

Instructions for use

KIR-TYPE Epitop-TYPE

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Low resolution

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



**10 Typings
ready to use prealiquoted**

REF	7105	KIR-TYPE
REF	7106	Epitop-TYPE

Contents

1. Product description	2
2. Material.....	3
2.1.1 Contents of the KIR-TYPE kit	3
2.1.2 Contents of the Epitop-TYPE kit	3
2.2 Requirements and supplementary material.....	3
2.3 Storage and stability	3
3. Data of performance	3
4. Test procedure	4
4.1 Safety conditions and special remarks.....	4
4.2 DNA isolation	4
4.3 Amplification	4
4.4 Gel electrophoresis	6
4.5 Documentation and interpretation	6
5. Warnings and precautions	6
6. Troubleshooting	7
7. References	8
8. Explanation of symbols used on Labelling	9

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 number of genes and strong 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 have 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 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 accompanying loss 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 that healthy tissue with a stable 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 can 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-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 (*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 worksheet possible. With each typing a certain number **prealiquoted** and **dried** reaction mixes including internal amplification control with a final volume of 10 μ l are used.

2. Material

2.1.1 Contents of the KIR-TYPE kit

- ◆ KIR-TYPE plates for KIR typing. The pre-dropped 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. The last mix includes the positive control (only the internal control primers). The lot number is printed on each plate.
- ◆ 10x PCR-buffer
- ◆ 8er strip-caps
- ◆ Instructions for use, worksheet, specificity table

2.1.2 Contents of the Epitop-TYPE kit

- ◆ Epitop-TYPE strips for the Epitop typing. 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.
- ◆ 10x PCR-buffer
- ◆ 8er strip-caps
- ◆ Instructions for use, worksheet

2.2 Requirements and supplementary material

- ◆ Taq Polymerase (5 U/ μ l), Happy Taq (REF 70976) or another Taq Polymerase validated with the KIR-TYPE / Epitop-TYPE kits by the user
Don't use a Hot-start Taq Polymerase please!
- ◆ **EXTRA-GENE I** Kit (REF 7059) (optional) for DNA extraction from blood /lymphocyte/ leucocytes or material for other DNA extraction methods
- ◆ piston pipettes (0.5-250 μ l)
- ◆ sterile tips with integrated filter
- ◆ DNA Cycler (list of the validated cyclers please see page 5)
- ◆ 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)
- ◆ UV source (220 - 310 nm)
- ◆ Gel documentation system

2.3. Storage and stability

The kit is delivered at ambient temperature. Upon receipt please store all reagents at $\leq -20^{\circ}\text{C}$ or $2\text{...}8^{\circ}\text{C}$ in the dark (**Avoid frequent changes of the storage temperature!**). Store in temperature monitored devices.

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.

3. Data of performance

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 worksheet. Updates will be done regularly.

The accuracy and reproducibility of the specificity of each primer mix was verified with DNA from control samples, with known KIR- / Epitop-specificities. Alleles, which are not included and because of their rareness could not be tested respectively, are indicated on the worksheet / specificity table.

A performance evaluation study was done for the KIR-TYPE kit and the 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.

The evaluation and quality control of the mixes were done with DNA samples, which were extracted by EXTRA GENE I (salting out method) or Qiagen kits (column based method).

KIR-TYPE and Epitop-TYPE are validated with the Happy Taq (REF 70976). When another Taq Polymerase is used, the enzyme must be validated with the KIR-TYPE / Epitop-TYPE kits by the user.

A reliable typing is guaranteed by using 50 - 80 ng DNA per reaction mix.

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 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

The **BAG EXTRA-GENE I** kit is most suitable for the DNA isolation since pure DNA can be obtained from whole blood in a short time without the use of toxic chemicals or solvents. Furthermore commercial column- or beads-based methods or other methods described in literature are suitable to provide DNA of sufficient purity. The presence of heparin potentially inhibits PCR [5]. Therefore EDTA or Citrate Blood is recommended for typing.

DNA should have the following purity indexes:

- $OD_{260}/OD_{280} = >1.5$ and <2.0 (indicator for contamination with RNA/proteins)
- $OD_{260}/OD_{230} = >1.8$ (indicator for contamination with salt, carbohydrate or organic solvents)

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 strips and the 10x PCR buffer from the kit.

2. Pipet the Master-Mix consisting of 10x 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.

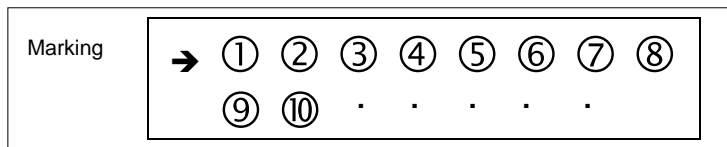
If the **contamination control** should be performed, produce the Master-Mix without the DNA solution first and pipet 10 μ l of this mix in the contamination control. Afterwards add the DNA solution to the remaining Master-Mix and mix well.

