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Gene-Specific PCR Typing of Killer Cell Immunoglobulin-Like Receptors

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Chapter 12

Gene-Specific PCR Typing of Killer Cell Immunoglobulin-Like Receptors

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Abstract

By interacting with specific HLA class I molecules, the killer cell immunoglobulin-like receptors (KIR) regulate the effector function of natural killer (NK) cells and subsets of CD8 T cells. The KIR receptors and HLA class I ligands are encoded by unlinked polymorphic gene families located on different human chromosomes, 19 and 6, respectively. The number and type of KIR genes are substantially variable between individuals, which may contribute to human diversity in responding to infection, malignancy and allogeneic transplants. PCR typing using sequence-specific primers (PCR-SSP) is the most commonly used method to determine KIR gene content. This chapter describes a step-by-step protocol for PCR-SSP typing to identify the presence and absence of all 16 known KIR genes. Moreover, the chapter provides the basic rules to verify the accuracy of KIR genotyping results and explains specific methods for the data analysis.

Key words Killer-cell immunoglobulin like receptors, KIR receptors, Natural killer (NK) cell receptors, KIR genotyping, KIR typing, Innate immune receptors, Leukocyte receptor complex (LRC)

1 Introduction

Killer cell immunoglobulin-like receptors (KIR) that recognize certain HLA class I molecules are expressed on natural killer (NK) cells and on subsets of T lymphocytes, mostly CD8 cells with memory phenotype [1–4]. Therefore, KIR receptors have the potential to contribute to both innate and adaptive immune responses against infections, tumors, and allogeneic transplants [5–7]. A family of 16 homologous genes clustered at the leukocyte receptor complex on chromosome 19q13.4 encodes KIR receptors [8–10]. Fourteen of which are functional genes encoding receptors that trigger either inhibition (3DL1-3, 2DL1-3, 2DL5) or activation (3DS1, 2DS1-2DS5) or both (2DL4) and two are pseudogenes (2DP1 and 3DP1) that do not encode a cell-surface receptor.

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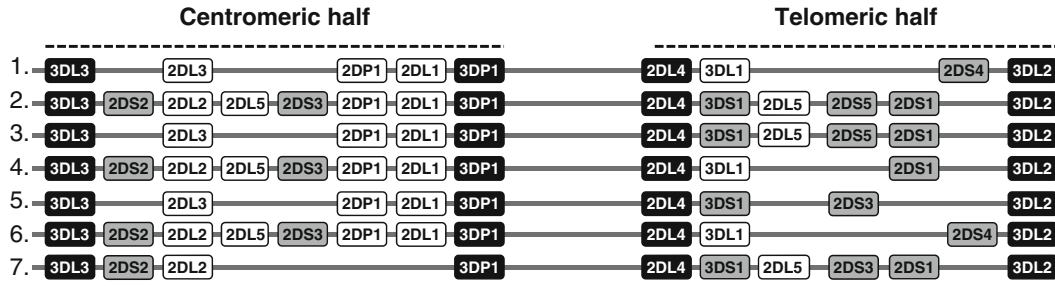

Fig. 1 KIR haplotypes have variable gene content. Map of selected KIR haplotypes is shown. Haplotype 1 represents group-A KIR haplotype and the remainder are the representative of over 30 known group-B haplotypes. The framework genes present in all haplotypes are shown in *black boxes*; genes encoding activating KIR are in *gray boxes*; and those for inhibitory receptors are in *white boxes*. KIR2DP1 and KIR3DP1 are pseudogenes that do not encode functional receptors. Inheritance of two distinct gene content haplotypes, one from each parent, produces substantial diversity in humans that may contribute to the individual's immunity

The number and type of KIR genes differ substantially between haplotypes, which are further diversified by the allelic polymorphism of most KIR genes [8, 11–16] (*see* Fig 1). Over 30 KIR haplotypes with distinct gene content have been characterized to date by sequencing genomic clones and haplotype segregation analysis in families [8, 13, 15–19]. They are broadly classified into two groups [8, 19, 20]: group A and B. Group-A haplotypes have relatively fixed gene content comprising KIR3DL3-2DL3-2DP1-2DL1-3DP1-2DL4-3DL1-2DS4-3DL2 (*see* Fig 1: haplotype 1). In contrast, group-B haplotypes have variable gene content comprising several genes and alleles that are not part of the A haplotype (*see* Fig 1: haplotypes 2–7). Particularly, KIR2DS1, 2DS2, 2DS3, 2DS5, 2DL2, 2DL5, and 3DS1 are associated only with group-B haplotypes, and thus B haplotypes generally encode more activating KIR receptors than the A haplotype that encodes a single activating receptor, KIR2DS4. The gene content varies dramatically between different group-B haplotypes. Only four KIR genes (KIR2DL4, 3DL2 and 3DL3, 3DP1) are invariably present on all KIR haplotypes (therefore ubiquitously present in all individuals) and thus they are referred to as "framework" genes [8]. Inheritance of paternal and maternal haplotypes comprising different KIR gene content generates substantial diversity between humans in their KIR gene profile. For example, homozygotes for group-A haplotypes (*see* Fig 1: haplotype 1) have only seven functional KIR genes, while heterozygotes for group-A and group-B haplotypes (*see* Fig 1: haplotypes 1 + 2) may have all 14 functional KIR genes.

