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# The production of KIR-Fc fusion proteins and their use in a multiplex HLA class I binding assay

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## Abstract

Soluble recombinant proteins that comprise the extracellular part of a surface expressed receptor attached to the Fc region of an IgG antibody have facilitated the determination of ligand specificity for an array of immune system receptors. Among such receptors is the family of killer cell immunoglobulin-like receptors (KIR) that recognize HLA class I ligands. These receptors, expressed on natural killer (NK) cells and T cells, play important roles in both immune defense and placental development in early pregnancy. Here we describe a method for the production of two domain KIR-Fc fusion proteins using baculovirus infected insect cells. This method is more scalable than traditional mammalian cell expression systems and produces efficiently folded proteins that carry posttranslational modifications found in native KIR. We also describe a multiplex binding assay using the Luminex platform that determines the avidity and specificity of two domain KIR-Fc for a panel of microbeads, each coated with one of 97 HLA class I allotypes. This assay is simple to perform, and represents a major improvement over the assays used previously, which were limited in the number of KIR and HLA class I combinations that could be assayed at any one time. The results obtained from this assay can be used to predict the response of NK cell and T cells when their KIR recognize HLA class I.

### Keywords

Natural Killer Cells; MHC; Comparative Immunology/Evolution; Antigens/Peptides/Epitopes

## 1. Introduction

Killer-cell immunoglobulin like receptors (KIR) are a family of germ-line encoded cell surface receptors that regulate the activity of natural killer (NK) cells and T cells in immunity and reproduction through interaction with HLA class I molecules (Parham and

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Moffett 2013). Both KIR and HLA are encoded by polymorphic genes, which have numerous alleles encoding unique KIR and HLA class I proteins, which are known as allotypes. HLA class I proteins are expressed on the surface of most cell types and present a diverse repertoire of peptides that furnish ligands for KIR and other immune system receptors (Bjorkman et al. 1987; Colonna et al. 1992; Colonna and Samaridis 1995; Moretta et al. 1993). The *HLA class I* locus contains three highly polymorphic genes, called *HLA-A, B* and *C. HLA-C* is the most recently evolved and the only one for which all the variant forms are KIR ligands (Guethlein et al. 2007; Older Aguilar et al. 2010; Older Aguilar et al. 2011). Dimorphism at position 80 in HLA-C defines two epitopes, C1 (asparagine 80) and C2 (lysine 80), which are ligands for two different forms of two-domain KIR (Mandelboim et al. 1996; Winter and Long 1997). *KIR2DL1* encodes methionine at position 44 and binds to C2 bearing HLA-C, *KIR2DL2/3* encodes lysine at position 44 and binds to C1 bearing HLA-C allotypes.

Because the genes encoding KIR and HLA class I are on different chromosomes, their independent segregation during meiosis produces diversity in the number and type of *KIR-HLA* gene combinations inherited by individuals (Norman et al. 2013; Wilson et al. 2000). Further, NK cells can express more than one KIR at a time (Lanier 1997; Valiante et al. 1997). This inherent diversity has complicated the investigation of the specific KIR-HLA class I interactions that modulate immune response. Development of soluble KIR proteins for which the reactivity for single HLA class I molecules was determined by direct binding assay, facilitated understanding of how particular receptor-ligand combinations contributed to NK cell reactivity (Winter et al. 1998). These recombinant proteins were made in a mammalian cell expression system by fusing the extracellular domains of a two-domain KIR with two Fc domains of a human IgG1 to form a soluble homodimer (Winter and Long 2000).

We have adapted this method for the production of soluble KIR-Fc fusion proteins by using baculovirus-infected insect cells. The advantage of this approach is that insect cells are simple to culture. They have short doubling times that facilitate scaling and they are capable of higher protein yields than mammalian cell systems of expression. Because of these advantages, the baculovirus-insect cell system is now one of the most widely used methods for the production of recombinant proteins (Hitchman et al. 2009). Although not equivalent to higher eukaryotic cells, most post-translational modifications are made correctly in insect cells, and proteins unable to be expressed in *E. coli* have been successfully expressed in the insect cell system (Victor et al. 2010). The baculovirus family are species-specific double-stranded DNA viruses that infect insects as their natural host (Kost and Condreay 1999). Once inserted into the host nucleus, the baculovirus is packaged into flexible nucleocapsids, into which foreign DNA may readily be inserted. The target gene, in this case the KIR-Fc fusion construct, is inserted into a transfer vector and positioned between sequences that are homologous to ones in the baculoviral genome. When the viral genome and transfer vector are transfected into insect cells, recombination occurs, and produces intact viral genomes harbouring the target gene sequence. The target gene replaces the non-essential baculoviral polyhedrin gene. The strong promoter of the polyhedrin gene is co-opted for production of recombinant target protein.

We have also developed a multiplex assay that tests the binding of soluble KIR-Fc to 97 HLA class I allotypes. This assay uses the Luminex platform, in which the antigenic targets are microbeads, each coated with a defined HLA class I allotype. Such beads were developed originally for studying the specificity of human alloantibodies (Pei et al. 1998; Pei et al. 2003), but our group has successfully adapted this platform for use with recombinant two-domain KIR-Fc fusion proteins and monoclonal antibodies (Hilton and Parham 2013; Moesta et al. 2008). By adjusting the relative concentration of two fluorescent dyes, a set of 100 individually identifiable beads is generated. Each bead is then coated with a different HLA class I allotype, allowing the results of the immunoassay to be correlated with HLA class I specificity.

The goal of the KIR-Fc HLA-bead binding assay is to determine the strength and specificity of the interactions between HLA class I and KIR using defined purified proteins. The results can be used to predict the reactivity of KIR expressing NK cells and T cells when their KIR recognize cognate HLA class I ligands. This assay represents a major advance from the cell-based direct binding assay in which the reactivity of only a few KIR and HLA class I combinations could be determined at any one time (Winter and Long 2000). Moreover, the KIR-Fc HLA bead-binding assay is designed to inform cellular assays of lymphocyte function in which receptor deficient effector NK cells transfected with a specific KIR are incubated with ligand-deficient target cells transfected with a specific HLA class I molecule (Moesta et al. 2008).