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

No. of mixes	Aqua dest.	10x PCR buffer	DNA-solution (25 - 40 ng/μl)	Happy Taq (5 U/μl)	whole volume	
1	7	1	2	0,08	10	μl
6	55	8	16	0,64	80	μl
22	180	26	52	2,1	260	μl
28	221	32	64	2,6	320	μl

- ⇒ The quantity of DNA should be 50 – 80 ng per mix. According to DNA-concentration, the amount of DNA and water must be varied. (e.g. for 22 mixes: 26 μl DNA solution (50 ng/μl) and 206 μl Aqua dest.).
- ⇒ If another Taq Polymerase shall be used, the enzyme must be validated with the KIR-TYPE kit / Epitop-TYPE kit by the user.

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 to avoid contamination. If cyclers with tightly closable lid are used, it is also possible to use reusable PCR mats. Slightly shake the plate/strip to dissolve the pellet at the bottom of the plate/strip. All PCR solution should settle on the bottom. If necessary the plate/strip should be briefly spun down.



4. Place the reaction tubes firmly into the thermal cycler and tighten the lid well. Start the PCR program. Overlaying of the reaction mixtures with mineral oil is **not** required if a heated and adjusted lid 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	

Validated Cycler types

PTC 100 / 200 / C1000 (MJ Research/ BioRad),

GeneAmp PCR-System 9700 (use heating rate of 9600 please), Veriti (ABI),

Mastercycler epGradient S (use “simulate Mastercycler gradient” function please) (Eppendorf)

Tprofessional (Biometra)

Please don't use an aluminium heating block (e.g. GeneAmp PCR-System 9700).

By using thermal cyclers with a very fast heating and cooling rate, it is recommended to use a reduced ramp rate (~2.5°C/sec).

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. If other models than the validated cyclers mentioned above are used they have to be validated by the user.

To optimize your machine use the following guide:

If there are **false positive** reactions (unspecific bands, additional types), increase the annealing temperature in 1°C steps.

If there are **false negative** reactions (bands missing), decrease the annealing temperature in 1°C steps and/or increase of the annealing times in 5 second steps and/or increase of the denaturation times in 5 second steps.

It's recommended to use exclusively cyclers that are calibrated regularly. For this the CYCLER CHECK kit is well suited (REF 7104, 71044).

The quality control tests were done on a PTC-200 resp. C1000 (MJ Research / BioRad), 9700 (ABI), Mastercycler epGradient S (Eppendorf) and Tprofessional (Biometra).

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 minutes. After the run has been completed, the complete gel is stained for 30 - 40 minutes 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 a suitable gel documentation system. Choose exposure time and aperture such that the bands are drawn sharp and stand out against the dark background.

Only bands that have the correct size compared to the DNA length standard should be considered positive. The correct sizes are given in the worksheets. 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!

If neither a specific band nor the internal control band appears, the result with the relevant mix can not be used for evaluation. Possible reasons for not evaluable 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 176 bp and/or 282 bp and/or 580 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).

Disposal of all samples, unused reagents and waste should be in accordance with country, federal, state and local regulations.

A declaration on Material Safety Data Sheets (MSDS) is available to download at www.bag-healthcare.com.

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
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 method
	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	staining too weak	repeat staining
gel background shines too bright	staining was too long, staining solution concentration too high	soak gel in H ₂ O or TBE, lower staining solution concentration
blurred band	electrophoresis buffer too hot or used up, wrong electrophoresis buffer, polymerisation of the gel not well	lower the voltage, use 0.5x TBE buffer, use completely polymerised gel

☆ 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

	Storage temperature / Temperature limitation
	Storage temperature / Lower limit of temperature
	Use by
	Consult instructions for use
	Sufficient for n tests
	Caution
	Manufacturer
CONT	Content, contains
CONTROL CC	Contamination Control
IFU	Instructions for use
IVD	For in vitro diagnostic use
KIR HLA-LIGAND TYPING	Intended use: Determination of KIR (Killer-cell Immunoglobulin-like Receptors)-HLA-Ligands
KIR TYPING	Intended use: Determination of KIR (Killer-cell Immunoglobulin-like Receptors)-Genotypes
LOT	Batch code
OR	Or
PCRBUF 10x	PCR buffer, 10x concentrated
PCRCAP	PCR caps
PCRPLATE	PCR plates
PCRSTRIP	PCR strips
REACTIONMIX	Reaction mixes
REF	Catalogue number
RTU	Ready to use
WORKSHEET	Worksheet

Instructions for use in other languages see:

<http://www.bag-healthcare.com> <http://service.bag-healthcare.com> or phone: +49 (0)6404-925-125



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