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TITLE:

Quantitative Semi-automated Typing of Killer-cell Immunoglobulin-like Receptor Genes

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KEYWORDS:

Immunogenetics, killer cell immunoglobulin-like receptor (KIRs), copy number variation, haplotype, human leukocyte antigen (HLA), natural killer cell, quantitative polymerase chain reaction (qPCR)

SUMMARY:

Quantitative killer cell immunoglobulin-like receptor (KIR) semi-automated typing (qKAT) is a simple, high-throughput, and cost-effective method to copy number type *KIR* genes for their application in population and disease association studies.

ABSTRACT:

Killer cell immunoglobulin-like receptors (KIRs) are a set of inhibitory and activating immune receptors, on natural killer (NK) and T cells, encoded by a polymorphic cluster of genes on chromosome 19. Their best-characterized ligands are the human leukocyte antigen (HLA) molecules that are encoded within the major histocompatibility complex (MHC) locus on chromosome 6. There is substantial evidence that they play a significant role in immunity, reproduction, and transplantation, making it crucial to have techniques that can accurately genotype them. However, high-sequence homology, as well as allelic and copy number variation, make it difficult to design methods that can accurately and efficiently genotype all *KIR* genes. Traditional methods are usually limited in the resolution of data obtained, throughput, cost-effectiveness, and the time taken for setting up and running the experiments. We describe a method called quantitative *KIR* semi-automated typing (qKAT), which is a high-throughput multiplex real-time polymerase chain reaction method that can determine the gene copy numbers for all genes in the *KIR* locus. qKAT is a simple high-throughput method that can provide

high-resolution *KIR* copy number data, which can be further used to infer the variations in the structurally polymorphic haplotypes that encompass them. This copy number and haplotype data can be beneficial for studies on large-scale disease associations, population genetics, as well as investigations on expression and functional interactions between *KIR* and *HLA*.

INTRODUCTION:

In humans, the killer immunoglobulin-like receptor (*KIR*) locus is mapped on the long arm of chromosome 19 within the leukocyte receptor complex (LRC). This locus is around 150 kb in length and includes 15 *KIR* genes arranged head-to-tail. The *KIR* loci that are currently known are *KIR2DL1*, *KIR2DL2*/*KIR2DL3*, *KIR2DL4*, *KIR2DL5A*, *KIR2DL5B*, *KIR2DS1-5*, *KIR3DL1*/*KIR3DS1*, *KIR3DL2-3,* and two pseudogenes, *KIR2DP1* and *KIR3DP1*. The *KIR* genes encode for two-dimensional (2D) and three-dimensional (3D) immunoglobulin-like domain receptors with short (S; activating) or long (L; inhibitory) cytoplasmic tails, which are expressed by natural killer (NK) cells and subsets of T cells. Copy number variation exhibited within the *KIR* locus forms diverse haplotypes with variable gene content[1]. Non-allelic homologous recombination (NAHR), facilitated by a close head-to-tail gene arrangement and high-sequence homology, is the mechanism proposed to be responsible for the haplotypic variability. Over 100 different haplotypes have been reported in populations worldwide[1-4]. All these haplotypes could be divided into two major groups: A and B haplotypes. The A haplotype contains 7 *KIR* genes: *KIR3DL3*, *KIR2DL1*, *KIR2DL3*, *KIR2DL4*, *KIR3DL1*, and *KIR3DL2*, which are inhibitory *KIR* genes, and the activating *KIR* gene *KIR2DS4*. However, up to 70% of Caucasian individuals who are homozygous for KIR haplotype A exclusively carry a non-functional "deletion" form of *KIR2DS4*[5,6]. All other *KIR* gene combinations form group B haplotypes, including at least one of the specific *KIR* genes *KIR2DS1*, *KIR2DS2*, *KIR2DS3*, *KIR2DS5*, *KIR3DS1*, *KIR2DL2*, and *KIR2DL5*, and typically include two or more activating *KIR* genes.

HLA Class I molecules have been identified as the ligands for certain inhibitory receptors (*KIR2DL1*, *KIR2DL2*, *KIR2DL3*, and *KIR3DL1*), activating receptors (*KIR2DS1*, *KIR2DS2*, *KIR2DS4*, *KIR2DS5*, and *KIR3DS1*), and for *KIR2DL4*, which is a unique *KIR* that contains long cytoplasmic tails like other inhibitory KIR receptors but also has a positively charged residue near the extracellular domain which is a common feature of other activating *KIR* receptors. The combination of variants within the *KIR* genes and the *HLA* genes influences receptor-ligand interaction that is shaping potential NK cell responsiveness at the individual level[7,8]. Evidence from genetic association studies has indicated that *KIR* plays a role in viral resistance (*e.g.*, human immunodeficiency virus [HIV][9] and hepatitis C virus [HCV][10]), the success of transplantation[11], the risk of pregnancy disorders and reproductive success[12,13], the protection against relapse after allogeneic hematopoietic stem cell transplantation (HSCT)[14-16], and the risk of cancers[17].

The combination of high-sequence homology and allelic and haplotypic diversity presents challenges in the task of accurately genotyping *KIR* genes. Conventional methods to type *KIR* genes include sequence-specific primer (SSP) polymerase chain reaction (PCR)[18-20], sequence-specific oligonucleotide probe (SSOP) PCR[21], and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)[22]. The drawbacks of these techniques are that they only provide partial insight into the genotype of an individual whilst also being laborious to

perform. Recently next-generation sequencing (NGS) has been applied to type the *KIR* locus specifically. While this method is very powerful, it can be expensive to run, and it is time-consuming to conduct in-depth analysis and data checks.

qKAT is a high-throughput quantitative PCR method. While conventional methods are laborious and time-consuming, this method makes it possible to run nearly 1,000 genomic DNA (gDNA) samples in five days and gives the *KIR* genotype, as well as the gene copy number. qKAT consists of ten multiplex reactions, each of which targets two *KIR* loci and one reference gene of a fixed copy number in the genome (*STAT6*) used for the relative quantification of the *KIR* gene copy number[23]. This assay has been successfully used in studies involving large population panels and disease cohorts on infectious diseases such as HCV, autoimmune conditions like type 1 diabetes, and pregnancy disorders such as preeclampsia, as well as providing a genetic underpinning to studies aimed at understanding the NK cell function[1,4,24-26].

