

Killer-cell immunoglobulin-like receptor genotyping and HLA killer-cell immunoglobulin-like receptor-ligand identification by real-time polymerase chain reaction

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Abstract

The effector function of natural killer (NK) cells is modulated by surface expression of a range of killer-cell immunoglobulin-like receptors (KIRs) that interact with human leukocyte antigen (HLA) class I ligands. We describe the use of real-time polymerase chain reaction (PCR) assays that allow easy and quick detection of 16 *KIR* genes and the presence/absence of KIR-ligands based on allelic discrimination at codon 80 in the *HLA-A/B Bw4* and *HLA-C C1/C2* genes. These methods overcome the tedious and expensive nature of conventional *KIR* genotyping and HLA class I typing using sequence-specific primer (SSP) PCR, sequence-specific oligonucleotide (SSO) hybridization or sequence-based typing (SBT). Using these two cost-effective assays, we measured the frequencies of KIRs, KIR-ligands and KIR/KIR-ligand pairs in a cohort of Black women recruited in South Africa.

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Introduction

Natural killer (NK) cells comprise approximately 10%–15% of peripheral blood lymphocytes, and their primary function is the detection and destruction of malignant and virus-infected cells (1, 2). A number of cell surface receptors that control the effector function of NK cells have been characterized, such as the killer-cell immunoglobulin-like receptors (KIRs), C-type lectins and natural cytotoxicity receptors (NCRs) (3, 4).

KIR proteins are encoded in the leukocyte receptor complex (LRC) located on chromosome 19, and are characterized by the presence of two-dimensional (2D) or three-dimensional (3D) extracellular immunoglobulin-like domains that determine ligand specificity (3, 5). In addition, KIRs fall into two broad groups with different cytoplasmic tail lengths. Receptors with long tails (L) suppress NK cell activation, whereas short-tail (S) receptors are stimulatory, inducing the secretion of interferon- γ (IFN- γ) and the release of perforin and granzyme (6).

Sixteen *KIR* genes have been identified; 7 are inhibitory (*KIR2DL1-3*, *KIR2DL5*, *KIR3DL1-3*), 6 are activating (*KIR2DS1-5* and *KIR3DS1*), 2 are pseudogenes (*KIR2DP1* and *KIR3DP1*) (5, 7) and 1 has both inhibitory and activating potential (*KIR2DL4*) (8). Certain KIRs, upon engagement with specific human leukocyte antigen (HLA) class I ligands, may either inhibit or stimulate NK cell activity (6, 9). *HLA* and *KIR* genes are highly polymorphic and map to chromosomes 6 and 19, respectively, and thus assort independently. On the basis of *KIR* gene combinations, two haplotype groups have been identified; haplotype A, with a fixed content of nine *KIR* genes (*KIR2DL1/3/4*, *KIR3DL1-3*, *KIR2DS4*, *KIR2DP1* and *KIR3DP1*) and haplotype B, with variable gene content. Collectively, this allows for a large range of KIR/HLA diversity (10).

KIR-HLA ligands (KIR-ligands) for *KIR2DL1-3*, *KIR2DS1* and *KIR2DS4* are HLA-C molecules. *KIR2DL2* and *KIR2DL3* recognize HLA-C group 1 (C1) allotypes, which have an asparagine at position 80 (C1:N⁸⁰), whereas *KIR2DL1* and *KIR2DS1* bind to the HLA-C group 2 (C2) allotypes, which have a lysine at position 80 (C2:K⁸⁰) (11). Whilst *KIR2DS4* can bind to both subsets of HLA-C C1 and C2 allotypes, it has also been found to bind to two HLA-A alleles (HLA-A*11:01 and A*11:02) (12). For *KIR3DL1* and putatively *KIR3DS1*, the ligands are HLA-B allotypes with the Bw4 motif designated by five variable amino acids spanning positions 77–83, with either isoleucine (Bw4:I⁸⁰) or threonine (Bw4:T⁸⁰) at position 80 (13, 14). In addition, HLA-B Bw4:T⁸⁰ variants can vary with alanine (Bw4:T⁸⁰A⁸¹) or leucine (Bw4:T⁸⁰L⁸¹) at position 81 (13–15). Some HLA-A allotypes also have the Bw4 motif (Bw4:I⁸⁰) and bind *KIR3DL1* and putatively *KIR3DS1*, whereas HLA-A allotypes lacking the Bw4 motif (non-Bw4:T⁸⁰) and HLA-B Bw6 allotypes (Bw6:N⁸⁰) are not known to bind any KIRs.

Specific KIR and KIR-ligand combinations have been associated with numerous infectious/autoimmune disease

outcomes as well as in pregnancy and transplantation complications (10, 16). The presence of specific KIRs with or without their corresponding ligands can influence susceptibility to disease and/or improved response to therapy (10, 17–19). Conventional *KIR* genotyping is done by sequence-specific primer polymerase chain reaction (SSP-PCR), and HLA class I typing is done by SSP-PCR, sequence-specific oligonucleotide (SSO) hybridization or sequence-based typing (SBT) assays. These methods are often cumbersome and expensive and require large quantities of high-quality genomic DNA. Amplified SSP products are visualized by agarose gel electrophoresis, which is time-consuming as well as hazardous if using ethidium bromide staining (16).

We have optimized and validated two real-time PCR assays for *KIR* genotyping and identification of KIR-ligands using samples previously characterized by SSP-PCR and SBT. The *KIR* genotyping assay is able to determine the presence/absence of 16 *KIR* genes, while the KIR-ligand assay identifies the presence/absence of KIR-ligands (HLA-A/B Bw4, and HLA-C C1/C2). We then applied these new methods to identify *KIR* genotype, KIR-ligand frequencies and KIR/KIR-ligand pairs in a small South African cohort.

