perkin Elmer)
- Nucleotide solution (10 mM): dATP, dGTP, dCTP, dTTP
- 0.2 ml PCR tubes, thin-walled (e.g. Perkin-Elmer)
- Thermocycler with heatable cover (e.g. Perkin-Elmer)
- Adjustable pipettes
- Filtered pipette tips

2.9.2 Gel electrophoresis

- Agarose (quality for molecular biology)
- Gel chamber (Minigel, e.g. 8 x 10 cm)
- 1 x TBE buffer
- Ethidium bromide solution (1 mg/ml)
  Caution: Ethidium bromide is mutagenic, poisonous and a potential carcinogen. Wear protective gloves when working with ethidium bromide (even in diluted form). If it touches the skin, wash off immediately with copious water. Waste management by burning.
- DNA length standard (e.g. 50-1000 bp marker) (not absolutely necessary)
- Magnetic stirrer with hot-plate or microwave
- Adjustable pipettes
- Polaroid camera (not absolutely necessary)
- Gel loading buffer
- UV transilluminator (~ 200-300 nm)
- Power supply

2.9.3 ELPHA SL

- Waterbath (46ºC)
- 1N sulphuric acid

  Caution: Sulphuric acid is a strong corrosive. Avoid contact with eyes, clothing or skin.

  Wear protective gloves.
- Elisa washer (e.g. Biotest Elisa Washer III, REF 840 370); alternatively, dispenser top with washing comb
- Eppendorf repeater pipette with 2.5 ml adapters
- Adjustable pipettes
- Multichannel pipette
- 2 ml reaction tubes for HLA-C, DRB LowRes, DRB1 Basic and DQB-Typing
- 5-10 ml reaction tubes for HLA-AB and DRB HiRes

2.9.4 ELPHA DL

- Waterbath (46ºC)
- Elisa washer (e.g. Biotest Elisa Washer III, REF 840 370); alternatively, dispenser top with washing comb
- Eppendorf repeater pipette with 2.5 ml adapters
- Adjustable pipettes
- Multichannel pipette
- 2 ml reaction tubes

2.9.5 Evaluation

- Elisa reader (ELPHA SL: 450 nm and 620 nm; ELPHA DL: 370 nm and 492 nm)
- Printer
- Biotest HLA Typing Software (REF 847 070)
- Operating system Windows 2000
  (The software can be used with other Windows operating systems but has not been tested)
  Min. available local disc storage: 100 MB
  Min. working storage: 128 MB

3. Specimen Collection and Preparation

3.1 DNA Isolation

Genomic DNA can be isolated from all nucleated cells. The simplest approach is the isolation from cell suspensions like coagulation inhibited peripheral blood, buffy coats or cultured cells. A vast range of protocols exist for the isolation of DNA from cells. For PCR-SSO testing, only those methods which provide DNA of adequate quality and quantity for PCR (OD_{260}/OD_{280} ratio is 1.7-1.8) should be considered, e.g. salting out method.

3.2 Specimen Collection and Preparation

Typing is performed using anticoagulated peripheral blood (e.g. sodium citrate, EDTA).

Caution: Blood samples may transmit infectious diseases, and should be handled with appropriate precautions. Blood specimens should never be pipetted by mouth. Gloves should be worn whenever handling blood samples. The generation of aerosols should be avoided. Working steps like mixing of blood or of cells derived from blood should be performed in a biosafety hood to contain aerosols. Work surfaces should be decontaminated daily and any contaminated items should be sterilized before disposal.

Contamination of the DNA by PCR inhibitors, such as haemoglobin, heparin, ethanol, etc. can result in serious interference with the PCR reaction. For this reason, heparinised blood should not be used as a starting material for DNA isolation. Use either citrate or EDTA blood. If the patient is on heparin therapy, use an alternative source of DNA.

3.3 DNA Quantity and Quality

The DNA sample to be used should be resuspended in sterile distilled water at a concentration of approx.
50-200 ng/µl. DNA **should not be re-suspended** in solutions containing chelating agents, **such as EDTA,** above 0.5 mM in concentration.
Determination of the concentration of DNA is performed by measuring the optical density (O.D.) at 260 nm (A_{260}). The value A_{260} = 1 (= O.D. 1.0) corresponds with approx. 50 ng/µl of double-strand DNA.
To determine the degree of contamination of the DNA with protein, an additional measurement is made at 280 nm and the quotient A_{260}/A_{280} calculated. Pure DNA will give an A_{260}/A_{280} ratio of 1.8 or higher. Values for A_{260}/A_{280} of less than 1.8 indicate contamination of the DNA by protein.
For good PCR-SSO results, DNA is required with an A_{260}/A_{280} quotient of 1.7 or higher.