PROTOCOL:

1. Preparation and Plating out of DNA

1.1. Accurately quantify the gDNA concentration using a spectrophotometric or fluorometric instrument.

1.2. Dilute DNA to 4 ng/µL on a 96-well deep-well plate. Include at least one control gDNA sample with a known copy number and one non-template control.

1.3. Centrifuge the 96-well plates at 450 x g for 2 min.

1.4. Using a liquid handling instrument, dispense each sample in quadruplicate onto 384-well qPCR plates so that every well has 10 ng of DNA (2.5 µL/well). Prepare at least ten 384-well plates, one for each qKAT reaction.

1.5. If gDNA is being dispensed from more than one 96-well plate, perform a full-volume wash with 2% bleach and ultrapure water to clean the needles of the liquid handling system between each 96-well plate of gDNA samples.

1.6. Air-dry the DNA by incubating the 384-well plates in a clean area at room temperature for at least 24 h.

2. Preparation of the Primers and Probes

Note: qKAT consists of ten multiplex reactions. Each reaction includes three primer pairs and three fluorescence-labeled probes that specifically amplify two *KIR* genes and one reference gene. The probes that were published in Jiang *et al.*[27] were modified so that the oligonucleotides are now labeled with ATTO dyes since they offer improved photostability and long signal lifetimes. Pre-aliquoted primer combinations are commercially available (see **Table of Materials**).

2.1. Prepare primer combinations for each reaction as per the dilutions given in **Table 1**.

2.2. Prepare probe combinations for each reaction as per **Table 1**. Test each individual probe prior to making the combination.

3. Preparation of the Master Mix

Note: The volumes mentioned below are for performing one qKAT reaction on a set of 10x 384-well plates.

3.1. Ensure that the gDNA samples plated on the 384-well plates are completely dry. Conduct all steps on ice and keep the reagents covered from exposure to light as much as possible since the fluorescence-labeled probes are photo- and thermo-sensitive.

3.2. Defrost the qPCR buffer, primer, and probe aliquots at 4 °C.

3.3. On ice, prepare a master mix for 10x 384-well plates by adding 18.86 mL of ultrapure water, 20 mL of qPCR buffer, 1,000 µL of preprepared primer combination, and 180 µL of preprepared probe combination (**Table 2**).

3.4. Distribute the master mix evenly across a 96-deep well plate using a multi-channel pipette, pipetting 415 µL into each well. Keep this plate in an ice box covered from light.

3.5. Using a liquid handling instrument, dispense 9.5 µL of the master mix into each well of the 384-well plate with dried gDNA. Seal the plate with a foil and immediately place it at 4 °C. Repeat this process for the remaining plates, ensuring that the needles of the liquid handling system are washed with water between each plate.

3.6. Centrifuge the 384-well plates at 450 x g for 3 min and incubate them at 4 °C overnight or between 6 - 12 h to resuspend the DNA and to dissipate any air bubbles.

4. qPCR Assay

4.1. Following the overnight incubation, centrifuge at 450 x g for 3 min to dissipate any remaining air bubbles.

4.2. For purposes of automation, connect the qPCR machine (*e.g.*, LightCycler 480) to a microplate handler (see **Table of Materials**). Program the microplate handler to place the plates into the qPCR machine from a cooled storage dock that is protected from light.

Note: The assays should, in theory, work on other qPCR machines with compatible optic settings.

4.3. Use the following cycling conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 15 s and 66 °C for 50 s, with data collection at 66 °C.

4.4. Once the run is complete, have the robot collect the plate from the qPCR machine and place it in the discard dock.

5. Post-run Analysis

5.1. After amplification, calculate the quantification cycle (Cq) values using either the second derivative maximum method or the Fit Points method with the software of the qPCR machine (see **Table of Materials**), following the steps below.

5.2. Open the qPCR software and, in the **Navigator** tab, open the saved reaction experiment file for one plate.

5.3. For the analysis using the second derivative maximum method, select the **Analysis** tab, and create a new analysis using **Abs Quant/Second Derivative Max method**.

5.3.1. In the **Create new analysis** window, select analysis type: **Abs Quant/Second Derivative Max method**, subset: **All Samples**, program: **Amplification**, name: **Rx-DFO** (where **x** is the reaction number).

5.3.2. Select **Filter Comb** and choose **VIC/HEX/Yellow555 (533-580)**. This ensures that the data collected for *STAT6* is selected.

5.3.3. Select **Colour Compensation** for VIC/HEX/Yellow555(533-580). Click **Calculate**. Repeat this for Fam (465-510) and Cy5/Cy5.5(618-660). Click **Save file**.

5.4. For the analysis using the Fit Points method, select **Abs Quant/Fit Points** in the **Analysis** tab.

5.4.1. In the **Create new analysis** window, select analysis type: **Abs Quant/Fit Points method**, subset: **All Samples**, program: **Amplification**, name: **RxF-DFO** (where **x** is the reaction number).

5.4.2. Select the correct filters and color compensations for *STAT6* and each of the *KIR* genes (Fam/Cy5). In the **Noiseband** tab, set the noise band to exclude the background noise.

5.4.3. In the **Analysis** tab, set the fit points to **3** and select **Show fit points**. Click **Calculate**. Click **Save file**.

6. Export of the Results

6.1. In the qPCR software, open the **Navigator** tab. Select **Results Batch Export**.

6.2. Open the folder in which the experiment files are saved and transfer the files into the right-hand side section of the window. Click **Next**. Select the name and the location of the export file.

6.3. Select Analysis type **Abs Quant/Second Derivative Max method** or **Abs Quant/Fit Points**. Click **Next**. Check that the name of the file, the export folder, and the analysis type are correct and click **Next** to start the export process.

6.4. Wait until the **Export Status** is **Ok**. The screen will automatically move to the next step. Check that all selected files have been exported successfully so that the number of files failed = 0. Click **Done**.

6.5. Use scripts split_file.pl and roche2sds.pl to split the exported plates into individual reactions for each plate.

Note: The scripts are provided on request/GitHub.