Methodology

Validation samples

Stored genomic DNA was available for 230 samples previously genotyped for *KIR* or *HLA* class I using commercial assays. The real-time PCR *KIR* genotyping assay was validated against 50 samples, consisting of 24 South African Black individuals and 26 Caucasians that were *KIR* genotyped by SSP-PCR. Importantly, all 16 *KIR* genes were represented in these samples. The real-time PCR KIR-ligand assay was validated against 220 samples that had been *HLA* typed by SBT, in which all variants at position 80 were represented. To accommodate HLA class I allelic variation, we tested samples from South African Black individuals ($n = 107$) and Caucasians ($n = 83$), as well as published HLA-A, -B and -C reference samples ($n = 30$) obtained from the International Histocompatibility Working Group (<http://www.ihwg.org>).

Study samples

Whole-blood ethylenediaminetetraacetic acid (EDTA) samples were collected from consenting Black women ($n = 81$) recruited from the Carletonville mining area, South Africa. A salting-out method (20) was used to extract genomic DNA from leukocytes isolated from 1 ml of whole blood. DNA concentration was measured on a nanodrop spectrophotometer (Thermo Scientific, Wilmington, MA). The study was approved by the Human Research Ethics Committee (Medical) at the University of the Witwatersrand and informed written consent was obtained from all participants.

KIR PCR primers and real-time PCR

To date, real-time PCR-based KIR genotyping and HLA KIR-ligand identification assays have been described by Alves *et al.* (21) and Koehler *et al.* (22). In our system, we wished to avoid the use of fluorescent probe chemistry and nested PCR methods used by Koehler *et al.* (22) in favor of SYBR[®] green chemistry. For our real-time KIR genotyping assay, we selected previously published KIR primer sequences (16, 21, 23), which we mapped to updated *KIR* gene alignments in the Immuno Polymorphism Database (IPD) database (24). A combination of primers was used to improve detection of KIR allelic variants, with the exception of KIR2DL3 and KIR3DP1, where a single primer set was used (Table 1). The KIR2DL3 primer set detects all known alleles; however, the pseudogene KIR3DP1 primer set does not detect KIR3DP1*004 and KIR3DP1*009 alleles. Furthermore, an internal control gene galactosylceramidase (*GALC*) (21) was included.

For real-time PCR amplification, each 5- μ l reaction contained 2 \times SYBR[®] Green master mix (Roche, Mannheim, Germany), 0.2 μ M KIR-specific primers, 0.2 μ M *GALC*-specific primers and 5 ng of genomic DNA. PCR cycling was performed in a 96-well format on an ABI PRISM[®] 7500 real-time instrument (Applied Biosystems, Foster City, CA) as follows: 10 min at 95°C, 30 cycles of 15 s at 95°C and 1 min at 60°C. Melt curve analysis was performed after cycling (Figure 1A) and could discriminate between *GALC* ($T_m = 74.89^\circ\text{C}$) and the KIR amplicons ($T_m = 78.55^\circ\text{C} - 85.9^\circ\text{C}$), thereby making it simple to identify the presence/absence of specific *KIR* genes. The presence of an internal control amplicon ensured that the absence of a KIR amplicon was a true result.

KIR-ligand primers and real-time PCR

We designed our KIR-ligand primers from HLA class I alignments available at the International Immunogenetics Project (IMGT)/HLA database (25) to discriminate Bw4 from Bw6 allotypes at codon 80. Primers were synthesized with 3' locked nucleic acid (LNA) bases (Exiqon, Vedbaek, Denmark) in order to increase template specificity (Table 2). Each PCR included an internal positive control gene albumin (*ALB*) (26).

For real-time PCR amplification, each 5- μ l reaction contained 2 \times SYBR[®] Green master mix (Roche), 0.2 μ M KIR-specific primers, 0.2 μ M *ALB*-specific primers and 5 ng of genomic DNA. PCR cycling was performed in a 96-well format on an ABI PRISM[®] 7500 real-time instrument (Applied Biosystems) as follows: 10 min at 95°C, 30 cycles of 15 s at 95°C, 5 s at 60°C and 1 min at 72°C. Melt curve analysis was performed after cycling (Figure 1B), and similar to the KIR assay, differences in melting temperature could discriminate between *ALB* ($T_m = 75.62^\circ\text{C}$) and the KIR-ligand amplicons ($T_m = 87.3^\circ\text{C} - 88.3^\circ\text{C}$).

High-resolution HLA typing

High-resolution (four-digit) HLA typing was performed on validation samples as follows: DNA was extracted from either peripheral blood mononuclear cells or granulocytes using the Pel-Freez DNA isolation kit (DynaL Biotech, Ullernchaussen, Norway). HLA-A, -B and -C typing was performed by DNA sequencing of exons 2, 3 and 4 using SBTexcellerator kits (Qiagen, Dusseldorf, Germany) on an ABI3100 Genetic Analyzer (Applied Biosystems) and ASSIGN-SBT v3.5 software (Conexio Genomics, Fremantle, Western Australia). Any ambiguities resulting from either polymorphisms outside the sequenced exons or identical heterozygote combinations were resolved using statistical methods (27).

SSP-PCR KIR genotyping

KIR genotyping was performed on validation samples using either a commercially available kit, sequence-specific primer *KIR* PCR genotyping kit (Olerup, Loewengasse, Austria) or a published protocol (16). These methods both detect the presence/absence of the following 16 *KIR* genes: *KIR2DL1-5*, *KIR2DS1-5*, *KIR3DL1-3*, *KIR3DS1*, *KIR2DP1* and *KIR3DP1*.

On the basis of individual *KIR* gene combinations, two genotype groups were stratified, the AA genotype with nine genes (*KIR2DL1/3/4*, *KIR3DL1-3*, *KIR2DS4*, *KIR2DP1* and *KIR3DP1*) and the Bx genotype, which can be a mixture of AB or BB haplotypes. In addition, *KIR* Bx genotype numbers were assigned according to published nomenclature on the Allele Frequencies database (28).

