Instructions for use

HISTO TYPE SSP Kits

Low resolution

CE 0123

Test kits for tissue typing of HLA alleles
(Class I: HLA-A, B, C and Class II: HLA-DR, DQ)
on a molecular genetic basis

IVD

20 Typings
ready to use prealiquoted

REF 70721: HISTO TYPE A low	**(24 mixes, red)**
REF 70731: HISTO TYPE B low	**(48 mixes, white)**
REF 70741: HISTO TYPE C low	**(24 mixes, yellow)**
REF 70751: HISTO TYPE DR low	**(24 mixes, purple)**
REF 70761: HISTO TYPE DR mini	**(8 mixes, red)**
REF 70771: HISTO TYPE DRB3/4/5	**(4 mixes, white)**
REF 70891: HISTO TYPE DQB low	**(8 mixes, white)**
REF 7098: HISTO TYPE ABDR	**(96 mixes, white)**
REF 7102: HISTO TYPE ABC	**(96 mixes, blue)**
REF 7103: HISTO TYPE DR/DQ	**(32 mixes, white)**

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1. Product description

With the use of Polymerase Chain Reaction (PCR) in HLA typing much progress has been made recently. Sequencing of HLA alleles [1] has facilitated clear typing at the DNA level with a high resolution and has many advantages over the traditionally used serologic methods.

The basic material for typing with **HISTO TYPE SSP kits** is purified DNA. The test procedure is done by using the Sequence Specific Primers (SSP) -PCR (see Fig. 1) [2,3]. This method is based on the fact that primer extension, and hence successful PCR relies on an exact match at the 3'-end of both primers. Therefore, only if the primers entirely match the target sequence is amplification obtained which is subsequently visualized by agarose gel electrophoresis.

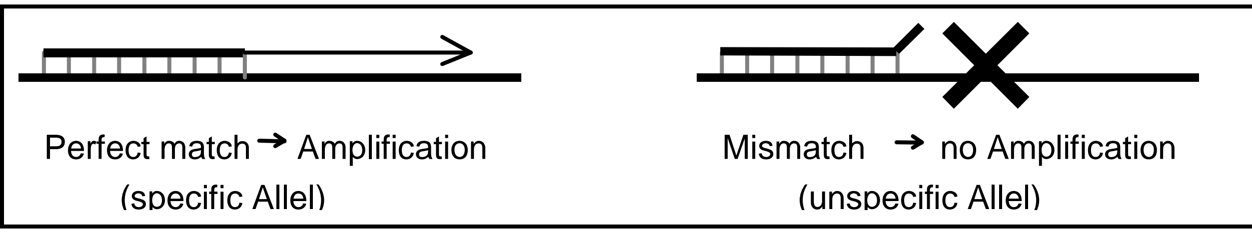

Fig. 1: Principle of SSP-PCR

The composition of the individual primer mixtures makes clear identification of the HLA Types indicated in the respective evaluation diagrams possible. With each typing a certain number (4, 8, 24, 32, 48 or 96) **prealiquoted** and **dried** reaction mixes including internal amplification control with a final volume of 10 µl are used.

2. Material

2.1 Contents of the HISTO TYPE SSP kits

- 20 HISTO TYPE plates sufficient for 20 HLA typings. The prealiqouted and dried reaction mixtures consist of allele specific primers, internal control primers (specific for the human G3PDH gene) and nucleotides. The first reaction mix is marked (please see mix-arrangement on page 7). In some HISTO TYPE products the contamination control is found in the last position of the plates (see attached lot specific specificity table and evaluation diagram). The lot number is printed on each plate/stripe.
- 24 x PCR reaction mixes contamination control with internal control primers and amplificate specific primers (not separately if the contamination control is integrated in the last position of the test plate).
- 10 x PCR-buffer sufficient for 20 typings
- Strip-caps or PCR foil sufficient for 20 typings
- instructions for use, specificity tabel, evaluation diagram and worksheet

2.2 Requirements and supplementary material

- Taq Polymerase (5 U/µl)
- **BAG EXTRA-GENE** (optional) Kit for DNA extraction from blood / lymphocytes / leucocytes or material for other DNA extraction methods
- piston pipettes (0,5-250 µl)
- sterile tips with integrated filter
- DNA Cycler (e.g. PTC 200 with a heated and adjusted cover, MJ Research/BioRad)