7. Copy Number Calculations

7.1. Open the copy number analysis software (*e.g.*, CopyCaller). Select **Import real-time PCR results file** and load text files created by **roche2sds.pl**.

7.2. Select **Analyze** and conduct the analysis by either selecting **calibrator sample with known copy number** or by selecting **most frequent copy number**. See **Table 5** for the most frequent copy number of *KIR* genes typically observed in Caucasian populations.

8. Data-quality Checks

8.1. Use R script **KIR_CNVdata_analysis_for_Excel_ver020215.R** to combine copy number data from all the plates into a spreadsheet.

Note: The scripts are provided on request/GitHub.

8.2. Recheck the raw data on the copy number analysis software for samples that do not conform to the known linkage disequilibrium (LD) for *KIR* genes (**Table 6**).

REPRESENTATIVE RESULTS:

Copy number analysis can be carried out by exporting the files to the copy number analysis software, which provides the predicted and estimated copy number based on the ΔΔCq method.

The copy number can be predicted either based on the known copy number of control DNA samples on the plate or by inputting the most frequent gene copy number (**Table 5**). **Figure 1** shows the results of a plate for a reaction that targets *KIR2DL4* and *KIR3DS1*, as well as the reference gene *STAT6.* The most frequent copy number for *KIR2DL4,* a framework gene in the *KIR* locus, is two copies, whereas the most frequent copy number for *KIR3DS1*, an activating gene, is

one copy. The results in the figure show the PCR amplification plots observed on the qPCR software and the copy number data generated from the qPCR data. As shown, the assay is able to distinguish between 0, 1, 2, 3, and 4 *KIR* gene copy numbers. The copy number analysis software also enables a viewing of the distribution of the copy number across the plate as a pie chart or a bar graph. The efficacy of the copy number prediction is lower for samples with a higher copy number.

The quality of all the materials used in the reactions, gDNA, buffer, primers, and probes, can affect the accuracy of the results obtained. However, discordance in results is most likely to be caused due to variation in the concentration of DNA across a plate. The purity of the extracted gDNA, which can be measured using the 260/280 and 260/230 ratios, can also have an effect on the quality. A 260/280 ratio of 1.8 - 2 and a 260/230 ratio of 2 - 2.2 are desirable. An uneven range of DNA concentrations across a plate can lead to a high variability in the threshold cycle (C_t) between samples and discordance in the range of the estimated copy number. The results in **Figure 2** show the effect the disparity between the C_t values across a plate can have on the accuracy in the prediction of the copy number. The red line indicates the range of the estimated copy number for a sample and, ideally, should be as close to an integer as possible.

The copy number data, once analyzed, can be exported as a spreadsheet file in a 96-well format. We used an R script (available on request) to combine the copy number data of all 10 plates that are run as a set into one spreadsheet. Published data about *KIRs* from mostly Caucasian populations enables the prediction of LD rules that exist between various genes in the *KIR* complex[1]. These predictions are used to conduct downstream checks on the copy number results obtained (**Table 6**). Samples that do not conform to the predicted LD between the genes might contain unusual polymorphism or haplotypic structural variations. A flowchart describing the protocol is shown in **Figure 3**.

A tool called *KIR* Haplotype Identifier (http://www.bioinformatics.cimr.cam.ac.uk/haplotypes/) was developed to facilitate the imputation of haplotypes from the data set. The imputation works on the basis of a list of reference haplotypes observed in a European-origin/Caucasian population[1]. However, the tool also allows for a custom set of reference haplotypes to be used instead. Three separate files are generated; the first file lists all haplotype combinations for a sample, the second file provides a trimmed list of the haplotypes combinations that have the highest combined frequencies, and the third file lists the samples that cannot be assigned haplotypes. Non-assignment of haplotypes could be used as an indicator of novel haplotypes.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative results of a plate for reaction number 4. (**A**) This panel shows amplification plots. (**B**) This panel shows copy number plots. (**C**) This panel shows the copy number distribution.

Figure 2: Representative results of a plate with a variable DNA concentration for reaction number 4. (**A**) This panel shows amplification plots. (**B**) This panel shows copy number plots.

Figure 3: Flowchart of the qKAT protocol.

Table 1: Combination and concentration of primers and probes used in each qKAT reaction[27].

Table 2: Volumes (µL) of 100 µM primer/probe stock solutions to make primer and probe combination aliquots.

Table 3: List of probes used in qKAT[1,27]. The fluorescent dyes used at the 5' end of the oligo probes P5b, P5b-2DL4, P9, and PSTAT6 were modified to ATTO dyes.

Table 4: Sequences of the primers used in qKAT[1,27].

Table 5: Most frequent copy number for *KIR* genes commonly observed in Caucasian/European-origin samples.

Table 6: Linkage disequilibrium between *KIR* genes commonly observed in Caucasian/European-origin populations can be used to check copy number data[1,27].

DISCUSSION:

We described a novel semi-automated high-throughput method, called qKAT, which facilitates copy number typing of *KIR* genes. The method is an improvement over conventional methods like SSP PCR, which are low-throughput and can only indicate the presence or absence of these highly polymorphic genes.

The accuracy of the copy number data obtained is dependent on multiple factors, including the quality and concentration-uniformity of the gDNA samples and the quality of the reagents. The quality and accuracy of the gDNA samples across a plate are extremely important since variations in concentration across the plate can result in errors in the calculation/prediction of the copy number. Since the assays were validated using European-origin sample sets, data from cohorts from other parts of the world require more thorough checks. This is to make sure that instances of allele dropout or non-specific primer/probe binding are not misinterpreted as copy number variation.

While the assays were designed and optimized to run as high-throughput, they can be modified to run fewer samples. The confidence metric in the copy number analysis software is affected when analyzing fewer samples, but this can be improved if control genomic DNA samples with a known *KIR* gene copy number are included on the plate and additional sample replicates are included.

The main aim behind the development of qKAT was to create a simple, high-throughput, high-resolution, and cost-effective method to genotype *KIRs* for disease association studies. This was successfully achieved since qKAT has been employed in investigating the role of *KIR* in several

large disease association studies, including a range of infectious diseases, autoimmune conditions, and pregnancy disorders[4,24-26].