Data analysis

For both sets of data (SSP-PCR, SBT and real-time PCR), carrier frequencies for the observed KIR and KIR-ligands were determined by direct counting (individuals positive for the gene divided by the individuals tested per population \times 100). Real-time PCR KIR and KIR-ligand assays were validated by direct comparison with SBT- and SSP-PCR-based typing results of the same samples.

Results

Validation of real-time PCR KIR assay

Using samples that represented the full spectrum of the *KIR* genes, we tested real-time PCR KIR assay on the KIR validation samples ($n = 50$) and compared our results to the gel-based (SSP-PCR) *KIR* genotyping kit (Olerup) and published methods (16). We found the real-time PCR KIR assay was 100% concordant with conventional SSP-PCR methods (Table 3).

Validation of real-time PCR KIR-ligand assay

In comparison with the commercial high-resolution SBT method, the real-time KIR-ligand assay correctly genotyped

Table 1 PCR primer sets used for real-time KIR genotyping assay

KIR gene	PCR well no.	Primers	Sequences (5'–3')	Primer binding site	Product T_m (°C)	Alleles not detected
2DL1	1	F1 ^a	GTTGGTCAGATGTCATGTTTGAA	Exon 4	80.36	—
		R1 ^a	GGTCCCTGCCAGGCTTGCG	Exon 4		
	2	F1 ^a	TGGACCAAGAGTCTGCAGGA	Exon 8	82.12	KIR2DL1*005
		R1 ^a	TGTTGTCTCCCTAGAAGACG	3'UTR		
2DL2	3	F1 ^a	CTGGCCCCACCCAGGTGCG	Exon 4	80.68	KIR2DL2*004, *00601, *00602, *00303, *004
		R1 ^a	GGACCGATGGAGAAGTTGGCT	Exon 4		
	4	F1 ^b	AAACCTTCTCTCTCAGCCCA	Exon 5	83.30	KIR2DL2*009
		R1 ^b	GCCCTGCAGAGAACCTACA	Exon 5		
2DL3	5	F ^b	AGACCCTCAGGAGGTGA	Exon 9	79.47	—
		R ^b	CAGGAGACAACCTTTGGATCA	Exon 9		
2DL4	6	F1 ^a	CAGGACAAGCCCTTCTGC	Exon 3	78.08	—
		R1 ^a	CTGGGTGCCGACCACT	Exon 3		
	7	F1 ^a	ACCTTCGCTTACAGCCCCG	Exon 5	84.21	—
		R1 ^a	CCTCACCTGTGACAGAAACAG	Exon 5		
2DL5	8	F1 ^a	GCGCTGTGGTGCCTCG	Exon 3	82.19	—
		R1 ^a	GACCACTCAATGGGGGAGC	Exon 3		
	9	F1 ^a	TGCAGCTCCAGGAGCTCA	Exon 5	83.09	—
		R1 ^a	GGGTCTGACCACTCATAGGGT	Exon 5		
2DS1	10	F1 ^b	TCTCCATCAGTCGCATGAG	Exon 4	79.34	—
		F2 ^b	TCTCCATCAGTCGCATGAA	Exon 4		
		R ^b	GGTCACTGGGAGCTGAC	Exon 4		
		F1 ^a	TTCTGCACAGAGGGGAAGTA	Exon 4	80.55	—
2DS2	11	R1 ^a	GGGTCACTGGGAGCTGACAA	Exon 4		
		F1 ^a	CGGGCCCCACGGTTT	Exon 5	83.47	KIR2DS2*00104
		R1 ^a	GGTCACTCGAGTTTGACCACTCA	Exon 5		
		2DS3	13	F1 ^c	AAACCTTCTCTCTCAGCCCA	Exon 5
R1 ^b	GCATCTGTAGGTTCTCCT			Exon 5		
	14	F1 ^a	CTATGACATGTACCATCTATCCAC	Exon 5	83.07	—
		R1 ^a	AAGCAGTGGGTCACTTGAC	Exon 5		
2DS4	15	F1 ^a	CTGGCCCTCCCAGGTCA	Exon 4	80.83	—
		R1 ^a	TCTGTAGGTTCTGCAAGGACAG	Exon 4		
	16	F1 ^a	GTTGAGGAGGAGAGAAT	Exon 5	84.35	—
		R1 ^a	GTTGACCACTCGTAGGGAGC	Exon 5		
2DS5	17	F1 ^a	TGATGGGGTCTCCAAGGG	Exon 4	81.54	KIR2DS5*003
		R1 ^a	TCCAGAGGGTCACTGGGC	Exon 4		
	18	F1 ^a	ACAGAGAGGGGACGTTTAACC	Exon 4	82.18	—
		R1 ^a	ATGTCCAGAGGGTCACTGGG	Exon 4		
2DP1	19	F1 ^a	GTCTGCCTGGCCAGCT	Exon 3	80.67	—
		R1 ^a	GTGTGAACCCGACATCTGTAC	Exon 3		
	20	F1 ^a	CCATCGGTCCCATGATGG	Exon 4	79.28	—
		R1 ^a	CACTGGGAGCTGACAACTGATG	Exon 4		
3DL1	21	F1 ^a	CGCTGTGGTGCCCTCGA	Exon 3	79.62	KIR3DL1*009, *042, *057
		R1 ^a	GGTGTGAACCCGACATG	Exon 3		
	22	F1 ^b	CCATCGGTCCCATGATGCT	Exon 4	79.13	—
		F2 ^b	CCATT GGTCCCATGATGCT	Exon 4		
		F3 ^b	TCCATCGGTCCCATGATGTT	Exon 4		
		R ^b	CCACGATGTCCAGGGGA	Exon 4		
3DL2	23	F1 ^a	CAAACCTTCCCTGTCTGCC	Exon 3	82.94	KIR3DL2*013, *014
		R1 ^a	GTGCCGACCAACCCAGTGA	Exon 3		
	24	F1 ^a	CCCATGAACGTAGGCTCCG	Exon 5	83.70	KIR3DL2*018
		R1 ^a	CACACGCAGGGCAGGG	Exon 5		
3DL3	25	F1 ^a	GTCAGGACAAGCCCTTCCTC	Exon 3	82.32	—
		R1 ^a	GAGTGTGGGTGTGAACTGCA	Exon 3		
	26	F1 ^a	TTCTGCACAGAGGGGATCA	Exon 4	82.47	—
		R1 ^a	GAGCCGACAACCTCATAGGGTA	Exon 4		

