Published in final edited form as:

Methods Mol Biol. 2010 ; 612: 365–375. doi:10.1007/978-1-60761-362-6_25.

KIR Genotyping by Multiplex PCR-SSP

Smita Kulkarni, Maureen P. Martin, and Mary Carrington

Abstract

Diversity across *KIR* haplotypes stems from differences in numbers of inhibitory and activating receptors, as well as allelic polymorphism of individual genes. The *KIR* locus has undergone large expansions and contractions over time and is believed to be coevolving with genes encoding its HLA class I ligands located within the *MHC* locus. *KIR* and *HLA* compound genotypes have been associated with susceptibility to or protection from infectious, autoimmune, reproductive, and malignant disorders. We describe here a simple and reliable multiplex PCR-SSP (sequence-specific priming) method for relatively rapid and inexpensive genotyping of 15 *KIR* genes using standard agarose gel electrophoresis.

Keywords

KIR genotyping; NK cell; *KIR* haplotypes; multiplex PCR; PCR-SSP

1. Introduction

Natural killer (NK) cells are central to the innate immune system and they represent the first line of defense against bacteria, parasites, viruses, and malignant cells (1). NK cells comprise 5–15% of circulating lymphocytes and are also found in tissues including the liver, peritoneal cavity, and placenta. They can mediate spontaneous killing of infected or transformed target cells and produce immunoregulatory cytokines and chemokines that stimulate the adaptive immune response (2, 3). In addition, they engage in interactions at the maternal–fetal interface that are critical for successful pregnancy (4).

NK cell activity is tightly controlled by activating and inhibitory interactions through numerous cell surface receptors. They respond to targets that express aberrant levels of MHC class I, and this involvement of MHC class I molecules in NK cell recognition of self vs nonself was originally described over two decades ago (5). MHC class I molecules are the essential signature of self that NK cell inhibitory receptors engage in order to avoid NK

© Humana Press, a part of Springer Science+Business Media, LLC 2010

³All primer mixes should be tested with positive and negative controls when new primer mixes are made from the stock primers.

⁵A fast ramping thermal cycler with a heated lid is recommended for best results. The 'step-down' program was designed for this protocol to increase specificity of the amplifications. It might be necessary to alter the PCR conditions slightly depending on the PCR machine being used.

⁶PCR machines should be checked regularly to ensure that all wells are amplifying uniformly. It is also important to ensure that the PCR plates fit snugly into the machine.

⁷The quality of dNTPs is important for reliable amplifications. Avoid repeated freeze–thawing; instead freeze in small aliquots after reconstitution.

⁸The highly homologous sequences of the *KIR* loci can lead to nonspecific amplification especially with increased numbers of cycles (>30) used for PCR.

¹⁰The gene content of some *KIR* haplotypes and sequence of some *KIR* alleles strongly suggest that unequal crossing-over in the region accounts for a substantial amount of the diversity at this locus (20–23). This could result in the production of unusual profiles where, for example, one or more of the framework genes are missing or chimeric genes are present.

cell attack of normal cells (5–7). The original ‘missing self’ hypothesis (8) proposed that downregulation of class I expression on target cells leads to spontaneous destruction by NK cells. Current knowledge of NK cell biology suggests that NK cells not only patrol for abnormal cells that lack MHC class I but also for those that over-express ligands for activating receptors such as altered MHC (‘altered self’) and non-MHC ligands (‘induced self’ or ‘nonself’) (9).

The killer cell immunoglobulin-like receptors (KIR) are one of the main types of MHC class I-specific receptors utilized by human NK cells, and they are comprised of both activating and inhibitory counterparts that are encoded by highly homologous sequences. The *KIR* locus maps to chromosome 19q13.4 within the leukocyte receptor complex (*LRC*) (10). The high sequence similarity of the *KIR* genes may facilitate the occurrence of non-allelic homologous recombination (NAHR) (11, 12) and probably partially explains expansion and contraction of *KIR* haplotypes (10). *KIR* haplotypes have been broadly classified into two groups referred to as A and B, both of which are found in most populations but sometimes at very different frequencies. Haplotype A is invariant in terms of gene content while haplotype B is quite variable (13) (Fig. 25.1). Currently, more than 40 different B haplotypes based on gene content have been described [summarized in (14)], and further diversity is afforded by allelic polymorphism of individual *KIR* genes such that it is unlikely that any two randomly selected individuals have identical *KIR* genotypes (15). The highly polymorphic and genetically unlinked *KIR* and *MHC* class I loci appear to have coevolved to give selective advantage to higher order mammals with respect to reproduction and resistance against infections. Thus, it is not surprising that an ever-increasing number of genetic studies have shown that combinations of *KIR* and *HLA* confer either protection against or susceptibility to infectious, reproductive, autoimmune, and malignant disorders (16). *KIR* genotypic variation across populations is also important from an evolutionary standpoint since differences may in part reflect a response to differences in exposure to infectious agents across populations (17).

