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Allele Level Sequencing of Killer Cell Immunoglobulin-Like Receptor Genes Using Oxford Nanopore Long Read Sequencing

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ABSTRACT

The human Killer cell Immunoglobulin-like Receptor (KIR) genes, found on chromosome 19, encode for cell surface protein receptors that, through interaction with their ligand, modulate the action of Natural Killer (NK) cells and some subsets of T lymphocytes. KIR genes exhibit extensive variation through variable gene content, copy number, and allele polymorphism. The combination of KIR genes and their ligands is implicated in various clinical settings including haematopoietic stem cell and solid organ transplant, and infectious disease progression. KIR gene content has been used in the selection of optimal stem cell donors with haplotype variations in recipient and donor giving differential clinical outcomes. With the introduction of massively parallel clonal next generation sequencing and single molecule long read third generation sequencing, allele level determination of KIR genotypes has become feasible. We describe a method for amplicon-based long read sequencing on the Oxford Nanopore Technologies platform that provides largely unambiguous allele level typing of KIR genes. The method was validated using DNA extracted from 48 10th International Histocompatibility Workshop (IHWS) cell lines with previously published allele level KIR genotypes and 176 Western Australian samples previously tested for the presence or absence of KIR genes. Our long-read sequencing method was able to accurately determine KIR alleles with an overall concordance of 97%–99% with the published data. Importantly, phasing ambiguity caused by the inability to phase heterozygous base positions over long stretches of gene sequence was resolved in several samples. Thus, our long read PCR sequencing strategy can be used to determine KIR genotypes at allele resolution level.

1 | Introduction

The Killer Cell Immunoglobulin like Receptor (KIR) genes, located on chromosome 19, encode a family of cell surface expressed transmembrane proteins that regulate the killing of virus infected or malignant cells by Natural Killer (NK) cells and some T cell subsets [1]. So far, 13 genes have been described which broadly fit into two groups: inhibitory or activating, depending on whether the protein has a long (L) or short (S) cytoplasmic tail: KIR2DL1, KIR2DL2/3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR3DL1S1, KIR3DL2, KIR3DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4 and KIR2DS5. In addition, there are two pseudogenes, KIR2DP1 and KIR3DP1.

The KIR genes show a high degree of variation in individual gene content and gene copy number. Framework genes KIR3DP1, KIR2DL4, KIR3DL2 and KIR3DL3 occur in nearly every individual. KIR haplotypes are classified into two groups, namely

A and B. The KIR A haplotype group has been defined as containing KIR2DS4 as the only activating KIR gene in combination with KIR3DL3, KIR2DL3, KIR2DL1, KIR2DL4, KIR3DL1, KIR3DL2, KIR2DP1 and KIR3DP1; whereas the B haplotype group shows more variation, carrying other KIR genes, sometimes including A haplotype specific genes and has more activating genes [2]. Thus, individual gene content varies between as little as eight and as many as 13 KIR genes.

Historically, KIR genes have mostly been genotyped to determine their presence or absence within the individual's genome [2, 3]. The presence or absence of haplotype group motifs has been used clinically to differentiate donors into 'neutral', 'better' or 'best' in the setting of acute myeloid leukaemia after HLA-matched or mismatched T-cell replete donor transplants, based on the beneficial effect of centromeric or telomeric B haplotypes in the donor [4]. More recently, advances in gene sequencing technology have stimulated much interest in allele level KIR gene sequencing, revealing a high degree of allelic polymorphism. As of April 2025, there were a total of 2219 alleles coding for 827 different proteins described in the Immuno-Polymorphism Database (IPD, Version 2.14) [5]. All KIR genes show a degree of allele polymorphism, with the highest number of alleles having been described in KIR2DL1 (428), KIR3DL1 (307), KIR3DL2 (252) and KIR3DL3 (232). KIR allele variation has the effect of further diversifying KIR haplotypes possessing the same set of genes [6].

So far, the application of high-resolution KIR genotyping has only been applied in the research domain. Allele polymorphism can have a functional impact on KIR where some alleles are not expressed at the cell surface; for example, *KIR3DL1*004* carries point mutations leading to intracellular retention [7]. Additionally, allele polymorphism may have an effect on the expressed receptor in terms of its ability to produce a strong or weak inhibitory or activating response and the strength of interaction with its ligand. Haematopoietic stem cell transplant (HSCT) donors with low inhibition KIR–KIR ligand combinations have been associated with lower relapse and higher survival, whereas combinations with higher expression and higher inhibitory activity were associated with higher relapse [8]. KIR2DL1 and KIR3DL1 have been preferentially studied in relation to HSCT and infectious disease outcomes due to their importance in NK cell allo-immunity and their interactions with their HLA ligands. Bari et al. have shown that allelic polymorphism in KIR2DL1 affects overall recipient survival and disease-free survival in paediatric allogeneic HSCT patients [9]. KIR3DL1 alleles have been segregated based on patterns of strong (**001*, **002*), weak (**005*, **007*) or lack (**004*) inhibition by target cells with HLA-B subtypes, and in AML patients who received HLA-compatible allografts, donor–recipient KIR3DL1/HLA-B subtype combinations that demonstrate weak or no inhibition were associated with significantly lower relapse and higher survival compared with strong inhibition combinations [10]. A recent study has shown that characterising individuals for the presence of a particular B haplotype defined by allele level genotyping showed a trend to significance for protecting from relapse [11]. KIR genotyping has been shown to be feasible in prospective HSCT donor selection where KIR3DL1 alleles of potential donors were differentiated into high and low expression groups [12]. In the context of solid organ transplantation, NK cells are known to infiltrate kidney allografts and occur at a higher frequency in the peripheral blood of patients with acute graft rejection. A role for inhibitory KIR–KIR ligand has been shown in the increased risk of chronic rejection and reduction in long-term graft survival [13]. KIR2DS4 and KIR2DS5 have been implicated in the outcome of kidney transplants in patients with glomerular nephritis [14], and individuals with a higher number of activating KIR genes exhibited increased NK cytotoxicity against their kidney grafts compared with individuals with no activating KIR specific for donor HLA [15]. The elucidation of the effect of KIR allele polymorphism on the outcomes of kidney transplantation is likely to shed further light on NK cell function in allografts. KIR, in the context of HIV infection, have been shown to exhibit an effect in a variety of settings, particularly in the context of their class I HLA ligands. Protection from HIV infection susceptibility and disease progression was shown to be associated with the presence of KIR3DS1 and its ligand HLA-B Bw4, where an isoleucine residue was present at amino acid position 80 [16]. In a recent study in individuals possessing *HLA-B*57:01*, a variant of the KIR3DL1 molecule has been shown to be associated with elite control of viral load and delay in disease progression [17]. The presence of a valine residue at position 47 of the KIR was significantly higher in elite controllers of disease.

A variety of methods have been employed to date to determine high-resolution KIR allele sequences [18], including full gene amplicon Illumina-based sequencing [19], probe capture Illumina-based sequencing [20], probe capture Pacific Biosciences-based sequencing [21], and CRISPR Cas9 enrichment Oxford Nanopore Technology [22]. The Oxford Nanopore Technologies (ONT) sequencing method uses a protein nanopore to produce fluctuations to an electric current as a fragment of DNA (or RNA) passes through it. The nanopores are engineered onto a flow cell which plugs into a USB powered device linked to a personal computer. Genomic, fragmented, or amplicon DNA is ligated to an adaptor molecule including a motor protein, which controls the translocation of the DNA strand through the nanopore. As the strand passes through the nanopore, the current is measured several thousand times per second. Changes in the voltage and raw signalling data are acquired by the MinKNOW software in real time and are subsequently processed using multiple bioinformatic tools to base call, demultiplex, and quality trim and stored in POD5 file format. We describe here a full gene PCR-based long read sequencing method using the ONT platform to sequence all KIR genes with the exception of KIR3DP1. We have generated allele-level genotyping results, defined as sequencing of the coding DNA regions.

2 | Materials and Method

2.1 | Sample Selection and DNA Extraction

DNA extracted from 48 EBV transformed 10th International Histocompatibility Workshop (IHWS) cell lines with known KIR allele genotyping results was selected based on the presence of material in our laboratory. The cell lines were from European ($n = 31$), Asian ($n = 5$), Undefined—Jewish ($n = 2$), African ($n = 1$) and Greater Middle Eastern ($n = 1$) ancestry,

TABLE 1 | Amplification primer sequences.

Mixture name	Primer name	5′–3′ sequence	KIR loci
MIX 1	UNI F	GCCAAATAACATCCTGTGCGCTGCTCAGCT	2DL1, 2DL2, 2DL3, 2DL5A, 2DL5B, 3DL1, 3DL2, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1, 2DP1
	IN HOUSE F	ACGGCTGCCTGTCTGCACAGACAGCACC	
	UNI R	TTGGAGAGGTGGGCAGGGGTCAAGTG	
MIX 2	KIR non2DL5 FOR	GTGTATGAGAGGTTGGATCTGAGACRTSTTT	2DL1, 2DL2, 2DL3, 3DL1, 3DL2, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DS1, 2DP1
	KIR 2DS4-5UTR FOR	GTGTTTGGGAGGTTGGATCTAAGACATGTTT	
	KIR 2DL1-5UTR FOR	GTGTTCGGGAGGTTGGATCTCAGACGTGTTT	
	KIR 2DS3_5UTR FOR	GTGTTCCGGAGGTTGGATCTGAGACGTGTTT	
	KIR non2DL5_3 REV	CTCYTGGYCAGYAGACAACTTTGGATCTGGYC	
	KIR2DS3_5_3UTR REV	CTCGTGGACAGAAGACAGCTTTGGATCTGGAC	
	KIR2DP1_3UTR REV	TCATGGGCAGGAGACAACTTTGGATCTGGAC	
MIX 3	KIR non2DL5 FOR	GTGTATGAGAGGTTGGATCTGAGACRTSTTT	2DL2, 2DL3
	KIR 2DL3-3UTR_REV	CTCATGGGCAGGAGACAACTTTGGATCAGGGC	
MIX 4	2DL4 F	CACATCGTCTGCACCGGTCAGTCGAGCCGA	2DL4, 3DL3
	3DL3 F	CTCACAACATCCTGTGTGCTGCTGAACTGA	
	UNI R	TTGGAGAGGTGGGCAGGGGTCAAGTG	

with eight unknown. All DNA samples were extracted in-house using the DNA Midi Kit (Qiagen, Germany) on the QIAsymphony SP instrument according to the vendor's protocol. The concentration and purity of extracted DNA were assessed by the optical density (OD) 260/280 ratio of 1.8–2.0 using the NanoDrop spectrophotometer (Thermo Scientific). Samples were then normalised to a concentration of 25 ng/μL. KIR allele genotypes were obtained from the literature for reference [19, 20]. The presence of *KIR2DP1*005* in the reference results was converted to *KIR2DP1*00201* due to the renaming of this allele [5].

