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BRIEF COMMUNICATION

High-throughput genotyping of KIR2DL2/L3, KIR3DL1/S1, and their HLA class I ligands using real-time PCR

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Abstract

Killer immunoglobulin-like receptors (KIRs) expressed on natural killer cells are critical components of innate immunity. Interactions between KIRs and their human leukocyte antigen (HLA) ligands have been shown to influence autoimmune and infectious disease course in defined populations. However, the low throughput and high cost of current methods impede confirmation of the universality of these findings. To support large epidemiology surveys, we developed a high-throughput real-time polymerase chain reaction-based assay to identify carriers of KIR3DL1, KIR3DS1, KIR2DL2, and KIR2DL3 and their HLA ligands. The platform performed with 100% sensitivity and specificity in detection of carrier and non-carrier on reference samples. The application of this platform will further clarify the nature and impact of the KIR–HLA epistatic interaction on disease course in large global population-based studies.

The killer immunoglobulin-like receptors (KIRs) expressed on the surface of natural killer (NK) cells exhibit a high level of genetic diversity (1). The KIR locus encodes a complex array of inhibitory and activating receptors, and their recognition of corresponding human leukocyte antigen (HLA) class I ligands is a key determinant of NK cytotoxic activity (2, 3). This cytotoxic activity is the result of a balance of signals transmitted by the activating and inhibitory KIRs in the presence of their corresponding HLA ligands on target cells (4, 5). The highly polymorphic nature of both KIR and HLA allows the potential to diversify NK-cell responses in individuals and across populations in response to infectious diseases. Recently, several epistatic interactions between KIRs and their HLA class I ligands have been found to be associated with a variety of outcomes of autoimmune and infectious diseases, highlighting the importance of their systematic characterization of world populations. As a variety of KIR–HLA epistatic interactions has been implicated in autoimmune and infectious disease pathogenesis or resistance (6–8), it has become increasingly necessary and important to genotype both KIR and their HLA ligands. It has been proposed that the KIR–HLA genotype determines two distinct classes of NK cells, the functionally competent 'licensed' NK cells that expresses KIR recognizing self-HLA class I molecules and another functionally incompetent 'unlicensed' class that does not (5, 9, 10). Therefore, determining the 'licensing' or functional competency of NK cells in future disease association studies in world populations will be highly relevant. In order to support the advancement of studies of the innate cellular immunity, we have developed and validated a high-throughput, cost-effective, sensitive, and

specific real-time polymerase chain reaction (PCR) genotyping platform for the typing of KIR and their HLA ligands in population-based studies.

Among different *KIR* genes, the activating KIR3DS1 and the inhibitory KIR3DL1 segregate as alleles of the KIR3DL1/S1 locus. KIR3DL1 and putatively KIR3DS1 are receptors for a subset of HLA-A and -B alleles, those carrying the Bw4 public epitope (11, 12). Bw4 serospecificity depends on variable amino acid residues at positions 77–83. HLA-B Bw4 allotypes are dimorphic at position 80, expressing either Ile or Thr, and KIR3DL1 has been shown to bind Bw4 80Ile variants with higher affinity than those carrying Bw4 80Thr (11). In turn, HLA-B Bw4 80Thr is dimorphic at position 81, expressing either Ala or Leu. All the HLA-A alleles expressing the Bw4 epitope bear Ile at position 80. At the HLA-B locus, alleles not bearing the Bw4 epitope express the Bw6 epitope (13), which is not known to interact with KIRs. Similarly, in the HLA-A locus, alleles not expressing the Bw4 epitope [i.e. Bw4(−)] do not exhibit interactions with KIRs.

KIR2DL2 and KIR2DL3, both inhibitory KIRs, segregate as alleles of the KIR2DL2/L3 locus and recognize HLA-C group C1 ligands with high and low affinity, respectively (14). Position 80 of HLA-C determines the mutually exclusive C1 or C2 grouping. C1 group alleles encode Asn at residue 80, while group C2 alleles encode Lys.

