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Table 1. Total number of alleles of 17 KIR genes.

KIR Gene	Number of Alleles	KIR Gene	Number of Alleles
KIR2DL1	111	KIR2DS4	39
KIR2DL2	34	KIR2DS5	24
KIR2DL3	64	KIR2DP1	40
KIR2DL4	109	KIR3DL1	184
KIR2DL5A	21	KIR3DL2	165
KIR2DL5B	37	KIR3DL3	166
KIR2DS1	17	KIR3DS1	39
KIR2DS2	24	KIR3DP1	29
KIR2DS3	16		

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Table 2. The percent identities between mRNA nucleotide sequences of KIRs.

.	2DL1*001	2DL2*0010101	2DL3*0010101	2DL4*00101	2DL5A*0010101	2DS1*001	2DS2*0010101	2DS3*00101	2DS4*0010101	2DS5*001	3DL1*0010101	3DL2*0010101	3DL3*00101
2DL2*0010101	96.94												
2DL3*0010101	95.80	97.75											
2DL4*00101	77.64	77.15	77.95										
2DL5A*0010101	79.88	79.79	80.44	88.12									
2DS1*001	96.38	94.41	95.08	75.67	77.12								
2DS2*0010101	94.74	96.82	97.49	76.23	78.57	96.50							
2DS3*00101	93.64	93.75	94.21	74.48	76.90	95.85	95.85						
2DS4*0010101	93.20	93.20	93.77	76.67	78.12	95.52	95.63	93.77					
2DS5*001	93.86	92.98	93.88	74.89	77.01	95.74	95.08	96.72	93.44				
3DL1*0010101	88.25	88.35	88.30	75.02	76.82	85.57	86.99	84.92	85.68	84.81			
3DL2*0010101	87.20	86.72	87.33	75.14	76.50	85.03	85.79	84.70	85.46	84.37	93.03		
3DL3*00101	83.81	83.70	84.70	72.63	73.95	82.41	83.10	80.90	82.75	81.37	86.70	87.43	
3DS1*010	84.59	84.82	85.10	70.69	72.11	84.64	86.12	84.30	84.76	84.07	97.34	91.32	84.64

1   **Table 3.** The reproducibility and cross reactivity assessment.

Template DNA copies	KIR2DL4			KIR3DL3			RPII		
	Mean	**SD**	**%CV**	**Mean**	**SD**	**%CV**	**Mean**	**SD**	**%CV**
Singleplex qRT-PCR ($n = 6$)									
5×10^1	37.91	0.80	2.11	41.79	2.68	6.41	37.08	0.70	1.89
5×10^2	35.24	0.54	1.55	35.26	0.45	1.29	33.80	0.74	2.19
5×10^3	32.01	0.63	1.96	32.50	0.42	1.30	30.08	0.34	1.12
5×10^4	29.39	0.63	2.14	29.17	0.44	1.52	26.54	0.18	0.66
5×10^5	25.59	0.31	1.20	26.02	0.59	2.26	23.77	0.24	1.01
5×10^6	22.30	0.61	2.72	22.92	0.69	3.00	20.68	0.26	1.26
5×10^7	18.81	0.39	2.08	19.46	0.86	4.40	17.06	0.40	2.37
5×10^8	16.11	0.57	3.57	16.14	0.80	4.96	14.36	0.10	0.72
Irrelevant plasmid	44.15	1.12	2.53	44.06	1.72	3.91	44.12	1.44	3.27
Cross-reactivity ($n = 3$)									
$5x10^7$ KIR2DL4	-	-	-	37.00	0.54	1.46	44.67	0.53	1.20
$5x10^7$ KIR3DL1	36.44	0.65	1.78	35.76	0.25	0.69	44.65	0.88	1.97
$5x10^7$ KIR3DL3	37.50	0.52	1.38	-	-	-	45.01	0	0
$5x10^8$ RPII	44.72	0.70	1.57	44.94	0.16	0.36	-	-	-

2   Mean; Mean of Cycle Time (C_T); SD; Standard deviation; % CV; The percentage of Coefficient of
3   Variation (between run).

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Table 4. Reproducibility assessment of reference gene in multiplex assays.