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DISCLOSURES:

The authors have nothing to disclose.

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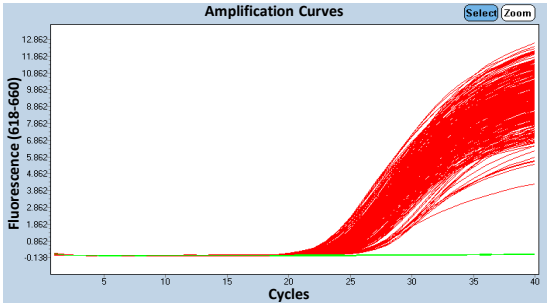
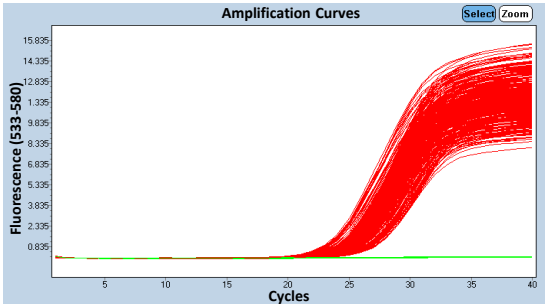
Figure 1

A

KIR2DL4



Stat6



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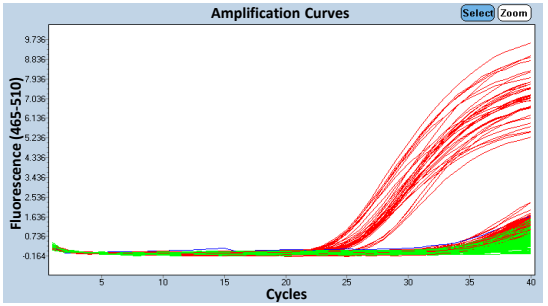

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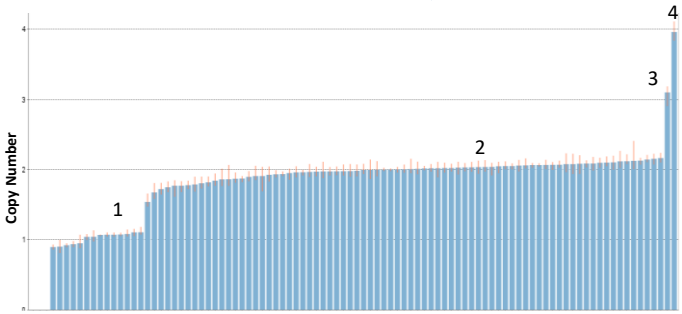
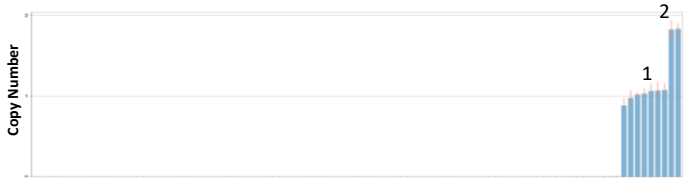
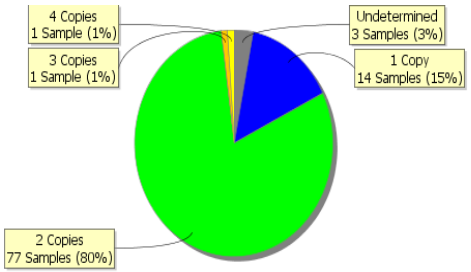




C



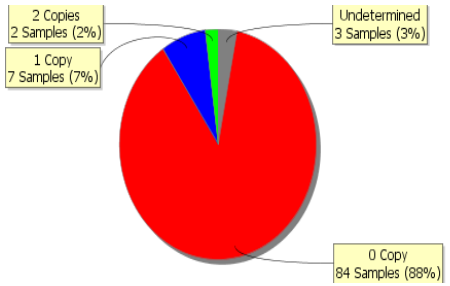
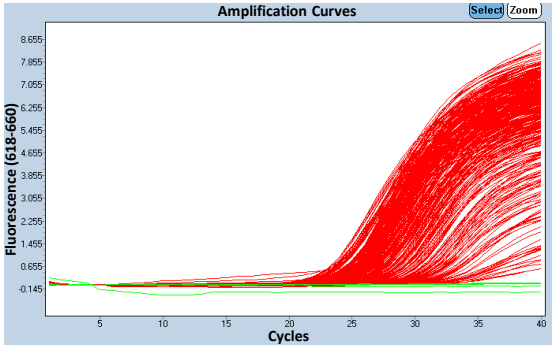