In addition, DNA from 176 Western Australian subjects that were part of a longitudinal population health study [23] and had been previously tested for KIR gene (except KIR2DP1 and KIR3DP1) content by an in-house PCR-SSP method [24] was selected at random where material was available. DNA had been previously extracted from whole blood using the DNA Mini Kit (Qiagen, Germany) on the QIAsymphony SP instrument according to the vendor's protocol. Samples were normalised to a concentration of 25 ng/μL.

2.2 | KIR Gene PCR and Primers

Primers specific for sites in the 5′ and 3′ untranslated regions in all KIR genes except KIR3DP1 were modified from a previously published method [19]. Nine forward primers and five reverse primers were used to amplify the whole gene region using four separate PCR mixtures. One mixture (MIX 1) amplifies all genes except KIR2DL4, KIR3DL3 and KIR3DP1. A separate mixture (MIX 2) amplifies all genes except KIR2DL5, KIR2DL4, KIR3DL3 and KIR3DP1. To increase the relative amount of sequencing target, a separate mixture (MIX 3) amplifies KIR2DL2 and KIR2DL3. A final mixture (MIX 4) amplifies KIR2DL4 and KIR3DL3. Table 1 shows the 5′ to 3′ nucleotide sequences of the primers. Table S1 shows the gDNA nucleotide start and end position and the approximate amplicon length for each locus. IPD-KIR release 2.13.0 was used to determine whether any polymorphic positions occur at the primer binding site.

For each PCR mixture, 4 μL of genomic DNA was mixed with 0.2 μM final concentration primer, 2.5 U of PrimeSTAR GXL DNA Polymerase (Scientifix, Clayton, Australia Cat# R044A), 10 μL 5× PrimeSTAR GXL buffer (Scientifix), 4 μL of 2.5 μM dNTP (Scientifix) and 28 μL molecular grade water (G-Biosciences, St. Louis, USA).

The mixtures were subjected to PCR as follows: an initial denaturation step of 2 min at 94°C was followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of primer annealing at 62°C and 12 min of extension at 68°C, and finally 10 min at 72°C. Cycling was carried out in an Eppendorf Mastercycler ×50s thermal cycler.

Amplicons were combined into a single pool for each sample in a ratio as follows—MIX 1: 5 μL; MIX 2: 20 μL; MIX 3: 15 μL; MIX 4: 5 μL. Amplicon pools were cleaned using 0.4× Agencourt AMPure beads (Beckman Coulter) on an automated liquid handler Microlab STAR line (Hamilton) and eluted in 35 μL of molecular grade water. Following cleanup, the amplicons were quantified using the Qubit high sensitivity

fluorometer (Thermo Fisher) and visualised on a 1% agarose gel to ensure the expected amplicon size had been achieved. Amplicon pools were then normalised to 20 ng/μL for library preparation.

2.3 | Library Preparation

Amplicons from the 48 IHWS DNA were sequenced in two separate libraries. Library preparation for ONT nanopore sequencing was performed following the recommended workflow for ligation sequencing, SQK-NBD114-96 chemistry. Each 250 ng amplicon mixture was subjected to an end-repair/dA-tailing step to repair blunt ends and add an 'Adenine' base to the 3′ end of the amplicon (New England Biolabs). A volume of 3.5 μL of the end-repaired amplicons was then barcoded using native barcodes (Oxford Nanopore) and pooled into a single mixture. The pool was cleaned using 0.4× Agencourt AMPure beads (Beckman Coulter) and eluted into 35 μL of molecular grade water. The pooled library was then subjected to adapter and motor protein ligation onto the prepared ends using NEB ligase enzyme (New England Biolabs). A final 0.4× Agencourt AMPure bead clean-up step was performed and washed with proprietary Long Fragment Buffer (LFB) and eluted in elution buffer (EB), both of which were provided in the kit. The final library was quantified using the Qubit high sensitivity fluorometer (Thermo Fisher) and diluted to 5 ng/μL. The final pooled libraries were loaded onto R10.4.1 flow cells as per recommendation from ONT. Sequencing data was collected as POD5 files for 48 h using the default running script for SQK-NBD119-96 chemistry by the MinKNOW data acquisition software (version 23.11.7). The Australian population samples were sequenced in 11 separate library pools using the same method as described above.

2.4 | Sequencing Data Quality Analysis

Quality metrics were obtained from the ONT run QC file using Nanoplot [25] in the Galaxy online bioinformatics environment [26].

The quality of raw reads in FASTQ format was assessed using FASTQC v.0.12.1 and reports compiled using MultiQC v.1.12. Sequencing accuracy of raw reads was estimated by comparing raw reads from 33 IHWS samples known to have *KIR2DL3*0010101*, chosen for its high frequency in the validation samples, to three separate full genomic reference sequences. Reference FASTA nucleic acid sequences for the human genome GRCh38 full analysis set (GCA_000001405.15) that combines the human decoy sequences from hs38d1 (GCA_000786075.2) with the GRCh38 'no alt' analysis set were downloaded from the NCBI. All chromosome 19 and alternatives were extracted from the reference genome to create a smaller reference (chr19_hs38dh.fasta) for this analysis. Reference FASTA nucleic acid sequences for the KIR genes (KIR_gen.fasta) were downloaded from the IPD-KIR Database [5]. Sequences corresponding to the *KIR2DL3*0010101* allele of the KIR2DL3 locus were extracted from the KIR gene reference into KIR2DL3_0010101.fasta. All sequences were indexed with SAMtools v.1.21 running htslib v.1.21 [27], makeblastdb v.2.16.0+ and LASTAL v.1542 [28] in preparation for mapping and alignment. Raw reads in FASTQ format from 33 samples were mapped into the reference sequences using minimap v.2.27-r1193 [29]. The three references used were: chr19_hs38dh.fasta—the chromosome 19 and its alternatives that contain KIR genes; KIR_gen.fasta—KIR gene reference sequences and KIR2DL3_0010101.fasta—*KIR2DL3*0010101* allele. The resulting alignments were converted into Binary Alignment Map (BAM) format, sorted and indexed using SAMtools. Read coverage, read depth and consensus statistics from the BAM files were calculated using SAMtools and BEDtools [30].

Reads were retained for further analysis if at least 80% of their sequence length aligned to the *KIR2DL3*0010101* reference allele, reflecting diagnostic-grade mapping confidence. These high-confidence reads were extracted from the BAM files and converted into FASTQ format using BEDtools, then subsequently converted into FASTA format using seqret, part of EMBOSS v6.6.0.0 [31]. Accuracy was determined by aligning the extracted FASTA reads mapped to target regions onto the reference sequence of the *KIR2DL3*0010101* allele using nucleotide–nucleotide BLAST v.2.12.0+ and LASTAL. All bioinformatics tools and packages were managed using conda v.25.3.1 and docker v.27.2.0.

2.5 | KIR Allele Calling

Raw POD5 files collected during sequencing were converted to FASTQ files using Dorado base-caller (v.7.2.13). The FASTQ read data were further filtered by a minimum length of 2 kb and a minimum Q score of 7, which corresponds to an estimated raw sequence accuracy of 80%, using NanoFilt (https://github.com/wdecoster/nanofilt). FASTQ files were analysed using NGSEngine v.3.2.0 (GenDX, Utrecht, The Netherlands) to determine which KIR alleles were present. Sequences were mapped against version 2.13.0 of KIR reference libraries and variants were called by NGSEngine accordingly. Intron polymorphisms were not analysed due to the complexity of intronic sequences with multiple indel and homopolymer positions. Thus, the genotypes generated were high resolution, including synonymous DNA substitutions within the coding region.

3 | Results

3.1 | Amplification of 10th IHWS DNA Samples

A total of 192 amplicons were generated from the 48 10th IHWS DNA samples. Amplicons were visualised after electrophoresis on a 1% agarose gel. Figure 1 shows a gel image of the four different amplicons from one DNA sample. Strong amplification bands can be seen with a size of ~12 kb in all four reaction mixtures.