The gold standard method for characterization of KIR and HLA ligand types is sequence-based typing (SBT); however, throughput, convenience, and cost limit its application for large population-based surveys. Other available typing technologies lack either the throughput or the resolution needed to conduct large-scale association studies. In this report, we describe the development and validation of a high-throughput, cost-effective, sensitive, and specific real-time PCR genotyping platform for the typing of KIR and their HLA ligands in population-based studies. This platform can identify carriers of KIR3DL1, KIR3DS1, KIR2DL2, and KIR2DL3, as well as their corresponding HLA class I ligands.

For the development and validation of the KIR typing platform, a reference panel of 48 specimens characterized by sequence-specific oligonucleotide probe (SSOP)–PCR and sequence-specific priming (SSP)–PCR methods was used (15). This KIR reference panel was available through the 13th International Histocompatibility Workshop. The HLA typing platform was developed and validated on a panel of specimens from Kampala, Uganda, previously genotyped by SBT (16), which represents all the addressed ligand motifs. Primers and TaqMan probes distinguishing KIR2DL2 from KIR2DL3 and KIR3DL1 from KIR3DS1 and their respective HLA class I ligands were designed based on the available sequence database (17).

Carriers of KIR2DL2, KIR2DL3, KIR3DL1, and KIR3DS1 were genotyped in separate SSP–real-time PCRs. Each typing reaction consisted of a sequence-specific reaction for the interrogated polymorphism and a parallel universal reaction that was used for internal standardization in a multiplex PCR format. Each of these genotyping PCR mixtures consisted of TaqMan 2× Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA), 400 nM of each forward and reverse sequence-specific primers, 400 nM of each forward and reverse universal primers, 250 nM of a locus-specific probe, 250 nM of a universal probe, and genomic DNA (1.25–25 ng) in a final volume of 6.25 µl. The universal primers for all KIR reactions targeted the *GAPDH* gene. All samples were run on a 384-well plate format with the following thermocycling program: 10 min at 95°C followed by 60 cycles of 15 s at 95°C and 1 min at 60°C. The intensity of each fluorescent probe was read automatically by the 7900HT Fast Real-time PCR System (Applied Biosystems) and analyzed and interpreted with SEQUENCE DETECTION SOFTWARE (version 2.2.2) as the cycle threshold (Ct), i.e., the number of cycles required to bring the fluorescent signal generated in the reaction above a set threshold. Samples that did not cross the threshold were manually assigned a Ct of 60.

Primer and probe sequences designed and used in each reaction are indicated in Table 1, and their application in each reaction mix is shown in Table 2. Some of the primers were taken from standard PCR-based protocols as published or were slightly modified to accommodate their application with real-time PCR, while others were newly developed. For several reactions, primers bearing locked nucleic acids (18) were used (Exiqon, Vedbaek, Denmark). These are a class of nucleic acid analogues that allow for PCR extension but have a more rigid configuration than standard primers, allowing for higher specificity, although sometimes at the expense of amplification efficiency. Their applicability for each reaction was determined empirically.

For HLA ligand typing, a 900- to 980-bp fragment encompassing exons 2 and 3 of HLA-A, -B, or -C was PCR amplified using locus-specific reagents targeting conserved regions of each respective *HLA* gene as previously described (19). The first-round PCR contained 10× PCR Gold Buffer (Applied Biosystems), 200 nM of each dNTP, 1.5 mM $MgCl_2$, 400 nM of each primer, 1.25 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and genomic DNA (20–100 ng) in a final volume of 50 µl. Thermocycling conditions were 10 min at 95°C, followed by 30 cycles of 30 s at 95°C, 1 min at 65°C, and 2 min at 72°C. PCR products were each diluted 1000-fold in molecular-grade water for use in subsequent genotyping reactions. Corresponding first-round dilutions were distributed into 2, 4, and 2 separate ligand typing SSP–real-time PCRs for the targeted variants in HLA-A, -B, and -C, respectively. Each ligand typing reaction was a multiplex real-time PCR designed to target both a ligand-specific region and a non-polymorphic region of the amplicon itself for internal

Table 1 PCR primers and probes used for KIR and KIR ligand identification by real-time PCR