➲ **The purity and concentration of the DNA is of decisive importance for optimal test results.**

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 results.

4. Procedure

4.1 PCR (Polymerase Chain Reaction)

4.1.1 Precautions

Because of the high efficiency of the method it is necessary to take precautions to avoid contamination. At the completion of 30 cycles, a 10^8 portion of the reaction volume (0.5 picoliter) contains the same number of copies as 50 µl of the starting mix. The slightest carry-over of PCR products into samples to be amplified can make an exact analysis impossible. In general, contamination will be detected by identifying more than 2 alleles in the ELPHA test.
If homozygous genomes are tested, there is a risk of undetected or unidentified alleles without a supporting family study.
To minimize the risk of contamination, it is strongly recommended to follow the minimal safety precautions listed below:

1. All working steps involving pre-PCR (preparation and storage of reagents and solutions, isolation and storage of DNA, set up of PCR) should be done in rooms separated from those where post-PCR work (analysis, storage and disposal of PCR products) is done.
2. Equipment and supplies from the post-PCR area should not be transferred to the pre-PCR area. The thermal cycler should be installed in the post-PCR area.
3. When setting up the PCR mixes, a separate set of micropipettes and aerosol free pipette tips should be used.

To detect contamination early, it is advisable to check all PCR reagents regularly by performing gel electrophoresis on control reaction mixtures without genomic DNA. Contaminated reagents should be disposed of immediately.

4.1.2 Preparation of nucleotide stock solution (dNTPs)

The 10 mM nucleotide solutions are diluted to give a stock solution ready for use:
400 µl of distilled water are mixed with 100 µl of dATP (10 mM), 100 µl of dGTP (10 mM), 100 µl of dCTP (10 mM) and 100 µl of dTTP (10 mM).
In the case of 100 mM nucleotide solutions, the volumes are adjusted accordingly: 10 µl of dATP, 10 µl of dGTP, 10 µl of dCTP and 10 µl of dTTP are mixed with 760 µl of distilled water.
The nucleotide stock solution (1.25 mM) is stored at least at -20ºC.

4.1.3 Preparation of the PCR reaction mixture

One (HLA-C, DRB LowRes) or two (HLA-AB, DRB HiRes, DQB typing) PCR reactions are performed for each DNA sample. The pipetting scheme of different master mixes is described in table 1 and table 2, respectively.

Table 1: Single label tests (SL)

Reagent	HLA-AB SL LowRes		HLA-C SL LowRes	DRB SL LowRes	DQB SL LowRes	
dH_2O	93,2 µl	93,2 µl	64,5 µl	60,5 µl	32,5 µl	32,5 µl
PCR buffer for HLA-ABC typing PCR BUF ABC	15 µl	15 µl	10 µl	-	-	-
PCR buffer for HLA-DR/DQ typing PCR BUF DR	-	-	-	10 µl	5 µl	5 µl
dNTPs (1.25 mM)	24 µl	24 µl	16 µl	16 µl	8 µl	8 µl
Primer mix PRIMER	HLA-A 9 µl	HLA-B 9 µl	HLA-C 5 µl	C 8 µl	A 2 µl	B 2µl
Taq DNA Polymerase	0.8 µl	0.8 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
sample DNA	8 µl	8 µl	4 µl	5 µl	2 µl	2 µl
total volume	150 µl	150 µl	100 µl	100 µl	50 µl	50 µl

Table 2: Double label tests (DL)

Reagent	HLA-AB DL LowRes		DRB DL HiRes		DRB1 DL All	DRB1 DL HiRes		DQB DL Extend
dH_2O	62,5 µl	62,5 µl	60,5 µl	60,5 µl	60,5 µl	60,5 µl	60,5 µl	67,5 µl
PCR buffer for HLA-ABC typing PCR BUF ABC	10 µl	10 µl	-	-	-			
PCR buffer for HLA-DR/DQ typing PCR BUF DR	-	-	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
dNTPs (1.25 mM)	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl	16 µl
Primer mix PRIMER	HLA-A 6 µl	HLA-B 6 µl	G 8 µl	T 8 µl	DRB1 All 8 µl	DRB1 G 8 µl	DRB1 T 8 µl	DQB DL 4 µl
Taq DNA Polymerase	0,5 µl	0,5 µl	0,5 µl	0,5 µl	0,5 µl	0,5 µl	0,5 µl	0,5 µl
sample DNA	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl	2 µl
total volume	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl

➲ For performing the ELPHA-DL DRB1 HiRes test it is recommended also to prepare the PCR for the DRB1 All test. In those cases where only one of the DRB1 HiRes primer mixes is positive, using the DRB1 All amplification (3 strips, see DRB1 All protocol in table 2) will achieve the same resolution as using the DRB1 HiRes amplification
However, if both DRB1 HiRes amplification are positive, a DRB1 HiRes typing using 6 strips has to be performed.
This procedure also ensures that samples with one failed DRB1 HiRes PCR reaction will not be mistyped as homozygous.