Pooled cleaned amplicons were quantitated using the Qubit high sensitivity quantitation assay. The average pooled amplicon

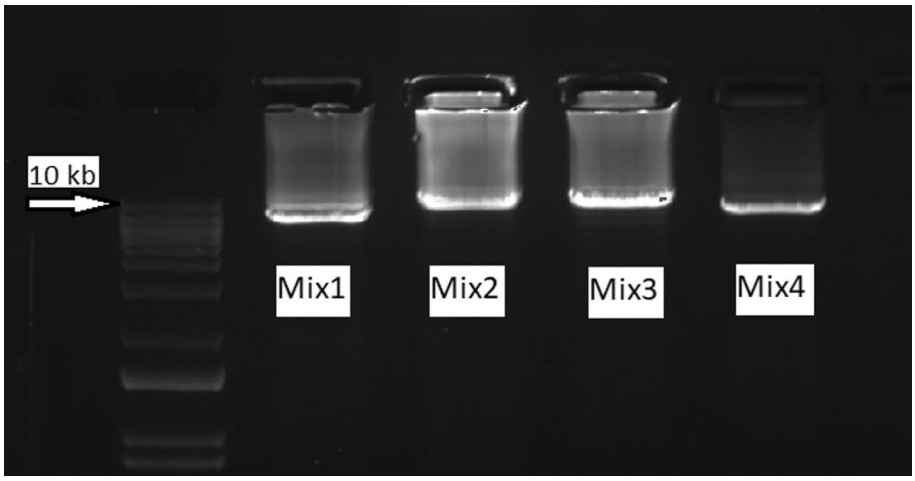

FIGURE 1 | Agarose Gel image of amplicons resulting from four KIR PCR mixtures.

TABLE 2 | Run quality metrics from NanoPlot.

Quality metric	Library 1	Library 2
Mean read length	10,276.1	9906.7
Mean read quality	16.1	15.8
Median read length	12,403	11,997
Median read quality	21.0	21.0
Number of reads	653,150	476,142
Read length N50	12,739	12,741
Total bases	6,711,844,023	4,716,994,757

DNA concentration was 40.72 ng/μL (range 6.86–87.6). A concentration of 20 ng/μL was required to be able to add 250 ng of amplicon to the library preparation stage. A total of 13 out of the 192 amplicons were below this amount, and these were used for library preparation with < 250 ng of input DNA.

3.2 | Library Preparation and Sequencing of 10th IHWS DNA Samples

The concentration of the two final libraries was 25.6 and 22.4 ng/μL, respectively. After sequencing for 48 h, the sequencing summary file was examined using Nanoplot on UseGalaxy.org.

Quality metrics were extracted from the ONT run QC file (Table 2). A total of 653,150 and 476,142 reads were obtained for each library, respectively, comprising of more than 11.4 billion bases. The mean read quality was 16.1 and 15.8, and the median read quality was 21 for both libraries; median read length was 12,403 and 11,997 bp, and the N50 read length was 12,739 and 12,741 bp. The mean percentage of reads with a quality score greater than Q5, Q7, Q10 and Q15 was 99.1, 96.3, 83.4 and 31.8, respectively. Figure 2 shows a histogram of read lengths showing the majority of reads around 10 kb.

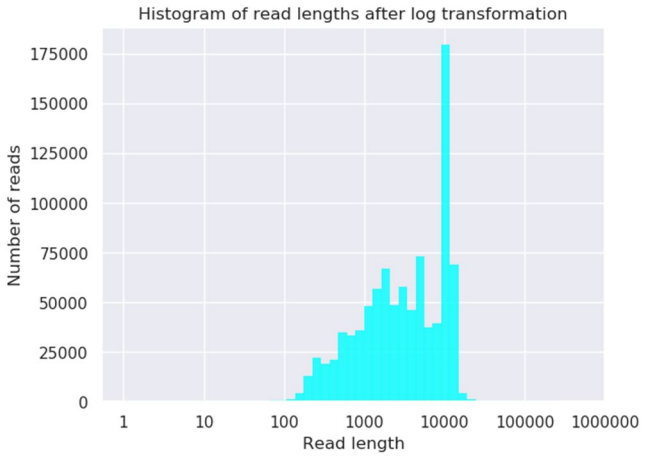

FIGURE 2 | Histogram of sequencing read lengths after log transformation.

3.3 | Alignment of ONT Reads Against *KIR2DL3*0010101* Reference Sequence, Raw Read Accuracy and Sequencing Error Rate

Datasets from 33 samples carrying the *KIR2DL3*0010101* allele, chosen for its relatively high frequency in the validation samples, were mapped to a reference sequence. The average raw-read accuracy for the 33 samples assessed against the *KIR2DL3*0010101* reference was 98.54% (LASTAL) and 98.43% (BLAST). The average consensus error rate compared with the KIR2DL3 reference was 0.015. The consensus error rate increased to 0.026 against chr19_hs38dh and 0.019 against KIR_gen references.

3.4 | KIR Allele Calling Using NGSEngine

Analysis performed using GenDX NGSEngine identified a total of 732 separate KIR alleles present in 480 genes detected in the 48 10th IHWS samples. Reference genotype data were available for all 48 samples. ONT sequencing detected 732 alleles compared with 736 in the published results. The extra alleles were detected in two cases of KIR2DL4 (E4181324 and

TABLE 3 | Number of reads and % mappability per sample as determined by NGSEngine.

Sample	*N* reads	Mappability
31227ABO	9650	98
AMIA	6139	98
BM9	7654	99
BM14	8100	99
BM15	8521	98
BM21	7185	97
BOB	7566	97
BOLETH	17,584	98
BTB	11,365	97
CB6B	4158	99
CALOGERO	6459	98
EJ23B	14,539	97
DEU	12,582	98
DBB	7922	98
E4181324	11,060	64
EJ23B	12,346	74
HO104	3258	96
HOR	6531	98
JBUSH	7702	99
JO528239	10,889	97
JVM	4643	98
KAS011	7700	97
KAS116	8964	98
SAVC	10,299	97
LBUF	7215	99
LUY	5466	98
MADURA	7066	98
MOU	12,601	99
MT14B	8492	99
PF4015	5656	98
PITOUT	9582	99
PLH	7261	99
RSH	20,367	98
RML	18,804	85
SA	13,425	98
SCHU	10,947	98
SLE005	12,516	98
SPO010	8746	98
SWEIG007	7687	99
T7526	2650	89
T7527	23,046	70
TAB	12,573	98
TEM	7151	98
WJR076	6066	98
WT8	7348	98
WT51	7540	99
WTBIS100	11,022	98
YAR	9451	98

WJR076) and two cases of KIR2DL5 (CB6B and WT51) where a third allele was reported in each case. Alleles of the framework genes KIR3DL3, KIR2DL4 and KIR3DL2 were detected in all 48 samples. The next most frequently detected locus was KIR2DL3, which was detected in 42 of the samples. The least frequent loci were KIR2DS3 and KIR2DS5, which were both present in only 12 of the 48 samples.

The number of reads and % mappability for each of the 10th IHWS samples is shown in Table 3. The average number of reads and % mappability was 8917 and 97.1%, respectively. For each locus, the total number detected, average number of reads, average read length, average mappability and average median read depth are shown in Table 4. The framework genes (2DL4, 3DL2 and 3DL3) were detected in every sample. The average number of reads varied by locus, with the lowest being KIR2DL5 at 186 and the highest being KIR2DP1 at 2791. The highest average median read depth was seen in KIR2DP1 at 2334. The highest average % mappability was seen in KIR2DL4 at 97%, whereas the lowest mappability was seen with KIR2DS4 at 60%, which also had the lowest average median read depth. The variation in average read numbers and median read depth between the different loci is due to an imbalance in either the relative amount of available target in the amplicon or sequencing efficiency. This is partially mitigated by the amplicon pooling step at a ratio designed to reduce the relative amount of KIR2DL5, KIR2DL4 and KIR3DL3 (which are overrepresented in the pre-pooled amplicons) in the final library preparation. The most likely reason for the over-representation of KIR2DL5 reads is the smaller amplicon size compared with other loci, being ~9235 bp compared with typically 10–16 kb for the other loci, which may be preferentially amplified during PCR. However, multiple copy number could also contribute to the relatively high number of reads seen. Low mappability may be due to reads not being able to be matched to a single locus and therefore being discarded by NGSEngine. This can happen when a high degree of similarity occurs between two or more loci, and reads closely match more than one locus. This can, in turn, lead to low read depth as reads are discarded from the analysis.

TABLE 4 | Average of number of reads, read length, mappability (%) and median read depth by KIR locus.

Locus	*N* detected	Average no. of reads	Average read length	Average mappability (%)	Average median read depth
KIR2DL1	44	564	12,383	79	404
KIR2DL2	18	687	13,073	88	573
KIR2DL3	43	1132	12,823	83	860
KIR2DL4	46	678	10,130	97	637
KIR2DL5	19	186	8702	88	159
KIR2DP1	44	2791	11,192	87	2334
KIR2DS1	15	343	12,775	83	264
KIR2DS2	19	403	12,365	83	320
KIR2DS3	12	282	12,170	76	180
KIR2DS4	43	202	12,108	60	98
KIR2DS5	12	261	12,046	71	168
KIR3DL1	43	1273	12,722	84	1016
KIR3DL2	46	894	14,901	88	733
KIR3DL3	46	513	11,031	91	452
KIR3DS1	17	351	12,961	86	281