Devices and material for gel electrophoresis

- DNA agarose
- 0.5 x TBE buffer (45 mM of Tris, 45 mM of boric acid, 0,5 mM of EDTA)
- Ethidium bromide (EtBr)
- submarine electrophoresis unit
- power supply (200-300 V, 200 mA)
- DNA-length standard (Cat.-No.: 7097)

Devices for interpretation and documentation

- ♦ UV source (220-310 nm)
- ♦ camera (e.g. Polaroid system) with films (Polaroid type 667)

2.3. Storage and stability

The kit is delivered unfrozen. Store all reagents at -20…-80℃ in the dark. The expiry date is indicated on the label of each reagent and is also valid for opened reagents. The expiry date indicated on the outer label refers to the reagent with the shortest stability contained in the kit. Thaw the 10 x PCR-buffer shortly before use.

3. Data of performance

Analytical Sensitivity:	A reliable typing is guaranteed by using 50 - 80 ng DNA per reaction mix
Diagnostical Specifity:	The composition of the primer mixture guarantees a reliable identification of the HLA-Types (based on the latest sequence data) indicated in the evaluation diagram. Updates will be done regular.

For every lot the specificity of each primer mix was verified with DNA from reference samples: each PCR reaction has been positively tested at least once. Alleles, which are not included and cause of their rareness not tested respectively, are indicated on the evaluation diagram or specificity table.

A study of performance was done for all HISTO TYPE SSP kits with at least 50 DNA samples. The comparison of the test results with other typings, done with SSP kits of another supplier, showed no discrepancy.

4. Test procedure

4.1 Safety conditions and special remarks

The PCR is a particularly sensitive method and should be performed by well trained personnel, experienced in molecular genetic techniques and histocompatibility testing. Transplantation guidelines as well as EFI standards should be followed in order to minimize the risk of false typings, in the particular case of discrepancies in serological and molecular genetic method.

Special safety conditions must be noted in order to avoid contamination and thus false reactions:

- Wear gloves during work (powder-free, if possible).
- Use new tips with each pipetting step (with integrated filter).
- Use separate working areas for pre-amplification (DNA isolation and preparation of the reactions) and post-amplification (gel electrophoresis, documentation). Preferably use two separate rooms.
- Use devices and other materials only at the respective places and do not exchange them.

4.2 DNA isolation

For a HLA-SSP typing of one patient 5-10 µg of DNA (corresponding to approximately 0.5 ml of blood) is required. E.g. the **BAG EXTRA-GENE** kit is most suitable for isolation since pure DNA can be obtained from whole blood in a short time without the use of toxic chemicals or solvents. Also, other methods described in literature [5] such as the chloroform-triethyl-ammonium-bromide (CTAB) method or phenol-chloroform purification are suitable to provide DNA of sufficient purity. The presence of heparin potentially inhibits PCR [6]. Therefore EDTA or Citrate Blood is recommended for typing. DNA should have a purity index (extinction ratio OD_{260}/OD_{280}) between 1.5 and 2.0.

4.3 Amplification

All prealiquoted and dried reaction mixtures already contain allele and control-specific primers and nucleotides. These are supplied dried down in the reaction vessel. Amplification parameters are optimized to a final volume of 10 µl.

1.: Remove the required number of HISTO TYPE HLA-SSP plates from - 20℃ and thaw the 10 x PCR-buffer at room temperature.

2.: Pipet the Master-Mix consisting of 10 x PCR-buffer, DNA solution, Taq-Polymerase and Aqua dest. and mix well. The different HISTO TYPE SSP Kits do all work with the same Master-Mix and can therefore be combined. The composition of the Master-Mix depending on the number of reaction mixes is given in Table 1 (p.6).

If a **contamination control** should be performed, produce the Master-Mix without the DNA solution first and pipet 10 µl of this mix in the contamination control. Afterwards add the DNA solution and distribute the Master-Mix on the predropped reaction mixes.