If DNA solutions of lower concentration are used, the amount of DNA and dH_2O can be adjusted.
If several typings are being performed simultaneously, it is advisable to prepare a corresponding multiple of the master mix in order to ensure the same conditions for all replicates. The mixture should always be prepared fresh for use.
The PCR program shown below is adapted to the thermocyclers of Perkin-Elmer. The heat/cooling rate of the PE 9700 is on average of 1°C/seconds for the sample and the temperature accuracy is ± 0.25°C over the range 35-100°C. Thermocyclers other as the recommended have to be user-validated.
Some thermocyclers are limited to a PCR volume of 100 µl. Larger PCR volumes should be divided to achieve an optimal amplification.

Program the thermocycler as follows:

	Temp.	Time	Cycles
⌈	95° C	5 min.	1 x
|			
⌊→	95° C	15 sec.	⌉
	↓		|
	55° C	15 sec.	| 30 x
	↓		|
⌈	72° C	15 sec.	⌋
|			
⌊→	72° C	6 min.	1 x
	4° C	∞	1 x

4.2 Gel electrophoresis

The quality and amount of PCR products should be checked in a 1.0% agarose gel.

4.2.1 Preparation of reagents

- **5 x TBE** (0.445 M tris-borate, 0.0125 M EDTA)
  54 g Tris(hydroxymethyl)-aminomethane
  27.5 g Boric acid (H_3BO_3)
  4.65 g Na_2-EDTA
  to 1000 ml with distilled water; store at RT.
- **Ethidium bromide solution** (1 mg/ml)
  Dissolve 10 mg of ethidium bromide in 10 ml dest. H_2O and store in the dark at 2...8ºC.
- **Gel loading buffer** (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll 400)
  25 mg Bromophenol blue
  25 mg Xylene cyanol
  2.5 g Ficoll 400
  to 10 ml with distilled water; store at 2...8ºC.
- **1 x TBE**
  final 1:5 dilution of the 5 x TBE buffer in demineralised water.

4.2.2 Procedure

Prepare 1.0% agarose gel by boiling 1 g agarose in 100 ml of 1 x TBE until the solution is clear. Cool the solution to <60ºC and mix with 4 µl of ethidium bromide solution. Place the sealed gel tray on a horizontal surface. Fill the agarose solution into the sealed gel holder with as few bubbles as possible, and place a comb in to form pockets of about 10 µl. After the gel has polymerized (about 30 minutes at RT), it is placed in the gel chamber filled with 1 x TBE. Remove the comb. The gel pockets should be covered with buffer. Mix 5 µl of each PCR sample with 2 µl of loading buffer and pipette into the gel pockets. To check the size of the PCR products, it is advisable to include a suitable molecular weight standard (e.g. 123 bp ladder) in the electrophoresis run. Electrophoresis is performed at 8 V/cm (electrode distance) over 15-20 minutes. The DNA bands can be observed on a UV transilluminator (with suitable protection of the eyes against UV light). The gel can be photographed with a Polaroid camera for documentation.

The HLA-A amplification should give products of 359 bp and 317 bp and the HLA-B amplification should give products of 424 bp and 438 bp.
The HLA-C amplification should give products of 418 bp and 468 bp.
The DRB LowRes amplification should give a product of 277 bp; in presence of a DR2 positive DNA an additional amplification product of 339 bp will be seen.
Both DRB HiRes amplifications (G+T) should give a product of 268 bp and 422 bp for the control band.
The DQB SL amplifications should give a product of 246 bp (A) and 231 bp (B) and each 422 bp for the control band.
The DQB DL amplification should give a producto of 270-285 bp.
The DRB1 All amplification should give PCR products of approx. 273 bp.
Both DRB1 HiRes amplifications (G and T) should give products of approx. 270 bp and 422 bp for the control band.