Template DNA	KIR2DL4			KIR3DL3			RPII		
Copies	Mean	SD	%CV	Mean	SD	%CV	Mean	SD	%CV
n = 3 (0.4 μM primer and probe)							**(5x10²) RPII**		
5×10^2	44.99	4.10	9.11	43.61	4.55	10.43	33.98	0.21	0.62
5×10^3	31.28	0.95	3.04	32.11	0.80	2.49	33.99	0.29	0.85
5×10^4	29.28	0.46	1.57	28.45	1.09	3.83	34.00	0.15	0.44
5×10^5	25.81	0.93	3.60	26.00	0.91	3.50	45.01	0	0
5×10^6	22.34	0.31	1.39	23.11	0.61	2.64	45.01	0	0
n = 3 (0.8 μM primer and probe)							**(5x10²) RPII**		
5×10^2	42.29	3.63	8.58	42.37	3.28	7.74	33.95	1.34	3.95
5×10^3	31.85	0.75	2.35	31.52	1.01	3.20	33.37	0.65	1.95
5×10^4	29.46	1.26	4.28	28.54	0.88	3.08	33.63	0.62	1.84
5×10^5	25.79	0.92	3.57	25.77	0.68	2.64	45.01	0	0
5×10^6	22.47	0.98	4.36	22.80	0.49	2.15	45.01	0	0
n = 6 (0.4 μM primer and probe)							**(5x10³) RPII**		
5×10^2	44.29	2.26	5.11	44.11	2.84	6.43	29.54	0.43	1.47
5×10^3	31.67	0.49	1.55	31.60	0.26	0.81			
5×10^4	29.11	0.46	1.59	28.72	0.53	1.84			
5×10^5	25.38	0.46	1.82	25.91	0.59	2.27			
5×10^6	22.27	0.57	2.55	22.55	0.53	2.33			

6
7 Mean; Mean of Cycle Time (C_T); SD; Standard deviation; %CV; The percentage of Coefficient of Variation (between run).

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                                10        20        30        40        50        60        70        80        90       100
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Primer and Probe KIR2DL4  ----TCAGGACAAGCCCTTCTGC------------------------------------------------------------------TGGGTTTAACAT
2DL4*0010201              GTGG.................TCTGCCTGGCCCAGCGCTGTGGTGCCTCAAGGAGGACACGTGACTCTTCGGTGTCACTATCGTCG...........
2DL5A*0010101            GTGG.........GCTGTCTGCCTGGCCCAGCGCTGTGGTGCCTCGAGGAGGACATGTGACTCTTCTGTGTCGCTCTCGTCT........C...
3DL1*0010101             GTGG.........A....CTGTCTGCCTGGCCCAGCGCTGTGGTGCCTCGAGGAGGACACGTGACTCTTCGGTGTCACTATCGTCA.A.........A
3DL2*0010101             GTGG.........A....CTGTCTGCCCGGCCCAGCACTGTGGTGCCTCGAGGAGGACACGTGGCTCTTCAGTGTCACTATCGTCG...........A
3DL3*00101               GTGG...........CT.TCTGCCTGGCCCGGCACTGTGGTGTCTGAAGGACAACATGTGACTCTTCAGTGTCGCTCTCGTCT..........GA
3DS1*010                 GTGG...............CTGTCTGCCTGGCCCAGCGCTGTGGTGCCTCGCGGAGGACACGTGACTCTTCGGTGTCACTATCGTCA.A.........A

                               110       120       130       140       150       160       170       180       190       200
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Primer and Probe KIR2DL4  CTTCACGCTGTAC--GAAAGATGGGGTCCCTGTC-----------------------------------------------------------------
2DL4*0010201             ..............AA...........CCTGAGCTCTACAACAGAATATTCTGGAACAGTTTCCTCATTAGCCCTGTGACCCCAGCACACGCA
2DL5A*0010101            .....GT....AAAG..........G......CCTGAGCTCTACAACAAAATATTCTGGAAGAGCATCCTCATGGGCCCTGTGACCCCTGCACACGCA
3DL1*0010101             T....T....A...AAAG....CA.AA...ACA.TCCCATCTTCCATGGCAGAATATTCCAGGAGAGCTTCAACATGAGCCCTGTGACCACAGCACATGCA
3DL2*0010101             T....T......AAAG....CA.AAG..AC..TCCCATCTTCCACGGCAGAATATTCCAGGAGAGCTTCATCATGGGCCCTGTGACCCCAGCACATGCA
3DL3*00101               A....GT....C.AAAG....C...A.G......CCTGAGCTCTACAACAGAATATTCCGGAACAGCTTTCTCATGGGCCCTGTGACCCCAGCACATGCA
3DS1*010                 T....T....A...AAAG....CA.AA...AC..TCCCATCTTCCATGGCAGAATATTCCAGGAGGGCTTCAACATGAGCCCTGTGACCACAGCACATGCA