Initial allele calls made by NGSEngine were concordant with those published [19, 20], in 695 (94.9%) out of the 732 alleles present in the 48 samples. Concordance was measured against the highest resolution provided by the published reference results, in 48% of cases at first field (allele group) and in 52% of cases at second field (synonymous substitution). Table 5 shows the KIR genotypes for the IHWS DNA samples along with reference genotypes and a summary of genotyping concordance for each gene. A total of 18 out of 48 genotypes were fully concordant for all KIR loci, according to the reference record (31227ABO, BTB, CALOGERO, DEU, EJ23B, JVM, LBF, MT14B, PITOUT, RSH, SA, SLE005, SWEIG007, T7527, WT100BIS, WT8, WT47 and YAR). The remaining samples showed results inconsistent with the published genotypes in a range of one to three separate loci. Observed results differed from expected results in the following genes: four results for KIR2DP1, ten results for KIR3DL3, five results for KIR2DL1 and KIR2DL4, two results for KIR3DS1 and KIR2DL5A and one result each for KIR2DS2, KIR2DL2, KIR2DS1, KIR2DS3 and KIR3DL1. No inconsistencies were identified in KIR2DL3, KIR2DL5B, KIR2DS5 and KIR3DL2 (Table 6). Eight KIR3DL3 results were inconsistent with expected results (AMAI, BOB, BOLETH, HO104, JO528239, KAS116, LUY and SCHU) due to phasing differences in the alleles called in the reference article compared with the ONT result obtained here. For example, in BOLETH, the reference result was listed as *KIR3DL3*00901*, **00602*; however, the result obtained using the ONT method was *KIR3DL3*00101*, **01501*. These genotypes differ in the phasing of a heterozygous position in exon 6, genomic DNA position 10725. At this position, *KIR3DL3*00901* has C, whereas *KIR3DL3*00602* has A; this heterozygous position is some 5558 bp distant from the nearest heterozygous position in exon 5. With short read sequencing, there is no possibility of phasing the two heterozygous positions; therefore, a genotype ambiguity exists. When this sample was confirmed using short read technology, the result obtained contained the allele combination reported in the reference article as well as the allele combination obtained with long read chemistry (Table 6). With ONT sequencing, all heterozygous positions in the two alleles are phased, and it is therefore definitive that the A at 10725 in exon 6 is present on the *KIR3DL3*00101* allele and the C is present on the *KIR3DL3*01501* allele, as shown in Figure 3. This highlights the benefit of long read ONT sequencing used in this study to resolve phasing polymorphism over long stretches of sequence. Concordantly phased genotypes of KIR3DL3 in two of the cells (HO104 and JO528239) obtained by this ONT sequencing method were also cited in an investigation by Bultitude, which sequenced KIR genes utilising the Pacific Biosciences long read platform [32]. Adding the eight phasing inconsistencies to the total of consistent observed/reference genotypes gives an accuracy of 96% for the ONT method.

A further 29 inconsistencies with the published genotype involved nucleotide differences matching alternative alleles; for example, in BOLETH, KIR2DP1, the previously published result was *KIR2DP1*00204*, **00201*, whereas the result obtained using ONT was **00204*, **00301*. *KIR2DP1*00201* and **00301* differ at two nucleotide positions: gDNA 22 A>G and 3683 C>G. The G at position 22 was detected at 56%, whereas the G at 3683 was detected at 47%, both being heterozygous positions, as shown in Figure 4. To determine whether the G base calls were errors produced by ONT sequencing, the amplicon was tested using our in-house Ion Torrent NGS workflow [33]. The bases at both positions were confirmed by Ion Torrent to be those detected by ONT, with the G at 22 detected at 57% and the G at 3683 detected at 55%. Although this does not rule out PCR-introduced errors, it does rule out ONT sequencing errors.

TABLE 5 | ONT sequencing based KIR genotypes for 48 tenth international histocompatibility workshop DNA samples.

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
31227ABO	*00101	*001	*001	*001	*002	3*00103	*00102	*00302	*00102		*002	*013	*00501	3*002		*002	*00101	*00201
IHW9061 REF	*00301						*002	*00401	*00501									*00701
31227ABO	*00101	*00101	*00101	*00101	*00201	3*00103	*00102	*00302	*00102		*00201	*01301	*00501	3*00201		*00201	*00101	*00201
ONT	*00301						*00201	*00401	*00501									*00701
AMAI IHW9010	*01309			*002			*003	*001	*00801		*001						*003	*00101
REF	*041			*005			*013	*01202	*00801		*001						*003	*00101
AMAI	*02702			*00201			*00301	*01202	*00801		*00101						*00301	*00101
ONT	*00101			*00501			*013	*00201	*00801									
BM09 IHW9068	*00207			*001			*002	*00302	*00102		*002						*00101	*00201
REF	*044			*001			*002	*00302	*00802		*00401						*004	*00501
BM09	*00207			*00101			*00201	*00302	*00102		*00201						*00101	*00201
ONT	*044								*00802		*00401						*00601	*00501
BM14 IHW9033	*00201			*001			*00203	*00302	*00102		*00501						*00101	*00103
REF	*00901			*001			*00201	*00302	*011		*01502						*010	*00201
BM14	*00201			*00101			*00203	*00302	*00102		*00501						*00101	*00103
ONT	*00901						*00201		*01101		*01502						*010	*00201
BM15 IHW9040	*00201			*001			*00203	*00302	*00501		*00401	*013	A*001		*002	*002	*006	*00301
REF	*00206			*001			*00201	*00302	*00802									*00701
BM15	*00201			*00101			*00203	*00302	*00501		*00402	*01301	A*00101		*00201	*00201	*00601	*00301
ONT	*00206						*00201		*00802									*0070101
BM21 IHW9043	*00902	*001	*001	*001	B*002	5*015	*00101	*00302	*011		*00501	*013	A*001	3*00103	*002		*010	*010
REF	*00301						*002	*00401	*00501									*00701
BM21	*00902	*00101	*00101	*00101	B*00201	5*00201	*00101	*00302	*01101		*00501	*01301	A*00101	3*00103	*00201		*010	*01001
ONT	*00301						*00201	*00401	*00501									*00701
BOB IHW9089[†]	*00101	*00101	*00301	+			*00301	*00302	*001		*002	*013	A*0010101	5*00201		*00201	*001	*00201
REF	*01303								*005									*00701
BOB	*00101	*00101	*00301	*00201			*00301	*00201	*00102		*00201	*01301	A*001010	5*00201		*00201	*00101	*00201

(Continues)

TABLE 5 | (Continued)

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
ONT	*01402								*00501									*00701
BOLETH	*00901			*001			*00204	*00302	*00802		*00401	*013	A*001		*002	*002	*006	*00501
IHW09031 REF	*00602			*002			*002	*001	*00501									*00701
BOLETH	*00101			*00101			*00204	*00302	*00802		*00401	*01301	A*00101		*00201	*00201	*00601	*00501
ONT	*01501			*00201			*00301	*00201	*00501									*00701
BTB IHW09067	*00206			*001			*00203	*00302	*00802		*00401						*006	*00301
REF	*00902			*030			*002	*00302	*011		*00501						*010	*010
BTB	*00206			*00101			*00201	*00302	*00802		*00401						*00601	*00301
ONT	*00902			*030			*00203		*01101		*00501						*010	*01001
CALOGERO IHW9084	*00207			*001			*002	*00302	*00801		*001						*003	*00101
REF	*017			*001			*002	*00302	*00802		*00401						*006	*076
CALOGERO	*0020701			*00110			*00201	*00302	*00801		*00101						*00301	*00101
ONT	*01001			*00101			*00201		*00802		*00401						*00601	*00501
CB6B IHW9060	*00301	*001	*001		B*002	3*00103	*012	*00401	*00501			*013	A*001	3*002	*002	*002		*00701
REF	*01403	*001	*003						*00501			*013	A*005			*002		*00701
CB6B	*00301	*00104	*00101		B*00201	3*00103	*00101	*00401	*00501			*01301	A*00101	3*00103	*002	*00201		*00701
ONT	*01403	*00101	*00301										—					
DBB IHW09052	*020			*001			*002	*00302	*00801		*001						*003	*00101
REF	*00801	*001	*001		B*002	3*00103	*00102	*00401	*00802		*00401						*006	*00301
DBB	*020	*00101	*00101	*00101	B*00201	3*00103	*00102	*00302	*00801		*00101						*00301	*00101
ONT	*00801						*00201	*00401	*00802		*00401						*00601	*00301
DEU IHW9025	*00101	*001	*001	*002			*003	*002	*00801		*001						*003	*010
REF	*01402								*011		*00501						*010	*011
DEU	*00101	*00101	*00101	*00201			*00301	*00201	*00801		*00101						*00301	*01001
ONT	*01402								*01101		*00501						*010	*01101
E4181324	*00206			*001			*00203	*00302	*00103	*00501	*002	*013					*00101	*00103

(Continues)

TABLE 5 | (Continued)

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
IHW09011 REF	*01501			*001			*00204	*00302	*00802		*00401						*006	*00501
E4181324	*00206			*00101			*00203	*00302	*00102	—	*00201	*01301					*00101	*00103
ONT	*01501						*00204		*00802		*00401						*00601	*00501
EJ23B IHW09085	*00206			*001			*00203	*00302	*011		*00501						*010	*00103
REF	*00901			*001			*00204	*00302	*00501			*013	A*001		*002	*002		*00701
EJ23B	*00206			*00101			*00203	*00302	*01101		*00501				*00201	*00201	*010	*00103
ONT	*00901						*00204	*00302	*00501			*01301	A*00101					*00701
HO104 IHW09082	*00902			*001			*002	*00302	*00102		*01502						*00101	*00201
REF	*01402	*001	*001		B*002	3*00103	*00102	*00401	*00501			*013	A*001		*002	*002		*00701
HO104	*0301	*00101	*00101	*00101	B*00201	3*00103	*00201	*00302	*00102		*01502		A*00101		*00201	*006	*00101	*00201
ONT	*00209						*00102	*00401	*00501			*01301						*00701
HOR IHW09053	*048			*002			*003	*002	*00501			*013	A*001		*002	*002		*00701
REF	*00102			*002			*003	*002	*00501			*013	A*001		*002	*002		*021
HOR	*04801			*00201			*00301	*00201	*00501			*01301	A*00101		*00201	*00201		*00701
ONT	*04802						*00301											*021
JBUSH IHW09035	*01102			*001			*003	*00302	*00102		*002						*00101	*00201
REF	*01302			*002			*002	*002	*00103		*008						*003	*00901
JBUSH	*01102			*00101			*00201	*00302	*00102		*00201						*00101	*00201
ONT	*01302			*00201			*00301	*00201	*00103		*00801						*00301	*00901
J0528239 IHW9041	*00901	*001	*001	*001	B*002	3*00103	*00102	*032N	*00801		*001		A*005	3*002		*002	*003	*00101
REF	*01601						*002	*00401	*00501									*00701
J0528239	*01501	*02101	*00101	*00101	B*00201	3*00103	*00102	*03201N	*00501		*00101		A*00501	3*00201		*00201	*00301	*00101
ONT	*00301						*00201	*00401	*00801									*00701
JVM IHW9039	*007	*001	*003	*001			*002	*00302	*00103		*001						*003	*00101
REF	*00801								*00801		*008							*00901
JVM	*00701	*00101	*00301	*00101			*00201	*00302	*00103		*00101						*00301	*00101
ONT	*00801								*00801		*00801							*00901