Locus	Name	Sequence[a] (5′–3′)	Location[b]
HLA-A	A226F01	GGCTCYCACTCCATGAGGTATTTC	203–226
HLA-A	A818F01	GGCAAGGATTACATCGCC	801–818
HLA-B	B224F01	GCTCCCACTCCATGAGGTATTTC	202–224
HLA-B	B751F01	CAGANSATGTAYGGCTGCGAC	731–751
HLA-C	C232F01	AGGCTCCCACTCCATGARGTATTTC	207–232
HLA-C	C798F01	CCTCCTCCGCGGGTAT	783–798
GAPDH	GAPDHF	ACCGGGAAGGAAATGAATGG	816–835
KIR2DL2	KIR2DL2F[c]	GGGGGAGGCACATGAAT	5376–5392
KIR2DL2	KIR2DL3F	CCCTCAGGAGGTGACATATG	13863–13883
KIR3DL1	KIR3DL1F[c]	CCTGGTGAAATCAGGAGAGAG	3316–3336
KIR3DS1	KIR3DS1F[d]	CAGCGCTGTGGTGCCTCGC	1859–1877
HLA-A	A652R01	GTAGTAGCCGCGCAGGG	652–669
HLA-A	A652R02	GGTTGTAGTATCGGAGCGCG[A]	652–673
HLA-A	A932R01	GAGCCMSTCCACGCAC	932–947
HLA-B	B439R01	TGTAGTAGCCGCGCAGGT	439–456
HLA-B	B439R02	GGTTGTAGTATCGGAGCGCG[G]	439–456
HLA-B	B439R03	GGTTGTAGTAGCGGAGCAGG[G]	439–456
HLA-B	B439R04	GGTTGTAGTATCGGAGCGCG[A]	439–456
HLA-B	B860R01	CTGGGTGATCTGAGCCGC	860–877
HLA-C	C439R01	GCAGTTTCCGCAGGT	439–453
HLA-C	C439R02	GCAGGTTCCGCAGG[C]	439–453
HLA-C	C948R01	GAGCCACTCCACGCAC	948–963
GAPDH	GAPDHR	GCAGGAGCGCAGGGTTAGT	862–880
KIR2DL2	KIR2DL2R	AAGCAGTGGGTCACTCGAGTT	5518–5538
KIR2DL3	KIR2DL3R[c]	GCAGGAGACAACTTTGGATCA	13996–14016
KIR3DL1	KIR3DL1R[c]	TGTAGGTCCCTGCAAGGGCAA	3480–3500
KIR3DS1	KIR3DS1R[d]	CTGTGACCATGATCACCAT	2091–2109
HLA-A	A278P01	FAM-CGCACAAACTGCATGTCGTCCACGTAGCC-BHQ1	278–306
HLA-A	A846P01	TET-TCTGAGCCGCCATGTCCGCCGC-BHQ1	846–867
HLA-B	B320P01	FAM-CWGTACGTGAGGTTCGACAGCGACGCC-BHQ1	294–320
HLA-B	B805P01	TET-CAAGGATTACATCGCCCTGAACGAGGACCTG-BHQ1	805–835
HLA-C	C380P01	FAM-CGCTTGTACTTCTGTGTCTCCCGGTCCCAATACTCC-BHQ1	380–415
HLA-C	C818P01	TET-AGRTCCTCGTTCAGGGCGATGTAATCCTTGC-BHQ1	818–848
GAPDH	GAPDHP01	FAM-ACCGGCAGGCTTTCCTAACGGCT-BHQ1	838–860
GAPDH	GAPDHP02	TET-ACCGGCAGGCTTTCCTAACGGCT-BHQ1	838–860
KIR2DL2	KIR2DL2P	FAM-TTCTCTGCAGGGCCCAAGGTCAACGG-BHQ1	5398–5423
KIR2DL3	KIR2DL3P	FAM-AGAGGCCCAAGACACCCCCAACAGA-BHQ1	13934–13959
KIR3DL1	KIR3DL1P	TET-ATCTCTAAGGACCCCTCACGCCTCGTTGGACA-BHQ1	3398–3429
KIR3DS1	KIR3DS1P	FAM-TGACTCTTCGGTGTCACTATCGTCATAGGTTTAACAATTTCAT-BHQ1	1888–1930

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

[a] Locked nucleic acid bases are depicted between brackets (Materials and methods).