                               210       220       230       240       250       260       270       280
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Primer and Probe KIR2DL4  GGGACCTACAGATGTCGAGGTTTTCACCCGCACTCCCCCACTGAGTGGTCGGCACCCAGCAACCCCCTGGTGATCATGGTCACAG
2DL4*0010201             GGGACCTACAGATGTCGAGGTTTTCACCCGCACTCCCCCACTGAGTGGTCGGCACCCAGCAACCCCCTGGTGATCATGGTCACAG
2DL5A*0010101            GGGACCTACAGATGTCGGGGGTTCACACCCACGCTCCCCCATTGAGTGGTCAGCACCCAGCAACCCCGTGGTGATCGTGGTCACAG
3DL1*0010101             GGGAACTACACATGTCGGGGGTTCACACCCACATCCCCCACTGGGTGGTCGGCACCCAGCAACCCCGTGGTGATCATGGTCACAG
3DL2*0010101             GGGACCTACAGATGTCGGGGTTCACGCCCACACTCCCTCACTGGGTGGTCGGCACCCAGCAACCCCCTGGTGATCATGGTCACAG
3DL3*00101               GGGACCTACAGATGTTGCAGTTCACACCCACACTCCCCCACTGGGTGGTCGGCACCCAGCAACCCTGTGGTGATCATGGTCACAG
3DS1*010                 GGGAACTACACATGTCGGGGTTCACACCCACACTCCCCCACTGGGTGGTCGGCACCCAGCAACCCCATGGTGATCATGGTCACAG
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(A)

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                                10        20        30        40        50        60        70        80        90       100
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Primer and Probe KIR3DL3  ----------------------------------------------------------------------GCAATGTTGGTCAGATGTCAG--------
3DL3*00101               GAGTCCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCCCCTGGTGAAATCGGGAGAGACGGTCATCCT.....................GTTTGAGCG
2DL1*001                 GAGTCCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCCCCTGGTGAAATCAGAAGAGACAGTCATCCT...................TGTTTGAACA
2DL2*0010101             GAGTCCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCGCCTGGTGAAATCAGAAGAGACAGTCATCCT..................GTTTGAGCA
2DL3*0010101             GAGTCCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCCCCTGGTGAAATCAGAAGAGACAGTCATCCT..................GTTTCAGCA
2DS1*001                 GAGTCCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCGCCTGGTGAAATCAGAAGAGACAGTCATCCT...................TGTTTGAACA
2DS2*0010101             GAGTCCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCCCCTGGTGAAATCAGAAGAGACAGTCATCCT..................GTTTGAGCA
2DS3*00101               GATTCCGCAGAAAACCTTCCCTCCTGGCCCACCCAGGTCGCCTGGTGAAATCAGAAGAGACAGTCATCCT..................TGTTTGAGCA
2DS4*0010101             GAGTCCACAGAAAACCTTCCTTCCTGGCCCTCCCAGGTCACCTGGTGAAATCAGAAGAGACAGTCATCCT...........G......TGTTTGAGCA
2DS5*001                 GATTCCGCAGAAAACCTTCCCTCCTGGCCCACCCAGGTCCCCTGGTGAAATCAGAAGAGACAGTCATCCT..................TGTTTGAGCA
3DL1*0010101             GAAACCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCCCCTGGTGAAATCAGGAGAGAGAGTCATCCT..............A...TGTTTGAGCA
3DL2*0010101             GAAACCACAGAAAACCTTCCCTCCTGGCCCACCCAGGGCCCCTGCTGAAATCAGGAGAGACGAGTCATCCT.................TGTTTGAGCA
3DS1*010                 GAAACCACAGAAAACCTTCCCTCCTGGCCCACCCAGGTCCCCTGGTGAAATCAGGAGAGAGAGTCATCCT..............A...TGTTTGAGCA