The HLA class I specificity of several KIR allotypes has been investigated using various assays. Our initial study with the multiplex bead-binding assay showed that KIR2DL2*001-Fc recognized HLA-C2 allotypes with higher avidity than its allotypic variant KIR2DL3*001-Fc (Moesta et al. 2008). A cellular cytotoxicity assay subsequently showed that KIR2DL2*001, but not KIR2DL3*001 effectively inhibited lysis when incubated with HLA class I deficient 221 cells transfected with the HLA-C2 allotype, HLA-C*04:01 (Moesta et al. 2008). Another group investigated a second allotypic variant, KIR2DL3*005 (Frazier et al. 2013) using a similar multiplex assay. They showed that KIR2DL3*005-Fc bound HLA-C1 allotypes with higher avidity than KIR2DL3*001-Fc. This result was concordant with a cellular assay in which NK cells expressing either 2DL3*005 or 2DL3*001 respectively were incubated with 221 cells expressing the C1 bearing allotype HLA-C*03:04. Natural killer cells expressing 2DL3*005 exhibited a more potent inhibitory signal than those transfected with 2DL3*001. (Frazier et al. 2013). A third type of assay, surface plasmon resonance, confirmed that the 2DL3*005 variant bound with greater avidity than the 2DL3*001 variant to the HLA-C1 allotype HLA-C*03:04 (Frazier et al. 2013).

In summary, we have developed a simplified method for the production of KIR-Fc and designed an assay that tests their binding to 97 HLA class I allotypes simultaneously. The assay is easy to perform and correlates well with more complicated experimental techniques such as cellular cytotoxicity and surface plasmon resonance that have traditionally been used to determine the avidity and specificity of KIR for HLA class I ligands.

## 2. Materials and Methods

### KIR-Fc fusion protein generation

This section describes the generation of a DNA insert, flanked by restriction sites, that encodes the first 224 amino acids of the KIR2D of interest and the Fc region of human IgG1 (Figure 1A). This insert is first cloned into the pAcGP67a transfer vector (Figure 1B). Subsequently it is co-transfected with linearized baculovirus into insect cells.

### 2.1 KIR-Fc fusion construct generation

Amplify the sequences encoding the D1, D2 and stem region of the selected KIR2D molecule by PCR (Primers from Figure 1D, upper panel). The forward primer annealing site is immediately downstream of the *KIR* signal sequence cleavage site and should contain a *BamHI* (New England BioLabs, Cat. #R0136) site. The reverse primer annealing site is immediately upstream of the transmembrane region and contains an *XbaI* (New England Biolabs, Cat. #R0145s) site.

Add a mix consisting of 20.2μl PCR grade sterile water, 2.5μl 10X cloned *Pfu* buffer, 0.5μl forward primer (10mM), 0.5μl reverse primer (10mM), 0.2μl dNTP mix (40mM) and 0.25μl HotStart TAQ polymerase (Kit from Qiagen, Cat. #203203) to 1μl of template DNA (10ng/μl) and perform PCR using the following conditions: (5min at 95°C) × 1, (30s at 94°C, 1min25s at 62°C, 40s at 72°C) × 35, (10min at 72°C) × 1, hold at 4°C. Digest the amplification products with *BamHI* and *XbaI* and ligate into the *BamH1* and *XbaI* digested pAcGP67a vector (BD Pharmigen, Cat. #554758). Amplify the sequences encoding the hinge, CH2 and CH3 regions of human IgG1 using a forward primer containing an *XbaI* site and a reverse primer containing a *NotI* (New England Biolabs, Cat. #R0189) site (Primers from Figure 1D, upper panel). Digest the amplification products with *XbaI* and *NotI* and ligate into the *XbaI* and *NotI* digested pAcGP67a vector. Transform the ligation products into *E.coli* competent cells (Kit from Invitrogen, Cat. #K4510-20). Following overnight incubation select individual *E.coli* colonies, clonally amplify and prepare transfection quality DNA using Qiagen columns (Kit from Qiagen, Cat. #27104). Sequence each clone to confirm that the desired KIR-Fc fusion gene is inserted in frame behind the GP67 secretion signal sequence (primers from Figure 1D, lower panel).

### 2.2 Site-directed mutagenesis of KIR-Fc constructs

Site-directed mutagenesis can be used to introduce non-synonymous mutations into the newly generated KIR-Fc fusion gene. This technique is useful for making comparisons of closely related KIR2D-Fc allotypes or to investigate the effect of alternative amino acids at a particular position on HLA class I recognition. Mutagenesis primers should have a melting temperature of at least 78°C and the desired mutation should be situated in the centre of the primer sequence. The website www.bioinformatics.org/primerx was used to design mutagenesis primers. Add a mix consisting of 40μl PCR grade sterile water, 5μl 10X cloned *Pfu* buffer (Agilent Technologies, Cat. #600153-82), 1μl forward primer (10mM), 1μl reverse primer (10mM), 1μl dNTP mix (40mM) and 1μl Turbo polymerase (Agilent Technologies, Cat. #600252-52) to 1μl of template DNA (50ng/μl) and perform PCR using the following conditions: (30s at 95°C) × 1, (30s at 95°C, 60s at 55°C, 15min at 68°C) × 20,

(7min at 72°C) × 1, hold at 4°C. A 15 min extension time is recommended, because of the large size of the pAcGP67a vector (9761bp). Following the PCR, add 1μl *DpnI* (New England BioLabs, Cat. #R0167S) to the reaction mixture and incubate for 30min at 37°C. *DpnI* digests the methylated template DNA and effectively increases the efficiency of mutated DNA transformation into competent *E.coli* cells. Following overnight incubation select individual *E.coli* colonies and then clonally amplify and prepare transfection quality DNA using Qiagen columns (Kit from Qiagen, Cat. #27104). Sequence each clone to confirm that the desired KIR-Fc fusion gene is inserted in frame with the GP67 secretion signal (primers from Figure 1D, lower panel).