4.3 ELPHA (Enzyme Linked Probe Hybridization Assay)

4.3.1 Preparation of reagents

ELPHA Reagent 48

1. Buffer 2xSSC/0.1%SDS: BUF SSC/SDS
   1 bottle (= 500 ml) of 20xSSC Buffer SSC 20x
   + 4000 ml distilled water
   + 1 bottle (= 500 ml) of 1% SDS Solution SDS 1%

2. Buffer A BUF A
   1 bottle (= 500 ml) of buffer A BUF A10x
   + 4500 ml distilled water

3. Buffer B BUF B
   (Conjugate dilution buffer)
   To prepare buffer B BUF B, heat buffer A BUF A to just below boiling point and transfer 50 ml to a bottle of blocking reagent BLOC REA. Shake well so that the blocking reagent BLOC REA is fully dissolved. 6 ml of buffer B BUF B are sufficient for 1 plate.
   Cool buffer B BUF B to room temperature before diluting the conjugate.

3. Conjugate dilution CONJDIL FITC
   (always use fresh)
   Before first use, centrifuge conjugate (FITC) CONJ FITC at high speed. Dilute conjugate (FITC) CONJ FITC 1:1000 in buffer B (at room temperature), i.e. **for 1 plate pipette 6 µl of conjugate (FITC) CONJ FITC into 6 ml of buffer B BUF B** and mix well.

   ➲ If the entire plate is not being used, it is advisable to prepare only the required amount of diluted conjugate CONJDIL FITC and to store the remaining buffer B BUF B at -20ºC or colder.

5. Solution for denaturation DENAT, solution for neutralization NEUTRAL and substrate solution (TMB) SUBS TMB are ready for use.

ELPHA Reagent DL Supp 48

1. Buffer A BUF A
   1 bottle (= 500 ml) of buffer A BUF A10x
   + 4500 ml distilled water

2. Buffer B BUF B
   (Conjugate dilution buffer)
   To prepare buffer B BUF B, heat buffer A BUF A to just below boiling point and transfer 50 ml to a bottle of blocking reagent BLOC REA. Shake well so that the blocking reagent BLOC REA is fully dissolved. 6 ml of buffer B BUF B are sufficient for 1 plate.
   Cool buffer B BUF B to room temperature before diluting the conjugate.

3. Conjugate dilution CONJDIL D
   (always use fresh)
   Before first use, centrifuge conjugate (D) CONJ D at high speed. Dilute conjugate (D) CONJ D 1:1000 in buffer B (at room temperature), i.e. **for 1 plate pipette 6 µl of conjugate (D) CONJ D into 6 ml of buffer B BUF B** and mix well.

   ➲ If the entire plate is not being used, it is advisable to prepare only the required amount of diluted conjugate CONJDIL D and to store the remaining buffer B BUF B at -20ºC or colder.

4. Substrate solution (TMB) SUBS TMB and POD inhibitor INHIB are ready for use.

4.3.2 Procedure of an ELPHA SL typing

Please bring the components of the kit to room temperature before use.

(1) The number of strips which are necessary for one typing is described in table 2.
Strips that are not required can be stored dry at 2...8ºC for later tests.

➲ **Please do not discard the desiccant contained in the ELPHA plate pouches. Once opened, plates should be kept in their original pouch along with desiccant. Reseal the pouches with tape or weld them together again.**

(2) Transfer x µl of PCR product in a reaction tube, add x µl of solution for denaturation DENAT (ready for use) and mix well.

(3) Incubate for 5 minutes at room temperature.
The double-stranded PCR products are denatured to single stranded products.

(4) Add x µl of solution for neutralization NEUTRAL (ready for use).
If not processed further immediately, it is advisable to place the samples on ice to prevent double-strand renaturation.

(5) Add x µl of distilled water and mix.

➲ See table 2 for the exact volume of x.

(6) Distribute 50 µl aliquots of diluted PCR product (Eppendorf repeater pipette with 2.5 ml combitip) in the corresponding wells of the microtestplate MTP. See table 2 for the assay's corresponding wells.

Table 3:

	HLA-AB SL LowRes	HLA-C SL LowRes	DRB SL LowRes	DQB SL LowRes
Strips per test	12 strips	4 strips	3 strips	3 strips
Transfer each 50 µl diluted PCR product in following strips (wells):	HLA-A in strip A1 – A5; HLA-B in strip B1 – B7	C1 – C4	1 – 3	A in strip 1 + 2 (well A1 – E2); B in strip 2 + 3 (well G2 – H3)
PCR Product	135 µl	80 µl	90 µl	40 µl
Solution for denaturation DENAT	750 µl	480 µl	400 µl	200 µl
Solution for neutralization NEUTRAL	750 µl	480 µl	400 µl	200 µl
Distilled water	1500 µl	960 µl	800 µl	400 µl

(7) Seal the strips with the self-adhesive foil, float the plates MTP in the pre-warmed waterbath and incubate for 45 minutes at 46 ± 1ºC.
Correct adjustment and constancy of the temperature is important for the hybridisation and for the following stringent washing step. Therefore it is strongly recommended to check the temperature before each incubation by means of a calibrated thermometer placed in the water.