[b] Locations based on reference sequences NC_000006.10 30018310-30021633 for HLA-A, NC_000006.10 C31432914-31429628 for HLA-B, NC_000006.10 31347834-31344508 for HLA-C, NC_000019.8|NC_000019:60019705-60034045 for KIR3DL1, NT_113949.1|Hs19_111668:70097-84658 for KIR3DS1, NG_005994.1|:30696-45237 for KIR2DL2, NC_000019.8|NC_000019:59941786-59956316 for KIR2DL3, and NC_000012.10|NC_000012:6513918-6517797 for GAPDH.

[c] Modified from previously published (25).

[d] Previously published (25).

standardization. Table 2 indicates the primers used for each of the typing reactions. Each reaction used PCR conditions equivalent to those described for KIR typing. In all cases, non-template controls were included where water substituted for genomic DNA.

The KIR typing reactions effectively distinguished carriers and non-carriers of KIR2DL2 and KIR2DL3 alleles (Figure 1). For example, the reactivity of the KIR reference panel with the KIR2DL2-discriminating reaction is presented as a scatter plot of Ct values from the KIR2DL2-allele-specific reaction plotted on the *x*-axis *vs* the Ct value of the internal standardization reaction plotted on the *y*-axis for each sample (Figure 1A). Samples carrying the KIR2DL2 allele formed a distinct cluster, with similar

Table 2 Primer and probe combinations used for KIR and KIR ligand typing reactions by real-time polymerase chain reaction

		Specific reaction			Internal standardization reaction		
Locus	Specificity	Forward primer[a]	Reverse primer[a]	Probe[a]	Forward primer	Reverse primer	Probe
HLA-A	HLA-A Bw4(−)	A226F01	A652R01	A278P01	A818F01	A932R01	A846P01
HLA-A	HLA-A Bw4(+)	A226F01	A652R02	A846P01	A818F01	A932R01	A846P01
HLA-B	HLA-B Bw6	B224F01	B439R01	B320P01	B751F01	B860R01	B805P01
HLA-B	HLA-B Bw4 80Thr 81Ala	B224F01	B439R02	B320P01	B751F01	B860R01	B805P01
HLA-B	HLA-B Bw4 80Thr 81Leu	B224F01	B439R03	B320P01	B751F01	B860R01	B805P01
HLA-B	HLA-B Bw4 80Ile	B224F01	B439R04	B320P01	B751F01	B860R01	B805P01
HLA-C	HLA-C group 2	C232F01	C439R01	C380P01	C798F01	C948R01	C818P01
HLA-C	HLA-C group 1	C232F01	C439R02	C380P01	C798F01	C948R01	C818P01
KIR3DL1/3DS1	KIR3DL1	KIR3DL1F	KIR3DLR	KIR3DL1P	GAPDHF	GAPDHR	GAPDHP
KIR3DL1/3DS1	KIR3DS1	KIR3DS1F	KIR3DS1R	KIR3DS1P	GAPDHF	GAPDHR	GAPDHP
KIR2DL2/2DL3	KIR2DL2	KIR2DL2F	KIR2DL2R	KIR2DL2P	GAPDHF	GAPDHR	GAPDHP
KIR2DL2/2DL3	KIR2DL3	KIR2DL3F	KIR2DL3R	KIR2DL3P	GAPDHF	GAPDHR	GAPDHP

HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor.
[a] Primer names refer to Table 1.

levels of amplification for both the internal standardization and the specific reactions. In contrast, samples that are non-carriers of the KIR2DL2 allele did not amplify a specific product yet amplified the internal standardization reaction comparable to carrier samples, forming a separate cluster. Non-template controls did not react with either the internal standard or the specific reactions. Similarly, the KIR2DL3, KIR3DS1, and KIR3DL1 carriers were effectively distinguished (Figure 1B–D). In the case of KIR3DL1-specific reaction, the carriers of this allele showed a similarly efficient reactivity for both the specific and the internal standardization reactions, while the single non-carrier of KIR3DL1 present in the reference panel exhibited significantly less efficient amplification in the specific reaction allowing for differentiation from the carrier group. The IHWG gold standard panel contains only 1/48 non-carriers of KIR3DL1. In order to further address the performance of the platform on this genotype, we supplemented the standard panel with additional KIR3DL1 non-carrier specimens (n = 4), and we were able to clearly differentiate KIR3DL1 carriers from non-carriers (Supplemental Figure S1).