### 2.3 Co-transfection of KIR-Fc containing transfer vector and baculovirus into insect cells

Having constructed the KIR-Fc gene, cloned it into the pAcGP67a transfer vector and mutated it if required, the construct must now be combined with linearized baculovirus and transfected into insect cells. For the initial transfection, the insect cells derived from *Spodoptera frugiperda* (*Sf9*) (Invitrogen, Cat. #11496-015) are used. Both the *Sf9* insect cells and baculovirus should be handled in a sterile laminar flow hood. The *Sf9* cells should be cultured in Sf-900 II media (Invitrogen, Cat. #10902-096) supplemented with 10% heat inactivated fetal bovine serum (FBS), 1% Penicillin-Streptomycin and 1% L-Glutamine, shaking at 120 rpm at 27°C. The *Sf9* cells should be maintained at between $4 \times 10^5$ and $2 \times 10^6$ cells/ml. When maintained at this density, *Sf9* cells have an approximate doubling time of 24h; a slower doubling time may signify unhealthy cells (see *section 2.11)*. For each KIR-Fc transfection, seed one well of a sterile six-well plate (BD Falcon, Cat. #353046) with 2ml *Sf9* cells at a density of $1 \times 10^6$ cells/ml and incubate for 60 mins at 27°C to allow adherence of *Sf9* cells. Concurrently, mix (gently using a pipette) 2μg of purified transfer vector DNA with KIR-Fc insert from step *2.1* with 0.5μg of linearized baculovirus DNA (Expression Systems, Cat. #91-002) in a sterile 2ml microcentrifuge tube and incubate for 5 mins at room temperature. This specific baculovirus has the chitinase and cathepsin loci deleted to remove a detrimental protease and reduce competition for resources for protein synthesis during late gene expression. Meanwhile, for each transfectant, mix 10μl Cellfectin II (Invitrogen, Cat. #10362-100) with 100μl un-supplemented Sf-900 II media (Invitrogen, Cat. #10902-096) and pipette to mix them together. Add this to the baculovirus/KIR-Fc construct mixture (immediately following the 5 min incubation), mix gently using a pipette and incubate for a further 30 mins at room temperature. Following the 30 min incubation, add 1.8ml un-supplemented Sf-900 II media to complete each KIR-Fc transfection mixture. In summary, each KIR-Fc transfection mixture should contain *Sf9* cells, KIR-Fc fusion gene DNA in pAcGP67a transfer vector, linearized baculovirus DNA, Cellfectin and un-supplemented Sf-900 II media.

Remove the 6-well plate from incubation and replace the medium covering the adherent cells with the KIR-Fc transfection mixture. Incubate, covered but not shaking, for 3–5 hours at 27°C. Remove the supernatant from each well and replace with 3ml Sf-900 II media (warmed to 27°C and supplemented with 10% heat inactivated FBS, 1% Penicillin-Streptomycin and 1% L-Glutamine). Incubate, covered and sealed, with laboratory film (Pechiney, Cat. #PM996), for 7 days at 27°C. Remove the supernatant from each well and transfer to a sterile 15ml tube. Separate the cells by centrifugation (1000g for 10 mins),

discard the pellet and transfer the supernatant to a sterile 15ml tube and store in the dark at 4°C. This first transfectant, termed *P0*, and each subsequent *Sf9* amplification supernatant (*P1–P3*) may be placed without further modification at −80°C for long-term storage.

### 2.4 Amplification of baculovirus in Sf9 cells – P1-3 production

Successive rounds of *Sf9* cell amplification are now used to amplify the baculovirus (now containing the KIR-Fc gene added in *step 2.3*). Add 30ml *Sf9* cells at a density of $1 \times 10^6$ cells/ml to a 125ml vented sterile Erlenmeyer flask (Thermo-Scientific, Cat. #4116-0125) and leave shaking at 115 rpm overnight at 27°C. The following day, add 500μl of *P0* virus stock to the flask and incubate for 4–5 days shaking at 120rpm at 27°C. Collect 14ml suspension from each flask and transfer to a sterile 15ml tube. Separate the cells by centrifugation (1000g for 5mins) and transfer each supernatant to a new sterile 15ml tube. Discard the pellet and store in the dark at 4°C. This forms the *P1* viral stock. A second (*P2*) or third (*P3*) round of amplification in which each round is seeded with 50μl of the preceding amplified supernatant, may be required to produce *Sf9* supernatant with a sufficiently high baculoviral titre for adequate protein production.

### Quantification of baculovirus amplification in Sf9 supernatant

Before proceeding it is necessary to quantify the amount of baculovirus generated from the amplification of the *Sf9* cells in *Sections 2.3* and *2.4*. The amount of baculovirus generated is critical, as insufficient baculoviral amplification will result in low or absent protein yield. An estimation of viral titre can be made in two ways: directly, using small-scale protein production (protein mini-prep) (see *Section 2.5*) or indirectly, by flow cytometry, assessing baculoviral-induced up-regulation of surface glycoprotein 64 (GP64) (see *Section 2.6*).

### 2.5 KIR-Fc fusion protein – mini-prep

This small-scale protein production tests the capacity of the baculoviral infected *Sf9* supernatant from *steps 2.3* and *2.4* to produce protein from *Trichopulsia ni (Hi5*) (Invitrogen, Cat. #B855-02) insect cells. *Hi5* cells are similar to *Sf9* cells but are optimized for recombinant protein production rather than baculoviral amplification. *Hi5* cells should be cultured, shaking at 27°C in Express Five serum free media (Invitrogen, Cat. #10486-025) supplemented with 1% L-Glutamine. The cell density should be kept at between $4 \times 10^5$ and $3 \times 10^6$ cells/ml. Aggregation of cells, which is more likely to occur at densities higher than $3 \times 10^6$ cells/ml, is a sign of unhealthy cells and will reduce the possible protein yield from any preparation in which they are used (see *Section 2.11*). Seed each well of a sterile 6-well tissue culture plate with 2ml *Hi5* cells at a density of $1 \times 10^6$ cells/ml. Add 100μl of the viral stock under test (*P1, P2* or *P3*) to each well, seal the plate with laboratory film and incubate for 48 hours (covered, shaking at 120 rpm, at 27°C). To serve as a negative control, one well should contain *Hi5* cells but no Sf9 supernatant. To serve as a positive control, one well should be transfected with an *Sf9* supernatant previously used to produce KIR-Fc protein. Transfer the contents of each well to a 2ml microcentrifuge tube and separate the cells by centrifugation (1000g for 5 mins). Discard the pellet and transfer 1.5ml of the supernatant to a clean 2ml microcentrifuge tube, add 150μl 10x Hepes Buffered Saline (HBS), pH 7.2 and

10μl protein A Sepharose bead slurry (Invitrogen, Cat. #101142). Rotate the mixture overnight at 4°C.