(Continues)

TABLE 5 | (Continued)

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
KAS011 IHW9009	*00901			*001			*00203	*002	*00103		*008	*013	A*001	5*002		*002	*003	*00701
REF	*01302			*002			*002	*00302	*00501									*00902
KAS011	*00901			*00101			*00203	*00201	*00103		*00801	*04901N	A*00101	5*00201		*00201	*00301	*00701
ONT	*01302			*00201			*00301	*00301	*00501									*00901
KAS116 IHW09003	*01302			*001			*002	*00302	*011		*00501						*010	*00103
REF	*01501			*001			*002	*00302	*011		*00501						*010	*010
KAS116	*00202			*00101			*00201	*00302	*01101		*00501						*010	*00103
ONT	*00602			*00101														*01001
LBF IHW09096	*00901			*001			*00203	*00302	*00102		*002						*00101	*00201
REF	*00301	*001	*003						*011		*00501						*010	*00103
LBF	*00901	*00101	*00301	*00101			*00203	*00302	*00102		*00201						*00101	*00201
ONT	*00301								*01101		*00501						*010	*00103
LUY IHW9070	*00101			*001			*002	*00302	*00801		*00401						*006	*00501
REF	*02701			*005			*016	*00302	*011		*00501						*010	*00103
LUY	*01302			*00101			*00201	*00101	*00801		*00401						*00601	*00501
ONT	*04101			*00501			*016	*00302	*01101		*00501						*010	*00103
MADURA IHW9069	*00301	*001	*003			3*002			*00103		*002						*00101	*00201
REF	*007	*001	*003		A*021				*00501			*013				*002		*00701
MADURA	*00301	*00101	*00301		A*02101	3*00201			*00102		*00201	*01301				*00201	*00101	*00201
ONT	*00701	*00101	*00301						*00501		*00201					*00201		*00701
MOU IHW9050	*00801			*001			*002	*00302	*00801		*001						*003	*01101
REF	*00207			*001			*002	*00302	*00801		*00401						*006	*01004
MOU	*00801			*00101			*00201	*00302	*00801		*00101						*00301	*01101
ONT	*00207			*00101			*00201	*00302	*00801		*00401						*00601	*01004
MT14B IHW9050	*00206			*001			*00203	*00302	*00103		*020						*00101	*00902
REF	*00206			*001			*00203	*00302	*00103		*020						*00101	*00902
MT14B	*00206			*00101			*00203	*00302	*00103		*02001						*00101	*00902

(Continues)

TABLE 5 | (Continued)

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
ONT	*00206			*00101			*00203	*00302	*00103		*02001						*00101	*00902
PF04015 IHW9088	*01402	*001	*003						*011		*00501						*010	*00103
REF	*01402	*001	*003						*011		*00501						*010	*00103
PF04015	*01402	*00101	*00101						*01101		*00501						*010	*0010301
ONT	*01402	*00101	*00101						*01101		*00501						*010	*0010301
PITOUT IHW09051	*017			*002			*003	*002	*00802		*00402						*006	*00301
REF	*01402	*001	*001						*011		*00501						*010	*00103
PITOUT	*01701	*00101	*00101	*00201			*00301	*00201	*00802		*00402						*00601	*00301
ONT	*01402								*01101		*00501						*010	*00103
PLH IHW9047	*00202			*001			*002	*00302	*00801		*001						*003	*00101
REF	*00901			*001			*002	*00302	*00103		*008						*003	*00901
PLH	*00202			*00101			*00201	*00302	*00103		*00101						*00301	*00101
ONT	*00901			*00101			*00201	*00302	*00801		*00801						*00301	*00901
RSH IHW9021	*00901			*001			*002	*00302	*011		*00501						*010	*010
REF	*00402	*001	*001		B*004		*009	*01201	*00103		*01701			5*006			*00101	*023
RSH	*00901	*00101	*00101	*00101	B*004		*00201	*00302	*00103		*00501			5*00601			*010	*01001
ONT	*00402						*009	*01201	*01101		*01701						*00101	*023
RML IHW09016	*00902			*001			*002	*00302	*00103		*01502						*00101	*00201
REF	*00402	*001	*003						*00501			*013	A*001		*002	*002		*00701
RML	*00902	*00101	*00301	*00101			*00201	*00302	*00102		*01502	*01301	A*00101		*00201	*00201	*00101	*00201
ONT	*00402								*00501									*00701
SA IHW9001	*00802			*001			*002	*00302	*00102		*01502						*00101	*00201
REF	*00901			*001			*002	*00302	*00102		*01502						*00101	*00201
SA	*00802			*00101			*00201	*00302	*00102		*01502						*00101	*00201
ONT	*00901			*00101			*00201	*00302	*00102		*01502						*00101	*00201
SAVC† IHW09034	*00801			*00101			*008	*0030201	*00102		*00401						*00601	*00202
REF	*00202						*002		*00802		*01502							*00301
SAVC	*00801			*00101			*00201	*00302	*00102		*01502						*006	*00201

(Continues)

TABLE 5 | (Continued)

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
ONT	*00202						*008		*00802		*00401						*001	*00301
SCHU IHW9013	*00602			*001			*002	*00302	*011		*00501						*010	*00103
REF	*00901			*001			*002	*00302	*00102		*01502						*00102	*00201
SCHU	*01501			*00101			*00201	*00302	*01101		*00501						*010	*00103
ONT	*00101			*00101			*00201	*00302	*00102		*01502						*00101	*00201
SLE005 IHW9059	*017			*001			*002	*00302	*011		*00501						*010	*00103
REF	*00101			*002			*003	*002	*00102		*01502						*00101	*00201
SLE005	*01701			*00101			*00201	*00302	*00102		*00501						*010	*00103
ONT	*00101			*00201			*00301	*00201	*01101		*01502						*00101	*00201
SP0010 IHW9036	*00206			*001			*00203	*00302	*011		*00501						*010	*00103
REF	*00206			*001			*00203	*00302	*011		*00501						*010	*00103
SPO010	*00206			*00101			*00203	*03701	*01101		*00501						*010	*00103
ONT	*00206			*00101			*00203	*03701	*01101		*00501						*010	*00103
SWEIG IHW9037	*00101			*001			*002	*00302	*00801		*001						*003	*00101
REF	*00101			*002			*003	*002	*00802		*039						*006	*00501
SWEIG007	*00101			*00101			*00201	*00302	*00801		*00101						*00301	*00101
ONT	*00101			*00201			*00301	*00201	*00802		*039						*00601	*00501
T7526 IHW9076	*00901			*001			*002	*00302	*00103		*01502		A*001				*00101	*00201
REF	*00901			*001			*002	*00302	*00501			*013		5*002		*002		*00701
T7526	*00901			*00101			*00201	*00302	*00102		*01502	*01301	A*00101	5*00201		*00201	*00101	*00201
ONT	*00901			*00101			*00201	*00302	*00501									*00701
T7527 IHW09077	*00801			*001			*002	*00302	*00103		*01502						*00101	*00201
REF	*00802	*001	*003						*00501			*013	A*005	3*002		*002		*00701
T7527	*00801	*00101	*00301	*00101			*00201	*00302	*00102		*01502	*01301	A*00501	3*00201		*00201	*00101	*00201
ONT	*00802								*00501									*00701
TAB089 IHW09066	*00802			*001			*019	*00302	*00801		*001						*003	*00101
REF	*01501			*001			*002	*00302	*011		*00501						*010	*010

(Continues)

TABLE 5 | (Continued)