Reactions distinguishing KIR ligands (i.e. serotypic variants of HLA-A, -B, and -C) were validated with a reference panel previously genotyped by SBT ($n = 175$) (16). To eliminate cross-reactivity among the highly homologous HLA loci, genotyping reactions were preceded by separate locus-specific first-round PCRs. Subsequent genotyping reactions consisted of SSP–real-time PCRs targeting the polymorphisms in exon 2 that define each serotype and an internal standardization reaction targeting an invariant region in exon 3. Figure 2 shows the reactivity of the panel with each genotyping reaction that distinguished carriers of HLA-A Bw4(+), HLA-A Bw4(−), HLA-B Bw6, HLA-B Bw4 80Ile, and HLA-B Bw4 80Thr. For all reactions, carriers and non-carriers of the interrogated serotype formed distinct clusters (Figure 2) characterized, respectively, by exhibiting similar reactivity in the specific and internal standardization reactions and reactivity in the internal standardization reaction in the absence of reactivity in the specific reaction.

Of note, reactions designed to identify carriers of alleles bearing the Bw4 80Thr epitope were partitioned into two separate typing reactions specific for either Bw4 80Thr, 81Ala or Bw4 80Thr, 81Leu. Variation in position 81 represented a sequence constraint, and therefore, two reactions were used to distinguish these variants. Each of these two HLA-B Bw4 80Thr typing reactions exhibited significant separation between carriers and non-carriers. For each of the 12 reactions that are part of the current typing platform, 100% sensitivity, specificity, positive predictive value, and negative predictive value were attained, establishing full concordance with gold standard typing methods (Table 3).

It is increasingly clear that epistatic interactions between KIR and their ligands have functional significance and can influence disease course including resolution of viral infection. Recently, several epidemiological studies have identified correlations between specific KIRs and their ligands with certain disease outcomes; notably, KIR3DS1 in combination with Bw4 80Ile has been associated with decreased progression to AIDS (8), while KIR2DL3 and HLA-C group C1 have been associated with increased clearance of hepatitis C virus (20). The currently described typing method combines sensitivity and specificity with a high-throughput platform using SSP–real-time PCR, which can facilitate rapid screening of carriers of KIR3DL1, KIR3DS1, KIR2DL2, and KIR2DL3 and the HLA alleles