The combined variation in gene content and allelic polymorphism results in unrelated individuals always having different KIR genotypes, which may individualizes immune response and thus

contribute to human health and disease. KIR genotyping has increasingly been used for epidemiological studies to show links between select KIR genes and the risk of developing certain human diseases [21]. Furthermore, donors with group-B KIR haplotypes are found to have improved relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia [22]. Moreover, the absence of donor HLA class I ligands for recipient inhibitory KIR was shown to be associated with reduced long-term graft survival in HLA-A, B, DR compatible kidney transplants [23–25].

Uhrberg et al. developed the first KIR genotyping method in 1997 using sequence-specific primers-based PCR (PCR-SSP) [20]. Since then, many KIR genotyping methods have been developed that use either the SSP strategy [26–36] or an approach that utilizes sequence-specific oligonucleotide hybridization of PCR-amplified products (PCR-SSO) [37]. The PCR-SSO is an acceptable method for high-volume sample testing, but requires substantial time for extensive post-PCR processing and complex interpretation, thus limiting its utility [37]. The reverse SSO method utilizing Luminex-technology is available from commercial vendors that simplifies the SSO assay but requires expensive reagents and a Luminex instrument [38]. The most commonly used method for KIR genotyping is SSP-PCR amplification because of its simple hands-on-procedure and straightforward interpretation. This chapter describes the step-by-step protocol of PCR-SSP typing method for KIR genotyping. Moreover, using our previously published data set [39], we describe the basic rules to verify the accuracy of genotyping results and approaches for the data analysis.

2 Materials

2.1 Facility

1. It is highly recommended that laboratories performing PCR amplification use physical barriers to prevent the contamination (carry-over) of DNA from previously amplified DNA product. It is ideal to have physically separate locations—one dedicated for pre-PCR work (i.e., DNA isolation and PCR set up), and another for post-PCR detection (i.e., separation of PCR-amplified DNA fragments).
2. Optimally, pre-PCR manipulations should be handled in a laminar flow hood to decrease the possibility of contamination.
3. Pre- and post-PCR work area should be equipped with a separate set of pipettors, lab coats, and other supplies.
4. Utilize disposable gloves and use new/sterile disposable plastic supplies.

2.2 Materials for PCR Amplification

1. *Samples*: High-quality whole genomic DNA sample extracted from peripheral blood or tissues using standard protocols (*see* **Notes 1** and **2**).
2. *Controls*: A panel of reference samples that include DNA standards positive and negative for each variable KIR gene (*see* **Note 3**).
3. *Primers*: Oligonucleotide primers for each KIR gene and for the internal positive control gene (*see* Table 1 for primer sequences) (*see* **Note 4**).
4. *Taq* DNA polymerase (5 U/μL) (*see* **Note 5**).
5. 10× PCR buffer II (10 mM Tris–HCl, 50 mM KCl) (*see* **Note 5**).
6. 100 μM of each dNTP mix.
7. 25 mM of $MgCl_2$.
8. Ultrapure PCR grade water.
9. Thermal cycler with 96-well block.
10. 96-well PCR plates.
11. Strips of eight PCR reaction tubes (0.2 mL volume).
12. Electronic single and multichannel (8 and 12 channel) repetitive pipettors and compatible tips to dispense multiple aliquots of the desired volume following a single aspiration.
13. Sterile disposable tubes (1.5 mL).
14. Vortex mixer.
15. Centrifuges capable of holding 1.5 mL tubes and 96-well PCR plates.

2.3 Materials for Gel Electrophoresis

1. Electrophoresis-grade agarose.
2. 10× TAE electrophoresis buffer (400 mM Tris, 200 mM acetic acid, 10 mM EDTA).
3. Orange G gel loading buffer (0.5 % Orange G, 20 % Ficoll, 100 mM EDTA).
4. 100 bp DNA ladder.
5. Ethidium bromide solution (10 mg/mL) (*see* **Note 6**).
6. Horizontal gel electrophoresis instrument with high-voltage power supply.
7. Gel-casting tray and 25-well combs with teeth appropriately separated for use with multichannel pipettors.
8. Gel-photo documentation system.
9. Microwave or heating apparatus to dissolve the agarose.