Separate the beads by centrifugation (2500g, 15min, 4°C) and discard the supernatant. Add 20μl Laemmli sample buffer (Bio-Rad, Cat. #161-0737) with 5% β-mercaptoethanol (Bio-Rad, Cat. #161-0710) and incubate the sample at 95°C for 10 minutes. Load the sample onto a 12% SDS-PAGE gel (Bio-Rad, Cat. #456-1043) and run at 150v for 1hr. Stain with Coomassie reagent (Bio-Rad, Cat. #161-0786) to identify an appropriately sized band. Although each KIR-Fc exists as a 102Kda homodimer in native format (Figure 1C), they run as a band of 51Kda monomers (Figure 3A) on an SDS gel. The reducing conditions in the sample application buffer disrupt the disulphide bonds that link the two monomers. The bands produced from successive *Sf9* amplifications of the same KIR-Fc construct should show increasing intensity. Amplification is considered sufficient when successive rounds of amplification produce bands of similar intensity. This indicates that the amplification of baculovirus from *Sf9* cells has reached capacity on SDS-PAGE.

### 2.6 Identification of baculovirus-infected Hi5 cells by flow cytometry

GP64 is a baculovirus encoded glycoprotein that is expressed on insect cells upon infection with baculovirus (Blissard and Rohrmann 1989). Because the titre of the transfecting supernatant corresponds to the degree of cell-surface expression of GP64, this can be used as a proxy to assess viral amplification in *Sf9* supernatant. Seed an appropriate number of wells of a sterile six-well tissue culture plate with 2ml *Hi5* cells at a density of $1 \times 10^6$ cells/ml. Add 100μl of *P1, P2* or *P3* viral stock to each well, seal the plate with laboratory film and incubate for 24 hours (covered, shaking at 120 rpm, at 27°C). To serve as a negative control, one well should contain *Hi5* cells but no Sf9 supernatant. To serve as a positive control for the ability of the Hi5 cells to express GP64, one well should be transfected with a high baculoviral titre *Sf9* supernatant. Mix the cells and add 250μl of the contents of the well to a 2ml microcentrifuge tube and separate the cells via centrifugation (50*g* for 2 mins). In the following steps, all wash solutions should be at 4°C. Resuspend the cells in flow cytometry buffer (FCB) (1x phosphate buffered saline [PBS - Cellgro, Cat. #21-031-CV] supplemented with 0.5% EDTA and 1% BSA) and wash twice. Following the second wash, resuspend the cells in 25μl FCB and stain the cells with 1μl anti-baculovirus GP64 antibody (eBioscience, Cat. #14-6995-81). Incubate for 30 mins at 4°C. Wash the cells a further three times in FCB, resuspending in 1ml FCB after the final wash. Flow cytometry is then used to detect the presence of the PE-conjugated antibody bound to cell surface GP64. Hi5 cells transfected with low viral titre *P0* or *P1* viral stocks show low or absent levels of surface GP64. Successive Sf9 amplifications of the same KIR-Fc construct should show increasing levels of cell-surface GP64 (Figure 2). Amplification is considered complete when successive rounds of amplification result in similar levels of cell-surface GP64. This indicates that the amplification of baculovirus from *Sf9* cells has reached capacity and typically occurs at the *P2* stage.

### 2.7 Harvest and purification of soluble KIR-Fc fusion proteins from baculovirus infected Hi5 cells

Once a suitable baculoviral *Sf9* stock has been produced and assessed for viral titre, a full-scale experiment to produce recombinant KIR-Fc protein can be performed. Grow a 1L *Hi5* cell culture to a density of $2 \times 10^6$ cells/ml. Add 1ml of high-titre *P2* or *P3* viral stock and incubate for 60 hours (covered, shaking at 120 rpm, 27°C). Separate cells by centrifugation (2500g for 15 mins, 4°C). Discard the pellet and pass the supernatant through a 0.2μm filter (Nalgene, Cat. #450-0020) into a sterile container. Add 100ml 10x HBS, pH 7.2 per 900ml filtered Hi5 supernatant. Add 1ml protein A Sepharose bead slurry and rotate bottles slowly overnight at 4°C.

In the following steps, all wash solutions should be at 4°C. Separate the protein A Sepharose beads from the *Hi5* supernatant by filtration using gravity flow through a Buchner funnel with fritted glass disc (Pyrex, Cat. #36060). Care should be taken to avoid the Sepharose beads running dry. Wash the beads with approximately 500ml of 1x PBS and transfer to an empty 13ml PD-10 column with a 20–85μm frit filter (GE Healthcare, Cat. #17-0435-01) using a serological pipette. The beads should collect on the frit filter as the wash solution passes through by gravity flow.

Elute the KIR-Fc fusion protein from the collected beads in the PD-10 column using 100mM glycine, pH 2.7 in 8 fractions of 800μl each, neutralizing by elution into 8 separate microcentrifuge tubes, each containing 200μl 1M Tris, pH 9.0. The protein content of each elution fraction should be determined by Bradford assay (Kit from Bio-Rad, Cat. #500-0001).

Wash a Sephadex G-25 desalting column (GE Healthcare, Cat. #17-0851-01) with 25ml 1x PBS. Load 2.5ml of the fractions having the highest protein concentration onto the desalting column. Add 3.5ml 1x PBS to the desalting column in 0.5ml aliquots and collect seven 0.5ml fractions in separate microcentrifuge tubes. Determine the protein content of each fraction by Bradford assay.

At this stage, it is best to estimate the purity of each eluted protein fraction by gel electrophoresis. Add 10μl of each KIR-Fc eluent to an equal volume of Laemmli sample buffer with 5% β-mercaptoethanol and incubate the sample at 95°C for 10 minutes. Transfer the sample onto a 15% SDS-PAGE gel and electrophorese at 150–200v for 30–60 minutes. Stain with Coomassie reagent to identify the appropriately sized band (Figure 3A).

### 2.8 Assessment of KIR-Fc integrity by flow cytometry

Having produced recombinant KIR-Fc protein from the full-scale prep as described in *Section 2.7*, it is now necessary to determine the integrity (ie. correct protein folding) of the protein. This is achieved by flow-cytometry, using monoclonal antibodies specific for KIR. In the following steps, all wash solutions should be kept at 4°C. Add 20μl of anti-human IgG-coated beads (Bangs Laboratories, Cat. #BM562) to 500μl KIR-Fc fusion protein (diluted in 1x PBS to 100μg/ml). Ensure that the beads are vortexed gently to resuspend them prior to their use. Incubate, shaking gently for 30 min at 4°C. Collect the beads by centrifugation (50*g* for 2 mins) and wash twice with FCB. Following the second wash,

resuspend the beads in 25μl FCB and add 2μl PE-conjugated mouse anti-human KIR antibody (KIR2DL1: Beckman Coulter, Cat # EB6-PE; KIR2DL2/3, Beckman Coulter, Cat # DX27-PE; Lineage III KIR: AbD Serotec, Cat # NKVFS1). Incubate, shaking gently for 30 min at 4°C. Collect the beads by centrifugation (50*g* for 2 mins) and wash a further two times with FCB. Resuspend the beads in 150μl FCB. Flow cytometry is then used to detect the presence of the PE conjugated anti-KIR antibody bound to individual IgG coated beads (Figure 3B).