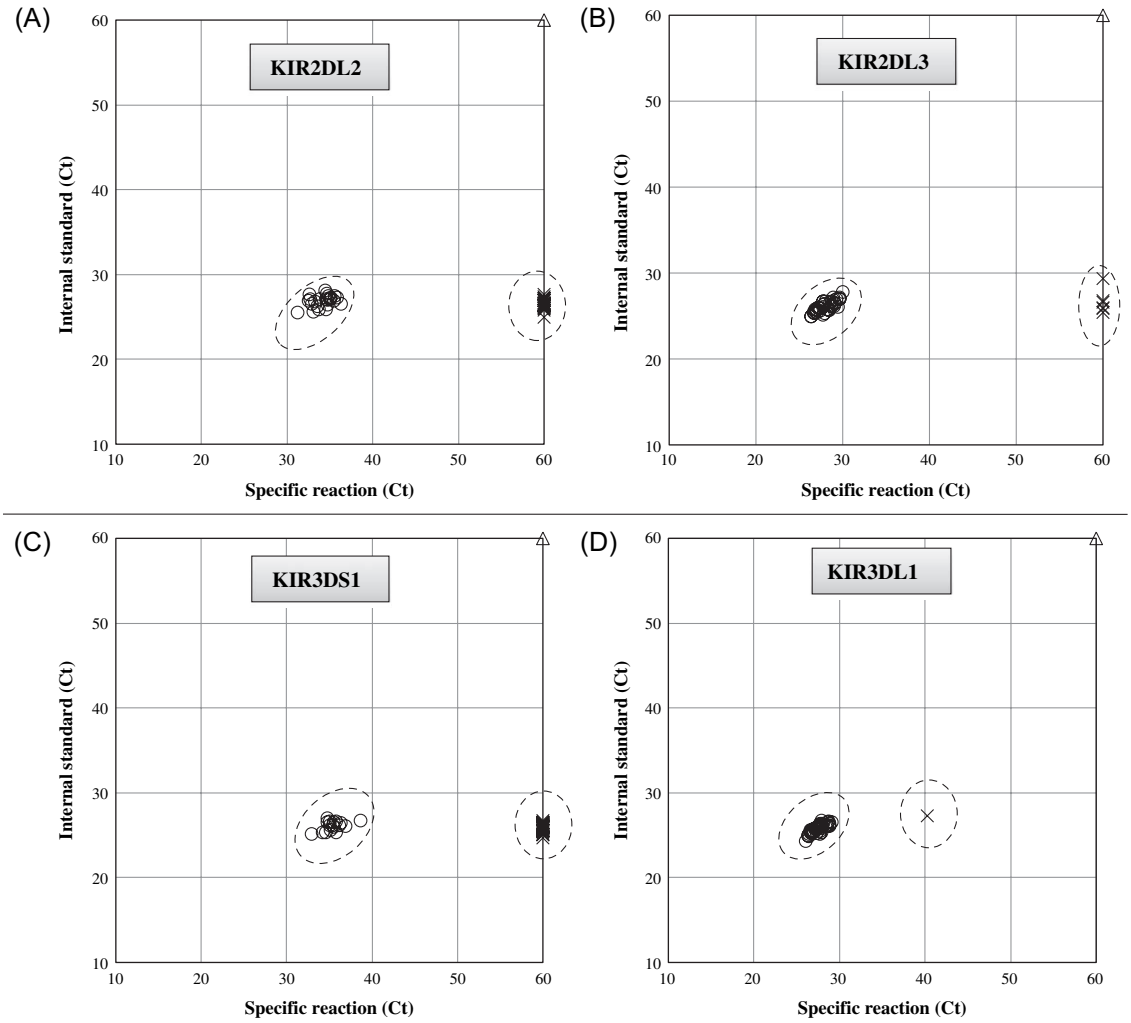

Figure 1 Real-time polymerase chain reaction-based reactivity of the killer immunoglobulin-like receptor (KIR) typing platform on a reference panel ($n = 48$). Data points represent reactivity in the allele specific (*x*-axis) and internal standardization reactions (*y*-axis) expressed as the measured cycle threshold value. Open circles depict carriers for (A) KIR2DL2, (B) KIR2DL3, (C) KIR3DS1, and (D) KIR3DL1. Crosses represent non-carriers for the indicated allele. Triangles indicated non-template controls. Carrier and non-carrier groups are each outlined.

encoding their associated ligands. Use of this platform allows the carrier frequency of any given world population to be determined with relatively few reactions, with the potential to enhance population-based disease association studies of these KIR and their HLA ligands.

Current KIR and KIR ligand typing methodologies, including SBT, are labor intensive and costly, while methodologies such as SSOP-PCR are time-consuming and not ideal for large-scale population-based studies. The use of real-time PCR provides a bridge between the high resolution obtained by gold standard methods and the high throughput needed to conduct extensive surveys. The platform has the potential for supplementation with additional reactions to account for other polymorphisms of interest. One of the limitations of the platform is that because of its assessment of single short regions in each KIR locus, naturally occurring rare interallele KIR recombinants (21–23) can be mistyped.

Real-time PCR offers the option for either a kinetic or an endpoint readout. In the current platform, we used the former readout as it has the advantage of providing additional information critical to SSP-PCR. SSP-PCR is based on the level and nature of sequence identity between primers and target (24), therefore determining the efficiency of the reaction, here reflected in Ct values, provided clear discrimination among genotypes (Figures 1 and 2).