This chapter will describe a method for multiplex *KIR* genotyping based on our previously published methodology (18). We have used the same primers but in multiplex combinations that can be resolved by gel electrophoresis. This method detects the presence/absence of *KIR* genes, thus providing *KIR* gene profiles.

2. Materials

2.1. PCR Amplification

1. Good-quality DNA from tissues or cells isolated using standard protocols at a concentration of 50–100 ng/μl (*see* Notes 1 and 2).
2. Oligonucleotide primer mixes containing forward and reverse primers (*see* Table 25.1 for primer sequences and Table 25.2 for list of primer mixes and primer concentrations).
3. 50 mM MgCl₂.
4. dNTP mix: 25 mM each of dATP, dTTP, dGTP, and dCTP.
5. 10× PCR buffer: 200 mM Tris–HCl, pH 8.4, and 500 mM KCl.

¹The quality of DNA is the most important requirement for reliable PCR-SSP results. DNA extracted from heparinized blood should be avoided, as heparin is a PCR inhibitor (19).

²A total of 100–200 ng (gel electrophoresis method) DNA is required for reliable amplification using the primer mixes described here. Increasing the number of cycles is likely to increase false-positive reactions. Therefore, if the amplification is not satisfactory because of DNA quality, it is recommended that each primer mix be amplified individually, as described in (18).

6. *Taq* DNA polymerase (see Note 4).
7. 384-Well PCR plates. Each sample will require 12 wells, thus allowing genotyping of 32 samples per plate.
8. Multichannel pipettors (8 and 16 channels).

2.2. Gel Electrophoresis

1. Orange G loading buffer: 0.5% Orange G, 20% Ficoll, and 100 mM ethylenediaminetetraacetic acid (EDTA).
2. 10× TAE electrophoresis buffer: 400 mM Tris, 200 mM acetic acid, and 10 mM EDTA.
3. 100 bp DNA ladder.
4. Electrophoresis grade agarose.
5. Horizontal electrophoresis chamber.
6. Gel casting tray with 50-well combs having teeth appropriately spaced to allow for loading with a multichannel pipettor. The Centipede™ horizontal model D3–D14 wide gel system (Woburn, MA) will accommodate two combs per gel allowing for electrophoresis of eight DNA samples, each with 12 PCR reactions.
7. 10 mg/ml ethidium bromide solution.
8. High-voltage power supply.
9. Photographic means of gel documentation (ultraviolet light source, camera).

3. Methods (KIR Genotyping by Gel Electrophoresis)

3.1. PCR

1. Using a multichannel pipette, dispense 1 µl of each primer mix into separate wells of the 384-well PCR plate. Each sample will require 12 wells in a horizontal row (e.g., A1–A12) such that 32 samples can be accommodated on one plate.
2. Prepare PCR cocktail of 60 µl (enough for 15 wells) for one sample as follows: 150–200 ng DNA, 7.5 µl 10× PCR buffer, 2.25 µl MgCl₂ (final PCR concentration 1.5 mM), 0.6 µl dNTP (final PCR concentration 200 µM), and 0.375 µl Platinum *taq* polymerase. Add water to make up the volume to 60 µl. Mix well by vortexing at low speed.
3. Dispense 4 µl of PCR cocktail for each DNA sample to each primer mix in the plate for a total PCR reaction volume of 5 µl.
4. Cover the plate with acetate film and centrifuge briefly to pull the contents of the well to the bottom of the plate.
5. The amplification is carried out in a programmable PCR thermal cycler with heated lid to minimize evaporation using the following program: 3 min at 94°C, 5 cycles of 15 s at 94°C, 15 s at 65°C, 30 s at 72°C; 21 cycles of 15 s at 94°C, 15 s at 60°C, 30 s at 72°C; 4 cycles of 15 s at 94°C, 1 min at 55°C, 2 min at 72°C followed by a final 7 min extension step at 72°C.

⁴Platinum *Taq* (Invitrogen) polymerase gives the most reliable and consistent results for this protocol.

3.2. Agarose Gel Electrophoresis

1. For one gel, prepare 150 ml of 3% w/v agarose in 1× TAE by heating until the agarose is completely solubilized.
2. Cool the mixture to 65°C and add 5 µl ethidium bromide and gently mix to avoid bubble formation.
3. Pour the gel mixture into the gel casting tray and insert the 50-well combs. Allow the gel to solidify (~20 min).
4. Submerge the solidified gel in the electrophoresis chamber filled with 800 ml of 1× TAE buffer. Remove the combs.
5. Add 5 µl of Orange G loading buffer to each PCR product and centrifuge briefly.
6. Load 2 µl of the 100 bp DNA ladder to the first and last well of each row of wells.
7. Using a 16-channel pipettor, load 10 µl of each PCR product into the gel.
8. Electrophorese for 70 min at 100 V or until the Orange G has migrated 6 cm.
9. Visualize the gel using a UV light source and photograph the gel for a permanent record (Fig. 25.2).