### 2.9 Multiplex assay to detect binding of soluble KIR proteins to HLA class I single antigen beads

The purified, functional KIR-Fc fusion proteins are now ready to be tested for their capacity to recognize HLA class I allotypes. Each KIR-Fc protein is first incubated with approximately 10,000 individual beads, each coated with one of 97 HLA class I allotypes; the goal being to test the binding of the KIR-Fc to each HLA class I allotype approximately 100 times. In the second step of the assay, a secondary antibody is added that binds to the Fc portion of the KIR-Fc. The Luminex reader is able to simultaneously detect the identity of the bead (which correlates with a specific HLA class I) and the fluorescence of the antibody, which indicates the amount of KIR-Fc bound to the bead.

For each KIR-Fc protein to be tested, pre-wet one well of a 96-well 0.65μm filter plate (Millipore, Cat. #MSDVN6510) with 200μl 1x PBS. Remove the PBS from the wells by vacuum aspiration (manifold from Qiagen, Cat. #19504). Add 50μl of soluble KIR protein (100μg/ml) and 3μl LABscreen microbeads (One Lambda, Cat. #LS1A04) to each pre-wetted well. Ensure that the beads are vortexed gently to resuspend them prior to aliquoting into each well. For each assay, 50μl W6/32 antibody (Biolegend, Cat. #311402) (50μg/ml) should be added to one well to control for antigen density on individual beads (see *Section 2.10*). Incubate the KIR-Fc proteins and W6/32 with the beads for 60 mins, shaking gently and covered at 4°C.

In the following steps, all wash and resuspension solutions should be at 4°C. Wash the beads in each well four times with 1x Luminex wash buffer (One Lambda, Cat. #LS1A04). For each wash, add 200μL of wash buffer to each well and gently pipette up and down several times. If more than one protein is being tested use a multi-channel pipette to ensure even washing of each KIR-Fc protein being tested. Care should be taken to avoid introducing air bubbles into the wells. Following each wash, aspirate the wash solution using a vacuum manifold, ensuring that the well does not become dry. The vacuum pressure should not exceed 100mmHg.

Following the first complete wash cycle (4 individual washes), resuspend the KIR-Fc fusion proteins in 100μl PBS with 1% PE-conjugated goat anti-human IgG-Fc antibody (One Lambda, Cat. #LS-AB2). Suspend the beads incubated with W6/32 in 100μl PBS with 1% PE-conjugated goat anti-mouse IgG-Fc antibody (BD Pharmigen, Cat. #550589). Incubate both KIR-Fc and W6/32 for a further 60 mins, shaking gently and covered at 4°C. Following the second incubation, wash the beads a further four times with 1x Luminex wash buffer, resuspend in 100μl 1x PBS and transfer into a 96-well, 250μl ‘V’ bottom microplate (Whatman, Cat. #7701-3250). The test plate should be transferred immediately into the

Luminex reader (with pre-warmed lasers and beadset template entered to avoid delay in starting the assay).

### 2.10 Calculating fluorescence relative to HLA class I antigen density

To correct for differences in the absolute amount of HLA class I annealed to each microbead, the binding of KIR-Fc fusion proteins to a specific HLA class I should be calculated relative to the amount of HLA class I as determined by binding of W6/32, an antibody that recognizes an epitope common to all HLA class I allotypes. The relative fluorescence ratio of a given KIR-Fc is calculated using the formula (KIR-Fc binding – negative control bead binding)/(W6/32 binding – negative control bead binding).

### 2.11 Common problems and their solutions

The most common problem encountered in the protocol is failure of amplification of baculovirus in *Sf9* cell preps and failure of protein production from *Hi5* cell preps. We have introduced several steps to test baculoviral amplification, the goal being to identify insufficient baculoviral amplification early and take steps to correct it to minimize lost time. A common cause of amplification failure is unhealthy *Sf9* or *Hi5* cells. The following steps can be used to ensure healthy cells and detect unhealthy cells should they occur.

*Sf9* cells should have a doubling time of between 24 and 30h. *Hi5* cells should have a doubling time of between 18 and 24h. Slow doubling times usually indicate that *Sf9* or *Hi5* cell cultures are unhealthy. Unhealthy cells will not amplify baculovirus successfully or produce adequate recombinant protein. Cell viability for both *Sf9* and *Hi5* cells, as determined by trypan blue staining, should be greater than 95% at all times. Both bacterial and fungal infections in the insect cell culture will reduce cell viability and doubling times. These can be prevented with good cell culture technique and the addition of antibiotics (1% Penicillin-streptomycin) and/or anti-fungals (0.25μg/ml Amphotericin B) to the culture medium. Cultures should be discarded immediately if there is evidence of microorganism contamination. A second reason for slow doubling is oxygen restriction. If the cell culture does not have sufficient surface area exposed to the air, cell growth will be retarded. This can be prevented by ensuring that flasks are filled no more than one-third (by volume) with culture medium. Ensuring that the flasks are shaken at between 120–150 rpm also ensures adequate oxygenation. Shaking at a higher rpm leads to cell damaging shearing stress and should be avoided. Further information on the culture of insect cells is available (Shrestha et al. 2008).

For the formation of intact baculovirus it is essential that transfer vector and linearized baculoviral DNA recombine during initial co-transfection of *Sf9* cells. We have obtained the best results with freshly isolated transfer vector and baculoviral DNA that has not been stored at 4°C for more than two weeks. Additionally, freeze/thaw cycles of the transfer vector should be avoided where possible. Because the linearized baculovirus is a large DNA fragment (~130Kb), it is particularly susceptible to shear stress; over-zealous pipetting during transfection should therefore be avoided to minimize DNA and maximize transfection success.

