HLA SSP Typing Kits

SSP reagent kit for DNA based HLA typing

Product	REF	Package		
HLA-A	800 111	24 Tests	CE 0197	
HLA-B	800 112	24 Tests	CE 0197	
HLA-DR	800 113	24 Tests	CE 0197	
HLA-C	800 114	24 Tests	CE	
HLA-DQ	800 115	24 Tests	CE	
HLA- DRDQ	800 116	24 Tests	CE 0197	CE
HLA-ABC	800 117	20 Tests	CE 0197	CE
HLA-ABDR	800 110	20 Tests	CE 0197	
HLA-ABDRDQ	800 118	20 Tests	CE 0197	CE

IVD

CE 0197

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Contents

1 Introduction

1.1 Intended Use

The HLA SSP Typing Kits are a “low resolution“ test system for the identification of HLA-A, B, C, DRB and DQB alleles from DNA using the PCR method.

1.2 Basic Principles

HLA means “Human Leukocyte Antigens“. This referred to a system of human tissue antigens. The antigens play an important role at the immune defense and thus in the diagnostic. They help the body to distinguish between own and foreign cells and activate the immune reaction at contact with foreign cells. If, by organ transplantation, the characteristics of the HLA system match insufficiently, it can cause the rejection of the organ by the patient. The more alike the HLA characteristics of the donor and patient are the lesser is the danger of rejection.

The principle of the polymerase chain reaction (PCR) is based on the amplification of DNA areas by elongating the sequence specific primers (SSP) with the help of thermo stable DNA polymerase. With oligonucleotids, whose sequences are completely complementary to the target sequence, the DNA sequence can be amplified more effectively than by primers with mismatches. Those cannot bind under the specific reaction terms to the DNA sequence and consequently there will be no elongating by the Taq DNA polymerase. At the developing of the SSP primer pairs it is important, that they are only complementary to one single allele or to a group of alleles. Primer pairs bind perfectly under controlled reaction conditions and produce an amplificate in the PCR reaction. Non-complementary primer pairs do not bind to the DNA and no amplification takes place.

1.3 Test Principle

Primer pairs are designed for typing the HLA class I genes A (24 primer pairs), B (48 primer pairs), C (16 primer pairs) and the class II genes DQB (8 primer pairs), DRB1 (21 primer pairs), DRB3(1 primer pair), DRB4(1 primer pair), DRB5(1 primer pair). For the combined testing of ABDRDQ the number of DRB1 and DQB primer pairs was reduced to 19 and 5 respectively. The PCR accomplished with these primers produce well-defined DNA fragments of different length. The Primer-Mixes also contain in addition to the Sequence Specific Primers (SSP) control primers of the human globin gene at a lower concentration. They act as an internal control for the PCR. They show if the PCR reaction had taken place and to exclude results based on inhibitors, non-sufficient DNA and displaced primers from other wells. For the PCR of the class I genes the length of the fragments is 1070 bp, and for PCR of the class II it is 429 bp. The amplified DNA fragments will be detected in an agarose gel by dyeing the double stranded DNA with ethidium bromide.

2 Reagents

2.1 Contents of the HLA SSP Typing Kits

- PCR Plates, containing the dried Primer/dNTP-Mixes. In one corner a black dot is applied as an orientation point.
- 2,5x PCR Mix (ready for use)
  The PCR Mix contains PCR buffer, cresol red, glycerol and detergents
- PCR cover seals (adhesive)
- Reaction pattern, primer position sheet, instructions for use, short instructions for use

2.2 Needed Materials, not provided by R.O.S.E. Europe GmbH

2.2.1 Sample Materials

- DNA
- UV spectrophotometer

2.2.2 PCR

- Taq-DNA polymerase (5 U/µl)
- Thermal cycler (e.g. GeneAmp® PCR 9600, GeneAmp® PCR 9700)
- Adjustable pipettes
- Multipipette
- Combitips
- Filtered sterile pipette tips
- Vortex
- Centrifuge
- Distilled water
- Vials

2.2.3 Gel Elektrophoresis

- Agarose
- Microwave or magnetic stirrer with hotplate
- Electrophoresis equipment
- Power supply
- Adjustable pipettes
- Filtered sterile pipette tips
- ethidium bromide (10mg/ml)
- DNA ladder (100-1000 bp marker)
- 1x TBE buffer
- Distilled water
- Gel documentation system

2.3 General Safety Instructions

- For in *in-vitro* diagnostic (IVD) use only
- The test has to be performed by well-trained and authorised qualified personnel.
- All reagents and samples should be handled as potentially infectious and the appropriate precautions should be taken.
- Use different pipettes for the Post- and the Pre-PCR area.
- Caution: **ethidium bromide** for dyeing the gels is **potentially carcinogenic**. Allow no contact to the skin; always wear special nitrile protective gloves while working.
- Caution: **Wear UV blocking glasses for eye protection**. Never look directly into the UV Light source while viewing or taking pictures of agarose gels.
- Do not use reagents past the expiration date printed on the label.
- Do not use reagents under suspicion of turbidity or microbial contamination.
- Used PCR plates are considered as potentially infectious and like the gels they should be destroyed according to the prevailing national guidelines.

