

UK

# HLA SSP Kits

SSP-Reagenzienkit für die HLA-Typisierung auf DNA-Basis  
Ready to use SSP reagent kit for DNA based HLA typing  
Trousse de réactifs SSP pour le typage HLA, basé sur l'ADN  
Kit di reagenti SSP per tipizzazione HLA basata sul DNA  
Juego de reactivos SSP para la tipificación del antígeno

IVD

For In Vitro Diagnostic Use

HLA-A SSP	REF	826 201	€ € 0197
HLA-B SSP	REF	826 206	€ € 0197
HLA-C SSP	REF	826 212	€ €
DRB SSP	REF	826 215	€ € 0197
DQB SSP	REF	826 220	€ €
ABDR SSPtray	REF	826 230	€ € 0197
ABC SSPtray	REF	826 240	€ € 0197

**Besondere Hinweise für den Anwender / Special remarks for the user / Remarques importantes pour l'utilisateur / Avvertenze per l'utilizzatore / Indicaciones importantes para el usuario:**

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<b>REF</b>	GB DE ES IT FR NL DK CZ	Article number Artikelnummer Artikulo numero Codice prodotto Référéncé du produit Artikelnummer Artikelnummer Katalogové číslo	<b>IVD</b>	GB DE ES IT FR NL DK CZ	For in vitro diagnostic use Nur zur in-vitro Diagnostik Sólo para el diagnóstico in vitro Solo per la diagnostica in vitro Pour le diagnostic in vitro Voor in vitro gebruik Til diagnostik brug i glas Použití pro in vitro diagnostiku			
<b>BLOCK</b>	GB DE ES IT FR NL DK CZ	PCR block PCR-Block PCR bloque PCR blocco PCR bloc PCR blok PCR blok PCR blok	<b>NC</b>	GB DE ES IT FR NL DK CZ	negative control Negativkontrolle control negativo controllo negativo contrôle négatif negatieve controle negativ kontrol negativní kontrola	<b>CK</b>	GB DE ES IT FR NL DK CZ	PCR cocktail PCR-Cocktail PCR coctel PCR cocktail PCR cocktail PCR cocktail PCR blanding PCR koktejl

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## 1. INTRODUCTION

### 1.1 Intended Use

HLA Sequence Specific Primers (SSP) kits are intended 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". 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 the most important HLA alleles are now known, variations in sequences can be identified at the DNA level with the help of synthetic oligonucleotides. Utilizing amplifications of genomic DNA (PCR, **P**olymerase **C**hain **R**eaction) together with specific primers pairs (SSP, **S**equence **S**pecific **P**rimers) it is possible to identify a large number of HLA alleles by molecular test methods.

### 1.3 Principle of the Test

HLA SSP Kits are test systems for typing HLA characteristics using PCR techniques. For typing, the SSP method uses allele-specific primers in the amplification reaction. The method is based on the principle that only primers whose sequences are perfectly complementary to that of the target sequence of a DNA sample present will bind to this DNA and produce an amplificate in the PCR reaction. Non-complementary primers, on the other hand, do not bind to the DNA and no amplification takes place.

The amplified DNA is determined using agarose gel electrophoresis. Successful amplification will generate a DNA fragment of defined length which appears as a band in the gel. If no amplification takes place, this band is missing.

The composition of the primers permits positive identification of the HLA characteristics. The field of application for this detection system is the determination of individual HLA alleles of organ donors and recipients and of patients receiving blood transfusion or blood component substitution therapy.

## 2. REAGENTS

### 2.1 Contents of HLA SSP Kits

The contents of HLA SSP Kits are sufficient for 24 test.