                               110       120       130       140       150       160       170       180       190       200
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Primer and Probe KIR3DL3  --------------------------------------------------------------TGCGGGTTCCCAGGTCAACTATTCCATGG----------
3DL3*00101               CTTCCTTCTGCACAGAGAGGGGATCACTGAGGACCCCTTGCGCCTCATTGGACAGCTCCACGA.....................GTCCCATG
2DL1*001                 CTTCCTTCTGCACAGAGAGGGGATGTTTAACGACACTTTGCGCCTCATTGGAGAACACCATGA..G..TC...A...C......TC.....CAGTCGCATG
2DL2*0010101             CTTCCTTCTGCACAGAGAGGGGAAGTTTAAGGACACTTTGCACCTCATTGGAGAGGCACCATGA..G..TC...A.A.C....TC.....C.GTCCCATG
2DL3*0010101             CTTCCTTCTGCACAGAGAGGGGAAGTTTAAGGACACTTTGCACCTCATTGGAGAGGCACCATGA..G..TC...A...C....TC....C.GTCCCATG
2DS1*001                 CTTCCTTCTGCACAGAGAGGGGATGTTTAACGACACTTTGCGCCTCATTGGAGAACACCATGA..G..TC...A...C......TC.....CAGTCGCATG
2DS2*0010101             CTTCCTTCTGCACAGAGAGGGGATATAAGGACACTTTGCACCTCATTGGAGAGGCACCATGA..G..TC...A...C......TC.....C.GTCGCATG
2DS3*00101               CTTCCTTCTGCACAGAGAGGGGACGTTTAACGACACTTTGCGCCTCATTGGAGAGGCACATTGA..G..TC...A...C....TC....C.GTCGCATG
2DS4*0010101             CTTCCTTCTGCACAGAGAGGGGAAGTTTAACAACACTTTGCACCTCATTGGAGAGGCACCATGA..G..T....A...C......TC.....T.GTCCCATG
2DS5*001                 CTTCCTTCTGCACAGAGAGGGGACGTTTAACAACACTTTGCGCCTCATTGGAGAGGCACATTGA..G..TC...A...G......TC....C.GTCGCATG
3DL1*0010101             CTTCTTTCTGCACAAAGAGGGGATCTCTAAGGACCCCTCACGCCTCGTTGGACAGATCCATGA..G..TC...A...C...T.TC.....C.GTCCCATG
3DL2*0010101             CTTCTTTCTGCACAGAGAGGGGATCTCTGAGGACCCCTCACGCCTCGTTGGACAGATCCATGA..G..TC...A...C......TC.....C.GTCCCTTG
3DS1*010                 CTTCTTTCTGCACAAAGAGTGGATCTCTAAGGACCCCTCACGCCTCGTTGGACAGATCCATGA..G..TC...A...C...T.TC.....C.GTTCCATG

                               210       220       230       240       250       260       270       280       290       300
                          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Primer and Probe KIR3DL3  --------------------------------------------------TACCCTATGAGTTGTCGGCT----------------------------
3DL3*00101               ACACCTGCCCTTGCAGGGACCTACAGATGCTTTGGTTCTGTCACTCACT.....................CCCAGTGACCCTCTGGACATCGTGGTCGTAG
2DL1*001                 ACGCAAGACCTGGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACTCC......C..G....A....CCCAGTGACCCTCTGGACATCGTGATCATAG
2DL2*0010101             ATGCAAGACCTTGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACTCC......C.......A....CCCAGTGACCCTCTGGACATCGTCATCACAG
2DL3*0010101             ATGCAAGACCTGGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACTCC......C.......A....CCCAGTGACCCTCTGGACATCGTCATCACAG
2DS1*001                 AGGCAAGACCTGGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACTCC......C.......A....CCCAGTGACCCTCTGGACATCGTGATCATAG
2DS2*0010101             ATGCAAGACCTTGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACTCC......C.......A....CCCAGTGACCCTCTGGACATCGTCATCACAG
2DS3*00101               AGGCAAGACCTGGCAGGGACCTACGGTTCTGTTCTGTCACTCC......C....T..A....CCCAGTGACCCTCTGGACATCGTGATCATAG
2DS4*0010101             ATGCCTGTCCTTGCAGGGAACCTACAGATGCTACGGTTCTGTTCCTCACTCC......C.......A....CCCAGTGACCCTCTGGACATGGTGATCATAG
2DS5*001                 ACACAAGACCTGGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACTCC......C.......A....GCCCAGTGACCCTCTGGACATCGTGATCACAG
3DL1*0010101             ATGCTTGCCCTTGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACACC......C.......A....CCCAGTGATCCCCTGGACATCGTGGTCACAG
3DL2*0010101             ATGCCTGTCCTTGCAGGGACCTACAGATGTTATGGTTCTGTTCCTCACTCC......C.......A....CCCAGTGACCCCCTGGACATCGTGATCACAG
3DS1*010                 ATGCGTGCCCTTGCAGGGACCTACAGATGCTACGGTTCTGTTACTCACACC......C.......A....CCCAGTGATCCCCTGGACATCGTGGTCACAG
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(B)