For Material Safety Data Sheets for the HLA SSP Typing Kits make application at the R.O.S.E. GmbH.

2.4 Storage and Shelf Life

The SSP PCR plates have to be stored at 2-8 °C. The expiration date is printed on all labels. Opened packages should be used up within 4 weeks. In the meantime they should be sealed with tape, so no dampness can intrude the plates.

3 Instrument Requirements

3.1 Thermal Cycler

The HLA SSP Typing Kits are designed for the GeneAmp® PCR System 9700 of ABI. If using thermal cyclers of another type or another manufacturers they have to be validated by the user. For application of thermal cyclers without adjustable pressure plate an additional adaptor mat has to be attached for optimal heat transfer between the heat cover and the PCR plate.

The PCR program is shown in the following table.
Additionally set up the Ramp Rate 9600 at the GeneAmp® PCR System 9700.

Cycle		Temp.	Time
Denaturation		94 °C	2 min
10 cycles	Denaturation	94 °C	10 sec
	Annealing and Extension	65 °C	1 min
20 cycles	Denaturation	94 °C	10 sec
	Annealing	61 °C	50 sec
	Extension	72 °C	30 sec
Elongation		72°C	2 min
Final Hold		4 °C	∞

3.2 Gel Electrophoresis

The accomplishment of the gel electrophoresis is described in chapter 5.

4 DNA Material

All samples should be handled as potentially infectious and the appropriate precautions should be taken.

4.1 Isolation of DNA

The DNA can be isolated from human cells containing nucleus by each favored method. The easiest way is using cell suspension like blood, buffy coat or cultured cells.

4.2 Preparation of the Samples

Do not use blood samples containing heparin. It will lead to disruption an inhibition of the PCR. Use anticoagulants such as EDTA or sodium citrate as an addition to peripheral blood. Apply no blood without anticoagulants or blood that is several times frozen and thawed. It decreases the quantity and quality of the DNA.

4.3 DNA Quality

To achieve optimal PCR SSP amplification and typing, detect the optical density of the DNA by a spectrophotometer before the DNA is to be used. Measure the adsorption at a wavelength of A_{260} and A_{280}. The pureness of the DNA can be calculated by the ratio A_{260}/A_{280}. It should be between 1,6 - 1,8. Values < 1,6 indicate a contamination with protein. Using the adsorption of the wavelength A_{260} it is possible to calculate back on the amount of the DNA. 1 OD is equivalent to approx. 50 ng DNA/µl.
The DNA can be stored at –20 °C for a long time without loosing quality.

4.4 DNA Quantity

Extracted DNA should be diluted with distilled, sterile water. We suggest diluting in 10 mM Tris-HCl pH 7,0.

Apply the DNA at a concentration of 75 ng/µl (± 25 ng/µl) in each reaction.

5 Accomplishment

5.1 Needed Materials

See
2.1 Contents of the HLA SSP Typing Kits
2.2 Needed materials, not provided by R.O.S.E. Europe

5.2 PCR

5.2.1 Preparation of the PCR

1. Preparing the master mix following the table below.

Amount of PCR-Reactions	**24**	**32**	**48**	**96**
PCR Mix (µl)	110	148	220	440
Taq DNA Polymerase [5 U/µl] (µl)	1,8	2,4	3,5	7
DNA [50 ng/reaction] (µl)	27,5	37	55	110
Water (bidest) (µl)	135,7	182,6	271,5	543

2. Pipette 10 µl of the master mix in every well on the PCR plate containing the dried primer/dNTP-Mixes. For this purpose we suggest to use a dispenser and carefully pipette the master mix on the wall at the top of the well. This way ensures that the specific primers are not carried from one well to another. The black point serves as an orientation point.

3. Spin down the solution and seal properly with the PCR adhesive foil.

4. Put the plate into the thermal cycler and start the program (see 3.1 thermal cycler).

5.3 Gel Electrophoresis

The PCR products are identified using agarose gel electrophoresis and detected through ethidium bromide intercalation under UV light.

5.3.1 Preparation of the Gel electrophoresis

1 L 10x TBE buffer:

Tris	108 g
Boric acid	55 g
0,5 M EDTA (pH 8,0)	40 ml

1:9 final dilution of the 10x TBE buffer solution in demineralised water. The solution is used for running buffer and the preparation of the gel.

