MACS molecular

KIR Typing Kit

Genotyping and gene expression profiling of human KIR genes

8 tests	130-092-551
24 tests	130-092-584

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1. Description

This product is for research use only.

Components	**2×96-well KIR Typing PCR Plate** containing lyophilized Enzyme Mix **8×600 µL Resuspension Buffer** **4 Sealing Foils** **1 Evaluation Form** Please use a copy of the Evaluation Form or download it from **www.miltenyibiotec.com**.
Capacity	8 tests ▲ **Note:** Kit for 24 tests contains 3× the kit components for 8 tests.
Product format	2×96-well KIR Typing PCR Plate containing lyophilized Enzyme Mix in 4×22 wells. Please note that the wells G3+H3, G6+H6, G9+H9, and G12+H12 are empty.
Storage	Store KIR Typing Kit contents protected from light at 2–8 °C. **Do not freeze!** The expiration dates are indicated on the labels.

1.1 Principle of the KIR Typing Kit

The KIR Typing Kit allows the detection of human killer cell immunoglobulin-like receptor (KIR) genes at the level of genomic DNA or mRNA. The presence or absence of KIR genes is analyzed by PCR technology using sequence-specific primers (SSPs). Carefully designed SSPs enable the detection of all known 15 human KIR genes plus two pseudo genes. Differentiation between 2DS4del versus 2DS4ins is also possible.

Every KIR typing PCR reaction contains an internal control, generated by a second pair of PCR primers. Thereby, two PCR fragments should be generated from the sample template: One is derived from the KIR-specific primers and the second is amplified by the internal control primers.

The Enzyme Mix is lyophilized in the wells of the 96-well KIR Typing PCR plate. To set up the KIR typing PCR reaction, the Resuspension Buffer is simply mixed with the template (genomic DNA or cDNA) and dispensed into the wells of the PCR plate. The Enzyme Mix contains Taq DNA-Polymerase and loading buffer; thus, the PCR products can be directly subjected to electrophoretic analysis in agarose gels (2%).

PCR with sequence-specific primers (SSP-PCR) is a method which amplifies and detects single-nucleotide polymorphisms. The SSP-PCR amplification is performed by using primers with at least one different base pair at the 3'-end of one or both primer oligonucleotides. PCR products are separated by electrophoresis in an agarose gel. No visible band in the gel indicates the absence of the analyzed target for the corresponding primer pair and a band of expected size the presence of the target gene under investigation.

1.2 Background information

KIRs can be found on natural killer (NK) cells which play a critical role in the innate immune response to viral infection and tumor cell lysis. Furthermore, NK cells have been implicated in the suppression of graft versus host disease, promotion of bone marrow engraftment, and mediation of graft versus leukemia effect.[1,2] The KIRs exist in various isoforms. For example, inhibitory KIRs interact with specific HLA class I molecules, predominantly HLA-C, on target cells.

Not all of the 15 KIR genes are present in every human individual. Further heterogeneity exists at the transcriptional level. Different subsets of NK cells may express different KIRs, even within one individual.

1.3 Applications

- Investigation of the KIR gene constellation on the DNA level and its expression profile on mRNA level in the sample of interest.

1.4 Reagent and instrument requirements

- Pipette and tips
- Plate holder
- Dispensing (electronic) pipettes
- Eight-channel pipette
- 96-well thermal cycler **with heated lid**
- TAE electrophoresis buffer (40 mM Tris base, 1 mM EDTA, 1.1 mL/L pure acetic acid)
- DNA grade agarose
- Ethidium bromide (10 mg/mL)

Caution: Ethidium bromide is a mutagen. Handle only with appropriate personal protection!

- DNA molecular weight marker (100–10,000 bp)
- Gel electrophoresis power supply and electrophoresis system
- Gel documentation system

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MACS Miltenyi Biotec

Miltenyi Biotec GmbH
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macs@miltenyibiotec.de
www.miltenyibiotec.com

Miltenyi Biotec Inc.
2303 Lindbergh Street, Auburn, CA 95602, USA
Phone 800 FOR MACS, +1 530 888 8871, Fax +1 877 591 1060
macs@miltenyibiotec.com

2. General protocol for the KIR Typing Kit

▲ Do not freeze the KIR Typing PCR Plate. When setting up the PCR reaction, keep the plate at room temperature (19–25 °C).