Table 1 *Continued*

KIR gene	PCR well no.	Primers	Sequences (5'–3')	Primer binding site	Product T_m (°C)	Alleles not detected		
3DS1	27	F1 ^a	AGCCTGCAGGGAACAGAAG	Exon 8	81.41	—		
		R1 ^a	GCCTGACTGTGGTGCTCG	3'UTR				
	28	F1 ^b	CATCGGTTCCATGATGCG	Exon 4	80.38	—		
		F2 ^b	CATCAGTTCATGATGCG	Exon 4				
		R ^b	CCACGATGTCCAGGGGA	Exon 4				
3DP1	29	F ^b	GTACGTCACCCTCCCATGATGTA	5'UTR	85.97	KIR3DP1*004, *009002		
		R ^b	GAAAACGGTGTTCGGAATAC	Exon 3				
GALC (control)	30	F ^c	TTACCCAGAGCCATATCGTTCT		74.89	—		
		R ^c	GTCTGCCATCACCACCTATT					

GALC, galactosylceramidase; KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction; UTR, untranslated region.

^aPrimers designed by Martin and Carrington (16).

^bPrimers designed by Vilches *et al.* (23).

^cPrimers designed by Alves *et al.* (21).

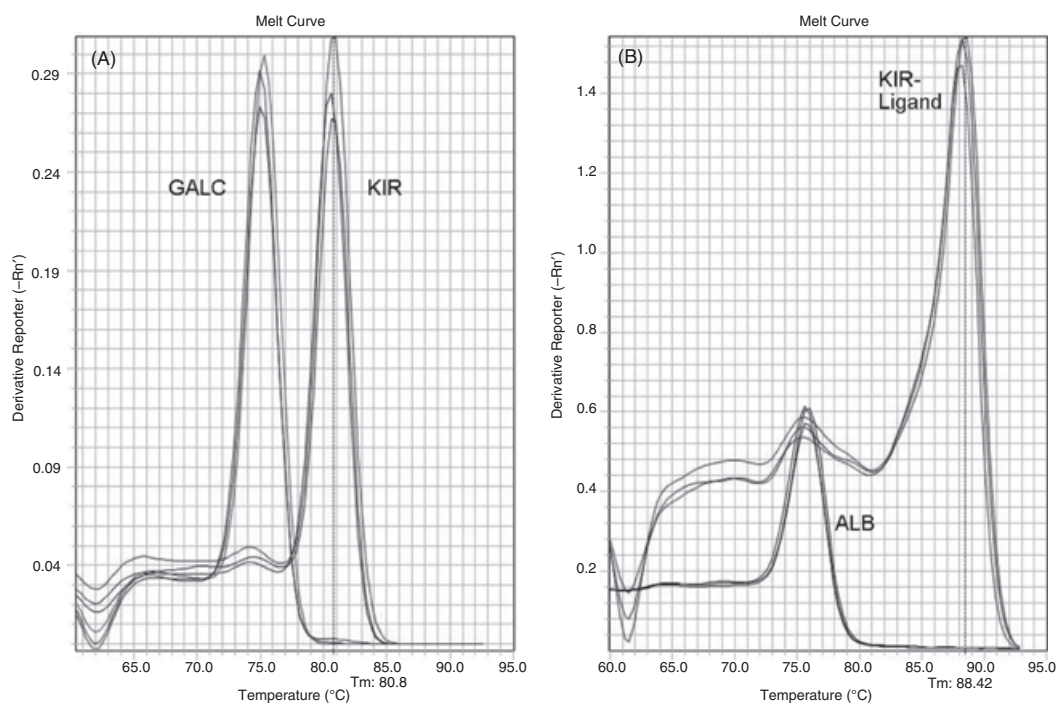


Figure 1 (A) Melt curve analysis of killer immunoglobulin-like receptor (KIR)-polymerase chain reaction (PCR) products melting to the right of the internal control galactosylceramidase (GALC) (74.89°C) and (B) KIR-ligand PCR amplicons melting to the right of the internal control albumin (ALB) (75.62°C).

99.1% (218/220) of individuals tested. All KIR-ligand primer sets, except HLA-B Bw4:T⁸⁰A⁸¹, were 100% concordant with SBT results (Table 4). Our assay failed to correctly assign HLA-B*38:02 to the Bw4:T⁸⁰A⁸¹ allotype for two DNA samples obtained from the IHWG database. The sample was retyped by SBT and confirmed to be correctly assigned based on exon 2, 3 and 4 sequences. In addition, the genomic sequences for HLA-B*38:02:01, B*38:02:02 and B*38:02:03 were available to confirm that the reverse primer binding site had no changes. SBT typing does not include the 5' untranslated region (5'UTR); however, genomic sequences

were available for HLA-B*38:01:01, B*38:02:01 and B*38:14 and confirmed that the forward primer was conserved except for a –7 mismatch. It is unlikely that this was the cause of a failed PCR amplification as the forward primer was found to successfully amplify other HLA-B alleles with the same mismatch. It is therefore possible that these two samples are undescribed B*38:02 alleles with changes in the 5'UTR. Nevertheless, the HLA-B*38:02 allele is prevalent at a low frequency in people of Taiwanese/Chinese descent (0.11% and 0.08%, respectively) and has not been reported within the African continent (28).