- 24 PCR-blocks **BLOCK** respectively, each consisting of PCR tubes in blocks that contain the dried primer/nucleotid mixtures. As an aid to identification, a black mark has been placed onto position 1 (=H1).
- PCR cocktail **CK** (ready for use)  
The cocktail contains PCR-buffer (final concentration: 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl, 0.001% gelatine), glycerol and cresol red.
- PCR cover strips or PCR cover seals.
- Worksheets, reaction pattern chart, primer position sheet

The negative control **NC** is packed separately as additional PCR tubes (colourless), if it is not included in the PCR block **BLOCK**.

## 2.2 Warning and Precautions

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**Caution:** The test must be performed by well-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 as potentially infectious. Do not pipette by mouth.

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

**Caution:** Do not use reagents with any evidence of turbidity or microbial contamination.

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

**Caution:** Biohazard Warning: The ethidium bromide used for staining of DNA is a potential carcinogen. Always wear protective gloves when handling stained gels. Waste management by burning.

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

**Caution:** All used PCR blocks should be treated as potentially infectious and should be destroyed according to the valid national guidelines.

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

See Material Safety Data Sheets for detailed information.

## 2.3 Storage and Shelf Life

The SSP reagents ([BLOCK], [CK], [NC]) must be stored at 2...8 °C. The expiry date is printed on the packaging of the kit components. PCR blocks are sealed in pouches.

## 2.4 Indications of Instability or Deterioration

Once the pouches have been opened, the remaining unused PCR blocks should be kept in their original pouch, resealed with tape, or heat sealed to prevent moisture accumulation during storage. Once opened PCR blocks should be used within 4 weeks.

## 3. Instrument Requirements

### 3.1 Programming the Thermal cycler

Program HLA SSP:

Initial Denaturing:	<b>94°C</b>	<b>2 min.</b>		
Denaturing:	<b>94°C</b>	<b>10 sec.</b>	]	<b>10 cycles</b>
Annealing & Extension:	<b>65°C</b>	<b>60 sec.</b>		
Denaturing:	<b>94°C</b>	<b>10 sec.</b>	]	<b>20 cycles</b>
Annealing:	<b>61°C</b>	<b>50 sec.</b>		
Extension:	<b>72°C</b>	<b>30 sec.</b>		

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.

Thermal cyclers which have no adjustable pressure plate required an adaptor mat in order to guarantee optimal heat transfer from the heat cover to the PCR tubes.

### 3.2 Gel Electrophoresis

Refer to electrophoresis in the Procedure Section 5.

## 4. Specimen Collection and Preparation

### 4.1 DNA Isolation

Genomic DNA may be obtained from all nucleated cells. The simplest method is to isolate DNA from cell suspensions (blood, buffy coat or cultured cells). A vast range of various protocols exist for the isolation of DNA from cells. For PCR-SSP testing, only those methods which provide DNA of adequate quality and quantity for PCR should be considered, e.g. salting out method (Ref.2).

Among the suppliers of commercial DNA extraction kits, suitable products include "Puregene" from Gentra Systems and "Super Quick Gene" from The Analytical Genetic Testing Center.

### 4.2 Specimen Collection and Preparation

#### 4.2.1 Sample

Typing is performed using anticoagulated peripheral blood (e.g. sodium citrate, EDTA). For blood sample storage and stability information, please refer to the technical information provided by the extraction test kit manufacturer and it has to be user-validated.

#### 4.2.2 Contamination

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

#### 4.2.3 Hemolyzed Specimen

Avoid the use of lipemic or hemolyzed 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.

#### 4.2.4 DNA Quantity

The DNA sample to be used should be resuspended in sterile distilled water at a concentration of approx.

100 ± 50 ng/µl. DNA **should not be re-suspended** in solutions containing chelating agents, **such as EDTA**, above 0.5 mM in concentration.

#### 4.2.5 DNA Quality

Determination of the concentration of DNA is performed by measuring the optical density (OD) at 260 nm ( $A_{260}$ ). The value  $A_{260} = 1$  (= OD 1.0) corresponds to approx. 50 µg/ml 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 ratio  $A_{260}/A_{280}$  is 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 an  $A_{260}/A_{280}$  ratio of 1.5, the percentage of protein in the DNA preparation is about 50 %.