KIR allele carrier frequencies vary in world populations; for example, the KIR3DS1 carrier frequency correlates inversely with increasing distance from East Africa (21). This platform allows rapid determination of carrier frequencies of KIR3DS1 and other KIR alleles in any population, which will expand the growing database of KIR

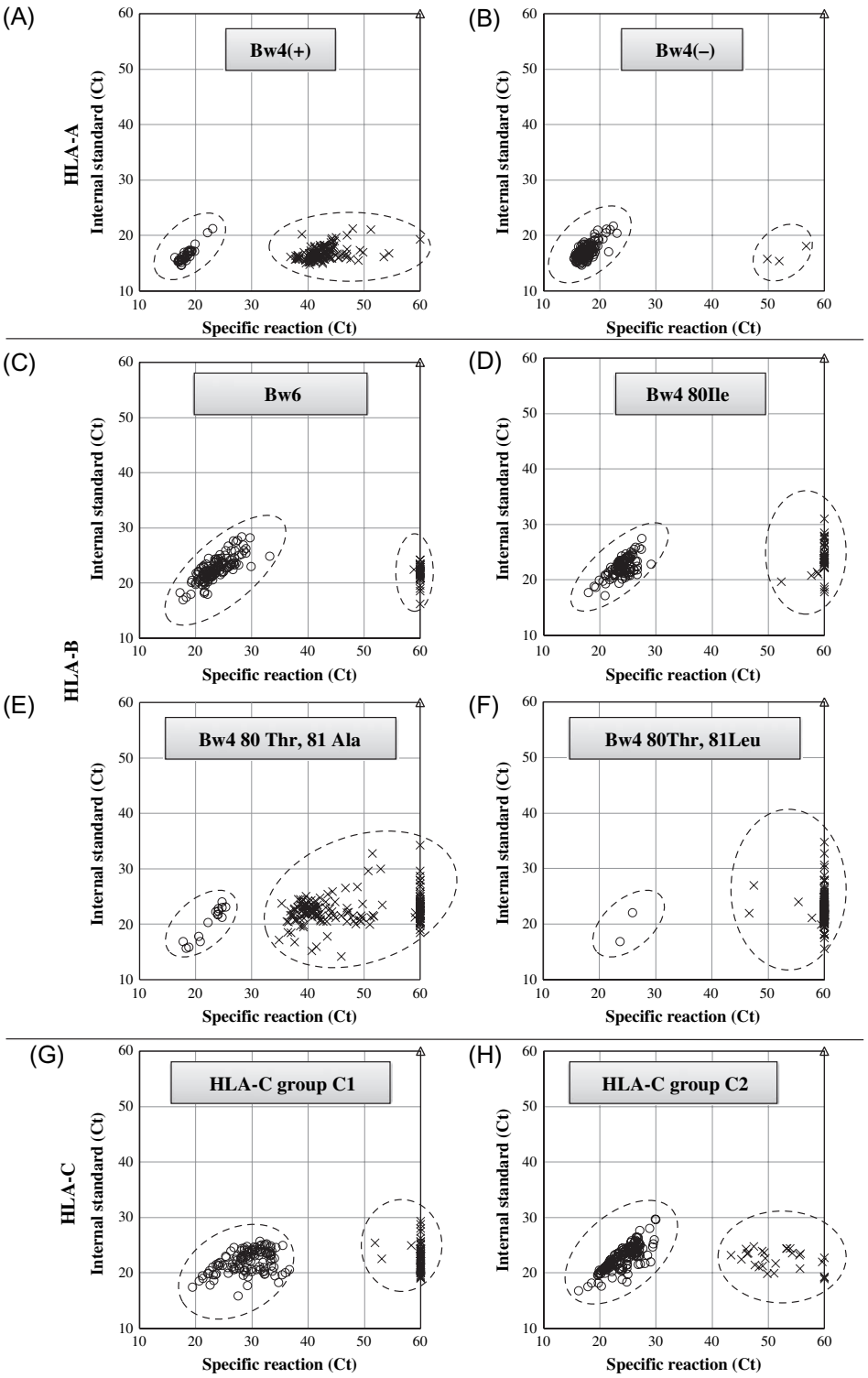

Figure 2 Performance of the real-time polymerase chain reaction-based typing platform for killer immunoglobulin-like receptor ligands. Reactivities for a sequence-based typing reference panel ($n = 175$) are shown. Each circle represents carriers of alleles bearing the serotype indicated for the given reaction: (A) HLA-A Bw4(+), (B) HLA-A Bw4(−), (C) HLA-B Bw6, (D) HLA-B Bw4 80Ile, (E) HLA-B Bw4 80Thr, 81Ala, (F) HLA-B Bw4 80Thr, 81Leu, (G) HLA-C group C1, and (H) HLA-C group C2. The crosses represent non-carriers of targeted motifs. Triangles indicated non-template controls. Carrier and non-carrier groups are each outlined.

Table 3 Performance of typing platform with reference panels

Reference panel	Targeted receptor or ligand	Carriers	Non-carriers	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
KIR panel ($n = 48$)	KIR3DL1	47	1	100	100	100	100
	KIR3DS1	17	31	100	100	100	100
	KIR2DL2	24	24	100	100	100	100
	KIR2DL3	42	6	100	100	100	100
HLA panel ($n = 175$)	HLA-A Bw4(−)	35	140	100	100	100	100
	HLA-A Bw4(+)	172	3	100	100	100	100
	HLA-B Bw6	140	35	100	100	100	100
	HLA-B Bw4 80Thr 81Ala	15	160	100	100	100	100
	HLA-B Bw4 80Thr 81Leu	2	173	100	100	100	100
	HLA-B Bw4 80Ile	105	70	100	100	100	100
	HLA-C group 2	148	27	100	100	100	100
	HLA-C group 1	119	56	100	100	100	100

HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor.

genotypes (http://www.allelefrequencies.net). This platform can also assist in the elucidation of the respective contributions of HLA-A Bw4 and HLA-B Bw4 alleles as ligands of KIR3DS1/L1 as the ligand genotype is assessed in separate reactions after preamplification of HLA-A or -B. Another relevant question that can be addressed with the current platform is the epidemiological significance of HLA-B Bw4 80Thr as a ligand of KIR3DL1/S1 (11), including the contribution of the dimorphic position 81, whose significance remains to be elucidated.