Table 2 PCR primer sets used for real-time PCR KIR-ligand genotyping assay

Gene		Primers	Sequences (5'–3') ^a	Product <i>T</i> _m (°C)	Reference
HLA-A	Bw4:I ⁸⁰	F	CCATTGGGTGTCGGGTTTC[C]	87.39	This study
		R ^b	CTCTGGTTGTAGTAGCGGAGCGC[A]		
	Non-Bw4:T ⁸⁰	F	AATCAGTGTGTCGCGGTC[G]	87.65	
		R	TGTAGTAGCCGCGCAGG[G]		
HLA-B	Bw6:N ⁸⁰	F	TCAGCGTCGCCGGGTCCC[A]	87.73	This study
		R	TGTAGTAGCCGCGCAGG[T]		
	Bw4:I ⁸⁰	F	ACCCGACTCAGAATCTCC[T]	87.96	
		R ^b	CTCTGGTTGTAGTAGCGGAGCGC[A]		
	Bw4:T ⁸⁰ A ⁸¹	F	ACCCGACTCAGAATCTCC[T]	88.08	
		R	TGTAGTAGCGGAGCGC[G]		
Bw4:T ⁸⁰ L ⁸¹	F	ACCCGACTCAGAATCTCC[T]	87.73		
	R	CTCTGGTTGTAGTAGCGGAGCAGG[G]			
HLA-C	C1:N ⁸⁰	F	AGCCAATCAGCGTCTCCGC[A]	88.34	This study
		R	GCTCTGGTTGTAGTAGCCGCGCAG[G]		
	C2:K ⁸⁰	F	CCATTGGGTGTCGGGTTCT[A]	88.06	
		R	GCTCTGGTTGTAGTAGCCGCGCAG[T]		
ALB (control)		F	TCGATGAGAAAACGCCAGTAA	75.62	(26)
		R	ATGGTCGCCTGTTACCAA		

ALB, albumin; HLA, human leukocyte antigen; KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction.

^a3' Locked nucleic acid bases are depicted between square brackets. Melting temperature (*T*_m) for each PCR product was determined automatically following melt curve analysis.

^bThe same reverse primer is used for detection of HLA-A and -B Bw4:I⁸⁰ alleles.

Table 3 Validation of real-time PCR KIR genotyping assay (*n* = 50)^a

KIR genes	Carriers/noncarriers		Concordant (%)
	SSP KIR PCR	Real-time KIR assay	
2DL1	48/2	48/2	100
2DL2	27/23	27/23	100
2DL3	41/9	41/9	100
2DL4	50/0	50/0	100
2DL5	26/24	26/24	100
2DS1	15/35	15/35	100
2DS2	27/23	27/23	100
2DS3	10/40	10/40	100
2DS4	47/7	47/7	100
2DS5	21/29	21/29	100
2DP1	48/2	48/2	100
3DL1	47/3	47/3	100
3DL2	50/0	50/0	100
3DL3	50/0	50/0	100
3DS1	13/37	13/37	100
3DP1	50/0	50/0	100

KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction; SSP, sequence-specific primer.

^aConcordance between assays was established by directly comparing the number of individuals who did/did not carry the KIR gene divided by the KIR genes present/absent × 100.

KIR genotyping in a South African population group

KIR genotyping using the real-time PCR KIR assay showed similar gene frequencies to other South African population groups studied (29, 30). We identified 20 different KIR genotype profiles (Table 5), 2 of which have not been previously reported (*Bx46* and *Bx70*) and 1 undescribed KIR

Table 4 Validation of real-time PCR KIR-ligand genotyping assay (*n* = 220)^a

HLA KIR-ligand	Carriers/noncarriers		
	SBT HLA sequence	Real-time KIR-ligand assay	Concordant (%)
HLA-A Bw4:I ⁸⁰	62/158	62/158	100
	Non-Bw4:T ⁸⁰	215/5	215/5
HLA-B Bw6:N ⁸⁰	180/40	180/40	100
	Bw4:I ⁸⁰	93/127	93/127
HLA-C C1:N ⁸⁰	49/171	47/171	95.9
	Bw4:T ⁸⁰ A ⁸¹	11/209	11/209
C2:K ⁸⁰	173/47	173/47	100
	161/59	161/59	100

HLA, human leukocyte antigen; KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction; SBT, sequence-based typing; SSP, sequence-specific primer.

^aConcordance between assays was established by directly comparing the number of individuals who did/did not carry the KIR-ligand gene divided by the KIR-ligand genes present/absent × 100.

Bx genotype with all KIR genes present with the exception of *KIR2DL2*, *KIR2DL3*, *KIR2DS1*, *KIR2DS2* and *KIR3DS1*.

KIR-ligand genotyping in a South African population group

Using the real-time PCR KIR-ligand assay, we determined the absence/presence of the HLA-A/B Bw4 and HLA-C C1/C2 motifs. At the HLA-A locus, 2.5% of individuals were homozygous for Bw4:I⁸⁰ (ligands for KIR3DL1/S1), 69.1% were homozygous for non-Bw4:T⁸⁰