2.1 Sample requirements and preparation

One-step mRNA isolation and cDNA synthesis

Protocol 1: For whole blood, we recommend to use PAXgene® Blood RNA Tubes (PreAnalytiX®) for RNA stabilization in combination with the µMACS™ One-step cDNA Kit (# 130-091-902). Alternatively, mRNA can be isolated by using the µMACS mRNA Isolation Kits (# 130-075-201, # 130-075-101).
A special protocol for cDNA synthesis with the µMACS One-step cDNA Kit or the µMACS mRNA Isolation Kit is available at www.miltenyibiotec.com/protocols.

Protocol 2: For other sample material, use the µMACS One-step cDNA Kit (# 130-091-902) or the µMACS mRNA Isolation Kit (# 130-075-201, # 130-090-276, # 130-075-101, # 130-090-277). For reliable mRNA expression profiling, up to 10 µg mRNA (approximately 5×10^6–1×10^7 cells) per test are needed.

▲ When working with small amounts of sample material, mRNA should be amplified prior to cDNA synthesis to ensure sufficient amounts of transcripts.

DNase digestion
Perform a DNase digestion of the RNA sample. Genomic DNA contamination could lead to false-positive results of the mRNA expression profile because primers may be located within one exon.

The KIR Typing Kit contains a genomic DNA control in well 20; any contamination of the mRNA with genomic DNA will result in a PCR product sized 260 bp.

Genomic DNA isolation
▲ Blood samples should be collected in a buffer containing EDTA; buffers with heparin may inhibit DNA amplification.

High-quality DNA is critical for optimal results. The OD values, measured by UV spectrophotometry, should range from 1.7–1.9. Genomic DNA isolation can be performed by any protocol that produces high-purity DNA, for example:
- Chemagic DNA Blood Kit (Chemagen)
- DNA Isolation Kit for Mammalian Blood (Roche Applied Sciences)
- QIAamp® DNA Blood Maxi Kit (QIAGEN®)

Sample preparation
▲ The DNA used should be intact. DNA should not be stored longer than three months because the integrity of the DNA might be affected by long-term storage.

Prepare template in 10 mM Tris / HCl pH 8 in these concentrations:

- Genomic DNA: 75–125 ng/µL (per typing 1.8–3.1 µg)
- cDNA from whole blood using protocol 1 (see 2.1): 2.5–10 ng/µL
- cDNA from other sources, e.g., isolated NK cells*: 5–10 ng/µL

▲ If it is necessary to amplify cDNA prior to KIR Typing, more (amplified) cDNA should be used for KIR typing. Thus, amount of cDNA might be increased, e.g., use 50–100 ng/µL if following protocol 2.

* Miltenyi Biotec provides various products for the isolation of NK cells, e.g., NK Cell Isolation Kit, human (# 130-092-657), CD56 MicroBeads, human (# 130-050-401), or Whole Blood CD56 MicroBeads, human (# 130-090-875).

2.2 Thermal cycler

Thermal cycler profile

Step 1	1 cycle	95 °C	60 s
Step 2	35 cycles for cDNA or		
	28 cycles for genomic DNA	94 °C	20 s
		63 °C	20 s
		72 °C	90 s
HOLD		4 °C	

Total reaction volume per well: 25 µL
The thermal cycler profile is optimized for Eppendorf and MJ Research cyclers with a temperature ramping rate of 3 °C / seconds. If other instruments are used, optimization of the thermal cycler profile may be necessary.

▲ Make sure all wells of the KIR Typing PCR Plate are in close contact to the heating block of the thermocycler used. Also ensure a tight closure of the lid. If necessary, use the appropriate adaptor delivered with your thermocycler. Insufficient or uneven conduction of heat to the individual KIR typing reactions will result in incomplete amplification and false results.