The most common problems associated with the Luminex assay are high background and inter-assay variability. We have found that the following precautions minimize these problems. In this context we define high background readings as those in which the negative control bead binding is greater than 1% of the highest positive reading obtained. During the wash and incubation phases of the protocol, care should be taken to minimize any warming of the reagents. All wash reagents should be chilled to 4°C and wash steps should be completed as quickly as possible to avoid unnecessary warming of the samples. This may mean reducing the number of KIR-Fc proteins under test in any one assay to expedite this phase.

Although not always the case, some KIR-Fc are not stable when stored at 4°C and their use in the assay can lead to high background readings. To control for this issue, divide each batch of protein into 100 μl aliquots and store them at −80°C. Similarly, HLA class I beads that have been thawed and stored at 4°C are not typically stable for more than 3 months. The beads should also be divided into 10μl aliquots and frozen at −80°C if they are unlikely to be used within this time frame. Each of these precautions also helps to reduce both high background and inter assay variability. Additionally, we include well characterized HLA-C1 and HLA-C2 receptors (e.g KIR2DL3*001-Fc and KIR2DL1*003-Fc respectively) in every assay as positive controls. Changes in the avidity and specificity of the KIR-Fc under test can then be related not only to W6/32, but also to these KIR-Fc controls.

In addition to the failure of baculoviral amplification described above, a further limitation of this system is that we have, as yet, been unable to produce functional three domain KIR using the Fc fusion system. Although the KIR3D protein is expressed by insect cells, it does not fold into a tertiary structure that binds anti-KIR antibody or to HLA class I. As such, the HLA class I reactivity of these receptors remains under investigation.

## 3. Results and Discussion

The purpose of this protocol is to provide a simplified method with which to produce and test the reactivity of soluble two-domain KIR-Fc fusion proteins. We chose to use an insect cell expression system because most post-translational modifications are made correctly in insect cells and the system is scalable, allowing production of large quantities of soluble recombinant protein in a comparatively short time. We have sought to reduce variability in final protein yield by implementing a series of quality controls at points during the baculoviral amplification phase. These methodological improvements allow us to better track baculoviral amplification in *Sf9* cells and prevent the *Hi5* preparations having insufficient protein yield.

We have also designed a novel assay that tests the binding of KIR-Fc to HLA class I allotypes. This assay simultaneously detects the binding of KIR to 97 HLA class I allotypes. As a result it holds a clear advantage over previous cell-based binding assays that allowed examination of only a few KIR-HLA class I interactions at any one time. The assay is sensitive over a two-log range, permitting both strong and weak reactions to be interpreted with confidence and correlated with structural polymorphisms in both KIR and HLA class I

(Frazier et al. 2013; Gendzekhadze et al. 2009; Graef et al. 2009; Hilton et al. 2012; Moesta et al. 2008).

The KIR-Fc HLA-bead binding assay has also been used to explore the binding characteristics of KIR in simian primates. The HLA class I specificity of primate KIR has been difficult to determine because of a comparative lack of cellular reagents. As such, the combination of methods described in this paper has led to a number of critical discoveries on the immunologic function and co-evolution of KIR and MHC class I. In Old World Monkeys, the lineage III *KIR* (precursor to the MHC-C receptors of higher primates) is represented by a single gene (Sambrook et al. 2005) while lineage II *KIR* genes have expanded and diversified. To identify their MHC epitope-specificity and avidity, a panel of rhesus macaque lineage II KIR-Fc was assayed using the methods present here (Older Aguilar et al. 2011). Although MHC-C is not present in macaques, their KIR recognize HLA-C epitopes more effectively than they recognize HLA-A and HLA-B, suggesting that MHC-C evolved to become a stronger ligand for KIR than HLA-A and -B. The emergence of *MHC-C* in the orangutan was accompanied by an expansion of lineage III KIR and their evolution as MHC-C receptors (Guethlein et al. 2007). All orangutan MHC-C allotypes have asparagine at position 80 and display the C1 epitope. Correspondingly, results from the KIR-Fc HLA bead binding assay showed that the orangutan has C1-specific KIR but no C2-specific KIR (Older Aguilar et al. 2010). In chimpanzees the *MHC-C* gene became fixed and the C2 epitope emerged. As a consequence, out of nine chimpanzee lineage III KIR genes (Abi-Rached et al. 2010), eight encode receptors with high avidity for HLA-C, comprising three C1-specific receptors and 5 C2-specific receptors (Moesta et al. 2009). Thus, results from the multiplex binding assay show that changes in the character of the *KIR* locus correlate with change in the *MHC* class I genes, suggesting co-evolution between these receptors and ligands and uncovering a progression in which the complexity of the *KIR* locus gets increasingly sophisticated across higher primate species.

### 3.1 Flow cytometry can be used to confirm baculoviral transfection

Surface glycoprotein GP64 is a baculovirus encoded glycoprotein that can be used as a marker for successful transfection and amplification of baculovirus in *Hi5* insect cells. (Blissard and Rohrmann 1989; Volkman and Goldsmith 1988). Figure 2 shows that GP64 expression is not detected on un-transfected *Hi5* cells but is expressed following transfection with high viral titre *Sf9* supernatant. *P0* viral stock was not sufficient to induce GP64 surface expression whereas surface expression was typically detected after transfection with *P1* viral stock and with each subsequent amplified viral stock (*P2* and *P3*) (Figure 2). Surface expression of GP64 was sensitive to the baculoviral titre with *P2* transfected *Hi5* cells showing a 40% increase in surface expression of GP64 as compared to *Hi5* cells transfected with *P1* viral stocks. That *P3* stocks induced only marginally greater GP64 surface expression than *P2* viral stocks suggests that GP64 is either maximally up-regulated by a given viral titre or *Sf9* cells reach maximal viral amplification between the second and third amplification rounds.

### 3.2 Confirmation of KIR-Fc integrity by flow cytometry

Both EB6 antibody, which recognizes KIR2DL1, and DX27 antibody, which recognizes KIR2DL2/3, bound to KIR-Fc immobilized on anti-human IgG flow cytometry beads (Figure 3B). This test provides a cost-effective way to test the integrity of both the KIR region and Fc region of the final fusion protein, as both are required to have folded correctly to produce a positive result in the Luminex binding assay.

### 3.3 Titration of KIR2D-Fc fusion proteins against HLA class I shows that 100μg/ml is an appropriate concentration to use in the binding assay

The binding of KIR-Fc fusion proteins to HLA class I increases with an increasing concentration of KIR-Fc fusion protein until binding becomes saturated at approximately 100μg/ml. Use of KIR-Fc at greater concentrations (100–400μg/ml) does not increase binding to HLA class I and avidity differences between individual allotypes are found to be consistent at this concentration. Additionally, this saturation point applies to between different HLA class I allotypes and for different naturally occurring and mutant KIR2D (Figure 3C).