For good PCR-SSP results, DNA is required with an  $A_{260}/A_{280}$  ratio of  $\geq 1.6$ .

- ☉ The purity and concentration 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 periods of time (over 1 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.

## 5. Procedure

### 5.1 Material Provided

See 2.1 contents of HLA SSP Kits

### 5.2 Additional Materials Required

#### 5.2.1 Sample Material (DNA)

- UV spectrophotometer

#### 5.2.2 PCR

- Taq DNA polymerase (5 U/μl, e.g. PE Applied Biosystems)
- Thermal cycler with heated cover (e.g. PE 9600, PE 9700 PE Applied Biosystems)
- Adjustable pipettes
- Eppendorf Multipipette with combitips (10 μl)
- Filtered pipette tips
- distilled water (dH<sub>2</sub>O)

#### 5.2.3 Gel electrophoresis

- Agarose (for molecular biology)
- Gel chamber (suitable for gel with at least 25 pockets)
- 5x TBE buffer
  - Tris(hydroxymethyl)-aminomethane (base)
  - Boric acid (H<sub>3</sub>BO<sub>3</sub>)
  - Disodium EDTA
- Ethidium bromide solution (10 mg/ml)
- DNA ladder (50 – 1000 bp marker)
- Magnetic stirrer with hotplate or microwave
- Adjustable pipettes
- Polaroid camera with UV filter and Polaroid film type 667
- UV transilluminator (approx. 200 – 300 nm)
- Power supply
- distilled water (dH<sub>2</sub>O)

### 5.3 PCR (Polymerase Chain Reaction)

#### 5.3.1 Precautionary measures

PCR is an extremely sensitive method which can efficiently amplify even the smallest amount of DNA. It follows from this that even traces of contaminating DNA in a sample can be amplified in the PCR reaction and falsify the test 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:

1. 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).
2. Post-PCR area:  
Thermal cycler, gel electrophoresis, evaluation, storage of amplified DNA.  
Equipment and consumables from the post-PCR area must not be taken into the pre-PCR area.
3. When working in the pre-PCR area, pipettes with aerosol protection should be used (filtered tips). It is recommended that a negative control be included in the test procedure as an indication of contamination with foreign DNA.

#### 5.3.2 Performing the HLA SSP typing test

(1) For HLA typing of one DNA sample PCR reactions with a reaction volume of 10 μl in each PCR tube are performed. The black mark serves as an aid for correct orientation of the PCR block **BLOCK** (position H1).

(2) In an Eppendorf reaction tube, prepare for **each typing test** a **master mix** containing the following components:

PCR cocktail **CK**  
Taq DNA polymerase (5 U/μl)  
dH<sub>2</sub>O  
(see the following table for different configurations)

Mix well and pipette 10 µl of this mixture to the negative control **NC**. After that add DNA (approx. 100 ± 50 ng/µl) and mix well.

See the following table for different master mix configurations:

Number of PCR reactions	Master mix for configuration with				
	8	18	24	48	96
PCR cocktail <b>CK</b>	44 µl	100 µl	120 µl	228 µl	440 µl
Taq DNA polymerase	0.7 µl	1.5 µl	1.8 µl	3.5 µl	7 µl
dH <sub>2</sub> O	55 µl	125 µl	150 µl	288 µl	550 µl
DNA (approx. 100 ± 50 ng/µl)	11 µl	25 µl	30 µl	57 µl	110 µl

- (3) From this master mix, pipette 10 µl to each of the dried primer mixes. This is best done using a multipipette. Care should be taken that the pipette tips do not make contact with the primer in order to avoid carry-over of the primer. For this reason, pipette the master mix to the walls of the well.
- (4) Seal properly the PCR strips using the PCR cover strips or seals supplied. Transfer the tray into the thermal cycler and start the PCR with the program HLA SSP.