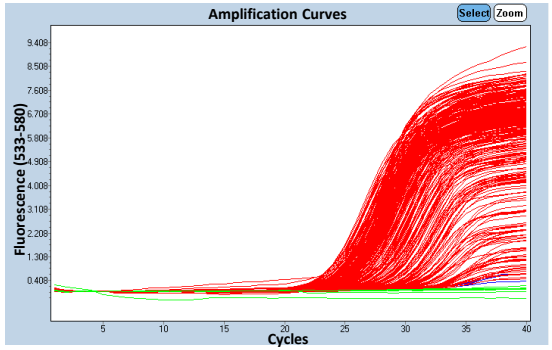
Figure 2

A

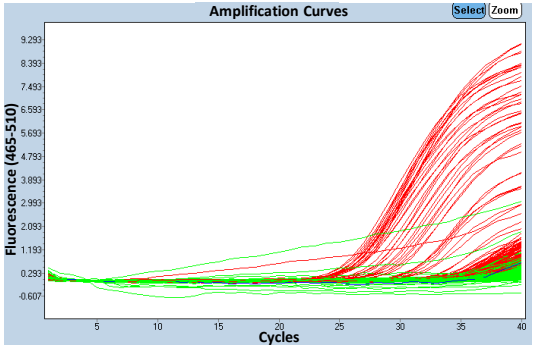
KIR2DL4



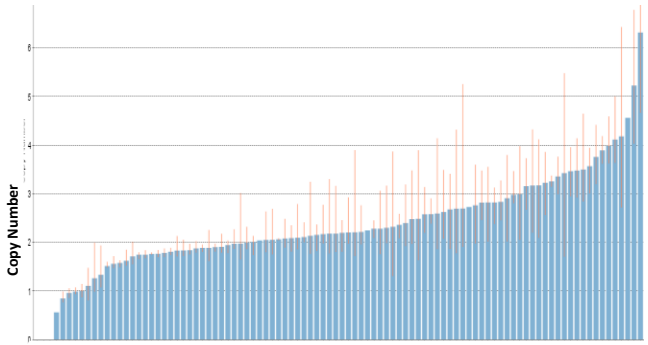
Stat6

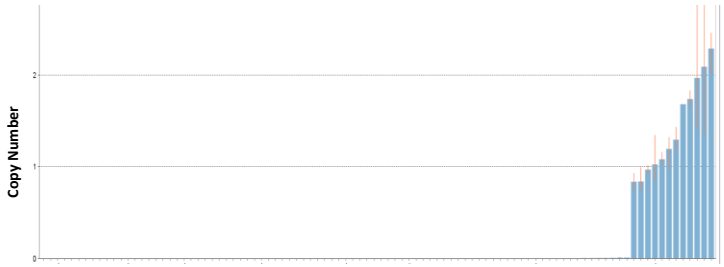


KIR3DS1



B





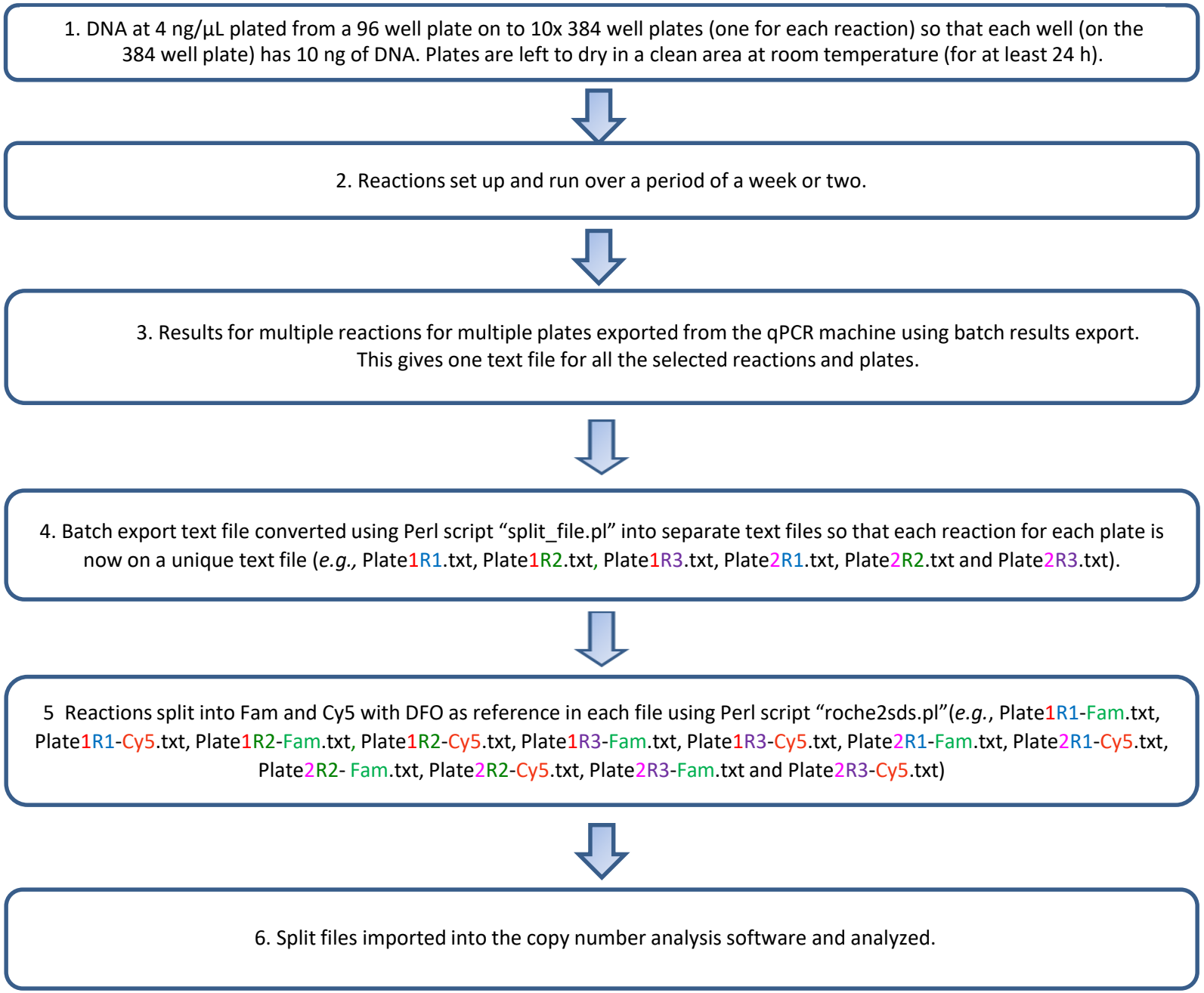


Figure 1 svg

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Video or Animated Figure

QKATplot_1.svg

Figure 2 svg

Click here to access/download

Video or Animated Figure

QKATplot_2.svg

Table 1

Assay	Genes	Forward Primers
No 1	***3DP1***	A4F
	2DL2	2DL2F4
	STAT6	STAT6F
No 2	***2DS2***	A4F
	2DL3	D1F
	STAT6	STAT6F
No 3	***3DL3***	A8F
	2DS4Del	2DS4Del
	STAT6	STAT6F
No 4	***3DL1e4***	B1F
	3DL1e9	D4F
	STAT6	STAT6F
No 5	***3DS1***	B2F
	2DL4	C1F
	STAT6	STAT6F
No 6	***2DL1***	B3F
	2DP1	D3F
	STAT6	STAT6F
No 7	***2DS1***	B4F
	2DL5	D2F
	STAT6	STAT6F
No 8	***2DS3***	B5F
	3DL2e9	D4F
	STAT6	STAT6F
No 9	***3DL2e4***	A1F
	2DS4FL	2DS4FL
	STAT6	STAT6F
No 10	***2DS5***	B6F2
	2DS4	C5F
	STAT6	STAT6F