Table 1
Oligonucleotide primers used for KIR genotyping

Name	Sequence (5′–3′)
Forward primers	
2DL1F	CCATCAGTCGCATGACG
2DL2F2	ACTTCCTTCTGCACA(C/G)AGAA
2DL3F3	CTTCATCGCTGGTGCTG
2DL4F1	CTGCATGCTGTGATTAGGTA
2DL5F	TGCCTCGAGGAGGACAT
3DL1F1	AT(C/T)GGTCCCATGATGCT
3DL2F1	TGCAGGAACCTACAGATGTTAT
3DL3F1	CACTGTGGTGTCTGAAGGAC
3DS1F	GGCAGAATATTCCAGGAGG
2DS1F1	CTCCATCAGTCGCATGAG
2DS1F2	CTCCATCAGTCGCATGAA
2DS2F	TGCACAGAGAGGGGAAGTA
2DS3F	TCACTCCCCCTATCAGTTT
2DS4F1	TCCTGCAATGTTGGTCG
2DS5F	AGAGAGGGGACGTTTAACC
2DP1F	TCTGTTACTCACTCCCCCA
3DP1F1	AGAGTATTCCGAAACACCG
PIC-F	ATGATGTTGACCTTTCCAGGG
Reverse primers	
2DL1R1	CCACTCGTATGGAGAGTCAT
2DL1R2	AATGTTCCGTTGACCTTGGT
2DL2R1	CCCTGCAGAGAACCTACA
2DL3R1	CAGGAGACAACTTTGGATCA
2DL4R1	CTGTTGAGGGTCTCTTGCT
2DL5R1	TCATAGGGTGAGTCATGGAG
3DL1R1	CTGAGAGAGAAGGTTTCTCATATG
3DL2R1	CTTGAGTTTGACCACACGC
3DL3R1	TCTCTGTGCAGAAGGAAGC
3DS1R1	GGCACGCATCATGGA
2DS1R	AGGGCCCAGAGGAAAGTT
2DS2R1	CGCTCTCTCCTGCCAA
2DS3R	GCATCTGTAGGTTCCTCCT
2DS4R1	ACGGAAACAAGCAGTGGA
2DS5R	GGAAAGAGCCGAAGCATC
2DS5RD	CAGAGGGTCACTGGGC
2DP1R	GGAAAGAGCCGAAGCATC
3DP1R1	CTGACAACTGATAGGGGGAA
PIC-R	ATTGTGTAACTTTTCATCAGTTGC

3 Methods

3.1 PCR Amplification Procedure

1. Determine the quality and quantity of DNA by UV spectrophotometry or other standard methods, and adjust the concentration of the DNA to ~100 ng/μL (*see* **Note 7**).
2. *Primer solution preparation*. The stock oligonucleotide primers are generally obtained in salt-Free lyophilized form. Before opening, spin the tube at 111,8 × *g* for 2 min to ensure that the oligonucleotides are at the bottom of the tube. Oligonucleotides should be resuspended in a sterile buffered solution (e.g., TE at pH 7.0) (*see* **Note 8**). Vortex oligonucleotides thoroughly after resuspension.
3. For optimal long-term use, it is recommended to prepare stock (100 μM) and working (10 μM) solutions of primers (*see* **Note 9**). For instance, if the lyophilized oligonucleotide primers are received at 59.68 nM (or 59,680 pM) add 596.8 μL water to prepare 100 μM stock solution. To make 500 μL of 10 μM working solution of KIR primer, mix 50 μL of 100 μM stock solution, and add to 450 μL water. Similarly, to make 500 μL of 5 μM working solution of internal positive control primer, mix 25 μL of 100 μM stock solution, and add to 475 μL water.
4. For routine use, prepare 16 distinct primer mixes as shown in Table 2 by combining working solution of four distinct primers in 1.5 mL tubes (*see* **Note 10**). Vortex to mix well and transfer 100 μL of each primer mix into 0.2 mL PCR tubes (or strips of eight tubes). Arrange them in the first two vertical rows of a 96-well PCR tube holder in the following order: well 1A (Mix-1), well 1B (Mix-2), well 1C (Mix-3), well 1D (Mix-4), well 1E (Mix-5), well 1F (Mix-6), well 1G (Mix-7), well 1H (Mix-8), well 2A (Mix-9), well 2B (Mix-10), well 2C (Mix-11), well 2D (Mix-12), well 2E (Mix-13), well 2F (Mix-14), well 2G (Mix-15), and well 2H (Mix-16). This allows the use of 8-channel repetitive pipettors to dispense the primers into the 96-well PCR plate.
5. Six DNA samples (one control and five test samples) can be typed using one 96-well PCR plate (*see* Fig 2). Using an 8-channel multiple repeating pipettor, dispense 3.6 μL of each primer mix as shown in Fig 2.
6. Prepare 193.8 μL of PCR master-mix for each DNA sample by adding the following components in a 1.5 mL tube: 108.46 μL of ultrapure PCR grade water, 25.5 μL of 10× PCR buffer II (final concentration 1×), 2.04 μL of 100 μM dNTP mix (final concentration 200 μM each), 30.6 μL of 25 mM $MgCl_2$ (final concentration 3.0 mM), 25.5 μL of