## 5.4 Gel Electrophoresis

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

### 5.4.1 Preparations of Reagents

- **5x TBE buffer** (0.445 M tris-borate, 0.0125 M EDTA):  
54 g Tris(hydroxymethyl)-aminomethane (base)  
27.5 g Boric acid (H<sub>3</sub>BO<sub>3</sub>)  
4.65 g Disodium EDTA  
ad 1000 ml with distilled water, store at room temperature.
- **Ethidium bromide solution** (10 mg/ml)  
Dissolve 100 mg of ethidium bromide in 10 ml of distilled water and store at 2...8 °C protected from light.  
**Caution: Ethidium bromide is mutagenic and toxic. Always wear protective gloves when working with ethidium bromide (also in diluted form). In case of contact with the skin, wash off immediately with copious amounts of water.**
- **1x TBE buffer**  
1:5 final dilution of the 5x TBE buffer solution in demineralised water.

### 5.4.2 Performing gel electrophoresis

A 2 % solution of agarose is prepared by boiling 5 g of agarose in 250 ml of 1x TBE until the solution becomes completely dissolved. By stirring the solution cool it down to < 60°C and add 4µl of ethidium bromide. Subsequently pour the agarose solution – devoid of bubbles - into a prepared and sealed gel tray. Place the combs (10µl pockets) and keep it at room temperature for at least 10 minutes.

After the agarose has solidified the gel is inserted into the gel chamber. The combs are removed and the gel is covered with 1 x TBE. The gel pockets should be completely covered by the buffer. Pipette the entire PCR mixtures (10 µl) into the gel pockets.

To be able to check the size of the PCR products, the use of an appropriate molecular weight standard (50-1000bp marker) is recommended for the electrophoresis.

The electrophoresis takes place in 15 to 25 minutes at 8V/cm (distance between electrodes). The migration distance of the cresol red should amount to 1-1.5cm.



### 5.4.3 Documentation

After the completion of the electrophoresis the gel is placed on an UV transilluminator and photographed for documentation and interpretation.

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

## 6. Results

### 6.1 Evaluation












The HLA primer mixture contains control primers which amplify a 1069 bp fragment of human growth hormone (HGH). The concentration of these primers 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 the presence and in the absence of an allele- or group-specific PCR fragment. The control band can therefore generally be seen in all PCR reactions. From time to time, the control band can appear weak or is completely missing in the presence of an allele-specific HLA PCR product. This is not a limitation to the method, as the specific band provides a check on the success of the PCR run.

The composition of the primers permits positive identification of the HLA characteristics. The interpretation is based on whether a specific band is present on the gel or not.

The size of the amplified DNA fragments 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 result sheet supplied and the typing result read off with the aid of the reaction pattern.

### Gel Interpretation

	positive reaction	positive reaction	negative reaction	non-amplification
Gel pocket				
Control band	none	-----		none
Specific band			none	none
Primer dimer				

### 6.2. Limitation of Procedure

- Depending on the quality and quantity of the DNA used, the intensity of the bands can vary. The quantity of the PCR product corresponds to the intensity of the bands that are made visible by the UV light.
- If 1 or 2 PCR amplifications fail, you must establish whether their positive evaluation leads to a typification result. If this is the case the test must be repeated. If the positive evaluation of the missing amplifications does not result in a typification result and if there is definite proof of the existence of two alleles, no further measures are required. However, if only one allele can be found the test must be repeated.
- The use of other Taq DNA polymerase than the one that has been recommended by PE Applied Biosystems can lead to failures as well as to non-specific false positive PCR amplifications.
- DNA samples may be used immediately after isolation or stored at  $-20^{\circ}\text{C}$  or below for extended periods of time (over 1 year) with no adverse effects on test results.
- Performance of the test can only be guaranteed if the enclosed instructions are strictly adhered to.
- This test should only be used for initial HLA typing. Other clinical and diagnostics findings should be used in addition, when determining suitability for transplant.
- Use of the Bio-Rad HLA SSP typing kit cannot resolve all combinations.