Concentration (nM)	Reverse Primers	Concentration (nM)
250	A5R	250
400	C3R2	600
200	STAT6R	200

400	A6R	400
400	D1R	400
200	STAT6R	200

500	A8R	500
250	2DS4R2	250
200	STAT6R	200

250	B1R	125
250	D4R2	500
200	STAT6R	200

250	B1R	250
200	C1R	200
200	STAT6R	200

500	B3R	125
250	D3R	500
200	STAT6R	200

500	B4R	250
500	D2R	500
200	STAT6R	200

250	B5R	250
250	D5R	125
200	STAT6R	200

200	A1R	200
250	2DS4R2	500
200	STAT6R	200

200	B6R3	200
250	C5R	250
200	STAT6R	200

Probes	Concentration (nM)
P4a	150
P5b	150
PSTAT6	150
P4a	200
P9	150
PSTAT6	150
P4a	150
P5b	150
PSTAT6	150
P4b	150
P9	150
PSTAT6	150
P4b	150
P5b-2DL4	150
PSTAT6	150
P4b	150
P9	150
PSTAT6	150
P4b	150
P9	150
PSTAT6	150
P4b	150
P9	150
PSTAT6	150
P4a	150
P5b	150
PSTAT6	150
P4b	150
P5b	150
PSTAT6	150

Table 2

Reaction					Primer Aliquots
R1	**3DP1**	A4F	A5R	2DL2F4	C3R2
	2DL2	100	100	160	240
R2	**2DS2**	A2F	A6R	D1F	D1R
	2DL3	160	160	160	160
R3	**3DL3**	A8F A8FB	A8R	2DS4DELF	2DS4R2
	2DS4DEL	100 100	200	100	100
R4	**3DL1E5**	B1F	B1R	D4F	D4R2
	3DL1E9	100	50	100	200
R5	**3DS1**	B2F	B1R	C1F	C1R
	2DL4	100	100	80	80
R6	**2DL1**	B3F	B3R	D3F	D3R
	2DP1	200	50	100	200
R7	**2DS1**	B4F	B4R	D2F	D2R
	2DL5	200	100	200	200
R8	**2DS3**	B5F	B5R	D4F	D5R
	3DL2E9	100	100	100	50
R9	**3DL2E4**	A1F	A1R	2DS4WTF	2DS4R2

	2DS4WT	80	80	100	200
R10	**2DS5**	B6F2	B6R3	C5F	C5R
	2DS4TOTAL	80	80	100	100

s (µL)				Probe Aliquots (µL)	
WATER	STAT6F	STAT6R		P4A	P5B
200	80	80		60	60
WATER	STAT6F	STAT6R		P4A	P9
160	80	80		**80**	60
				Note: need 20 µL less water in th	
WATER	STAT6F	STAT6R		P4A	P5B
200	80	80		60	60
WATER	STAT6F	STAT6R		P4B	P9
350	80	80		60	60
WATER	STAT6F	STAT6R		P4B	P5B-2L4
440	80	80		60	60
WATER	STAT6F	STAT6R		P4B	P9
250	80	80		60	60
WATER	STAT6F	STAT6R		P4B	P9
100	80	80		60	60
WATER	STAT6F	STAT6R		P4B	P9
450	80	80		60	60
WATER	STAT6F	STAT6R		P4A	P5B

340	80	80		60	60
WATER	STAT6F	STAT6R		P4B	P5B
440	80	80		60	60

PSTAT6
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ıe MasterMix
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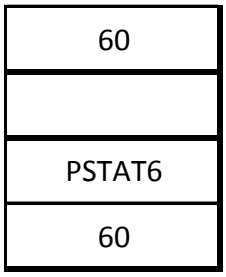
60
PSTAT6
60

Table 3

Name	Direction	5´ modification	3´ modification
P4a	Sense	FAM	BHQ-1
P4b	Antisense	FAM	BHQ-1
P5b	Sense	ATTO647N	BHQ-2
P5b-2DL4	Sense	ATTO647N	BHQ-2
P9	Sense	ATTO647N	BHQ-2
PSTAT6		ATTO550	BHQ-2

Sequence	Length	Tm a	GC%	Exon
TCATCCTGCAATGTTGGTCAGATGTCA	27	60	44.4	4
AACAGAACCGTAGCATCTGTAGGTCCCT	28	62	50	4
AACATTCCAGGCCGACTTTCCTCTG	25	60	52	5
AACATTCCAGGCCGACTTCCCTCTG	25	61	56	5
CCCTTCTCAGAGGCCCAAGACACC	24	60	62.5	9
CTGATTCCTCCATGAGCATGCAGCTT	26	62	50	

Position b
425-451
576-603
828-852
828-852
1246-1269

Table 4 Click here to access/download;Table;58646Table_4.xlsx

Gene	Primers	Direction
3DL2e4	A1F	Forward
	A1R	Reverse
3DP1	A4F	Forward
	A5R	Reverse
2DS2	A2F	Forward
	A6R	Reverse
3DL3	A8Fa	Forward
	A8Fb	Forward
	A8R	Reverse
3DL1e4	B1F	Forward
	B1R	Reverse
3DS1	B2F	Forward
	B1R	Reverse
2DL1	B3F	Forward
	B3R	Reverse
2DS1	B4F	Forward
	B4R	Reverse
2DS3	B5F	Forward
	B5R	Reverse
2DS5	B6F2	Forward
	B6R3	Reverse
2DL4	C1F	Forward
	C1R	Reverse

2DL2	2DL2F4	Forward
	C3R2	Reverse
2DS4	C5F	Forward
	C5R	Reverse
2DS4Del	2DS4Del	Forward
	2DS4R2	Reverse
2DS4FL	2DS4FL	Forward
	2DS4R2	Reverse
2DL3	D1F	Forward
	D1R	Reverse
2DL5	D2F	Forward
	D2R	Reverse
2DP1	D3F	Forward
	D3R	Reverse
3DL1e9	D4F	Forward
	D4R2	Reverse
3DL2e9	D4F	Forward
	D5R	Reverse
STAT6	STAT6F	Forward
	STAT6R	Reverse