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
TAB089	*00802			*00101			*019	*00302	*00801		*00101						*00301	*00101
ONT	*01503						*00201		*01101		*00501						*010	*01001
TEM IHW9057	*00101			L3*002			*003	*002	*00801		*001						*003	*00101
REF	*00301	*001	*001		B*002	3*008	*012	*00401	*00602		*007						*004	*008
TEM	*00101	*003	*00101	*00201	B*00201	3*008	*00101	*00201	*00602		*00101						*00301	*00101
ONT	*00301						*00301	*00401	*00801		*00701						*00401	*008
WJR076 IHW9012	*00902			*001			*002	*00302	*00102	*011	*002	*013					*00101	*00201
REF	*01404	*001	*001		B*002	3*00103	*00102	*00401	*00501		*00501						*010	*00103
WJR076	*00902	*00101	*00101	*00101	B*00201	3*00103	*00102	*00302	*00102	—	*00201	*0130101					*00101	*00201
ONT	*01404						*00201	*00401	*01101		*00501						*010	*00103
WT100BIS	*00901			*001			*00203	*00302	*00802		*00401						*006	*00501
IHW09006 REF	*00301	*001	*001		B*002	3*00103	*00102	*00401	*011		*00501						*010	*00103
WT100BIS	*00901	*00101	*00101	*00101	B*00201	3*00103	*00203	*00302	*00802		*00401						*00601	*00501
ONT	*00301						*00102	*00401	*01101		*00501						*010	*00103
WT8 IHW9017				*001					*00102		*002						*00101	*002
REF				*002					*006		*007						*004	*008
WT8	*044			*00101			*00201	*00101	*00102		*00201						*00101	*008
ONT	*01302			*00201			*00301	*00101	*00602		*00701						*00401	*00201
WT47 IHW09063	*00301	*001	*001		B*002	3*00103	*00101	*00401	*00501			*013	A*001		*002	*002		*00701
REF	*00301	*001	*001		B*002	3*00103	*00101	*00401	*00501			*013	A*001		*002	*002		*00701
WT47	*00301	*00101	*00101		B*00201	3*00103	*00101	*00401	*00501			*01301	A*00101		*00201	*00201		*00701
ONT																		
WT51 IHW9029	*00103			*002		3*00103	*018	*002	*00501			*013	A*001	5*002		*002		*00701
REF	*036	*001	*001		B*002		*004	*00401	*00501			*013	A*005	3*002		*002		*00701
WT51	*00103	*00101	*00101	*00201		3*00103	*004	*00201	*00501			*04901N	A*00101	5*00201		*00202		*00701
ONT	*036				B*00201		*018	*00401	*00501			*01301		3*00201		*00201		*00701
YAR IHW9026	*044			*001			*002	*00302	*00102		*002						*00101	*00201

(Continues)

TABLE 5 | (Continued)

Cell	3DL3	2DS2	2DL2	2DL3	2DL5B	2DS3/5c	2DP1	2DL1	2DL4	2DL4	3DL1	3DS1	2DL5A	2DS3/5t	2DS5	2DS1	2DS4	3DL2
REF	*00102			*002			*003	*002	*011		*00501						*010	*00103
YAR	*044			*00101			*00201	*00302	*00102		*00201						*010	*00103
ONT	*00102			*00201			*00301	*00201	*01101		*00501						*00101	*00201
Number of inconsistencies per locus	10	1	1	0	0	1	4	5	5		1	2	2	1	0	1	3	0

Note: Reference results (REF) are presented above the ONT result (ONT). All reference genotypes taken from Norman et al. (2017) except '†' taken from Maniangou et al. 2018. Differences between the ONT and REF result are highlighted grey in the ONT result.

In private correspondence with the authors of the reference genotypes, the published genotypes [20] of KIR2DP1 required manual curation, resulting in a higher chance of incorrect calls. This could account for four of the total 44 inconsistencies. In several other instances, only one or two nucleotide differences account for the different allele calls. For example, KIR2DL1 in AMAI, the reference genotype was *KIR2DL1*001*, **01202*, whereas our observed result was *KIR2DL1*00201*, **01202*. The difference between *KIR2DL1*00101* and *KIR2DL1*00201* is a SNP in exon 1, gDNA position 13 of G>T. Our observed sequence showed a 50% balance of G/T at this position, strongly indicating the presence of *KIR2DL1*00201* and not *KIR2DL1*00101*. Furthermore, the detected T was in phase with two other positions spanning almost the whole gene (exon 1, gDNA 3522-C; and exon 6, gDNA 13166-A) both with 50% nucleotide balance. The chance of the observed T at gDNA 13 being due to PCR or sequencing error, with an observed noise level of only around 1% (data not shown) for this sample, we believe is very low.

In the case of SPO010, the reference genotype for KIR2DL1 was *KIR2DL1*00302*, **00302*, whereas the observed was *KIR2DL1*03701*. Cross-referencing the IPD-KIR database entry for *KIR2DL1*03701* shows that the sequence of this allele was derived from SPO010 [34], indicating that our observed result was correct. In a further three examples of inconsistencies, our ONT result was agreed with by results cited by Bultitude [32].

Four of the inconsistencies relate to the inability of NGSEngine to clearly identify more than two alleles per locus. In CB6B, the published references report three distinct alleles at the KIR2DL5 locus (*KIR2DL5A*001*/*A*005*/*B*002*), whereas our method was only able to clearly identify *KIR2DL5A*001*/*B*002*. Similarly, in WT51, the reference shows three distinct alleles at the KIR2DL5 locus (*KIR2DL5A*001*/*A*005*/*B*002*), whereas our method was only able to clearly identify *KIR2DL5A*001*/*B*002*. In both cases, the balance in the two alleles identified by NGSEngine is 66/33 rather than an expected 50/50 for a bi-allele heterozygote. All heterozygous positions are shared between the three alleles, and therefore no indication of a third allele can be seen in NGSEngine. In E4181324, the published reference reported the presence of three distinct alleles at KIR2DL4 (*KIR2DL4*00103*, **00802* and **00501*), whereas our method identified *KIR2DL4*00102* and **00802*. Similarly, in WJR076, the published reference reports *KIR2DL4*00102*, **00501* and **011*, whereas the ONT method only identified *KIR2DL4*00102* and **00501*. Again, in both cases, the balance of the alleles identified is 66/33 instead of the expected 50/50. In addition, unlike the KIR2DL5 inconsistencies described above, mismatches are identified between the observed sequence and the reference for the two alleles identified. When these mismatches are compared with the 'missing alleles', in both cases, the nucleotides fit the reference for these missing alleles at the mismatched positions. This demonstrates that the alleles are present in the sequence reads; however, NGSEngine is not able to correctly identify the alleles to which these reads belong. Only by knowing the alleles expected to be present can this elucidation be performed, and imputing the presence of the third allele is not straightforward or reliable. However, in all four cases of 'failure' of NGSEngine to display a third present allele, the third allele described by the reference publication was identified in a list of ambiguities under the 'Genotype ranking' list presented by NGSEngine. Together

TABLE 6 | Details of discordance between published reference genotypes and those obtained with ONT sequencing.

Sample	KIR	Reference result	ONT result	gDNA position	Nucleotide difference	Ion Torrent result	Comment
AMAI	3DL3	*01309, *041	*00101, 07202	10725, 10806, 11443	Two positions in Ex6 and one in Ex8 are phased with Ex3 and 4 positions to give the observed result. Reference result has opposite phasing	Not performed	Not confirmed
AMAI	2DL1	*001, *01202	*00201, *01202	13	gDNA 13G/G>G/T at 50%	Not performed	Observed has a T at pos 13 of Ex1 whereas the reference *00101 has a G. The observed T at this position is phased with a heterozygous position in Ex3
BM09	2DS4	*00101, *004	*00101, *00601	5045, 6729, 6825	gDNA 5045 A>G at 99%; gDNA 6729 T>A at 99%; gDNA 6825 C>A at 99%	Not performed	Observed has 3 positions clearly different to reference and is well phased at other heterozygous positions
BM15	3DL1	*00401	*00402	13601	gDNA 13601 C>T at 84%	Not performed	Observed allele has T which is detected at a depth of 486.
BM21	2DS5	*015	*00201	13671	gDNA 13671 G>A at 89%	Not performed	Observed allele has A which is detected at a depth of 71
BOB	3DL3	*00101, *01303	*00101, *01402	10725 and 10735	A/T and T/C in Ex6 is phased with positions in Ex5; reference genotype has opposite phasing	*00101, *01303 or *00101, *01402	Ion Torrent cannot resolve the phasing ambiguity

(Continues)

TABLE 6 | (Continued)

Sample	KIR	Reference result	ONT result	gDNA position	Nucleotide difference	Ion Torrent result	Comment
BOB	2DL1	*00302	*00201	13, 3522, 3566 and 5345	gDNA 13 G>T; 3522 G>C; 3566 G>A; 5345T>C all above 90%	Not performed	Not confirmed
BOLETH	3DL3	*00901, *00602	*00101, *01501	10725	C/A in Ex6 is phased with positions in Ex5; reference genotype has opposite phasing	*00901, *00602 or *00101, *01501	Ion Torrent cannot resolve the phasing ambiguity
BOLETH	2DP1	*00204, *002	*00204, *00301	22 and 3683	gDNA 22 A/A>A/G at 25%; gDNA 3683 C/C>C/G at 47%	*0204, *00301	gDNA 22 is G at 57% and gDNA 3683 is G at 55% by Ion Torrent confirming the *00301
BOLETH	2DL1	*00302, *001	*00302, *002	13	gDNA 130 G/ G>T/G-T at 37%	*00302, *002	gDNA 13 is T at 56% by Ion Torrent confirming *00201
CB6B	2DP1	*012	*00101	3627	gDNA 3627 G>C at 97%	Not performed	Not confirmed
CB6B	2DL5	A*001, A*005, B*002	A*001, B*002	Only 2 alleles detected. A*005 only differs from B*002 by 1 nt in ex1 gDNA 16. 5A*001 also has A at this position, thus cannot detect A*005 when A*001 and B*002 are present		Not performed	Not confirmed
CB6B	2DS3	*002	*00103	13494	gDNA 13494 A>G 80%	Not performed	Not confirmed
E4181324	2DL4	*00103, *00802, *00501	*00102, *00802	Only 2 alleles detected *00102, *00802 should be A/A at gDNA 1257, but a G is present at 35%. *00501 has a G present at this position. A 3rd allele is thus indicated by the mismatch with the result obtained by ONT		2DL4*00102, *00802	Mismatch at gDNA 1257 at 30% consistent with presence of *00501 also heterozygotes are 30%
HO104	2DS1	*002	*006	13090	gDNA 13090 G>A at 99%	Not performed	Not confirmed

(Continues)

TABLE 6 | (Continued)