Table 5 Real-time PCR KIR genotyping in a South African Cohort ($n = 81$)^a

KIR genotype	KIR genes																No. of KIR genes	Genotype frequency N (%)
	2DL1	2DL2	2DL3	2DL4	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	2DP1	3DL1	3DL2	3DL3	3DS1	3DP1		
AA1	■	□	□	□	□	□	□	□	■	□	■	■	■	■	■	■	9	20 (24.7)
Bx21	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	13	11 (13.6)
Bx5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	13	7 (8.6)
Bx10	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	11	6 (7.4)
Bx20	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	12	6 (7.4)
Bx112	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	13	5 (6.2)
Bx73	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	15	5 (6.2)
Bx92	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	13	4 (4.9)
Bx228	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	12	3 (3.7)
Bx6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	16	2 (2.5)
Bx9	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	14	2 (2.5)
Bx91	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	14	2 (2.5)
Bx118	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	14	1 (1.2)
Bx27	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	12	1 (1.2)
Bx32	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	11	1 (1.2)
Bx35	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	12	1 (1.2)
Bx4	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	11	1 (1.2)
Bx46	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	13	1 (1.2)
Bx70	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	14	1 (1.2)
Bx*	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	11	1 (1.2)
Gene frequency N (%)	81 (100)	56 (69)	60 (74)	81 (100)	54 (67)	19 (23)	50 (62)	24 (30)	80 (99)	46 (57)	81 (100)	80 (99)	81 (100)	81 (100)	10 (12)	81 (100)		

KIR, killer-cell immunoglobulin-like receptor; PCR, polymerase chain reaction.

^aFrequency of each KIR gene is expressed as a percentage and is defined as the number of individuals having the gene divided by the number of individuals in the cohort ($n = 81$). Genotype frequency is defined by the number of individuals having a particular genotype divided by the number of individuals within the cohort. The black boxes indicate the presence of the gene, whereas the open boxes indicate the absence of the gene. Bx* is a new KIR genotype that has not been reported.

and the remaining 28.4% were heterozygotes (Figure 2). At the HLA-B locus, 40.7% of individuals were Bw6:N⁸⁰ homozygous. Of the remainder who carried a Bw4 allele (ligands for KIR3DL1/S1), 48.1% were heterozygous for Bw6:N⁸⁰/Bw4:I⁸⁰, 3.7% were Bw4:I⁸⁰/T⁸⁰A⁸¹ heterozygotes and 7% were Bw4:I⁸⁰ homozygotes. The Bw4:I⁸⁰/T⁸⁰L⁸¹ was detected in 1.2% of individuals, while no Bw4:T⁸⁰L⁸¹ homozygotes or Bw4:T⁸⁰L⁸¹/Bw6:N⁸⁰ heterozygotes were detected in this population. At the HLA-C locus, 16% of individuals were homozygous for C1:N⁸⁰, 38% were homozygous for C2:K⁸⁰ and the remaining 46% were heterozygotes.

KIR/KIR-ligand pairs in a South African population group

KIR and KIR-ligand data, when taken together, allow receptor-ligand combinations to be determined (Table 6).

Within this relatively small South African cohort ($n = 81$), the frequency of activating KIR/KIR-ligand pairs was lower than that of the inhibitory KIR/KIR-ligand pairs. Activating KIR2DS1 paired with the HLA-C C2 ligand was found at a frequency of 16% and KIR3DS1 paired with the putative HLA-B Bw4 ligand was present in 6.2% of individuals. Inhibitory KIR genes (KIR2DL1/2/3 and KIR3DL1) and their ligands HLA-C C2, HLA-C C1, HLA-C C1 and HLA-A/B Bw4 were present at frequencies of 84%, 44%, 44% and 58%, respectively.

Discussion

We have developed a fast and effective tool to determine KIR and KIR-ligand profiles using real-time PCR and SYBR[®] Green chemistry. Our KIR and KIR-ligand assays were validated against commercial and published methods and

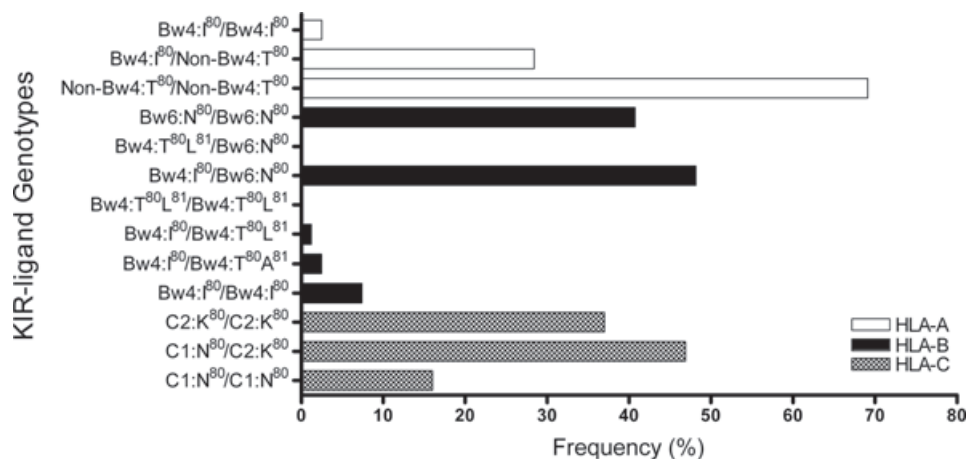


Figure 2 Frequency of killer immunoglobulin-like receptor (KIR)-ligand allele combinations in a South African Cohort ($n = 81$). On the basis of allelic discrimination at codon 80, the KIR-ligand assay can identify the presence/absence of KIR-ligands human leukocyte antigen (HLA)-A (Bw4:⁸⁰I and non-Bw4:⁸⁰T) and HLA-B (Bw4:⁸⁰I, Bw4:⁸⁰T⁸¹L⁸¹ and Bw6:⁸⁰N) and HLA-C (C1 and C2).

Table 6 KIR/KIR-ligand pairs and frequencies in a South African cohort ($n = 81$)^a

KIR gene	KIR gene N (%)	KIR-ligand	KIR-ligand N (%)	KIR + KIR-ligand frequency N (%)	
Inhibitory	2DL1	81 (100)	C2	68 (84)	68 (84.0)
	2DL2	56 (69)	C1	51 (63)	36 (44.4)
	2DL3	60 (74)	C1	51 (63)	36 (44.4)
	3DL1	80 (99)	Bw4	58 (72)	47 (58.0)
Activating	2DS1	19 (23)	C2	68 (84)	13 (16.0)
	3DS1	10 (12)	Bw4 ^b	58 (72)	5 (6.2)

KIR, killer-cell immunoglobulin-like receptor.