Sequence (5´-3´)	Length	Tm	GC%
GCCCCTGCTGAAATCAGG	18	52	61.1
CTGCAAGGACAGGCATCAA	19	53	52.6
GTCCCCTGGTGAAATCAGA	19	49	52.6
GTGAGGCGCAAAGTGTCA	18	52	55.6
GTCGCCTGGTGAAATCAGA	19	49	52.6
TGAGGTGCAAAGTGTCCTTAT	21	51	42.9
GTGAAATCGGGAGAGACG	18	50	55.6
GGTGAAATCAGGAGAGACG	19	50	52.6
AGTTGACCTGGGAACCCG	18	51	61.1
CATCGGTCCCATGATGCT	18	51	55.6
GGGAGCTGACAACTGATAGG	20	52	55
CATCGGTTCCATGATGCG	18	51	55.6
GGGAGCTGACAACTGATAGG	20	52	55
TTCTCCATCAGTCGCATGAC	20	52	50
GTCACTGGGAGCTGACAC	18	50	61.1
TCTCCATCAGTCGCATGAA	19	51	47.4
GGTCACTGGGAGCTGAC	17	49	64.7
CTCCATCGGTCGCATGAG	18	53	61.1
GGGTCACTGGGAGCTGAA	18	51	61.1
AGAGAGGGGACGTTTAACC	19	50	52.6
TCCAGAGGGTCACTGGGC	18	53	66.7
GCAGTGCCCAGCATCAAT	18	52	55.6
CCGAAGCATCTGTAGGTCT	19	52	52.6

GAGGTGGAGGCCCATGAAT	19	52	57.9
TCGAGTTTGACCACTCGTAT	20	51	45
TCCCTGCAGTGCGCAGC	17	57	70.6
TTGACCACTCGTAGGGAGC	19	52	57.9
CCTTGTCCTGCAGCTCCAT	19	54	57.9
TGACGGAAACAAGCAGTGGA	20	53	50
CCGGAGCTCCTATGACATG	19	53	57.9
TGACGGAAACAAGCAGTGGA	20	53	50
AGACCCTCAGGAGGTGA	17	48	58.8
CAGGAGACAACTTTGGATCA	20	50	45
CACTGCGTTTTCACACAGAC	20	52	50
GGCAGGAGACAATGATCTT	19	49	47.4
CCTCAGGAGGTGACATACGT	20	53	55
TTGGAAGTTCCGTGTACACT	20	50	45
CACAGTTGGATCACTGCGT	19	52	52.6
CCGTGTACAAGATGGTATCTGTA	23	53	43.5
CACAGTTGGATCACTGCGT	19	52	52.6
GACCTGACTGTGGTGCTCG	19	54	63.2
CCAGATGCCTACCATGGTGC	20	54	60
CCATCTGCACAGACCACTCC	20	54	60

Exon	Position	Amplicon (bp)
4	399-416	179
	559-577	
4	398-416	112
	492-509	
4	398-416	111
	488-508	
4	406-423	139
	405-423	
	526-543	
4	549-566	85
	614-633	
4	549-566	85
	614-633	
4	544-563	96
	622-639	
4	545-563	96
	624-640	
4	546-563	96
	624-641	
4	475-493	173
	630-647	
5	808-825	83
	872-890	

5	778-796	151
	909-928	
5	803-819	120
	904-922	
5	750-768	203
	933-952	
5	744-762	209
	933-952	
9	1180-1196	156
	1316-1335	
9	1214-1233	120
	1315-1333	
9	1184-1203	121
	1285-1304	
9	1203-1221	93
	1273-1295	
9	1203-1221	156
	1340-1358	
		129

Alleles might be missed
3DL2*008, *021, *027, *038.
3DL2*048
None
None
None
None
None
3DL3*054, 3DL3*00905.
None
3DL1*00505,3DL1*006, 3DL1*054, 3DL1*086, 3DL1*089
3DL1*00502
3DS1*047; may pick up 3DL1*054.
None
2DL1*020, 2DL1*028
2DL1*023, 2DL1*029, 2DL1*030
2DS1*001
None
None
None
None
2DS5*003
None
2DL4*018, 2DL4*019

2DL2*009; 782G changed to A.
None
None
2DS4*013
None
None
None
None
None
2DL3*010, 2DL3*017, 2DL3*01801 and 2DL3*01802
2DL5B*011 and 2DL5B*020
None
None
None
3DL1*061, 3DL1*068
3DL1*05901, 3DL1*05902, 3DL1*060,3DL1*061,3DL1*064,3DL1*065,3DL1*094N,3DL1*098
None
None

Table 5

KIR gene	3DL3	2DS2	2DL2	2DL3	2DP1
Most frequent copy number	2	1	1	2	2

2DL1	3DP1	2DL4	3DL1EX9	3DL1EX9	3DS1	2DL5	2DS3
2	2	2	2	2	1	1	1

2DS5	2DS1	2DS4Total	2DS4FL	2DS4DEL	3DL2ex4	3DL2EX9
1	1	2	1	1	2	2

Table 6

1
2
3
4
5
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10

Linkage disequilibrium rules for qKAT based on European populations

KIR3DL3, KIR3DP1,KIR2DL4 and *KIR3DL2* are framework genes present on both haplotypes.
KIR2DS2 and *KIR2DL2* are in LD with each other
KIR2DL2 and *KIR2DL3* are alleles of the same gene
KIR2DP1 and *KIR2DL1* are in LD with each other
Exon 4 of *KIR3DL1* and *KIR3DL2* is equal to exon 9 of *KIR3DL1* and *KIR3DL2* respectively.
KIR3DL1 and *KIR3DS1* are alleles
KIR2DS3 and *KIR2DS5* are in LD with *KIR2DL5*
KIR3DS1 and *KIR2DS1* are in LD
Presence of *KIR2DS1* and *KIR2DS4T* otal is mutually exclusive on a haplotype
KIR2DS4FL and *KIR2DS4del* are variants of *KIR2DS4TOTAL*