5.3.2 Performing Gel Electrophoresis

- Preparing a 2 % agarose gel:
  For one gel use 2 g agarose in 100 g TBE buffer. Cook the solution till the agarose is completely dissolved. Cool the solution down to approximately 50 °C and fill up with distilled water to 100 g. Add 1 µl ethidium bromide solution [10 mg/ml]. Caution: **ethidium bromide is potentially carcinogenic**. No contact to the skin, always wear special protective gloves!

Pour the agarose solution free of bubbles into a prepared gel tray, insert the combs for 96 wells and keep it at room temperature for at least 15 minutes.

- Performing the electrophoresis:
  After the gel is solidified, transfer it into the gel chamber, remove the comb and cover it completely with 1x TBE-buffer. Transfer the complete volume of the PCR from the plate (10 µl) into the gel lane (take notice of the marking on the plate). To check the size of the DNA fragments use an appropriate molecular weight standard (100-1000 bp) (optional). Connect the chamber to a power supply and accomplish the electrophoresis with the settings 8 V/cm (electrode gap) ~ 20 min. The migration distance of the bands should amount to 1-1,5 cm.

- Interpretation:
  Following the electrophoresis the products are made visible by UV Light and photographed for interpretation by a gel documentation system.
  Caution: **Wear UV blocking glasses for eye protection**. Never look directly into the UV Light source while viewing or taking pictures of the gels.

6 Interpretation

6.1 Results

The interpretation should be performed with the delivered worksheet. The usage of the right version has to be observed, do not use a version older than six month. For the current version of the worksheet make application at the R.O.S.E. Europe GmbH.

In addition to the Sequence Specific Primers (SSP) the primer mixes also contain control primers of the human globin gene at a lower concentration. They serve as an internal control for the PCR. For the HLA-A, HLA-B and HLA-C PCR the length of this fragment is 1070 bp, and for the HLA-DRB and HLA-DQ PCR it is 429 bp. The control band should be present in every successful PCR. If the SSP product is positive, the control band might be weaker or even entirely missing, because the reaction with the positive primer in the well is prefered.

Gel Interpretation:

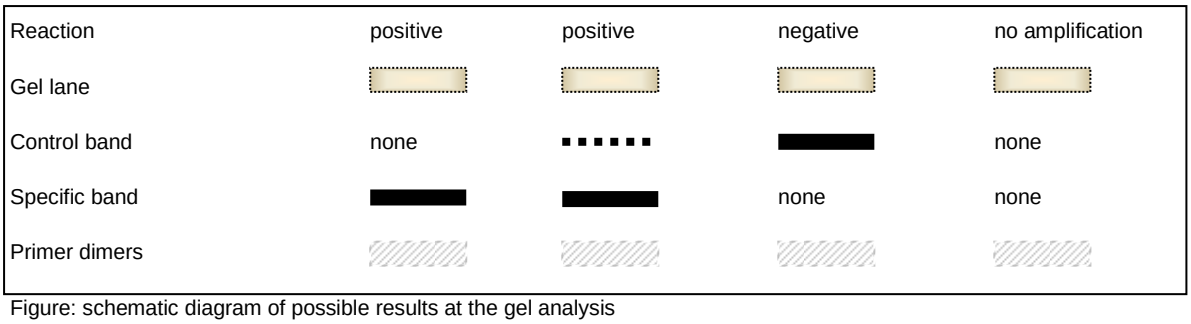

Figure: schematic diagram of possible results at the gel analysis

For the interpretation of the results it is important if a band is present in the gel or not. The composition of those positive bands identifies the HLA characteristics. The size of the fragments (using a molecular weight standard) can help to interpret the results, but it is not essential for the evaluation of the test. This way it is ensured that the results are not based upon false positive bands caused by unspecific reactions or specific primers carried from one well to another.

The interpretation should be performed with the delivered worksheet.

6.2 Quality Control

The quality control is important and essential for correct HLA typing.

Progression of the quality control at R.O.S.E. Europe GmbH

- Control of the primer using a selected DNA panel
- Testing of the kit elements
- Verifying of the whole kit

For accomplishment of a reception control of the lot use DNA with an allele combination covering as much as possible of the primer mixes.

6.3 Reliability of the HLA-DR SSP Typing Kit

The R.O.S.E. Europe GmbH regularly partakes at an interlaboraty comparison with the HLA SSP Typing Kits (External Testing). Additionally the Kit is verified with already tested DNA accumulated from China (In House Testing). The Chinese DNA was tested with other commercially available SSP Kits.
Results:

In House Testing

	DNA	Conformance	Deviation
Number	98	98	0
Percentage		100%	0%

External Testing:

	DNA	Conformance	Deviation
Number	91	91	0
Percentage		100%	0%

There is a 100% conformance with the results of the HLA SSP Typing Kits and the results of other SSO and SSP methods.