^aFrequency of each KIR/KIR-ligand combination is expressed as a percentage and defined as the number of individuals having the paired KIR/KIR-ligand (N) divided by the number of individuals within the cohort ($n = 81$).

^bPutative KIR-ligand for KIR3DS1 based on homology to KIR3DL1.

were found to be 100% and 99.1% concordant, respectively. The advantage of our assays compared to the commercial gel-based KIR SSP-PCR and HLA SBT methods, as well as published KIR/KIR-ligand typing methods, includes its low input DNA concentration, simple technique and ease of interpretation of results. To enhance detection of *KIR* genes that may contain sequence variation in the primer binding sites, multiple primers were used for all except three genes (Table 1). In addition, each of the assays uses a single internal positive control gene with a melting peak significantly lower than the KIR and KIR-ligand amplicon melting peaks. This enables proper discrimination between the absence of a target gene and a failed PCR. Most importantly, the KIR and KIR-ligand assays are very cost-effective, costing as little as 15%–20% of the price of commercial KIR and SBT

typing kits, provided the users have access to a real-time PCR instrument. However, with respect to the HLA SBT typing one must keep in mind the level of allele-specific detail that sequencing provides as opposed to the real-time KIR-ligand allotype designations (*HLA-A/B Bw4* and *HLA-C C1/C2*). Our assay is a single-round detection using SYBR[®] Green chemistry and melting curve analysis, which is cheaper and simpler than other real-time PCR methods that make use of fluorescent probes and multiple amplification steps (22).

Limitations of the KIR and KIR-ligand assay designs are that due to the large number of *KIR* and *HLA* genes described and allelic variation within these, certain alleles can be either undetected or misclassified. The KIR primers used detect all known allelic variants, except the pseudogene KIR3DP1 primer set which does not detect KIR3DP1*004 and KIR3DP1*00902. The KIR-ligand assay failed to detect HLA-B*38:02 as a Bw4:⁸⁰T⁸¹L⁸¹ allotype, but was not likely due to a primer mismatch. Furthermore, the KIR-ligand primers were designed to discriminate Bw4 from Bw6 at codon 80. By definition, amino-acid residues at positions 77 and 80–83 are important in defining the Bw4 (NXXIALR, NXXTALR, SXXIALR, DXXTLLR and SXXTLLR) and Bw6 (SXXNLRG and GXXNLRG) amino-acid motifs (14, 31). Therefore, a limitation of our approach is that unclassified HLA-B alleles such as HLA-B*44:11, B*51:50, B*57:08 and HLA-A*24:61 that contain amino-acid changes within the Bw4 motifs will be classified as Bw4. Likewise, unclassified HLA-B alleles such as HLA-B*07:11, B*07:57, B*08:17, B*35:56, B*39:20, B*40:37, B*55:12, B*78:06, B*95:35, B*08:29, B*27:42, B*037:05, B*25:52, B*37:14, B*47:03, B*40:76 and B*41:05 that have amino-acid changes that are divergent from the Bw6 motifs will be classified as Bw6. Lastly, a number of HLA-B alleles encode the C1 amino-acid motif (B*07:13, B*07:15, B*08:15, B*15:57, B*18:06,

B*35:74, B*39:27, B*40:73, B*46:01-18, B*55:03, B*67:02 and B*73:01) and will be misclassified as Bw6 allotypes by our assay. Therefore, complimentary HLA typing would be necessary in the geographic areas where these alleles have high frequencies, such as HLA-B*46 in Asia. Likewise for KIR2DS4 ligand identification, which consists of HLA-A*11:01/02 and a subset of HLA-C alleles, further HLA typing would be necessary.

When applied to a small South African sample set, we found similar KIR gene frequencies to other published studies (29, 30), and identified an unreported KIR genotype profile (with all KIR genes present with the exception of KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2 and KIR3DS1). In addition, paired KIR and KIR-ligand combinations could be determined showing a dominance of inhibitory compared to stimulatory KIR receptor pairings. Specific combinations of HLA and KIRs have been associated with higher risk for autoimmune disease (32, 33) and differential risk for certain infectious diseases (17, 19), thus it remains to be seen how the representation of activating/inhibitory KIR genes and their ligand pairs might impact on disease susceptibility/resistance in our population.

Conventional KIR and HLA genotyping methodologies involve gel-based SSP-PCR and SBT methods, respectively. These methods can be expensive, labor intensive and time-consuming. We describe an optimized and validated real-time PCR-based KIR and KIR-ligand assays that show high concordance with commercial KIR and HLA typing methods. Melt curve analysis offers straightforward determination of the presence/absence of KIRs or known HLA ligands for KIRs, thereby generating results much faster than conventional methods. In addition, the assay is inexpensive and allows for high-throughput data generation. Together, these two assays have relevance to understanding NK cell function in disease as well as in complications with pregnancy or bone marrow transplantation.

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Conflict of interest

We disclose that there are no conflicts of interest or any commercial links pertinent to this article.