Table 1: Composition of the Master-Mix depending on the number of reaction mixes:

no. of mixes	Aqua dest.	10 x PCR buffer	DNA-solution (50-80 ng/µl)	Taq-Polymerase (5 U/µl)	whole volume	
1	8	1	1	0.08	10	µl
4	63	8	8	0,6	80	µl
8	79	10	10	0.8	100	µl
24	222	28	28	2.2	280	µl
30	269	34	34	2.7	340	µl
32	285	36	36	2.9	360	µl
48	412	52	52	4.2	520	µl
54	459	58	58	4.6	580	µl
56	475	60	60	4.8	600	µl
72	618	78	78	6.2	780	µl
80	681	86	86	6.9	860	µl
96	808	102	102	8.2	1020	µl

⇨ for different DNA concentrations, the quantities of DNA and water must be varied accordingly (e.g. for 24 mixes: 11,7 µl DNA solution (120 ng/µl) and 238 µl Aqua dest.).

3.: After vortexing add **10 µl** of this mixture immediately to the pre-dropped and dried reaction mixtures. Change the tip after each pipetting step. Tightly close the tubes with the respective caps. Ensure that you do not touch the inner side of the caps and the upper edges of the tubes with the fingers so that contamination is avoided. If cyclers with tightly closable lid are used, it is also possible to use reusable PCR mats.

Marking	➔ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⑨ ⑩ · · · · ·

Slightly shake the plate downwards to dissolve the blue pellet at the bottom of the plate. All PCR solution should settle on the bottom.

4.: Place the reaction tubes into the thermal cycler and tighten lid so that the reaction vessels do not warp in heating. Start the PCR programme. Overlaying of the reaction mixtures with mineral oil is **not** required if a heated and adjusted cover is used!

Amplification parameters:

Programme-Step	**Temp.**	**Time**	**No. of Cycles**
First Denaturation	96°C	**5 Min**	1 Cycle
Denaturation	96°C	20 Sec	5 Cycles
Annealing+Extension	68°C	1 Min	
Denaturation	96°C	20 Sec	10 Cycles
Annealing	64°C	50 Sec	
Extension	72°C	45 Sec	
Denaturation	96°C	20 Sec	15 Cycles
Annealing	61°C	50 Sec	
Extension	72°C	45 Sec	
Final Extension	72°C	5 Min	1 Cycle

Cycler types:

PTC 100 / 200 (MJ Research/ BioRad), GeneAmp PCR-System 9600 / 9700 (use heating rate of 9600 please) (ABI) and Mastercycler epGradient S (Eppendorf)

By using thermal cyclers with a very fast heat- and coolingrate, it's recommended to use a slower heat- and coolingrate.

Since cyclers of different manufacturers perform differently and even individual machines of one type may be calibrated differently, it may be necessary to optimize the amplification parameters.

To optimize your machine use the following guide:
With **false positive** reactions (unspecific bands, additional types): Increase of the annealing temperature in 1℃ steps.
With **false negative** reactions (bands missing): Decrease of the annealing temperature in 1℃ steps and/or increase of the annealing periods in 5 second steps and/or increase of the denaturation periods in 5 second steps.

It's recommended to use only regularly calibrated cyclers. For this the BAG-Cycler Check kit is very suitable (Cat.-No.: 7104).
The quality control tests were done on a PTC-200 resp. 100 (MJ Research), 9700 (ABI) and Mastercycler epGradient S (Eppendorf)

4.4 Gel electrophoresis

Separation of the amplification products is done by electrophoresis via a (horizontal) agarose gel. As electrophoresis buffer, 0.5 x TBE (45 mM of tris, 45 mM of boric acid, 0.5 mM of EDTA) buffer is recommended. The gel concentration should be 2.0 - 2.5% of agarose. Allow the gel to polymerize at least 30 minutes before sample loading. After amplification has been finished, take the samples out of the thermal cycler and load the complete reaction mixtures carefully in each slot of the gel. In addition, apply 10 µl of the DNA length standard for size comparison. Electrophoretic separation is done at 10-12 V/cm (with 20 cm distance between the electrodes approx. 200-240 V), for 20-40 min.. After the run has been completed, the complete gel is stained in an ethidiumbromide (EtBr) solution (approx. 0.5 µg/ml of EtBr in H_2O or TBE buffer) for 30-40 min.. As alternative, EtBr (0.5 µg/ml) can also be added to the electrophoresis buffer or the agarose gel. If required, excess of EtBr can be removed by soaking the gel in H_2O or 0.5 x TBE buffer for 20-30 minutes.