Table 2
Primer mix composition for KIR genotyping

Primer mix	KIR gene	Gene-specific primers working solution (10 μM)		Positive internal control (PIC) primers working solution (5 μM)		Genomic PCR product size (bp)	Missing sequences[a]
		Forward	Reverse	Forward (PIC-F)	Reverse (PIC-R)		
1	2DL1	150 μL of 2DL1F	75 μL of 2DL1R1 + 75 μL of 2DL1R2	30 μL	30 μL	1,903 and/or 1,818	
2	2DL2	150 μL of 2DL2F2	150 μL of 2DL2R1	30 μL	30 μL	1,877	004, 009
3	2DL3	150 μL of 2DL3F3	150 μL of 2DL3R1	30 μL	30 μL	816	0010, 00102
4	2DL4	150 μL of 2DL4F1	150 μL of 2DL4R1	30 μL	30 μL	695	
5	2DL5	150 μL of 2DL5F	150 μL of 2DL5R1	30 μL	30 μL	1,151	
6	3DL1	150 μL of 3DL1F1	150 μL of 3DL1R1	30 μL	30 μL	1,661	037, 040, 054
7	3DL2	150 μL of 3DL2F1	150 μL of 3DL2R1	30 μL	30 μL	1,882	031, 048
8	3DL3	150 μL of 3DL3F1	150 μL of 3DL3R1	30 μL	30 μL	1,905	019 , 030, 031
9	3DS1	150 μL of 3DS1F	150 μL of 3DS1R1	30 μL	30 μL	1,847	
10	2DS1	75 μL of 2DS1F1 + 75 μL of 2DS1F2	150 μL of 2DS1R	30 μL	30 μL	1,922 and/or 1,897	008
11	2DS2	150 μL of 2DS2F	150 μL of 2DS2R1	30 μL	30 μL	1,781	00104
12	2DS3	150 μL of 2DS3F	150 μL of 2DS3R	30 μL	30 μL	1,812	
13	2DS4	150 μL of 2DS4F1	150 μL of 2DS4R1	30 μL	30 μL	2,050	
14	2DS5	150 μL of 2DS5F	75 μL of 2DS5R+ 75 μL of 2DS5RD	30 μL	30 μL	1,952 and/or 180	003, 009, 011
15	2DP1	150 μL of 2DP1F	150 μL of 2DP1R	30 μL	30 μL	1,825	
16	3DP1	150 μL of 3DP1F1	150 μL of 3DP1R1	30 μL	30 μL	1,900	

[a]Missing sequences are based on KIR allignment from IPD-KIR database available at http://www.ebi.ac.uk/ipd/kir/ (Release 2.4.0., 15 April 2011)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Mix-1	Mix-9	Mix-1	Mix-9	Mix-1	Mix-9	Mix-1	Mix-9	Mix-1	Mix-9	Mix-1	Mix-9
B	Mix-2	Mix-10	Mix-2	Mix-10	Mix-2	Mix-10	Mix-2	Mix-10	Mix-2	Mix-10	Mix-2	Mix-10
C	Mix-3	Mix-11	Mix-3	Mix-11	Mix-3	Mix-11	Mix-3	Mix-11	Mix-3	Mix-11	Mix-3	Mix-11
D	Mix-4	Mix-12	Mix-4	Mix-12	Mix-4	Mix-12	Mix-4	Mix-12	Mix-4	Mix-12	Mix-4	Mix-12
E	Mix-5	Mix-13	Mix-5	Mix-13	Mix-5	Mix-13	Mix-5	Mix-13	Mix-5	Mix-13	Mix-5	Mix-13
F	Mix-6	Mix-14	Mix-6	Mix-14	Mix-6	Mix-14	Mix-6	Mix-14	Mix-6	Mix-14	Mix-6	Mix-14
G	Mix-7	Mix-15	Mix-7	Mix-15	Mix-7	Mix-15	Mix-7	Mix-15	Mix-7	Mix-15	Mix-7	Mix-15
H	Mix-8	Mix-16	Mix-8	Mix-16	Mix-8	Mix-16	Mix-8	Mix-16	Mix-8	Mix-16	Mix-8	Mix-16

Control **Sample-1** **Sample-2** **Sample-3** **Sample-4** **Sample-5**

Fig. 2 The template map of 96-well PCR plate indicating the position of primer mixes 1–16 for each DNA sample. The composition of each primer mix is listed in Table 2

DNA (around 100 ng/μL), and 1.7 μL of Taq DNA polymerase. Vortex and centrifuge briefly.