The reference panels used contained both carriers and non-carriers of each KIR or HLA allele variant, as indicated in Table 3. Although these reference panels contained only two carriers of alleles bearing HLA-B Bw4 80Thr, 81Leu, and one non-carrier of KIR3DL1, all these samples were correctly typed. Larger sample sets from populations where these alleles are more frequent will be important for establishing the capacity of the platform to consistently type these rare ligands. Future implementation of this platform in other world populations should therefore be able to clearly differentiate carriers and non-carrier of each allele addressed by the platform.

In conclusion, the platform performed with high sensitivity, specificity, positive predictive value, and negative predictive value, showing that this assay provides a high-throughput cost-effective alternative to gold standard methods for the typing of large sample sets. The platform can provide much needed genetic information, not currently approachable by other technologies, which will contribute to the understanding of host variation and its effects on autoimmune and infectious diseases.

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Supporting information

Additional Supporting Information may be found in the online version of this article.

Figure S1. KIR3DL1/S1 genotyping by SSP-PCR and real-time SSP-PCR. (A) KIR3DL1/S1 genotyping of additional samples ($n = 8$) was performed by SSP-PCR as previously described with two sets of primers designed for each locus and an internal control (25). A non-template control (NTC) was also included. Genotyping reactions for KIR3DS1 (i and ii) and KIR3DL1 (iii and iv) are shown with corresponding PCR product sizes. With the same set of samples, real-time PCR-based reactivity of the KIR typing platform for (B) KIR3DS1 and (C) KIR3DL1 are shown. Data points represent reactivity in the allele specific (x-axis) and internal standardization reactions (y-axis) expressed as the measured Ct value. As determined by the SSP-PCR genotyping reactions, genotypes are listed with their respective identifying symbol. Carrier and non-carrier groups are each outlined. See text for details.

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