### 3.4 Multiplex Luminex assay comparing the binding of W6/32, KIR2DL1-Fc and KIR2DL3-Fc to HLA class I coated microbeads

KIR2DL1-Fc binds specifically and with high avidity to HLA-C2 but not HLA-C1 or any HLA-A or HLA-B allotypes (Figure 3D). HLA-C2 allotypes display a range of avidity for KIR2DL1 with HLA-C*15:02 and HLA-C*04:01 having the highest and lowest avidities respectively (Figure 3D). KIR2DL3-Fc binds specifically to HLA-C1 but does not bind to HLA-C2 or any HLA-A allotype. Unlike KIR2DL1, KIR2DL3 does bind to two unusual HLA-B allotypes (HLA-B*46:01 and HLA-B*73:01) that have the C1 epitope (Figure 3D). HLA-C1 allotypes have a range of avidity for KIR2DL3 with HLA-C*03:04 and HLA-C*16:01 having the highest and lowest avidities, respectively. Both HLA-B*46:01 and HLA*73:01 bind with high avidity to KIR2DL3.

### 3.5 KIR-Fc fusion proteins remain functional following long-term storage at −80°C

Each 1 litre prep of *Hi5* cells yields between 2 and 4 mg of KIR-Fc protein. Given that the KIR-Fc are used at a dilution of 100ug/ml in the multiplex binding assay, there is typically an excess of KIR-Fc reagent. KIR-Fc remain stable at 4°C for 1–6 months, however, to allow repetition of a particular experiment over longer time periods and to provide stable positive controls, we investigated the storage of KIR-Fc aliquots at −80°C (Figure 3E). KIR-Fc show similar binding in the multiplex binding assay up to 12 months following initial freezing. Freezing the KIR-Fc is assumed to prevent the protein degradation, aggregation and misfolding that occurs at an unpredictable rate when stored at 4°C.

## 4. Conclusions

We have described the production of KIR-Fc fusion proteins in an insect cell expression system and their interaction in a multiplex binding assay with a panel of 97 HLA class I allotypes. KIR-Fc production in insect cells is relatively simple allowing production of large amounts of recombinant protein in around 20 days. The assay is sensitive enough to

discriminate between single amino acid substitutions in the extracellular domains of the KIR molecule and has, as a result, greatly facilitated investigation of even closely related KIR allotypes. Furthermore, the results of this direct binding assay appear to correlate well with the results obtained in the limited cellular assays that were used to discover the KIR and first investigate their specificity for HLA class I.

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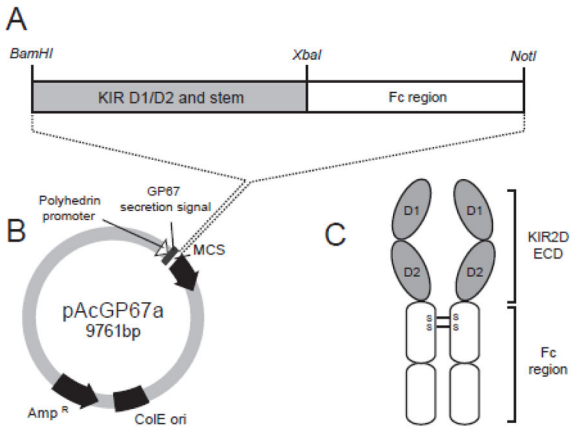
## Highlights

- KIR and HLA class I ligand interactions modulate NK cell reactivity
- Soluble recombinant KIR-Fc fusion proteins are made efficiently in insect cells
- Production method described is simple and scalable
- KIR-Fc used in multiplex HLA class I binding assay to characterize ligand specificity
- Results inform functional and evolutionary studies of NK cell immunity




D

| | Direction | Target | Rest. Site | Sequence (5' - 3') | GC content (%) | Tm (°C) | Length (bp) |
|---|---|---|---|---|---|---|---|
| Amplification | F | *KIR2DL1/2/3/S1/2* | *BamHI* | TAGGATCCCCATGAGGGAGTCCACAG | 57.7 | 62.8 | 26 |
| | F | *KIR2DS3/5* | *BamHI* | TAGGATCCCCATGAGGGATTCCGCAG | 53.8 | 61.7 | 26 |
| | F | *KIR2DS4* | *BamHI* | TAGGATCCCCAGGAGGAGTCCACAG | 57.7 | 62.7 | 26 |
| | R | *KIR2DL1/2* | *XbaI* | GTGCAGGTGTCTAGAGTTACCGGT | 54.2 | 60.2 | 24 |
| | R | *KIR2DL3/S2* | *XbaI* | ATGCAGGTGTCTAGAGTTACCGGT | 50.0 | 59.5 | 24 |
| | R | *KIR2DS1/4* | *XbaI* | ATGTAGGTGTCTAGAGTTACCGGT | 45.8 | 56.7 | 24 |
| | R | *KIR2DS3* | *XbaI* | GTGTAGGTGTCTAGAGTTACCGGT | 50.0 | 57.4 | 24 |
| | F | *Fc* | *XbaI* | TCTAGACACCTGCACGAGCCCAAATCTTCTGACAAAACTCACACATGCCC | 50.0 | 69.6 | 50 |
| | R | *Fc* | *NotI* | ATGCGGCCGCTCATTTACCGGAGACAGGGAG | 61.3 | 68.9 | 31 |
| Sequencing | F | pAcGP67a | - | TTTACTGTTTTCGTAACAGTTTTG | 29.2 | 50.3 | 24 |
| | R | pAcGP67a | - | GGAAGCTGTCTTCCATGAAGCG | 54.5 | 58.3 | 22 |

**Figure 1.**

(A) Schematic diagram showing the configuration of a recombinant KIR-Fc fusion gene. The recombinant fusion gene consisting of the D1, D2 and stem domains of a KIR2D molecule (grey box) and the Fc region of a human IgG1 antibody (white box) is cloned using *BamH1* and *Not1* restriction sites into the multiple cloning site (MCS) of the pAcGP67a vector (B) in frame behind the GP67 secretion signal sequence. Transfection into insect cells produces a soluble recombinant KIR-Fc dimer (C) consisting of the D1, D2 and stem regions (extracellular domain – ECD) of the KIR molecule (grey ovals) and the Fc region (white ovals) of the IgG1 antibody. (S-S) shows the location of the disulphide bonds that lead to formation of a dimer.