Sample	KIR	Reference result	ONT result	gDNA position	Nucleotide difference	Ion Torrent result	Comment
HO104	3DL3	*00902, *01402	*030101, *00209	10725, 10733 and 10725	3 positions in Ex6 phasing opposite with reference genotype	*00902, *01402 or *030101, *00209	Ion Torrent cannot resolve the phasing ambiguity
HOR	3DL3	*00102, *48	*04801, *04802	1615 and 1676	gDNA 1615 C>T at 96% and gDNA 1676 C>T at 97%	Not performed	Not confirmed
J0528239	3DL3	*00901, *01601	*01501, *00301	10725, 10735	2 positions in Ex6 phasing opposite with reference genotype	Not performed	Not confirmed
J0528239	2DS2	*001	*02101	12977	gDNA 12977 C>T at 80%	Not performed	Not confirmed
KAS011	2DP1	*00203, *002	*00203, *00301	22, 3683	gDNA 22 A>G at 50% and gDNA 3683 C>G at 50%	Not performed	Not confirmed
KAS011	3DS1	*013	*04901N	3717, 3718	gDNA 3717 G>A at 95%; 3718 Gdel at 94%	Not performed	Not confirmed
KAS116	3DL3	*01302, *01501	*00202, *00602	10725	C/A in Ex6 is phased with positions in Ex5 phasing opposite with reference genotype	*01302, *01501 or *00202, *00602	Ion Torrent cannot resolve the phasing ambiguity
LUY	3DL3	*00101, *02701	*01302, *04101	10725, 10806, 11443	3 heterozgous positions in Ex6 and Ex8 phasing opposite with reference genotype	Not performed	Not confirmed
LUY	2DL1	*00302	*00101, *00302	3522, 3556, 5345	gDNA 3522 G>C; gDNA 3556 G>A; gDNA 5345 T>C	Not performed	3 heterozygous positions are phased and well balanced

(Continues)

TABLE 6 | (Continued)

Sample	KIR	Reference result	ONT result	gDNA position	Nucleotide difference	Ion Torrent result	Comment
MADURA	2DL4	*00103, *00501	*00102, *00501	10392	gDNA 10392 G>A	Not performed	Observed A is phased with 3 other heterozygous positions
PF04015	2DL2	*003	*00101	5520 and 13952	gDNA 5520 C>T 81% and gDNA A>G 81%	Not performed	Not confirmed
RML	2DL4	*00103, *00501	*00102, *00501	10392	gDNA 10392 G/G>A/G-A is at 44%	*00102, *00501	gDNA 10392 is A by Ion Torrent making the allele *00102 and not *00103
SAVC	2DS4	*00601	*001, *00601	6747–6768	22 nucleotide indel ex4	*001, *00601	22 nucleotide indel present by Ion Torrent sequencing
SCHU	3DL3	*00602, *00901	*00101, *01501	10725	C/A in Ex6 is phased with positions in Ex5 phasing opposite with reference genotype	Not performed	Not confirmed
SCHU	2DP1	*002, *005	*00201, *00201	*005 deleted—renamed to *00201		—	—
SCHU	2DS4	*006, *001	*00102, *010	4812, 5054, 6782	gDNA 4812 G/G>A/G at 46%; gDNA 5054 G/G>G/A at 43% and gDNA 6782 G>A at 99%	Not performed	Not confirmed
SPO0010	2DL1	*00302, *00302	*03701	13150	gDNA 13150 G>A at 87%	Not performed	IMGT database states this cell provided the sequence for KIR2DL1*03701

(Continues)

TABLE 6 | (Continued)

Sample	KIR	Reference result	ONT result	gDNA position	Nucleotide difference	Ion Torrent result	Comment
T7527	2DL4	*00103, *00501	*00102, *00501	10392	gDNA 10392 G/G>A/G, A is 44%	*00102, *00501	gDNA 10392 is A by Ion Torrent making the allele *00102 and not *00103
TAB089	3DL3	*00802, *1501	*00802, *01503	10733	gDNA 10733 T/C>T/T-T is at 96%	Not performed	Not confirmed
TEM	2DP1	*003, *012	*00101, *00301	3627	gDNA 3627 G>C at 97%	Not performed	Not confirmed
WJR076	2DL4	*00102, *00501, *011	*00102, *00501	Only two alleles detected; gDNA 9571 and 9620 show heterozygosity or mismatch depending on whether *00501 or *011 is selected, this indicates presence of third allele.		Not performed	Not confirmed
WT51	3DS1	*013	*04901N, *01301	3717, 3718	gDNA 3717 G/G>G/A at 49%; 3718 G/G G/del at 51%	Not performed	Not confirmed
WT51	2DL5	A*001, A*005, B*002	A*001, B*002	Only 2 alleles detected. A*005 only differs from B*002 by 1 nt in ex1 gDNA 16. 5A*001 also has A at this position, thus cannot detect A*005 when A*001 and B*002 are present.		Not performed	Not confirmed

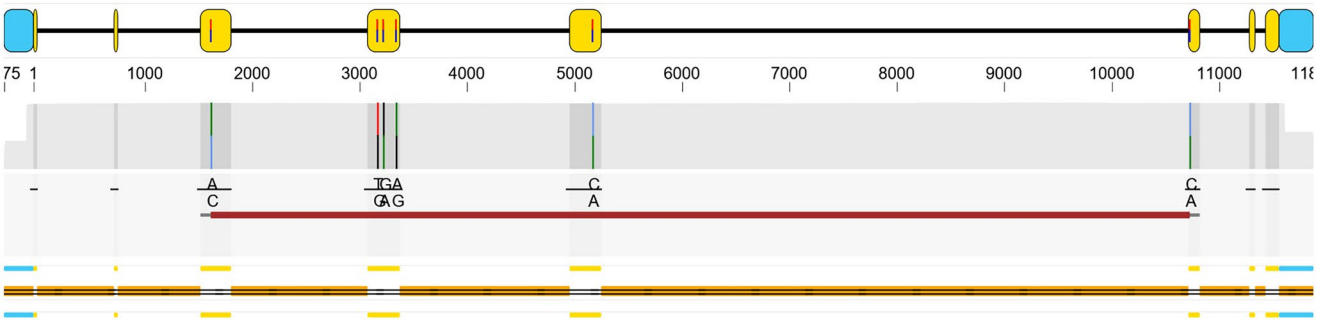

FIGURE 3 | NGSEngine sequence alignment of KIR3DL3 in BOLETH showing phasing of heterozygous position in exon 6.

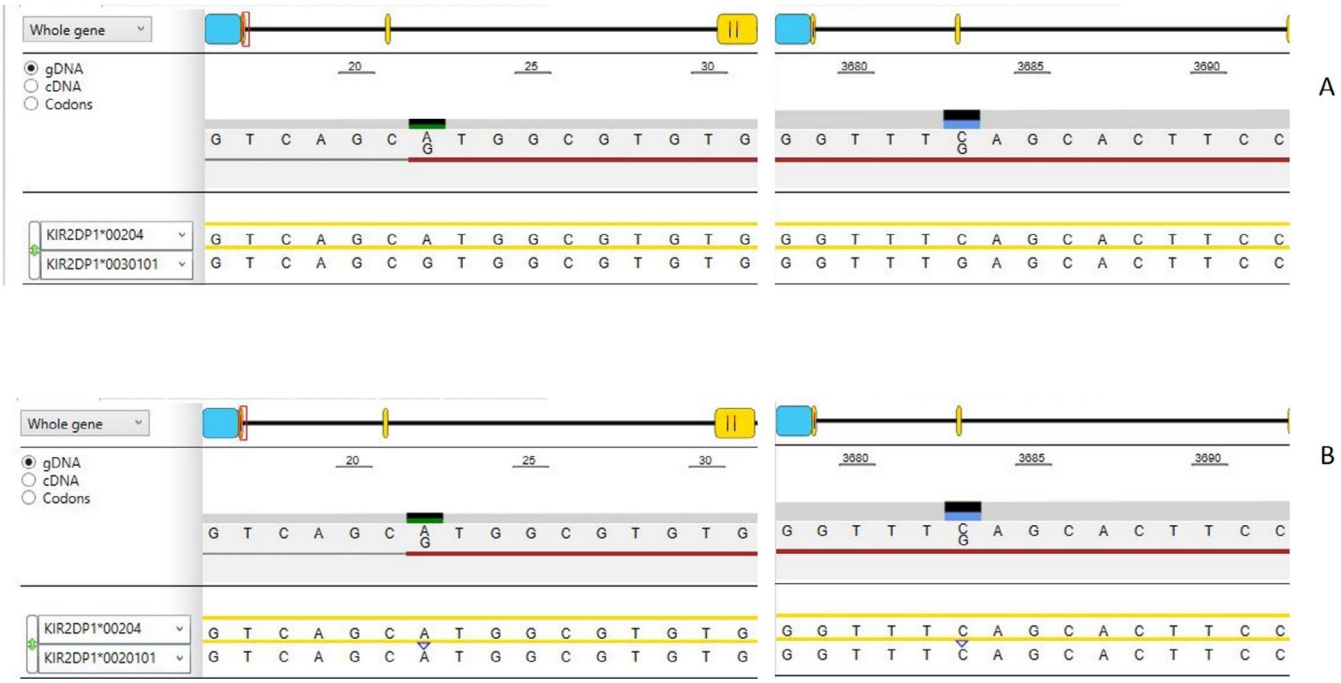

FIGURE 4 | Phased heterozygous positions in KIR2DP1 show differences with the expected reference leading to a discrepant genotype call. Panel A shows the heterozygous bases at positions 22 and 3683 matching genotype *KIR2DP1*00204*, **00301*, whereas they are mismatched (indicated by blue inverted triangle) with the previously published reference result (*KIR2DP1*00204*, **00201*).

with the clear 2:1 ratio of heterozygous reads, this may be used to identify a third allele with some reliability after further validation, as has been done in previous studies determining KIR gene copy number [35].