2.3 PCR and agarose gel electrophoresis

Resuspension Buffer is provided in aliquots (600 µL) for one test. One aliquot is dispensed in 22 wells (25 µL per well, refer to figure 1).

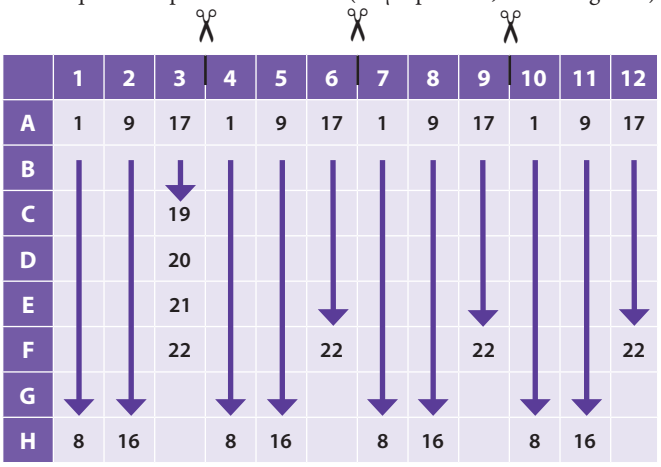

Figure 1: 96-well KIR Typing PCR Plate, for four tests (22 wells per KIR typing). All wells contain lyophilized Enzyme Mix, except PCR plate positions G3–H3, G6–H6, G9–H9, and G12–H12.

PCR wells 1–19 contain KIR-specific primers plus positive control primers. Thus, two PCR fragments can be amplified.

PCR well 20 is the control for genomic DNA contamination in cDNA templates. Only DNA contamination will generate a band of 260 bp.

PCR well 21 is the positive control of PCR reaction (only control-specific primers will generate a single band of 400 bp).

PCR well 22 serves as a negative control, do not add DNA template.

Set up PCR experiment

1. Take one KIR Typing PCR Plate from refrigerator. Ensure that the lyophilized Enzyme Mix is at the bottom of each well. To place the lyophilizate at the tip of the well, gently slap the plate onto the bench.

   ▲ **Note:** Only plates at room temperature will be closed optimally by sealing foil.

   ▲ **Note:** If you perform KIR typing of only one sample, use a pair of scissors to cut the PCR plate as indicated in figure 1.

2. Place the plate in a plate holder and carefully remove cover foil.
3. Use one aliquot of Resuspension Buffer per experiment. First, set up negative control (without DNA): Add 25 µL of Resuspension Buffer to well no. 22 (plate positions F3, F6, F9, or F12).
4. Dilute the template to the following final concentration.
   - Genomic DNA: 75–125 ng/µL (per typing 1.8–3.1 µg)
   - cDNA from whole blood using protocol 1 (see 2.1): 2.5–10 ng/µL
   - cDNA from other sources, e.g., isolated NK cells: 5–10 ng/µL
5. Add 25 µL of the diluted genomic DNA or cDNA to the remaining Resuspension Buffer (approximately 575 µL) and vortex briefly.
6. For each test, dispense 25 µL Resuspension Buffer-DNA mix per well (21 wells per test). Pipette the buffer-DNA mix closely to the top of the well onto the sidewalls. Please pay attention not to touch the lyophilized Enzyme Mix with the pipette tip!

   ▲ **Note:** The lyophilized Enzyme Mix will turn from yellow to purple.
7. Remove back side from an adhesive Sealing Foil, place foil over the top of the PCR plate, and press it onto the plate. Make sure that the plate is completely sealed.

   ▲ **Note:** It is very important that the wells are tightly closed, especially at cut-off edges. High liquid loss may cause failure of the PCR reaction.
8. Check that the PCR mix is at the bottom of each well. Gently shake down the liquid to place it at the bottom, or centrifuge shortly. Begin thermal cycling as specified in section 2.2.
9. After thermal cycling, remove plate and proceed directly to gel electrophoresis.
10. Load a molecular weight marker and 8–10 µL per sample onto a 2% agarose gel.

    ▲ **Note:** Run the gel electrophoresis until the pink dye is in the middle of the gel. The purple dye runs at an equivalent of 500 bp.