Figure 1. Comparative multiple-sequence alignments of primer and probe sequences. Primers and probes for KIR2DL4 (**A**) and KIR3DL3 (**B**) were aligned with mRNA nucleotide sequences of other KIRs retrieved from the IPD Database using the BioEdit version 7.0.4. Primers and new probes designed between the forward and reverse primers in this study were shown as well as the target sequences for hybridization. Probes and primers located in exon 4 of KIR2DL4 are at the nucleotide positions 5-23, 89-113 and 116-134, and in exon 3 of KIR3DL3 at the nucleotide positions 71-91, 164-193 and 250-269, respectively. A dot represents a nucleotide identity to the reference sequence and a dash represents a gap inserted to obtain optimal alignments.
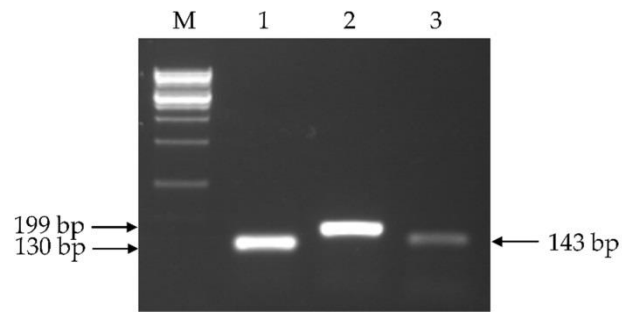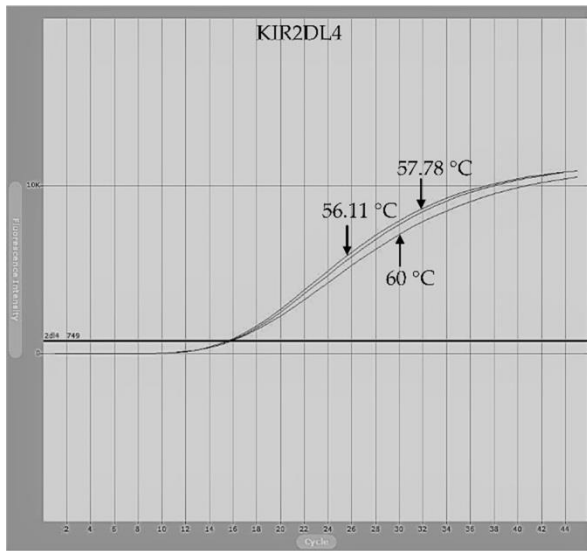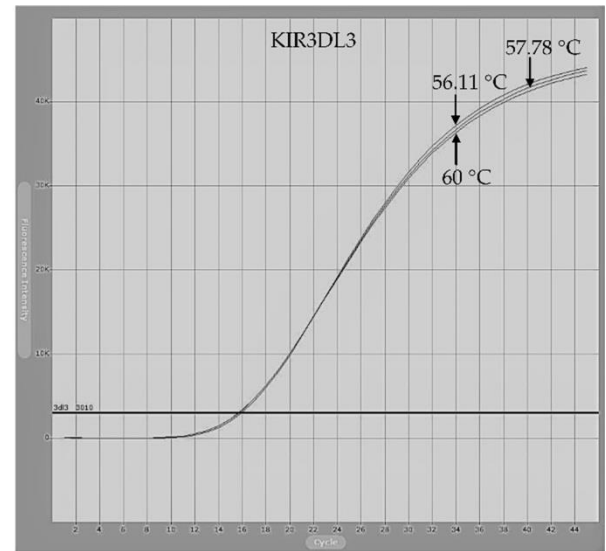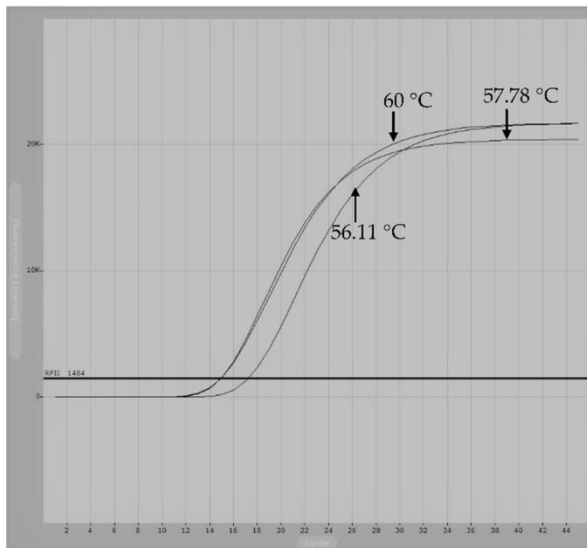
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Figure 2. Amplification of segmented cDNA of KIR2DL4, KIR3DL3 and RPII by RT-PCR assay. Specific primers were used to generate the 130, 199 and 143bp fragments specific to KIR2DL4, KIR3DL3 and RPII, respectively. M represents the 100 bp DNA ladder. Lane 1: segmented cDNA of KIR2DL4, lane 2: segmented cDNA of KIR3DL3 and lane 3: segmented cDNA of RPII.