➲ During the incubation period, prepare a sufficient volume of blocking reagent BLOC REA (=buffer B BUF B, preparation see chapter 4.3.1). Not used buffer B BUF B can be stored at -20°C or colder.

(8) Remove the microtest plate MTP from the waterbath to terminate the hybridisation step. Peel off the foil.

➲ **It is important to proceed to the next wash step immediately to avoid background. Extended exposure of the tray to room temperature will result in non-specific hybridisation and/or high background.**

Discard the hybridization solution and wash the plates MTP twice with 200 µl of 2xSSC/0.1%SDS BUF SSC/SDS (preparation see chapter 4.3.1)per well. After each washing step, remove the liquid by gently tapping on absorbent paper. After the 2nd washing step, pipette 200 µl of 2 x SSC/0.1% SDS BUF SSC/SDS in each well.
Unbound probes are removed in this washing step.

- ➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates MTP in the washer directly after incubation in the waterbath and start the "ELPHA_SSC" program.

(9) Place a paper towel on the top of the tray to dry the rims. Cover the plate MTP with a new foil and incubate for 15 minutes at 46 ± 1ºC in the waterbath.
In this stringency washing step, probes that are not 100% homologous are removed from the DNA. For this reason it is essential to maintain the temperature precisely. It should therefore be checked again.

(10) After the incubation time, discard the washing buffer BUF SSC/SDS and wash the plate MTP three times with 200 µl of buffer A BUF A (preparation see chapter 4.3.1) per well according to the washing procedure described above.

- ➲ It is important to proceed to the next wash step immediately to avoid background. Extended exposure of the tray to room temperature will result in non-specific hybridisation and/or high background.
- ➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates MTP in the washer directly after incubation in the waterbath and start the "ELPHA_A" program.

(11) Distribute 50 µl of the freshly prepared conjugate dilution CONJDIL FITC (preparation see chapter 4.3.1) per well with a Multichannel pipette and incubate for 30 minutes at room temperature.
The POD-coupled FITC antibody accumulates on the bound, FITC-labelled probes.

(12) Discard the conjugate dilution CONJDIL FITC and wash the plate MTP 3 times with 200 µl of buffer A BUF A per well.

- ➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates MTP in the washer directly after incubation in the waterbath and start the "ELPHA_A" program.

(13) Distribute 50 µl of substrate solution SUBS TMB (ready for use) per well with a Multichannel pipette and incubate for 15 minutes at room temperature in the dark.
The substrate (TMB) reacts with the antibody-coupled enzyme (POD) and the colour changes from colourless to blue.

- ➲ **It is important to bring the substrate SUBS TMB to room temperature and shake the bottle before use. Do not pour the remnants of the substrate solution SUBS TMB back into the stock bottle. Minimize direct pipetting from the stock bottle (contamination!).**
- ➲ Caution: The incubation time and temperature is critical after the addition of substrate. Do not exceed the defined incubation time!

(14) Distribute 50 µl of 1 N sulphuric acid per well with a Multichannel pipette.
The reaction is stopped; the colour changes from blue to yellow.

(15) Quantitate the results in each well in the plate photometrically by measuring the light absorption at 450 nm in an Elisa reader (with a reference filter at 620 nm). Record the O.D. Alternatively, visually observe each well in the plate for colour development and record results.

4.3.3 Procedure of an ELPHA-DL typing

Please bring the components of the kit to room temperature before use.

(1) The number of strips which are necessary for one typing is described in table 2.
Strips that are not required can be stored dry at 2...8ºC for later tests.

➲ **Please do not discard the desiccant contained in the ELPHA plate pouches. Once opened, plates should be kept in their original pouch along with desiccant. Reseal the pouches with tape or weld them together again.**

(2) Transfer x µl of PCR product in a reaction tube, add x µl of solution for denaturation DENAT (ready for use) and mix well.

(3) Incubate for 5 minutes at room temperature.
The double-stranded PCR products are denatured to single stranded products.

(4) Add x µl of solution for neutralization NEUTRAL (ready for use).
If not processed further immediately, it is advisable to place the samples on ice to prevent double-strand renaturation.

(5) Add x µl of distilled water and mix.

➲ See table 2 for the exact volumes of x.

(6) Distribute 50 µl aliquots of diluted PCR product (Eppendorf repeater pipette with 2.5 ml combitip) in the corresponding wells of the microtest plate MTP. See table 2 for the assay's corresponding wells.