7. Using a single channel pipettor, add 11.4 μL of PCR mix in each well for each sample (total PCR volume = 15 μL).
8. Cover plates with acetate film and centrifuge briefly to ensure that all the liquid is at the bottom of the wells. Place in the thermal cycler (*see* **Note 11**).
9. Perform PCR amplification under the following thermal cycling conditions: initial denaturation for 3 min at 95 °C; then 5 cycles of 94 °C for 20 s, 65 °C for 20 s, and 72 °C for 90 s; then 35 cycles of 94 °C for 20 s, 61 °C for 20 s, and 72 °C for 90 s; with final extension at 72 °C for 10 min.
10. Once the PCR thermal cycling is complete, remove the PCR plate and proceed with gel electrophoresis.

3.2 Gel Electrophoresis Procedure

1. Prepare 150 mL of 2 % agarose in 1× TAE per gel and heat until the agarose has completely gone into solution.
2. Cool gel mixture to 65 °C, add ~5 μL ethidium bromide, and gently mix to avoid bubble formation.
3. Pour gel mixture into the gel-casting tray, insert four 25-well combs, and allow the gel to solidify for 30 min.
4. Fill the electrophoresis chamber with appropriate volume of 1× TAE buffer and submerge the gel into the chamber. Gently remove the combs.

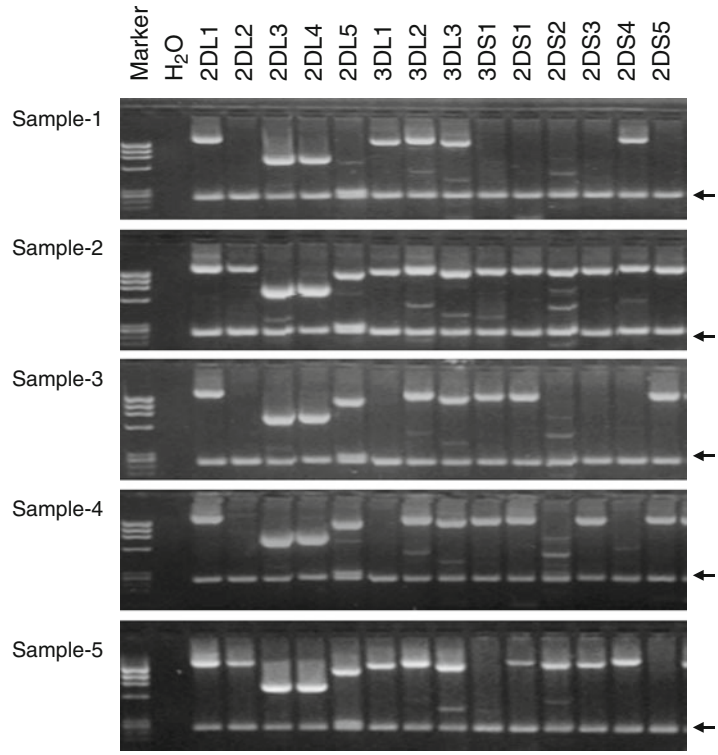

Fig. 3 KIR genotyping by the gene-specific PCR amplification method. Agarose gel pictures showing the PCR typing results of five representative DNA standards obtained from the UCLA KIR Exchange Program. Subject-1 has fewer genes (AA homozygote) and subject-2 has all known KIR genes (AB heterozygote). *Arrows* indicate internal positive control bands specific to an invariant gene

5. Add 5 μL Orange G gel loading buffer to each PCR well, mix, and centrifuge briefly.
6. Load 2 μL of the 100 bp DNA ladder to the first well of each row.
7. Using a 12-channel pipettor, load 10 μL of each PCR product into the gel.
8. Electrophorese for 30 min at 100 V or until the Orange G has migrated 3 cm.
9. Visualize the gel using a UV light source and photograph the gel for a permanent record. The KIR genotyping result of five unrelated samples by our SSP-PCR typing is depicted in Fig 3.