4.5 Documentation and interpretation

For documentation, visualize the PCR amplification using an UV transilluminator (220-310 nm) and photograph it with a suitable camera, film and filters (e.g. polaroid, film type 667). Choose exposure time and aperture such that the bands are drawn sharp and stand out against the dark background (approximates aperture 11, exposure time 1 second).

For interpretation use the specificity table and evaluation diagram (see extra sheet); only bands that have the correct size compared to the DNA length standard should be considered positive. The correct sizes are given in the table resp. diagram. In all lanes without allele-specific amplification, the **1070 bp** internal control should be clearly visible. In most cases where there is allele-specific amplification the internal control is weaker or completely disappears! For improper results see troubleshooting (6.).

No band should be visible in the **contamination control**. If there is a contamination with genomic DNA there will be a band at 282 bp. Additional bands may occur at 78 bp, 104 bp, 176 bp and around 580 bp. If there is a contamination with amplificates bands will occur at 78 bp and/or 104 bp and/or 282 bp.

5. Warnings and Precautions

Ethidiumbromide is a powerful mutagen. Wear gloves when handling gels or solutions containing EtBr! Note the instructions for use and warnings and precautions of the manufacturer! The transilluminator radiates very short-wave UV light which may cause burns of the skin and the retina. Use a UV face safety mask!

All for extraction of DNA used biological material, e.g. blood or human tissue, should be handled as potentially infectious. When handling biological material appropriate safety precautions are recommended (do not pipet by mouth; wear disposable gloves while handling biological material and performing the test; disinfect hands when finished the test).

Biological material should be inactivated before disposal (e.g. in an autoclave). Disposables should be autoclaved or incinerated after use.

Spillage of potentially infectious materials should be removed immediately with absorbent paper tissue and the contaminated areas swabbed with a suitable standard disinfectant or 70% alcohol. Material used to clean spills, including gloves, should be inactivated before disposal (e.g. in an autoclave).

6. Troubleshooting

Problem	Possible Reason	Solution
no amplification, length standard visible	DNA contaminated with PCR-inhibitors	repeat DNA isolation, try different methods
	DNA concentration too high/too low	alter DNA concentration, repeat DNA isolation
	enzyme is missing or concentration too low	repeat typing, alter enzyme concentration
	DNA from heparinized blood	repeat typing with EDTA or citrate blood
	wrong amplification parameters	optimize the amplification parameters (see 4.3) ☆
repeated failure in single lanes (no amplification- control)	leak in reaction tubes; water loss and change in concentration during PCR	close tubes tight with caps; use other reaction tubes
unspecific amplification, additional bands, (additional bands of the wrong size must be neglected)	contamination with amplification products	repeat typing, ensure exact working
	DNA contaminated with salts	repeat DNA isolation, try different methods
	DNA concentration too high	use less DNA
	enzyme concentration too high	use less enzyme
	wrong amplification parameters	optimize the amplification parameters (see 4.3) ☆
evaluation shows more than 2 specificities	carry-over contamination (amplification products!) new allel	check typing mixtures (no DNA added) ensure exact working
no or only very weak bands visible, length standard invisible	EtBr staining too weak	repeat staining
gel background shines too bright	staining was too long, EtBr concentration too high	soak gel in H_2O or TBE lower EtBr concentration
blurred band	electrophoresis buffer too hot wrong electrophoresis buffer	lower the voltage use 0,5x TBE buffer

☆ When using the equipment and materials listed, optimisation of the amplification parameters should be looked upon as a last resort. In most cases, it is possible to evaluate the test by eliminating the additional bands caused by size discrepancies.

7. References

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8. Explanation of symbols used on Labelling

Symbol	Meaning
IVD	For in vitro diagnostic use
	Storage temperature
LOT	Batch code
	Use by
REF	Catalogue number
	Consult instructions for use