Ensure a sufficient resolution by a long gel run to support a clear differentiation of amplicons.

▲ **Note:** Change of color from purple to yellow during the PCR reaction is due to liquid loss; usually, this will not impair the interpretation of results. If there is a high loss of fluid, please refer to 4. Troubleshooting.

3. Interpretation of gel electrophoresis results

3.1 Evaluation Form

1. After agarose gel electrophoresis, fix the photo of the agarose gel on the Evaluation Form in the designated field.
2. Check lanes 1 to 19 for KIR-specific PCR products of the expected size mentioned in the column "Amplicon size (bp)". If the KIR-band is clearly visible, mark the field in the column "Typing" (1–4) with a "+" (plus). Mark all other lanes with a "–" (minus).

   ▲ **Note:** Extremely weak bands are rather negative than positive results due to cross-reactivity.
3. Lanes 1–19 should also show the control band of 400 bp. The control primers are present in lower concentration to favor the allele-specific reaction.
4. In lane 20, the genomic DNA control, DNA contaminations in cDNA templates will produce a band of 260 bp. In lane 21, the positive control, a product of 400 bp should be visible.
5. Lane 22, the negative control, should remain empty.

3.2 Example of application

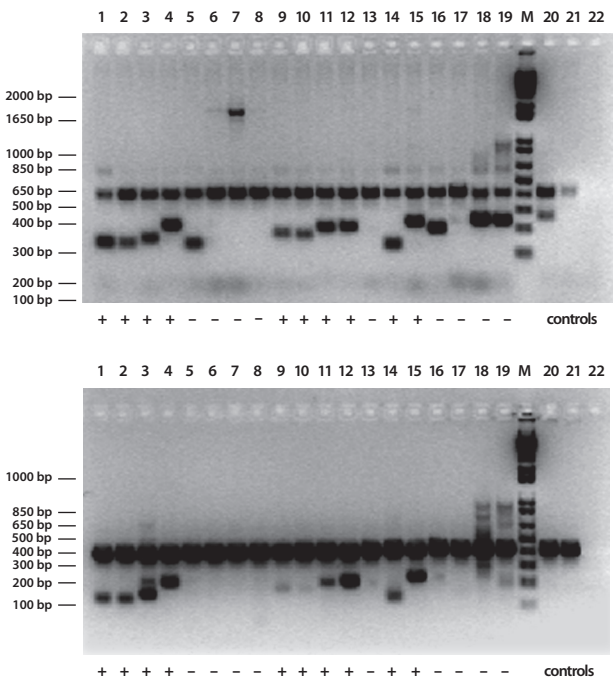

Figure 2: Gel fractionation of PCR products from whole-blood templates: A) genomic DNA; B) mRNA-derived cDNA (5 mL).

	Gene name	Plate pos.[1]	Amplicon size (bp)
1	2DL1	A1	148
2	2DL2	B1	145
3	2DL3	C1	161
4	2DL4	D1	221
5	2DL5all: 2DL5A; 2DL5B *	E1	136
6	2DL5A *	F1	DNA: ~1820 RNA: 325
7	2DL5B *	G1	DNA: ~1805 RNA: 286
8	2DS1	H1	148
9	2DS2	A2	177
10	2DS3	B2	172
11	2DS4del	C2	204
12	2DS4ins	D2	209
13	2DS5	E2	179
14	3DL1 **	F2	131
15	3DL2	G2	232
16	3DL3	H2	204
17	3DS1 **	A2	149
18	2DP1	B3	240
19	3DP1	C3	237
20	Genomic DNA control for contamination	D3	260
21	Positive control (β-actin)	E3	400
22	Negative control	F3	–

[1] These plate positions describe the first KIR typing test only.

* 2DL5: KIR2DL5 was found to embrace two distinct but closely related genes which were then distinguished by the names 2DL5A and 2DL5B. The recently discovered alleles 2DL5A*005 differs from the allele 2DL5B*002 in one base pair only. 2DL5B*002 is not expressed. 2DL5 is duplicated on some haplotypes.