3.3 Interpretation of Gel Results

1. Each PCR well includes a unique set of primers designed to have perfect matches with a single KIR gene and produce a product with a particular known size (*see* Table 2). Under strictly controlled PCR conditions, perfectly matched primer pairs result in the amplification of target sequences (i.e., a posi-

tive reaction) while mismatched primer pairs do not result in amplification (i.e., a negative reaction). In addition to KIR gene-specific primers, each PCR reaction includes a positive internal control primer pair which amplifies a 256 bp fragment from a conserved Polyposis coli gene. The presence of the 256 bp positive internal control band is used to confirm the success of each PCR reaction (*see* Fig 3). In the presence of a positive typing band, the product of the internal control primer pair may be weak or absent due to the differences in concentration and melting temperatures between the specific primer pairs and the internal control primer pair.

2. Interpretation of the PCR-SSP typing results is relatively simple and straightforward, and is done basically detecting an amplified product of the correct size by gel electrophoresis. Determine the approximate molecular weight of each PCR product by comparing the mobility against the DNA ladder.
3. Check if the typing results of the control DNA is consistent with the known typing.
4. Record the results in a Microsoft Excel spreadsheet indicating which genes are present (identified by number 8) and which genes are absent (identified by number 1) for each sample. Figure 4 illustrates the KIR genotyping raw data for a set of 26 samples (S-1 to S-26) that we recently published [39]. We use this data set as an exemplar to describe data analysis methods (hereafter called the exemplar data set).

3.4 KIR Genotyping Data Analysis

1. Rearrange the order of columns (data of different KIRs) using the cut and paste option in Microsoft Excel to sort genes that are associated with group-A haplotypes (2DL1, 2DL3, 3DL1, and 2DS4), group-B haplotypes (2DS2, 2DL2, 2DS3, 2DL5, 3DS1, 2DS5, and 2DS1), then framework/pseudogenes (2DP1, 3DP1, 2DL4, 3DL2, and 3DL3) as shown in Fig 5. Then, using the custom sort option in Microsoft Excel, sort the rows (data of different samples) to select samples with similar KIR genotypes. *See* Fig 5 for the sorted raw data for exemplar data set presented in Fig 4.
2. *Verification of raw data*. False-negative results are a common problem associated with the gene-specific PCR amplification-based KIR genotyping. We recommend reviewing the raw data vigorously to verify if it agrees to the following basic rules:
   (a) Four framework genes (KIR3DL3, 3DP1, 2DL4, and 3DL2) must be present in each sample.
   (b) KIR2DL3 and 2DL2 behave as alleles of same locus, and thus subjects negative for both 2DL3 and 2DL2 are questionable.

Sample I.D.	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	3DS1	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DP1
S-1	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8
S-2	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-3	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8
S-4	8	1	8	8	8	1	8	8	8	8	1	1	8	8	8	8
S-5	8	8	8	8	1	8	8	8	1	1	8	1	8	1	8	8
S-6	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-7	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-8	8	1	8	8	8	1	8	8	8	8	1	1	1	8	8	8
S-9	8	1	8	8	8	8	8	8	8	8	1	8	8	1	8	8
S-10	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-11	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8
S-12	8	1	8	8	8	1	8	8	8	8	1	8	8	1	8	8
S-13	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-14	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-15	8	8	8	8	8	1	8	8	1	8	8	1	8	1	1	8
S-16	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8
S-17	8	1	8	8	1	1	8	8	1	1	1	1	8	1	8	8
S-18	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-19	8	1	8	8	8	8	8	8	8	8	1	1	8	8	8	8
S-20	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8
S-21	8	8	8	8	8	8	8	8	8	8	8	1	8	8	8	8
S-22	8	1	8	8	8	8	8	8	8	8	1	8	8	1	8	8
S-23	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8
S-24	8	1	8	8	1	1	8	8	1	1	1	1	8	1	8	8
S-25	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8
S-26	8	1	8	8	1	8	8	8	1	1	1	1	8	1	8	8

Fig. 4 Microsoft Excel spreadsheet indicating which genes are present (identified by number 8) and which genes are absent (identified by number 1) for a set of 26 exemplar samples (S-1 to S-26) that we have recently published [39]

(c) KIR3DL1 and 3DS1 behave as alleles of same locus, and subjects negative for both of these KIRs are extremely infrequent.

(d) KIR2DS4 negatives are generally negative for KIR3DL1 (likely BB genotype carriers).

(e) KIR2DS2 has strong linkage disequilibrium with 2DL2, and therefore genotype with KIR2DS2^{Pos} but KIR2DL2^{Neg} is rare.

Genotyping results of any samples that do not confirm these basic rules, as well as those with ambiguous and uncertain typing results must be retyped using an alternative typing method. Since the KIR gene family has been the subject of rapid evolution [40, 41], several genotypes with unusual gene content and recombinant genes are reported as the consequence of unequal cross-overs [42–44]. Therefore, it is critical to retype using an alternative typing method to confirm if a sample carries an unusual/rare KIR genotype.