Copy number check
KIR3DL3, KIR3DP1,KIR2DL4 and *KIR3DL2* = 2
2DS2 =2DL2
*2DL2 +2DL3 =*2
2DP1 =2DL1
3DL1ex4 =3DL1ex9 AND *3DL2ex4 =3DL2ex9*
*3DL1 +3DS1 =*2
2DS3 +2DS5 =2DL5
3DS1 =2DS1
*2DS1 +2DS4TOTAL =*2
2DS4FL +2DS4DEL =2DS4TOTAL

Table of Materials

Click here to access/download;Table of Materials;Table_Materials.xlsx

REAGENTS

EQUIPMENT

SOFTWARE

REAGENTS
Oligonucleotides
Probes labelled with ATTO dyes
SensiFAST Probe No-ROX Kit
MilliQ water

EQUIPMENT
Centrifuge with a swinging bucket rotor
NanoDrop
OR
QuBit Fluorometer
Matrix Hydra
LightCycler 480 II Instrument 384-well
Twister II Microplate Handler with MéCour Thermal Plate Stacker (MéCour)
Vortex mixer
Single-channel pipettes (volume range: 0.5–10 µL, 2–20 µL, 20–200 µL, 200–1,000 µL; 1-10 mL)
RNase- and DNase-free pipette tips filtered (10 µL, 20 µL, 200 µL, 1,000 µL, 10 mL)
StarTub PS Reagent Reservoir, 55 mL
50 mL Centrifuge Tube
96-well deep well plate
LC480 384 Multi-well plates
LightCycler 480 Sealing Foil

NAME
Roche LightCycler 480 Software v1.5
Applied Biosystems CopyCaller Software v2.1
KIR haplotype identifier

COMPANY	CATALOGUE NUMBER	SEQUENCES
Sigma	Custom order	Listed in Table 4
Sigma	Custom order	Listed in Table 3
Bioline	BIO-86020	–
–	–	–

COMPANY	CATALOGUE NUMBER
Eppendorf(or equivalent)	Eppendorf 5810R or equivalent system
Thermo Scientific	ND-2000
Life Technologies	Q33216
Thermo Scientific	109611
Roche	05015243001
Caliper Life Sciences	204135
Biosan	BS-010201-AAA
Gilson(or equivalent)	F144801, F123600, F123615, F123602, F161201
Starlab (or equivalent)	S1111-3810, S1120-1810, S1120-8810, S1111-6810, I1054-0001
STARLAB	E2310-1010
STARLAB	E1450-0200
Fisher Scientific	12194162
Roche	04729749001
Roche	04729757001

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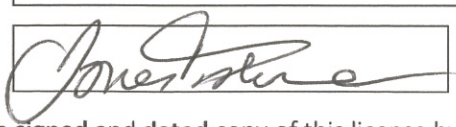
Signature:	
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Reply to Reviewers

Thank you for your useful comments.

Description of responses/corrections to reviewer comments are in italics.

Reviewer #1:

1. KIR2DL2 and KIR2DL3, as well as KIR3DL1 and KIR3DS1, segregate as alleles of the same locus. Accordingly, the sentence reporting KIR gene number (lines 19-20) should be revised.

This sentence now reads "The KIR loci that are currently known are KIR2DL1, KIR2DL2/KIR2DL3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1-5, KIR3DL1/KIR3DS1, KIR3DL2-3 and two pseudogenes, KIR2DP1 and KIR3DP1."

2. Introduction fails to report knowledge about some KIR/KIR ligand pairs (lines 35-36). In particular, it has been demonstrated that HLA Cl-I molecules are also recognized by KIR2DL4, KIR2DS2 and KIR3DS1 (Rajagopalan 1999 J. Exp. Med., Liu 2014 Proc. Natl. Acad. Sci. USA, David, 2013 J. Immunol., Burian 2016 PLoS One, Carlomagno 2017 Front. Immunol.)

Introduction has been modified and appropriate references added to relay this information; "HLA Class I molecules have been identified as the ligands for certain inhibitory receptors (KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1), activating receptors (KIR2DS1, KIR2DS2, KIR2DS4, KIR2DS5 and KIR3DS1) receptors and for KIR2DL4 which is a unique KIR that contains long cytoplasmic tails like other inhibitory KIR receptors but also has a positively charged residue near the extracellular domain which is a common feature of other activating KIR receptors. The combination of variants within the KIR genes and the HLA genes influence receptor-ligand interaction shaping potential NK cell responsiveness at the individual level[7,8]."

3. Pseudogenes are not mentioned in the introduction section but are both target in the protocol. A sentence about KIR2DP1 and KIR3DP1 could be added in the introduction.

The pseudogenes are now included in the introduction; "The KIR loci that are currently known are KIR2DL1, KIR2DL2/KIR2DL3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1-5, KIR3DL1/KIR3DS1, KIR3DL2-3 and two pseudogenes, KIR2DP1 and KIR3DP1."

4. Ramping temperature (i.e. °C/sec) can be included in the description of cycling conditions.

Non-applicable – temperature ramping was not used in the qPCR cycling program.

5. The Y-axis labels in Figures 1B and 2B are difficult to read. Please increase the font size.

The font size has been increased.

6. Last step of flowchart should be identified with the number 6.

Corrected.

7. Reference at line 38 is indicated using author name and publication year, please use the corresponding reference list number. *Corrected*

8. The authors should carefully revise the references according to journal style
-Last page number is not always correctly indicated (i.e. ref. 1, 4, 6, 9, 13).
Our citations are consistent with the journals. Please note that PLoS Genetics references do not contain page numbers.
-In my opinion reference 13 supports sentence at lines 37-38. A study reporting relevance of donor B content analysis in allogenic hematopoietic stem cell transplantation could be added (i.e. Cooley, 2010 Blood).
This reference has now been used to support this sentence.
-Title and page numbers of reference 16 are missing.
This reference has been replaced by three more relevant publications:
Vilches et al. 2007, Ashouri et al. 2009 and Martin et al. 2008.
-References 23 and 24 are identical.
Duplicate reference has been removed.

Reviewer #2:

9. Compliance with the MIQE guidelines (1) should be checked.

After careful review, we believe the manuscript is fully compliant with MIQE guidelines in relation to qPCR of genomic DNA.