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** KIR3DL1 and KIR3DS1: Citation IPD–KIR Sequence Database: Evidence exists indicating that the 3DL1 and 3DS1 genes behave as alleles of the same gene. It is possible that at some time in the future the alleles of these genes will be combined under one gene name. To avoid confusion, it was decided to name the alleles of both genes in a single numerical series; thus, 3DL1*001-3DL1*009 are followed by 3DS1*010-3DS1*014.

For a current list of allele specificities please refer to the Evaluation Form or to www.miltenyibiotec.com.

4. Troubleshooting

Problem	Possible cause	Solution
No or faint PCR products	Not sufficient DNA used.	Use amounts as indicated. Genomic DNA: 75–125 ng/µL (per typing 1.8–3.1 µg); cDNA from mRNA using protocol 1 (section 2.1): 2.5–10 ng/µL; cDNA and amplified cDNA from mRNA using protocol 2 (section 2.1): 50–100 ng/µL, e.g., white blood cells; cDNA from NK cells*: 5–10 ng/µL
	DNA not pure enough.	Make sure that A260/A280 is within 1.7–1.9; otherwise repeat DNA preparation. Use freshly prepared DNA.
	Not sufficient cDNA generated by reverse transcription.	Repeat reverse transcription with high-quality RNA.
	PCR inhibitor may be present, e.g., EDTA above 0.5 mM.	Use a DNA preparation with lower EDTA concentrations than 0.2 mM.
	No or not sufficient ethidium bromide is used in the agarose gel.	Generate a new agarose gel with ethidium bromide concentration of 0.5 µg/mL.
	The color of the PCR mix may turn from purple to yellow after amplification due to loss of fluid. That may be caused by a not tightly–closed reaction well. If the loss is too high, the PCR reaction is suppressed.	Repeat test and make sure that the PCR foil tightly closes the wells of the PCR plate.
	Lyophilized Enzyme Mixes might have been humidified, resulting in loss of activity of DNA polymerase.	Store the PCR plates with the lyophilized mix always in dry and cool environment (4 °C). The KIR Typing PCR Plates are sealed with silica gel pads inside of the reclosable bag.
No positive PCR control	The positive control PCR product may not amplify efficiently due to substrate competition. An absent positive control in addition to no specific KIR band indicates failed PCR reaction.	The typing habe to be repeated if failed reactions are observed.
PCR bands of unexpected size	The use of genomic DNA template in a concentration higher than 130 ng/µL per reaction may cause smear and non-specific PCR products.	Use the indicated amounts of template. Genomic DNA: 75–125 ng/µL (per typing 1.8–3.1 µg); cDNA from mRNA using protocol 1 (section 2.1): 2.5–10 ng/µL; cDNA and amplified cDNA from mRNA generated with protocol 2 (section 2.1): 50–100 ng/µL, e.g., white blood cells; cDNA from NK cells*: 5–10 ng/µL
	A PCR signal in the negative control (lane 22) indicates DNA contamination.	For genotyping: check pipette tips and use only sterile filter tips. For mRNA profiling: mRNA/cDNA is contaminated with DNA. Perform DNase digestion prior to reverse transcription.
	If only one single PCR product of non-KIR-specific size occurs, do not assess it as positive. If an additional band to the expected-size KIR product appears, it is considered as positive.	Check PCR product sizes and declare only bands as positive which have the correct size. Usage of PAXgene® System may reduce artefactual PCR products.
	Free primers will form a diffuse band below 50 bp. Primer dimers (<80 bp) usually appear as a fuzzy band in the agarose gel above the primer band, but below KIR-specific products.	Disregard these bands.

*see page 2, end of section 2.1

5. References

1. Ruggeri, L. *et al.* (2005) Natural killer cell alloreactivity for leukemia therapy. J. Immunother. 28: 175–182.
2. Arase, H. *et al.* (2002) Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. Science 296: 1323–1326.
3. Phillips, J. and Eberwine, J. H. (1996) Antisense RNA amplification: a linear amplification method for analyzing the mRNA population from single living cells. Methods 10: 283–288.

Warranty

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