	Group-A haplotype associated KIR				Group-B haplotype associated KIR							Framework/Psuedogenes					Genotype	
Sample I.D.	2DL1	2DL3	3DL1	2DS4	2DS2	2DL2	2DS3	2DL5	3DS1	2DS5	2DS1	2DP1	3DP1	2DL4	3DL2	3DL3	Number	Haplogroup
S-11	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-16	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-20	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-23	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-25	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-26	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-1	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-3	8	8	8	8	1	1	1	1	1	1	1	8	8	8	8	8	1	AA
S-17	8	8	1	8	1	1	1	1	1	1	1	8	8	8	8	8	2	AA
S-24	8	8	1	8	1	1	1	1	1	1	1	8	8	8	8	8	2	AA
S-4	8	8	1	8	1	1	1	8	8	8	8	8	8	8	8	8	3	AB
S-2	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-6	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-7	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-10	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-13	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-14	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-18	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-19	8	8	8	8	1	1	1	8	8	8	8	8	8	8	8	8	4	AB
S-21	8	8	8	8	8	8	1	8	8	8	8	8	8	8	8	8	5	AB
S-8	8	8	1	1	1	1	1	8	8	8	8	8	8	8	8	8	6	BB
S-9	8	8	8	8	1	1	8	8	8	1	8	8	8	8	8	8	7	AB
S-22	8	8	8	8	1	1	8	8	8	1	8	8	8	8	8	8	7	AB
S-12	8	8	1	8	1	1	8	8	8	1	8	8	8	8	8	8	8	BB
S-15	8	8	1	8	8	8	1	8	1	1	8	1	8	8	8	8	9	BB
S-5	8	8	8	8	8	8	1	1	1	1	1	8	8	8	8	8	10	AB
Number of carriers	26	26	20	25	3	3	3	15	14	11	15	25	26	26	26	26		
% of carriers	100	100	76.9	96.1	11.5	11.5	11.5	57.7	53.7	42.3	57.7	96.1	100	100	100	100		

Fig. 5 The raw data shorted on genes associated with group-A haplotypes (2DL1, 2DL3, 3DL1, and 2DS4), group-B haplotypes (2DS2, 2DL2, 2DS3, 2DL5, 3DS1, 2DS5, and 2DS1), framework/pseudogenes (2DP1, 3DP1, 2DL4, 3DL2, and 3DL3) as well as by KIR gene content. The carrier frequency of each KIR gene is determined by dividing the number of individuals positive for the gene by the total number of individuals tested in the panel, and then multiplying by 100 (shown in the *bottom row*). The KIR haplotypes are predicted on the basis of presence and absence of certain KIR genes (*see* **step 5** under Subheading 3.4). The presence of the T4 gene cluster (positive for KIR2DL5-3DS1-2DS5-2DS1) is shaded in *gray*

3. *Determination of KIR gene frequencies.* The percentage of individuals carrying each KIR gene in the study group is determined by direct counting (individuals positive for the gene divided by the individuals tested in the study group × 100). The percent carrier frequencies of each KIR gene within the exemplar data set are provided in the bottom row of Fig 5.
4. *KIR genotype frequency determination.* KIR gene content of a given individual is conventionally called the "KIR genotype,"

which is variable among individuals. Within the exemplar data set, ten distinct KIR genotypes are detected (*see* Fig 5). The percent frequency of each KIR genotype can be determined by direct counting of individuals carrying a particular genotype divided by the total number individuals tested in the study group × 100. For example, genotype #1 in the exemplar data set (*see* Fig 5) is determined to be 30.8 % (i.e., 8/26 × 100).