References

- Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol* 2001; **22**: 633–40.
- Moretta A, Bottino C, Mingari MC, Biassoni R, Moretta L. What is a natural killer cell? *Nat Immunol* 2002; **3**: 6–8.
- Middleton D, Curran M, Maxwell L. Natural killer cells and their receptors. *Transpl Immunol* 2002; **10**: 147–64.
- Vilches C, Parham P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* 2002; **20**: 217–51.
- Williams AP, Bateman AR, Khakoo SI. Hanging in the balance. KIR and their role in disease. *Mol Interv* 2005; **5**: 226–40.
- Barbour JD, Sriram U, Caillier SJ, Levy JA, Hecht FM, Oksenberg JR. Synergy or independence? Deciphering the interaction of HLA class I and NK cell KIR alleles in early HIV-1 disease progression. *PLoS Pathog* 2007; **3**: e43.
- Uhrberg M. The KIR gene family: life in the fast lane of evolution. *Eur J Immunol* 2005; **35**: 10–5.
- Faure M, Long EO. KIR2DL4 (CD158d), an NK cell-activating receptor with inhibitory potential. *J Immunol* 2002; **168**: 6208–14.
- Caligiuri MA. Human natural killer cells. *Blood* 2008; **112**: 461–9.
- Kulkarni S, Martin MP, Carrington M. The Yin and Yang of HLA and KIR in human disease. *Semin Immunol* 2008; **20**: 343–52.
- Mandelboim O, Reyburn HT, Vales-Gomez M *et al.* Protection from lysis by natural killer cells of group 1 and 2 specificity is mediated by residue 80 in human histocompatibility leukocyte antigen C alleles and also occurs with empty major histocompatibility complex molecules. *J Exp Med* 1996; **184**: 913–22.
- Graef T, Moesta AK, Norman PJ *et al.* KIR2DS4 is a product of gene conversion with KIR3DL2 that introduced specificity for HLA-A*11 while diminishing avidity for HLA-C. *J Exp Med* 2009; **206**: 2557–72.

13. Cella M, Longo A, Ferrara GB, Strominger JL, Colonna M. NK3-specific natural killer cells are selectively inhibited by Bw4-positive HLA alleles with isoleucine 80. *J Exp Med* 1994; **180**: 1235–42.
14. Gumperz JE, Litwin V, Phillips JH, Lanier LL, Parham P. The Bw4 public epitope of HLA-B molecules confers reactivity with natural killer cell clones that express NKB1, a putative HLA receptor. *J Exp Med* 1995; **181**: 1133–44.
15. Muller CA, Engler-Blum G, Gekeler V, Steiert I, Weiss E, Schmidt H. Genetic and serological heterogeneity of the supertypic HLA-B locus specificities Bw4 and Bw6. *Immunogenetics* 1989; **30**: 200–7.
16. Martin MP, Carrington M. KIR locus polymorphisms: genotyping and disease association analysis. *Methods Mol Biol* 2008; **415**: 49–64.
17. Jennes W, Verheyden S, Demanet C *et al.* Cutting edge: resistance to HIV-1 infection among African female sex workers is associated with inhibitory KIR in the absence of their HLA ligands. *J Immunol* 2006; **177**: 6588–92.
18. Gao X, Jiao Y, Wang L *et al.* Inhibitory KIR and specific HLA-C gene combinations confer susceptibility to or protection against chronic hepatitis B. *Clin Immunol* 2010; **137**: 139–46.
19. Vidal-Castineira JR, Lopez-Vazquez A, Diaz-Pena R *et al.* Effect of killer immunoglobulin-like receptors in the response to combined treatment in patients with chronic hepatitis C virus infection. *J Virol* 2010; **84**: 475–81.
20. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16**: 1215.
21. Alves LG, Rajalingam R, Canavez F. A novel real-time PCR method for KIR genotyping. *Tissue Antigens* 2009; **73**: 188–91.
22. Koehler RN, Walsh AM, Moqueet N *et al.* High-throughput genotyping of KIR2DL2/L3, KIR3DL1/S1, and their HLA class I ligands using real-time PCR. *Tissue Antigens* 2009; **74**: 73–80.
23. Vilches C, Castano J, Gomez-Lozano N, Estefania E. Facilitation of KIR genotyping by a PCR-SSP method that amplifies short DNA fragments. *Tissue Antigens* 2007; **70**: 415–22.
24. Robinson J, Waller M, Stoehr P, Marsh S. IPD – the Immuno Polymorphism Database. *Nucleic Acids Res* 2005; **33**: D523–6.
25. Robinson J, Malik A, Parham P, Bodmer J, Marsh S. IMGT/HLA – a sequence database for the human major histocompatibility complex. *Tissue Antigens* 2000; **55**: 280–7.
26. Douek DC, Brenchley JM, Betts MR *et al.* HIV preferentially infects HIV-specific CD4+ T cells. *Nature* 2002; **417**: 95–8.
27. Listgarten J, Brumme Z, Kadie C *et al.* Statistical resolution of ambiguous HLA typing data. *PLoS Comput Biol* 2008; **4**: e1000016.
28. Gonzalez-Galarza F, Christmas S, Middleton D, Jones A. Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. *Nucleic Acids Res* 2011; **39**: D913–9.
29. Wong AH, Williams K, Reddy S *et al.* Alterations in natural killer cell receptor profiles during HIV type 1 disease progression among chronically infected South African adults. *AIDS Res Hum Retroviruses* 2010; **26**: 459–69.
30. Paximadis M, Minevich G, Winchester R *et al.* KIR-HLA and maternal-infant HIV-1 transmission in sub-Saharan Africa. *Plos One* 2011; **6**: e16541.
31. Gumperz JE, Barber LD, Valiante NM *et al.* Conserved and variable residues within the Bw4 motif of HLA-B make separable contributions to recognition by the NKB1 killer cell-inhibitory receptor. *J Immunol* 1997; **158**: 5237–41.
32. Ramos-Lopez E, Scholten F, Aminkeng F *et al.* Association of KIR2DL2 polymorphism rs2756923 with type 1 diabetes and preliminary evidence for lack of inhibition through HLA-C1 ligand binding. *Tissue Antigens* 2009; **73**: 599–603.
33. Salim PH, Jobim M, Bredemeier M *et al.* Killer cell immunoglobulin-like receptor (KIR) genes in systemic sclerosis. *Clin Exp Immunol* 2010; **160**: 325–30.