(A)

(B)

(C)

Figure 3. The optimization with varying annealing temperature for qRT-PCR. Standard plasmids (10^8 copies/reaction) were performed with primers and probes of KIR2DL4 (**A**), KIR3DL3 (**B**) and RPII (**C**) at different temperature of 56.11, 57.78 and 60 °C by real-time PCR.
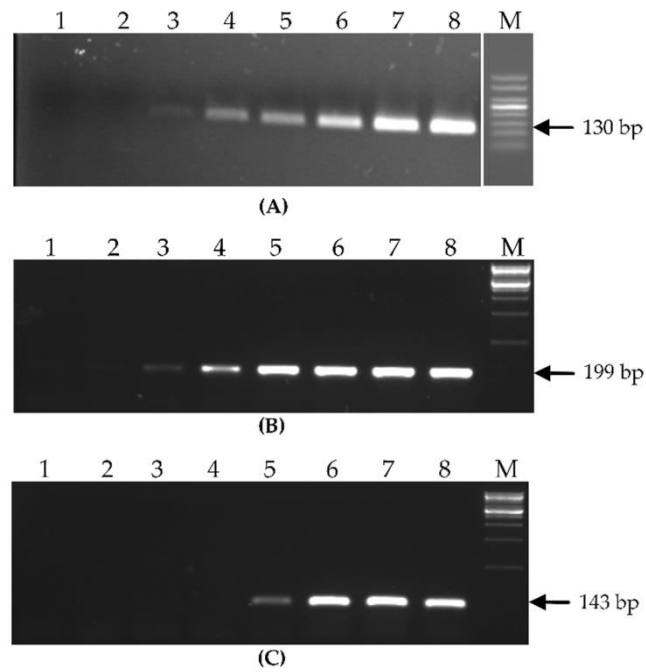
Figure S4. The sensitivity of conventional end-point PCR assay for the serial dilutions of standard plasmid DNA. Specific primers for KIR2DL4 (**A**), KIR3DL3 (**B**) and RPII (**C**) were used to amplify the standard plasmid DNA in ten-fold dilution series. Lane 1-8: standard plasmid ranged from 101 to 108 copies/reaction. M represents the 100 bp DNA ladder except 50 bp ladder for KIR2DL4.

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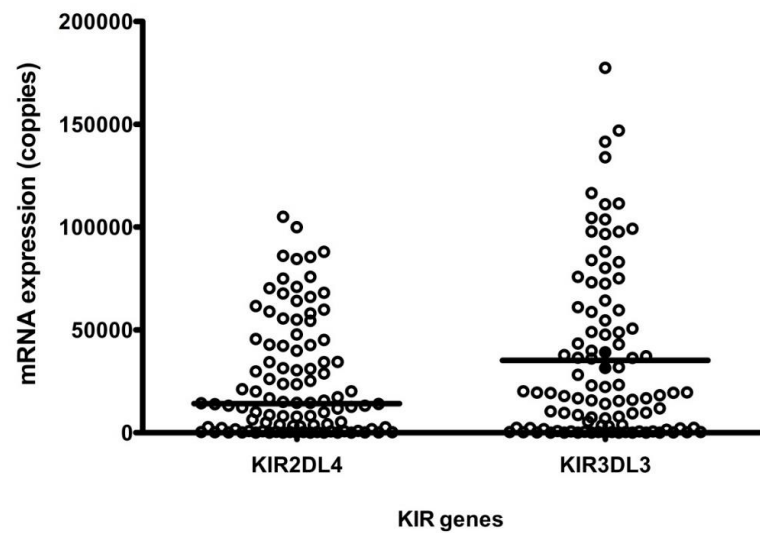
Figure 5. KIRs gene expression on clinical samples. A schematic showed frequencies of KIR2DL4 and KIR3DL3 expression in the 100 healthy donors. The data showed as the median copy numbers.