3.3. Interpretation of Result

Determination of *KIR* genotype using this method depends on presence or absence of detectable amplification product for each gene-specific primer pair (see Notes 9–11). It is important to have a good quality gel picture for accurate judgment of the product sizes, as each well contains multiple products of varying size. The most important product is the positive internal control amplicon, which should be present in all the samples. Each *KIR* gene is amplified using two pairs of specific primers and products should be detected for both sets of primers. If the amplification by the two gene-specific primer pairs is discrepant for any gene, the reaction should be repeated. Detectable bands as well as absence of product can be recorded in a spreadsheet such as Microsoft Excel. Some alleles of the *KIR2DS4* gene have a 22 bp deletion in exon 5 that can be detected by the *KIR2DS4* primers in primer mix 8. The full-length product is 219 bp while the deletion variant is 197 bp (see Fig. 25.2B).

Acknowledgments

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

References

1. Trinchieri G. Biology of natural killer cells. *Adv Immunol.* 1989; 47:187–376. [PubMed: 2683611]

⁹Some primers miss a couple of alleles of a given gene as noted in Table 25.1 and others amplify one allele of another gene. Furthermore, there are likely to be yet undiscovered alleles of a gene that might not amplify with the current set of primers and alternatively some of these might cross-react with alleles of other *KIR* genes. We strongly recommend routine checks of the *KIR* database (<http://www.ebi.ac.uk/ipd/kir>) for new alleles which might require designing new primers. It should also be remembered that most of the available *KIR* gene sequences have been obtained from Caucasians and thus may not include alleles that are prevalent in other populations. This highlights the importance of using more than one pair of primers for amplification of each gene.

¹¹Some of the *KIR* loci exhibit strong negative and positive linkage disequilibrium between them. For example, some gene pairs such as *KIR2DL2/2DS2*, *KIR2DL1/2DP1*, and *KIR3DL1/2DS4* are almost always found together across different haplotypes. This information is very useful in validation of the genotyping results.

2. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. *Trends Immunol.* 2001; 22:633–640. [PubMed: 11698225]
3. Nguyen KB, Salazar-Mather TP, Dalod MY, Van Deusen JB, Wei XQ, Liew FY, et al. Coordinated and distinct roles for IFN- α beta, IL-12, and IL-15 regulation of NK cell responses to viral infection. *J Immunol.* 2002; 169:4279–4287. [PubMed: 12370359]
4. Trowsdale J, Moffett A. NK receptor interactions with MHC class I molecules in pregnancy. *Semin Immunol.* 2008; 6:317–320. [PubMed: 18656382]
5. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature.* 1986; 319:675–678. [PubMed: 3951539]
6. Shimizu Y, DeMars R. Demonstration by class I gene transfer that reduced susceptibility of human cells to natural killer cell-mediated lysis is inversely correlated with HLA class I antigen expression. *Eur J Immunol.* 1989; 19:447–451. [PubMed: 2707298]
7. Storkus WJ, Alexander J, Payne JA, Dawson JR, Cresswell P. Reversal of natural killing susceptibility in target cells expressing transfected class I HLA genes. *Proc Natl Acad Sci U S A.* 1989; 86:2361–2364. [PubMed: 2784569]
8. Ljunggren HG, Karre K. In search of the ‘missing self’: MHC molecules and NK cell recognition. *Immunol Today.* 1990; 11:237–244. [PubMed: 2201309]
9. Lanier LL. NK cell recognition. *Annu Rev Immunol.* 2005; 23:225–274. [PubMed: 15771571]
10. Wilson MJ, Torkar M, Haude A, Milne S, Jones T, Sheer D, et al. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A.* 2000; 97:4778–4783. [PubMed: 10781084]
11. Lupski JR. Charcot-Marie-Tooth disease: lessons in genetic mechanisms. *Mol Med.* 1998; 4:3–11. [PubMed: 9513184]
12. Carrington M, Cullen M. Justified chauvinism: advances in defining meiotic recombination through sperm typing. *Trends Genet.* 2004; 20:196–205. [PubMed: 15041174]
13. Uhrberg M, Valiante NM, Shum BP, Shilling HG, Lienert-Weidenbach K, Corliss B, et al. Human diversity in killer cell inhibitory receptor genes. *Immunity.* 1997; 7:753–763. [PubMed: 9430221]
14. Khakoo SI, Carrington M. KIR and disease: a model system or system of models? *Immunol Rev.* 2006; 214:186–201. [PubMed: 17100885]
15. Shilling HG, Young N, Guethlein LA, Cheng NW, Gardiner CM, Tyan D, et al. Genetic control of human NK cell repertoire. *J Immunol.* 2002; 169:239–247. [PubMed: 12077250]
16. Kulkarni S, Martin MP, Carrington M. The Yin and Yang of HLA and KIR in human disease. *Semin Immunol.* 2008; 6:343–352. [PubMed: 18635379]
17. Carrington M, Martin MP. The impact of variation at the KIR gene cluster on human disease. *Curr Top Microbiol Immunol.* 2006; 298:225–257. [PubMed: 16329188]
18. Martin MP, Carrington M. KIR locus polymorphisms: genotyping and disease association analysis. *Methods Mol Biol.* 2008; 415:49–64. [PubMed: 18370147]
19. Satsangi J, Jewell DP, Welsh K, Bunce M, Bell JI. Effect of heparin on polymerase chain reaction. *Lancet.* 1994; 343:1509–1510. [PubMed: 7911214]
20. Shilling HG, Lienert-Weidenbach K, Valiante NM, Uhrberg M, Parham P. Evidence for recombination as a mechanism for KIR diversification. *Immunogenetics.* 1998; 48:413–416. [PubMed: 9799338]
21. Martin MP, Bashirova A, Traherne J, Trowsdale J, Carrington M. Cutting edge: expansion of the KIR locus by unequal crossing over. *J Immunol.* 2003; 171:2192–2195. [PubMed: 12928362]
22. Gomez-Lozano N, Estefania E, Williams F, Halfpenny I, Middleton D, Solis R, et al. The silent KIR3DP1 gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the KIR3DP1, KIR2DL4 and KIR3DL1/KIR3DS1 genes are duplicated. *Eur J Immunol.* 2005; 35:16–24. [PubMed: 15580659]
23. Shilling HG, Lienert-Weidenbach K, Valiante NM, Uhrberg M, Parham P. Evidence for recombination as a mechanism for KIR diversification. *Immunogenetics.* 1998; 48:413–416. [PubMed: 9799338]