### 6.3 Quality Control

Each manufactured lot is checked against a panel of DNA samples representative of specific detected by the primers and it is available upon request.

Quality control of new lot numbers could be performed by typing a combination of known heterozygote allele combinations which will react with most of the primer mixes in the kit.

**6.4 Specific Performance Characteristics**

The Bio-Rad SSP Typing kit was compared with a SSP typing kit and a SSO typing system by testing 167 specimens (random transplant candidates and potential donors) at two geographically distinct locations. There was 100% concordance (167/167) between the Bio-Rad SSP Typing kit results and the results obtained using the SSP and SSO tests.

	Concordant	Discordant	Total	%
N	167	0	167	100
%	100%	0%		

N=Number of samples

## 7. Troubleshooting

PROBLEM	CAUSE	SOLUTION
No visible bands.	Ethidium bromide is missing in the gel.	Redye gel in the dye bath (1xTBE buffer with 0.5 µg/ml ethidium bromide).
MW standard visible PCR products not visible	Incorrect PCR preparation: Forgotten to add DNA or Taq-Polymerase	Repeat the PCR preparation
	Wrong temperature cycle conditions	Check PCR program in the thermal cycler. The indicated program applies to the recommended thermal cyclers PE 9600, PE 9700. Other thermal cyclers than the recommended ones must be validated by the user. Other thermal cyclers than the recommended ones may have different heating and cooling speeds. <b>Possible variations of the PCR program:</b> If non-specific amplifications occur, more stringent conditions can be established by incrementally increasing the annealing temperature by 1°C. On the other hand, false negative amplifications can be caused by PCR conditions that are too stringent. In such a case an incremental decrease in the annealing temperature by 1°C is recommended. Increase denaturation time from 10 to 20 seconds. Increase extension time from 30 to 60 seconds.
Failure of one or several PCR amplifications (control bands and specific amplifications).		If 1 or 2 PCR amplifications fail, you must check whether their positive evaluation leads to a typification result. If this is the case you must repeat the test. If the positive evaluation of the missing amplifications does not lead to a typification result and if there is definite proof of the presence of two alleles, no further measures need to be introduced. However, if only 1 allele is found, the test must be repeated. If more than 2 PCR amplifications fail, the test must be repeated.
Weak specific bands in the gel; no or only very weak control bands.	Use of insufficient amount of DNA.	Double the amount of DNA (reduce dH <sub>2</sub> O in preparation); use around 100 ng DNA per PCR preparation.
	PCR inhibitors such as ethanol, haemoglobin, heparin, beads contained in preparation.	Use EDTA or citrate blood as base material; (after the DNA pellet has been washed with ethanol, ensure that it is sufficiently dry).
	pH value of the DNA solution is too acid (PCR cocktail changes colour after adding DNA)	Precipitate DNA once more and dissolve in aqua dest.
	Wrong temperature cycle conditions.	See above
Weak specific bands in the gel,	DNA concentration are too high	Dilute the DNA; overly high concentrations of DNA or DNA that has not been dissolved properly can in some cases lead to a failure of the specific bands.
	DNA dissolved in a buffer that contains an inhibitor for the PCR reaction	Precipitate DNA once more and dissolve in aqua dest.
	Wrong temperature cycle conditions	See above
Control bands exist, <b>specific bands exist but in addition</b> one or several weak non-specific bands occur ( <b>false positive reactions</b> )	DNA concentrations are too high	Dilute the DNA.
	Cross reactions with other alleles	Check the reaction pattern.
	<b>Insufficient DNA quality or contamination of DNA</b>	Isolate new DNA
Bands in the PCR mix of the negative control	<b>By mistake DNA was pipeted into the mix of the negative control</b>	<b>Possibly repeat preparation or make a note of it in the evaluation documents</b>
	Contamination of the reagents	Exchange reagents

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