Table 4

	HLA-AB DL LowRes	DRB DL HiRes	DRB1 DL All	DRB1 DL HiRes	DQB DL Extend
Strips per test	6 strips	2x3 strips	3 strips	2x3 strips	2 strips
Transfer each 50 µl diluted PCR product in following strips (wells):	HLA-A in wells A1 – D3 (red coloured) HLA-B in wells E3 – H6 (blue coloured)	G in the first set of strips 1 – 3; T in the second set of strips 1 – 3	strip 1-3	DRB1G in the first set of strips 1 – 3; DRB1T in the second set of strips 1 – 3	strip 1-2
PCR Product	90 µl	90 µl	90 µl	90 µl	90 µl
Solution for denaturation DENAT	400 µl	400 µl	400 µl	400 µl	250 µl
Solution for neutralization NEUTRAL	400 µl	400 µl	400 µl	400 µl	250 µl
Distilled water	900 µl	800 µl	800 µl	800 µl	400 µl

(7) Seal the strips with the self-adhesive sealer, float the plates MTP in the pre-warmed waterbath and incubate for 45 minutes at 46 ± 1ºC.
Correct adjustment and constancy of the temperature is important for the hybridisation and for the following stringent washing step. Therefore it is strongly recommended to check the temperature before each incubation by means of a calibrated thermometer placed in the water.

➲ During the incubation period, prepare a sufficient volume of blocking reagent BLOC REA (=buffer B BUF B, preparation see chapter 4.3.1). Not used buffer B BUF B can be stored at -20°C or colder.

(8) Remove the microtest plate MTP from the waterbath to terminate the hybridisation step. Peel off the foil.

➲ It is important to proceed to the next wash step immediately to avoid background.

Extended exposure of the tray to room temperature will result in non-specific hybridisation and/or high background.

Discard the hybridisation solution and wash the plates [MTP] twice with 200 µl of 2xSSC/0.1%SDS [BUF SSC/SDS] (preparation see chapter 4.3.1) per well. After each washing step, remove the liquid by gently tapping on absorbent paper. After the 2nd washing step, pipette 200 µl of 2 x SSC/0.1% SDS [BUF SSC/SDS] in each well.
Unbound probes are removed in this washing step.

➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates [MTP] in the washer directly after incubation in the waterbath and start the "ELPHA_SSC" program.

(9) Place a paper towel on the top of the tray to dry the rims. Cover the plate [MTP] with a new sealer and incubate for 15 minutes at 46 ± 1ºC in the waterbath.
In this stringency washing step, probes that are not 100% homologous are removed from the DNA.
For this reason it is essential to maintain the temperature precisely. It should therefore be checked again.

(10) After the incubation time, discard the washing buffer [BUF SSC/SDS] and wash the plate [MTP] three times with 200 µl of buffer A [BUF A] (preparation see chapter 4.3.1) per well according to the washing procedure described above.

➲ It is important to proceed to the next wash step immediately to avoid background.
Extended exposure of the tray to room temperature will result in non-specific hybridisation and/or high background.

➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates [MTP] in the washer directly after incubation in the waterbath and start the "ELPHA_A" program.

(11) Distribute 50 µl of the freshly prepared [CONJDIL D] (preparation see chapter 4.3.1) per well with a multichannel pipette and incubate for 30 minutes at room temperature.
The POD-coupled anti-DIG antibody accumulates on the bound, DIG-labelled probes.

(12) Discard the [CONJDIL D] and wash the plate 3 times with 200 µl of [BUF A] per well.

➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates in the washer and start directly the "ELPHA_A" program.

(13) Distribute 50 µl of [SUBS TMB] (ready for use) per well with a multichannel pipette and incubate for 15 minutes at room temperature in the dark.
The substrate (TMB) reacts with the antibody-coupled enzyme (POD) and the colour changes from colourless to blue.

➲ **It is important to bring the substrate to room temperature and shake the bottle before use. To avoid contamination of stock solution: Do not pour the remnants of the solution back into the stock bottle. Minimize direct pipetting from the stock bottle.**

➲ Caution: The incubation time and temperature is critical after the addition of substrate. Do not exceed the defined incubation time!

(14) The first readout of the plate has to be done exactly after 15 minutes substrate incubation in the Elisa reader at 370 nm (with a reference filter at 492 nm).

(15) Distribute 50 µl of POD inhibitor [INHIB] (ready for use) per well with a multichannel pipette and incubate for 4-6 minutes at room temperature.
The anti-DIG antibody coupled enzyme (POD) is inhibited.