5. *Prediction of KIR haplogroups from genotyping data.* The group-A and group-B KIR haplotypes can be predicted from the KIR genotyping data (*see* Fig 5). Individuals having only genes of the group-A KIR haplotypes (KIR3DL3-2DL3-2DL1-2DP1-3DP1-2DL4-3DL1-2DS4-3DL2) are considered to be homozygous for the A-haplotype and assigned as AA genotype carriers. Please note that some group-A KIR haplotypes may have deleted one or more of these genes and thus can produce a short KIR genotype. For example, the samples S-17 and S-24 in Fig 5 appear to be homozygous for short A-haplotypes that miss the KIR3DL1 gene. Individuals lacking any of the four A-haplotype associated genes (KIR2DL1, 2DL3, 3DL1 and 2DS4) that have a known function and carry one or more group-B haplotype associated genes are considered to be homozygous for group-B haplotypes, and assigned as the carriers of BB genotypes. All other individuals are regarded to be heterozygous for A and B haplotypes and assigned as AB genotype carriers. The individuals with AB genotypes have all nine genes present on the A-haplotype, as well as one or more B-haplotype specific genes (2DL2, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, and 3DS1). The AB and BB genotypes are collectively referred together as Bx genotypes [45].
6. *Prediction of group-A and group-B haplotypes.* Frequencies of A and B haplotypes are calculated using the following formula: group-A = $2n$AA + nAB/2 N and group-B = $2n$BB + nAB/2 N, where nAA, nAB, and nBB are the numbers of AA, AB, and BB genotypes and N is the total number of individuals tested within the study group.
7. *Classification of KIR genotypes on the basis of centromeric and telomeric gene-clusters.* Based on the linkage disequilibrium, we discovered two frequently occurring gene-clusters [46]. One cluster comprises KIR2DS2-2DL2-2DS3-2DL5 genes and is located at the centromeric half of the KIR gene complex, while another cluster comprises KIR3DS1-2DL5-2DS1-2DS5 genes and is located at the telomeric half of the complex (*see* Fig 1). For simplicity we call these clusters C4 and T4, in which "C" represents centromeric, "T" represents telomeric, and "4" indicates number of genes. On the basis of the presence and

absence of C4 and T4 clusters, the Bx genotypes are further divided into the following four subsets: C4Tx (presence of C4 and absence of T4), CxT4 (absence of C4 and presence of T4), C4T4 (presence of both C4 and T4), CxTx (absence of both C4 and T4). These Bx subsets are substantially variable in activating KIR gene content, and their frequencies differ significantly between human populations [47].

8. The function of the inhibitory KIR receptors depends on the availability of their specific cognate HLA class I ligands. Given that KIR genes at chromosome 19q13.4 and HLA genes at chromosome 6p21.3 are polymorphic and display significant variations, the independent segregation of these unlinked gene families produce diversity in the number and type of KIR-HLA pairs inherited in individuals [48], which could potentially influence the health and disease status of a given individual [21]. Therefore, it is critical to type for the KIR-binding HLA class I motif to determine if specific combinations of KIR-HLA genes are associated with specific diseases.

4 Notes

1. DNA extraction is the first step in the KIR genotyping method. Preparation of high quality DNA is critical for amplification of KIR genes since the length of the PCR-amplified fragments of most KIR genes is in the range of 2,000 base pairs.
2. Heparin has been shown to inhibit some PCR reactions and therefore heparinized blood should be avoided. EDTA or Citrate (ACD) anticoagulant is preferred.
3. It is critical to include sufficient control DNA standards (controls should represent 10 % of the test samples) to confirm the accuracy and reliability of positive/negative KIR genotyping results. The control panel must include positive and negative DNA standards for each variable KIR gene. The UCLA International KIR Exchange Program provides a comprehensive set of KIR genotyping control standards (http://www.hla.ucla.edu/pdf/KIR_brochure.pdf) that fulfills these requirements.
4. Custom oligonucleotide primers may be purchased from commercial vendors. Each primer is designed to carry a 3′ residue matching a unique position conserved on all known sequences of a given KIR gene. The primers recognize most of the sequences submitted to date in the IPD-KIR database available at http://www.ebi.ac.uk/ipd/kir/ (Release 2.4.0., 15 April 2011). Primer lengths are adjusted to result in annealing temperatures between 59 °C and 67 °C to enable PCR amplification of all KIR genes under the same PCR thermal cycling conditions.

5. We obtained the best results with AmpliTaq DNA polymerase and 10× PCR buffer II (Applied Biosystem, Foster city, California).
6. Ethidium bromide is a carcinogen. Handle with appropriate personal protective equipment including gloves, gown, and eye protection.
7. PCR amplification may fail if the DNA is contaminated with cellular proteins. Since the heme proteins of red blood cells are known to inhibit PCR amplification, many DNA isolation methods, particularly the salting out method [49] requires red cell removal prior to DNA extraction.
8. Oligonucleotides may not readily dissolve in sterile, distilled water. Adding NaOH to the water until the pH rises to 7.0 may help. If the oligonucleotides are resuspended at pH < 7.0 (deionized water may have a pH as low as 5.0), the oligonucleotide could begin to degrade and may lose functionality within a couple of weeks.
9. The 100 μM stock primer solution can be stored long term at −20 °C, while the 10 μM working primer solution can be stored at 4 °C for 3–4 months.
10. If you see too many nonspecific bands with 2DS5 (primer mix 14), re-run the 2DS5 PCR with two different primer combinations, one with 2DS5F and 2DS5R (produce 1,952 bp product) and another with 2DS5F and 2DS5RD (produce 180 bp product).
11. Ensure that the sealer covers the PCR plate properly and the plate fits snugly into the thermal cycler to avoid evaporation and amplification failure.

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