

Instructions for use
**KIR-TYPE /
Epitop-TYPE**
Low resolution

CE

Test kit for the typing of KIR-Genotypes and their HLA-ligands
on a molecular genetic basis

IVD

**10 Typings
ready to use prealiquoted**

**REF 7105: KIR-TYPE
REF 7106: Epitop-TYPE**

**(22 mixes, blue)
(6 mixes, green)**

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1. Product description

Natural killer cells (NK) and subpopulations of T-lymphocytes with a CD8⁺ memory phenotype (1) or $\gamma\delta$ T-cell-receptors express inhibitory and activating *Killer-cell Immunoglobulin-like Receptors* (KIRs). Due to differences in the amount of genes and to a distinctive polymorphism of single genes, the gene region of the KIR receptors shows a high variability within single individuals (3, 4). Meanwhile defined HLA class I molecules had been identified as ligands for single KIR receptors (5, 6). The inhibitory KIR2DL1 receptor binds to alleles of the HLA-C group 2 molecules, which has the amino acids Asn⁷⁷ and Lys⁸⁰, the KIR2DL2 / KIR2DL3 receptors to alleles of the HLA-C group 1 molecules, with the amino acids Ser⁷⁷ and Asn⁸⁰, and KIR3DL1 has an affinity to HLA-B alleles with a Bw4 epitope at the amino acid position 77-83 of the α 1 helix. The inhibitory receptor KIR3DL2 binds to alleles of the HLA-A*03 and *11 groups (7). The ligands for the activating KIR receptors are not documented sufficiently – though it is postulated, that they have an affinity to the same HLA-B and HLA-C molecules as their related inhibitory receptors.

The most accepted model of a NK cell activation at the moment, is the presumption, that the reactivity of the NK cells is controlled by a balance between inhibitory and activating signals. Thus an activation of NK cells could be occur due to a reduction of inhibitory signals or to an increased ligand binding of activating receptors. In case of transformation processes (e.g. tumor diseases or virus infections) with an accompanied lost of the HLA expression as a ligand, the missing inhibitory signals result in an activation of the NK cells and lysis of the target cell. This observation forms the basis of the missing-self hypothesis, in that healthy tissue with a stabile HLA expression is spared of a NK cell activation (8).

Particularly a large number of studies has demonstrated that HLA/KIR disparity leads to donor versus recipient NK cell reactivity in bone marrow transplantation resulting in the reduction of Graft versus Host Disease (GvHD) and relapses (9). Furthermore defined KIR genotypes could be associated with autoimmune diseases (e.g. Psoriasis), reduced progression of full-blown AIDS in HIV patients, the risk of preeclampsia and acute rejection after an allogeneic kidney transplantation.(10-14).

The **KIR-TYPE** kit allows the genotyping of 14 KIR genes and 2 pseudogenes. On the other hand the **Epitop-TYPE** kit detects the alleles of the HLA specificities HLA HLA-Cw Asn⁸⁰, HLA-Cw Lys⁸⁰, HLA-B Bw4^{Threo}, HLA-B Bw4^{Iso} and HLA-A Bw4.

The detection of the single KIR receptors / KIR HLA ligandes is performed by applying the PCR-SSP^① (*PCR-sequence-specific primers*) method (see Fig. 1) (13, 15).

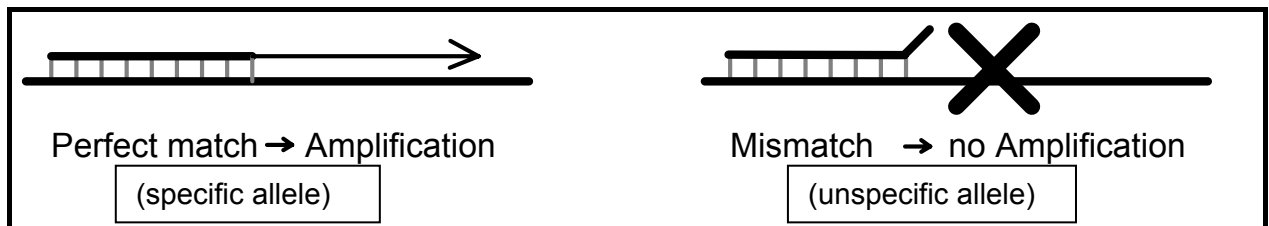


Fig. 1: Principle of SSP-PCR

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.

The selection of the sequence specific primers enables the detection of the single KIR / HLA genes on genomic DNA basis.

The composition of the individual primer mixtures makes clear identification of the KIR genotypes / HLA specificities indicated in the respective evaluation diagram possible. With each typing a certain number **prealiquoted** and **dried** reaction mixes including internal amplification control with a final volume of 10 µl are used.

2. Material

2.1.1 Contents of the KIR-TYPE kit

- ◆ 10 KIR-TYPE plates sufficient for 10 KIR typings. The predropped and dried reaction mixtures consist of allele specific primers, internal control primers (chromosome 1 specific sequence) and nucleotides.

The first reaction mix is marked and contains the contamination control / negative control with internal control primers and amplificate specific primers (see lot-specific specificity table and evaluation diagram) The lot number is printed on each plate.