In a total of 5 out of the 44 inconsistent allele calls (BOLETH KIR2DP1, KIR2DL1; RML KIR2DL4; SAVC KIR2DS4; T7527 KIR2DL4), Ion Torrent sequencing of the amplicons generated by our method confirmed that the ONT sequencing method provided the correct result, and the previously published typing results were likely incorrect. A further 5 cases (BOB KIR3DL3; BOLETH KIR3DL3; E4181324 KIR2DL4; HO104 KIR3DL3; KAS116 KIR3DL3) were also sequenced by Ion Torrent but this was unable to resolve the inconsistency, in the case of KIR3DL3 this is due to the Ion Torrent platform utilising short read sequencing chemistry and therefore not being able to phase heterozygous positions resulting in ambiguous genotypes, and in the case of KIR2DL4, this is due to the presence of a third allele in the sample. The remaining 34 inconsistencies were not able to be confirmed using Ion Torrent due to a lack of reagents. The ONT result, in four cases, is highly likely incorrect due to the inability of the NGSEngine to detect the presence of a third allele. Therefore, we believe the final genotyping error rate between our method and the previously published result is at worst 19 (2.8%) and at best 4 (0.1%) out of 732 alleles. Hence the final accuracy of our method is between 97.2% and 99.5%.

3.5 | Comparison of KIR Gene Content Determined by ONT Sequencing and PCR-SSP

KIR genotypes were obtained in all 176 Western Australian population DNA samples. A total of 1884 KIR genes were detected in the 176 samples. Framework genes KIR3DL3, KIR2DL4 and KIR3DL2 were detected in all 176 samples. The presence or absence of KIR genes was compared with the results obtained previously by PCR-SSP. Results were fully concordant at the gene level, that is, the presence or absence of the gene was found to be the same by both methods in all samples,

for all KIR loci except KIR2DS4. In one case of KIR2DS4 (BSN9402240), the gene was not observed by ONT sequencing but was present in the PCR-SSP result. The haplotype structure was consistent with the absence of KIR2DS4, in that the genes signifying that two different B haplotypes were present (KIR2DL5A and KIR2DL5B and two KIR2DS3 alleles). The total sequencing reads for this sample were 4648, which is on the low side; hence, there is a possibility that KIR2DS4 has been incorrectly omitted. Furthermore, the PCR-SSP was repeated and KIR2DS4 was detected in the repeat result. It is possible that there is a polymorphism at the KIR2DS4 primer binding site preventing amplification of the full gene target in this individual.

Overall, the concordance between the two methods was 99.95%. It is conceivable, particularly in the case of closely homologous genes such as KIR2DS3 and KIR2DS5, that sequencing reads are mapped to the wrong gene by the analysis software, leading to the incorrect detection of a gene; however, where total sequencing read numbers are above 5000 for a sample, and the read depth and mappability for each detected locus are good, the risk of an incorrectly detected gene is low. Miscalling due to incorrect mapping reads did not occur in this sample set, evidenced by the very high concordance. The risk of a gene failing to be detected is higher, as PCR drop-out is an ever-present possibility; however, where read numbers are appropriate, the risk is low, as seen in the high concordance of the 10th IHWS DNA sample results.

4 | Discussion

We describe here a method for full-length sequencing of KIR genes. Our method was highly accurate, showing 93% concordance with published results, but with an accuracy of more like 97.2%–99.5% when considering the alleles present in the samples. Of the 44 inconsistent results, we have shown that our ONT results are most likely correct in 25 cases, and 19 inconsistent results are yet to be resolved. KIR allele results obtained using the ONT method were only incorrect in 4 out of 44 of these inconsistent results, which in all four cases were due to the NGSEngine software being unable to call the presence of three alleles at a single locus, a deficiency that should be corrected in subsequent software versions. In addition, when compared with a PCR-SSP method for detection of the presence or absence of KIR genes, ONT sequencing was consistent in 99.76% of 1884 detected genes.

In contrast to the method described by Maniangou et al. [19], our method utilises long range PCR in combination with long read ONT sequencing, which advantageously allows accurate mapping of sequences with a high degree of similarity and the ability to resolve phasing ambiguity. The method was validated using DNA samples from the 10th International Histocompatibility Workshop with known KIR allele genotypes and showed excellent concordance with the previously published data. Unambiguous genotypes were obtained for all but one locus in the 48 samples genotyped, including those where phasing ambiguities were evident in the previously published data, for example, HO104 and BOLETH. This shows a significant advantage over short read technology methods.

The method described here does have several shortfalls. An amplicon-based sequencing strategy depends on knowledge of the primer binding sites, and any polymorphism in these sites may result in loss of amplification of an allele. An examination of sequence alignments of all alleles with known 5′ and 3′ UTR sequence at the commencement of this study showed that all alleles in the reference database should be amplified. However, similar to other amplicon-based strategies, homozygosity should be confirmed by an alternative method. In addition, the amplification of multiple genes in a single reaction may lead to preferential amplification of certain genes or alleles over others, leading to potential misassignment; we therefore performed extensive testing to ensure the assay was performing as expected. In contrast, a PCR amplicon approach does have the advantage of relative simplicity compared with other published methods. For example, the use of CRISPR-Cas9 to enrich KIR target sequences by Bruijnesteijn et al. [22] is not feasible for most routine diagnostic immunogenetics laboratories. Whereas the study by Bruijnesteijn was able to define KIR haplotypes at allele level resolution and elegantly used the long-read capability of ONT sequencing to achieve this, the purpose of the study was to characterise haplotype complexity, not as a routine allele resolution genotyping method that can be employed in the diagnostic transplant immunology laboratory. We believe we have employed ONT sequencing to achieve this.

The lack of determination of copy number variation (CNV) represents a limitation with the currently described analysis workflow. The current version of NGSengine is only able to consider a maximum of two KIR alleles per gene and therefore there is a risk that this data will not be analysed correctly. In some cases, the presence of a third allele can be imputed where a mismatch is detected in the consensus sequence compared with the two reference alleles considered by the software. Where this mismatch is present in another allele in the KIR library the possibility that the allele is present cannot be excluded. Alternatively, however, the mismatch could be due to the presence of a novel allele. CNV is also very useful for determining haplotype structure and knowing whether the haplotypes present both contain an apparently homozygous allele, or whether that allele is only on one haplotype (hemizygous). However, CNV presents a challenge to all PCR amplicon based sequencing methods where the number of sequence reads obtained may not be at a natural balance for any particular locus. Another issue is that KIR3DP1 was not included in this workflow since as a relatively small gene (4–5 kb) over-efficient sequencing out-competes the other larger (9–17 kb) KIR loci. It would be easy to sequence this locus separately using the method described here, and primer sequences for generating the required amplicon have been described [19]. As a pseudogene, the clinical significance of KIR3DP1 is less clearly understood, although it has been shown to be useful as a marker for haplotype content and could thus be used as a surrogate for copy number analysis for other KIR loci [36].

The known problem of distinguishing homopolymer length demonstrated by ONT sequencing was evident in the analysis of KIR3DS1 results by NGSengine. *KIR3DS1*010* has a string of 7 A nucleotides between position 13360 and 13366, whereas *KIR3DS1*013* has a string of 8 A nucleotides. These alleles also differ by a single nucleotide in exon 1. NGSengine presents the KIR3DS1 result as **010* with a mismatch in exon 1. This allele

call must therefore be 'edited' to ignore the deletion at 13366, making the result **013* and solving the mismatch in exon 1. This editing was not required at any other homopolymer region in any other KIR allele. However, improvements with the release of the R10 nanopore flow cells have significantly reduced the errors seen around homopolymer regions [37].

Many alleles differ by only a single nucleotide, which presents a challenge, for example, KIR3DL2 in DBB, exon 3 gDNA position 1789, **00301* has a G whereas **00101* has a C, and all other exon positions are the same. The allelic balance of G/C at this position is nearly 50/50, with a read depth of 141 giving high confidence in the heterozygous nature in this example. Heterozygous allele balance was examined by determining the average percentage of the 2nd most frequently detected base in samples known to be heterozygous for a particular locus (Table 7), with closer to 50% equating to a closer balance of the heterozygous base position(s). The range of average percentage of the 2nd most frequently detected base was 36%–48%. Due to good heterozygous allele balance, background 2nd base calls can essentially be ignored where their percentage is significantly lower. In the International Workshop cell line validation data set, the average noise identified by NGSEngine for all KIR loci was 14.9%.

In conclusion, our novel ONT KIR sequencing method described here allows for easy implementation of accurate full gene high-resolution KIR allele typing. This method was validated on well-characterised IHWS workshop cell samples with highly concordant results, and furthermore even detected errors in the published KIR sequence of some of these workshop samples. Rapid, scalable and accurate sequencing of KIR gene polymorphism may lead to further understanding of NK cell function in human diseases such as virus infections, transplantation and cancer.

TABLE 7 | Average % 2nd most frequently detected base.

KIR locus	Average % 2nd base
3DL3	45.7
2DS2	48
2DL2	47
2DL3	36
2DL5A/B	40.5
2DP1	42.6
2DL1	42
2DL4	43
3DL1	37.6
2DS1	—
2DS3	—
2DS5	—
2DS4	39.7
3DL2	40.6

Author Contributions

Jonathan Downing and Daniel Abromeit developed the method workflow, performed the research and analysed the data. Linh Truong provided practical support for the method development. Fredrick Mobegi performed bioinformatics analysis. Dianne De Santis provided expert technical supervision and scientific guidance. Jonathan Downing wrote the manuscript. Patricia Martinez, Lloyd D'Orsogna and Dianne De Santis reviewed the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** gDNA primer start and end positions and approximate amplicon size.