7 Limitation of the Procedure

1) Because of the high sensibility of the PCR the purity of the reagents should be obtained by working accurately.
2) To achieve correct typing, please adhere strictly to the Instructions for Use.
3) The HLA SSP Typing Kits are not able to detect all possible allele combination.
4) The HLA SSP Typing Kits are designed for the GeneAmp® PCR System 9700 of ABI. If using thermal cyclers of another type or another manufacturers they have to be validated by the user. Thermal cycler of other manufacturer can be used if they are licensed or feature equal or similar specification to the GeneAmp® PCR System 9700. We suggest, if using thermal cyclers of another type or another manufacturers to validate them.
5) This test system serves only as an initial HLA typing. Before starting transplantation or something similar consult other diagnostic and medical results to verify the outcome.
6) Check at a failure by 1 or 2 product bands how they would affect the interpretation if it were positives or negatives bands. Is this case unsure and if more than 3 failures occur, the test has to be repeated.
   The DNA should be applied in the specified quality and quantity, precisely because the intensity of the bands under UV Light depends on the amount of the received products.

8 Troubleshooting

Problem	Cause	Solution
No amplification (Absence of the control bands and the PCR products)	DNA is missing or the concentration is too low	Check, if the used amount of DNA is correct. Repeat the preparation and the PCR
	PCR inhibitors ➢ Blood containing heparin ➢ Buffer containing EDTA ➢ Proteins, ethanol, ...	➢ Do not use blood samples containing heparin. ➢ Repeat DNA extraction and dissolve DNA in sterile water ➢ Check DNA quality, if necessary extract again.
No visible bands	Ethidium bromide is missing in the gel	Put the Gel in a bath containing 1x TBE buffer and ethidium bromide to dye the gel afterwards.
Continuously weakening bands	Ethidium bromide is overage	Exchange ethidium bromide
	UV light source is defect	Exchange UV light source
Molecular weight standard is visible, PCR products are not visible	Taq polymerase or DNA is missing	Repeat the preparation and the PCR
	Wrong cycle parameters	Check the cycle parameters (Validated for ABI 9700), Calibrate thermal cycler/ if using other models please validate.
Failure of one or several PCR products		Check at a failure by 1 or 2 products how they would affect the interpretation if it were positives or negatives bands. Is this case unsure and if more than 3 failures occur, the test has to be repeated.
	Evaporation of the PCR solution during performing the PCR	Take care that the plate is completely sealed by the adhesive foil.
	Loss by pipetting	Accurate working.
	No effectual mixing of the master mix	Mix thoroughly before dispensing the master mix into the plate.
Weak specific bands and none or weak internal control bands	PCR inhibitors ➢ Blood containing heparin ➢ Buffer containing EDTA ➢ Proteins, ethanol, ...	➢ Do not use blood samples containing heparin. ➢ Repeat DNA extraction and dissolve DNA in sterile water ➢ Check DNA quality, if necessary extract again.
	PH value of the DNA solution too acid (PCR Mix changes color after adding DNA)	Repeat DNA extraction and dissolve DNA in sterile water.
	Wrong cycle parameters	Check the cycle parameters (Validated for ABI 9700), Calibrate thermal cycler/ if using other models please validate.
	DNA concentration is too low	Repeat the preparation and the PCR with a higher concentration of DNA.
	Taq polymerase: Either too low concentration or insufficient quality	Use another Taq polymerase or a higher concentration.
False positive	DNA concentration is too high	Dilute the DNA.
	Cross reaction with other alleles	Check the reaction pattern and the software.
	Annealing temperature is too low	Check the cycle parameters (Validated for ABI 9700), Calibrate thermal cycler/ if using other models please validate.
	Contamination or impure DNA	Exchange tips, reagents and vials. Check DNA quality, if necessary extract again.
False negative	Annealing temperature is too high	Check the cycle parameters (Validated for ABI 9700), Calibrate thermal cycler/ if using other models please validate.
Impure, blurred bands	Buffer of the gel electrophoresis is too warm	Cool down buffer. Use less voltage.
	Comb posses fat beaks	Using combs with thinner beaks.
No clear conclusion	New Allele; no interpretation with the reaction pattern or software possible	Check if the charge number of the reaction pattern chart is correct or if a new software version can be achieved (www.rose-europe.de).
	„False positive“ amplification	Check the length of the fragments by the molecular weight standard for a primer dimer or a contamination.
Weak control bands, unspecific bands caused by a very short DNA-Fragment	Formation of Primer-Dimers	Can happen at special primer-mixes. If gel is readable, don’t repeat PCR.

9 Bibliography

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