- ◆ Mix 22 includes the positive control (only the internal control primers)
- ◆ 10 x PCR-buffer sufficient for 10 typings
- ◆ 8er strip-caps à 12 sufficient for 10 typings
- ◆ instructions for use, worksheet with specificity table and evaluation diagram

2.1.1 Contents of the Epitop-TYPE kit

- ◆ 10 Epitop-TYPE stripes sufficient for 10 Epitop typings. The predropped and dried reaction mixtures consist of allele specific primers, internal control primers (chromosome 1 specific sequence) and nucleotides.

The first reaction mix is marked (print of the lot number).

- ◆ The last mix includes the contamination control / negative control (see lot-specific specificity table and evaluation diagram)
- ◆ 10 x PCR-buffer sufficient for 10 typings
- ◆ 8er strip-caps à 12 sufficient for 10 typings
- ◆ Instructions for use, worksheet with specificity table and evaluation diagram

2.2 Requirements and supplementary material

- ◆ Taq Polymerase (5 U/μl) (e.g. Qiagen)
- ◆ **BAG EXTRA-GENE** kit (optional) 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
- ◆ DNA-length standard (REF 7097)
- ◆ power supply (200-300 V, 200 mA)

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°C 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 Specificity: The composition of the primer mixture guarantees a reliable identification of the KIR genotypes / HLA specificities (based on the latest sequence data) indicated in the evaluation diagram. Updates will be done regularly.

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 the KIR-TYPE / Epitop-TYPE kit 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- / DGI-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 observed 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 the typing of one patient at least 1,5 - 3 µg of DNA (corresponding to approximately 0.2 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 [16] such as the chloroform-triethyl-ammonium-bromide (CTAB) method or phenolchloro-form purification are suitable to provide DNA of sufficient purity. The presence of heparin potentially inhibits PCR [17]. 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 reaction mixtures already contain allele and control-specific primers and nucleotides. These are supplied dried down in the reaction vessels. Amplification parameters are optimized to a final volume of 10 μ l.

- 1.: Remove the required number of KIR-TYPE plates / EpiTop-TYPE stripes from - 20°C 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 KIR-TYPE / EpiTop-TYPE kit works with the same Master-Mix as other HISTO TYPE SSP Kits do and can therefore be combined. The composition of the Master-Mix is given in Table 1 (p.8).

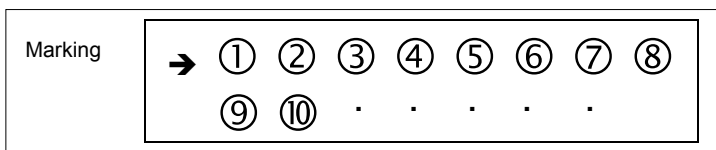
If the **contamination control** should be performed, produce the Master-Mix without the DNA solution first and pipet 10 μ l of this mix in the contamination control. Afterwards add the DNA solution and distribute the Master-Mix on the predropped reaction mixes.

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/ μ l)	Taq-Polymerase (5 U/ μ l)	whole volume	
1	8	1	1	0,08	10	μ l
6	63	8	8	0,64	80	μl
22	206	26	26	2,1	260	μl
28	253	32	32	2,6	320	μ l
30	269	34	34	2,7	340	μ l
46	396	50	50	4,0	500	μ l
54	459	58	58	4,6	580	μ l
70	602	76	76	6,1	760	μ l
118	982	124	124	9,9	1240	μ l

⇒ for different DNA concentrations, the quantities of DNA and water must be varied accordingly.
(e.g. for 22 mixes: 10,8 μ l DNA solution (120 ng/ μ l) and 195 μ l Aqua dest.).

3.: After vortexing add **10 µl** of this mixture immediately to the predropped 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.

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 program. 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	94°C	2 Min	1 Cycle
Denaturation	94°C	15 Sec	10 Cycles
Annealing	65°C	50 Sec	
Extension	72°C	45 Sec	
Denaturation	94°C	15 Sec	20 Cycles
Annealing	61°C	50 Sec	
Extension	72°C	30 Sec	

Cycler types:

PTC 100 / 200
(MJ Research/ BioRad)
and
GeneAmp PCR-System
9600 / 9700 (use the heating rate of 9600!!!)
(ABI)

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°C steps.

With **false negative** reactions (bands missing): Decrease of the annealing temperature in 1°C 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 (REF 7104).

The quality control tests were done on a PTC-200 resp. 100 (MJ Research) and 9700 (ABI).

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 for 30-40 min. in an ethidiumbromide (EtBr) solution (approx. 0.5 µg/ml of EtBr in H₂O or TBE buffer). 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 H₂O 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 worksheet with the specificity table and evaluation diagram. 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 **659 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 amplicates 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 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 allele	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 ₂ O 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

	For in vitro diagnostic use
	Storage temperature
	Batch code
	Use by
	Catalogue number
	Consult instructions for use

Notice to Purchaser: Disclaimer of License

① The SSP-Method correspond with the covered name ARMS™ of the firm ZENECA, Manchester. This method is covered under the european patent number 0 332 435 B1 and is used with the consent of the firm ZENECA.



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