(D) Table listing the primers for amplification and sequencing of KIR-Fc fusion genes representing inhibitory *KIR2DL1*, *2DL2/3* and activating *KIR2DS1*, *2DS2*, *2DS3*, *2DS4* and *2DS5*. The properties of each primer, including the GC content, melting temperature (Tm) and length are listed to the right. Restriction sites are shown in bold when present in a primer sequence.

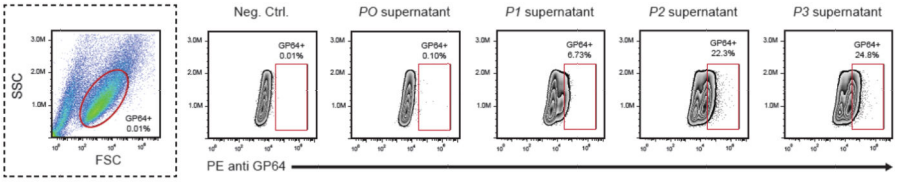



**Figure 2.**

(A) GP64 is up-regulated on the surface of *Hi5* cells following transfection with baculovirus infected *Sf9* supernatant. Shown is the gating strategy and representative flow cytometry plots from *Hi5* cells transfected with Sf9 supernatant from *P0*, *P1*, *P2* and *P3* viral stocks and stained with PE conjugated anti-GP64 antibody. *P0* viral stocks do not induce up-regulation of GP64 on the surface of *Hi5* cells. Transfection of *Hi5* cells with *P1* viral stocks induces partial up-regulation of GP64 whereas transfection of *Hi5* cells with *P2* or *P3* viral stocks maximally up-regulates GP64.

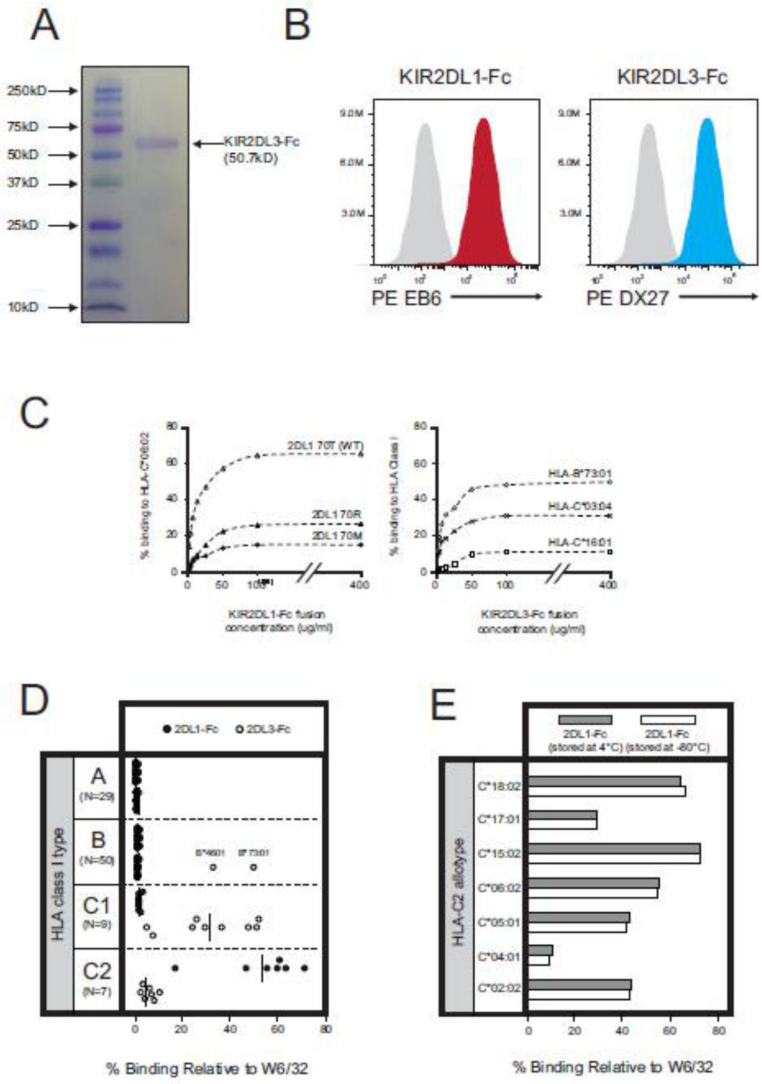



**Figure 3.**

(A) Shown is a reducing (SDS) gel stained with Coomasie Blue with a protein ladder in the left column (Precision plus Protein Kaleidescope Standards, Bio-Rad, Cat # 1610375) and a band corresponding to KIR2DL3-Fc at 50.7kDa in the right column. The gel was loaded with 15 μl of KIR2DL3-Fc protein (100μg/ml).

(B) KIR2D-Fc bound to IgG coated beads (Bangs Laboratories) are stained with KIR specific antibodies to assess their integrity. Representative flow cytometry plots showing staining of KIR2DL1 (left panel, red) and KIR2DL3-Fc (right panel, blue) fusion proteins with EB6 and DX27 antibodies respectively. (C) Titrations of the binding of KIR2D to beads coated with HLA class I molecules. Each of three KIR2DL1-Fc fusion proteins distinguished by substitutions at residue 70 bind to HLA-C*06:02 with different avidities. Both mutant and wild type KIR2DL1 show saturated binding at concentrations greater than 100μg/ml. KIR2DL3-Fc binds to three HLA class I allotypes (HLA-B*73:01, HLA-C*03:04 and HLA-C*16:01) with different avidities. The binding of KIR2DL3-Fc to each allotype becomes saturated at concentrations above 100μg/ml. (D) KIR2DL1-Fc binds to HLA-C2

bearing allotypes but not to HLA-C1, HLA-A or HLA-B allotypes. KIR2DL3-Fc binds to HLA-C1 allotypes and to HLA-C2 allotypes with lower avidity. KIR2DL3-Fc also binds to two HLA-B allotypes that encode the C1 epitope. (E) KIR-Fc fusion proteins are amenable to long-term storage at −80°C. The binding of KIR2DL1-Fc stored at −80°C for 12 months is compared to 2DL1-Fc stored at 4°C.