(16) Discard the POD inhibitor [INHIB] and wash the plate 3 times with 200 µl of [BUF A] per well.

➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates in the washer and start directly the "ELPHA_A" program.

(17) Distribute 50 µl of the freshly prepared [CONJDIL][FITC] (preparation see chapter 4.3.1) per well with a multichannel pipette and incubate for 30 minutes at room temperature.
The POD-coupled FITC antibody accumulates on the bound, FITC-labelled probes.

(18) Discard the [CONJDIL][FITC] and wash the plate 3 times with 200 µl of buffer A [BUF][A] per well.

➲ Alternatively, washing can be done with the Biotest Elisa Washer III or equivalent. If this washer is used, place the plates in the washer directly after incubation in the waterbath and start the "ELPHA_A" program.

(19) Distribute 50 µl of substrate solution [SUBS][TMB] (ready for use) per well with a multichannel pipette and incubate for 15 minutes at room temperature in the dark.
The substrate (TMB) reacts with the antibody-coupled enzyme (POD) and the colour changes from colourless to blue.

(20) The second readout of the plate has to be done exactly after 15 minutes substrate incubation in the Elisa reader at 370 nm (with a reference filter at 492 nm).

4.3.4 Results SL

Visual Observation	Photometric
Positive = visible yellow colour	Positive reaction = O.D. equal to or greater than 0.200
Negative = no visible colour	Negative reaction = O.D. less than 0.200

The results can be interpreted by comparing the reaction pattern with the evaluation diagram.

➲ The test components are balanced so that all O.D. values over 0.200 are generally to be considered as a positive result. For some probes the cut-off values differ from the standard setting of 0.200. The relevant positions are detailed in the lot information sheet. During installation of lot-specific updates of the ELPHA Typing Software cut-offs are automatically adjusted to modified values. Since the signal intensity also depends on a number of other parameters that are not influenced by the reagents contained in the kit (e.g. DNA isolation, PCR procedure), variations of intensity cannot be excluded. With an adequately low background (e.g. <0.070), positive signals of weaker amplifications (e.g. ≥0.150) can also be clearly recognized as such.

4.3.5 Results DL

All O.D. values over 0.160/0.140 are generally to be considered as a positive result. The results can be interpreted by comparing the reaction pattern with the evaluation diagram or by using the Biotest HLA typing software ([REF] 847 070).With the lot information, which is available from the internet (www.biotest.de), the lot dependant typing software can be started. For some probes the cut-off values differ from the standard setting. The relevant positions are detailed in the lot information sheet. During installation of lot-specific updates of the HLA Typing Software cut-offs are automatically adjusted to modified values.

➲ In very rare cases where all alleles (incl. alleles of pseudogenes) belong to one of the two amplification groups (GGT or GTG), the amplification with no positive reactions may show negative results also with the positive control. In such cases the interpretation of the positive control should be corrected manually in the software to "positive" in order to obtain a result without a missing position.

➲ The test components are balanced so that all O.D. values over 0.160/0.140 (DL) are generally to be considered as a positive result. Since the signal intensity also depends on a number of other parameters that are not influenced by the reagents contained in the kit (e.g. DNA isolation, PCR procedure), variations of intensity cannot be excluded. With an adequately low background (e.g. <0.090), positive signals of weaker amplifications (e.g. ≥0.130) can also be clearly recognized as such. For automatic evaluations the cut off value can be changed in the software for single probes or the automatic cutoff function can be used (described in the HLA Typing Software manual).

4.4 Limitations of procedure

1. Intensity of positive reactions will vary due to the quality (amount) of the PCR product. Quality of the PCR product will directly affect the raw scores obtained through the ELISA reader. In case of weak amplifications may not be achieved; therefore the test should be repeated.
2. Extracted DNA stored at 2...8°C for prolonged periods (> week) may not amplify due to enzyme digestion of the DNA.
3. Colour development, after substrate addition, for longer than 15 minutes, may result in excess background.
4. Performance of the test can only be guaranteed if the enclosed instructions are strictly adhered to
5. This test should only be used for initial HLA typing. Other clinical and diagnostic findings should be used in addition, when determining suitability for transplant.

4.5 Quality Control

A positive control probe is included in each test. The positive control reacts with a sequence conserved among all alleles in the corresponding gene loci. For the exact position see the reaction pattern included. For the test to be valid a positive result must be obtained with the positive control, if not it should be repeated.
Quality control of new lot numbers could be performed by typing a combination of known cells which will react with every probe in the kit.

Example: Quality control of a Biotest ELPHA DRB SL LowRes kit

DRB1		**DRB3**	**DRB4**	**DRB5**
0101	1102	02		
1501	1001			01
04c	1201	02	01	
0701	1401	02	01	
04a	09012		01	
03011	1601/02	01		02
0401	0801		01	
0701	1302	03	01	

4.6 Specific Performance Characteristics

The Biotest ELPHA DRB SL LowRes kit and commercially available anti-HLA serological tests were compared by testing 406 specimens (random transplant candidates and potential donors) at three geographically distinct locations. There was 90% concordance (365/406) between the Biotest ELPHA DRB SL LowRes kit results and the results obtained using the anti-HLA serological tests.

	Concordant	**Discordant**	**Total**	**%**
N	365	41	406	100
%	90%	10%		

N = Number of samples

Upon further testing to resolve the discordants using additional methods (standardized in-house DNA methods), there was 100% concordance (406/406).

There was 100% concordance (406/406) between results interpreted after visual observation of colour development and results interpreted after photometric measurement of colour development.

	Concordant	**Discordant**	**Total**
N	406	0	406
%	100	0	100

N = Number of samples

5. Trouble Shooting

PROBLEM	CAUSE	SOLUTION
1. Gel electrophoresis		
No bands are visible.	Ethidium bromide is absent from the gel.	Stain the gel again in a dye bath (50 µl of EB stock solution /100 ml of 1xTBE buffer).
Bands of the MW standard not visible; PCR product is visible.	Not enough MW standard DNA applied.	Check the concentration of the MW standard DNA.
MW standard is visible; PCR product is not visible.	Components missing from the PCR reaction mixture.	Repeat the PCR, taking care to add all components to the reaction mixture.
	Nucleotides could be too old or were not stored at least -20ºC.	Prepare a fresh nucleotide stock solution; prepare a new PCR reaction mixture.
	Thermocycler program	Check the PCR program of the thermocycler. Thermocyclers other as the recommended may have different heating and cooling rates (heating rate ≥1.5º/sec). In this case it is advisable to increase the steps from 15 to 30 seconds.
	DNA isolated from heparinised blood.	Check whether heparinised blood has to be used in the DNA extraction procedure applied. Use DNA extraction methods which give heparin-free DNA, e.g. sequential lysis (see section 4.), or use EDTA blood or citrated blood as the starting material.
Weak bands for PCR products.	Poor DNA quality Weak amplification	Start the ELPHA test. If poor results are also obtained in the ELPHA test, see under point ELPHA.
	Incorrect amount of DNA was added.	Check the amount of DNA in the preparation: DNA present in 1 µl should be clearly detectable in the gel. The DNA should be free of inhibitors such as haemoglobin, salts, proteins, ethanol and heparin. Use an alternative DNA extraction method.
2. ELPHA		
No detectable signals	PCR product not added.	Check the PCR product on the agarose gel.
	Error in denaturation and/or neutralization.	Check the pH of the PCR product. If the pH is not neutral, the test must be repeated, taking care to add solutions 1 and 2.
	Error in hybridization and/or stringency washing conditions.	Check the water bath temperature with an external thermometer.

PROBLEM	CAUSE	SOLUTION
	Error in colour development.	Check the Peroxidase activity in a mixture of 1 µl conjugate dilution CONJDIL FITC and 50 µl substrate SUBS TMB; the colourless substrate must turn blue in a short time.
	Conjugate CONJ FITC is not added to buffer B.	Provided that sulphuric acid has not been added, the plate MTP can be washed with buffer A BUF A (200 µl per well), the conjugate dilution CONJDIL FITC is then added and the procedure continued as usual.
Only the positive control is positive.	Wrong PCR product was applied.	Repeat the test.
Weak signals	Weak amplification	Check the parameters of amplification and the quality of DNA. If the background value is $\leq$0.070, reduce the cut-off to 0.150 or use the automatic cutoff function
Single signals weak	Water bath temperature too high.	Check the water bath temperature with an external thermometer.
False positive signals	Carry over due to pipetting errors.	Repeat the typing.
	Water bath temperature too low.	Check the water bath temperature with an external thermometer.
Average background OD>0.100	Poor washing conditions.	Ensure that the plate is dried after washing with buffer A BUF A. Check the washer settings.
	Substrate incubation - too long - warmer than room temp. - not in the dark	Change the conditions of the substrate incubation.
The entire plate is positive.	Conjugate CONJDIL FITC has not been washed out	Provided that sulphuric acid has not been added, the plate MTP can be washed with buffer A BUF A (200 µl per well) and continue with substrate addition.

6. References

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Nomenclature: http://www.anthonynolan.org.uk/HIG/nomenc.html
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