Fig. 25.1.

The leukocyte receptor complex on chromosome 19q13.4. The *KIR* gene cluster is located within the leukocyte receptor complex. The *KIR* locus is expanded to show the order of the genes on chromosome 19. Haplotypes A and B are labeled as such. The A haplotype is fixed in terms of gene content, while B haplotype is characterized by variable gene numbers. *Black boxes* represent inhibitory receptors, *hatched boxes* represent activating receptors, *open boxes* represent pseudogenes, and *gray boxes* represent framework loci present on virtually all haplotypes.

Fig. 25.2.

KIR genotyping by multiplex PCR-SSP and agarose gel electrophoresis. **(A)** Agarose gel electrophoresis depicting the presence of all the *KIR* genes tested. **(B)** Gel electrophoresis results from an individual homozygous for haplotype A. This individual possesses one truncated (197 bp) and one full-length copy (219 bp) of *KIR2DS4* as shown by the presence of the appropriately sized bands in primer mix 8. **(C)** Gel electrophoresis results from an individual homozygous for haplotype B. The order of primer mixes (see Table 25.2) used for **(B)** and **(C)** is as shown in **(A)**. **(D)** Genotyping results for the samples shown in **(A–C)** (samples 1–3). *M* = 100 bp molecular weight marker.

Table 25.1

KIR genotyping primers

Gene	Primers	Sequences	Exon	Size (bp)	Comments
2DL1	2DL1_A	F1 GTTGGTCAGATGTCATGTTTGAA	4	146	
		R1 GGTCCCTGCCAGGCTTGGC	4		
2DL1	2DL1_B	F2 TGGACCAAGAGTCTGCAGGA	8	~330	Misses*005, Sees 2DL2*004
		R2 TGTGTCTCCCTAGAAAGACG	3'UTR		
2DL2	2DL2_A	F1 CTGGCCCCACCCAGGTCTG	4	173	Misses*004
		R1 GGACCGATGGAGAAAGTTGGCT	4		
2DL2	2DL2_B	F2 GAGGGGAGGCCCATGAAT	5	151	
		R2 TCGAGTTTGAACCACTCGTAT	5		
2DL3	2DL3_A	F1 CTTTCATCGCTGGTCTG	7	~550	Misses*007
		R1 AGGCTCTTGGTCCATTACAA	8		
2DL3	2DL3_B	F2 TCCTTCATCGCTGGTCTG	7	~800	
		R2 GGCAGGAGACAACTTTGGATCA	9		
2DL4	2DL4_A	F1 CAGGACAAGCCCTTCTGC	3	254	
		R1 CTGGGTGCCGACCACT	3		
2DL4	2DL4_B	F2 ACCTTCGCTTACAGCCCG	5	288	
		R2 CCTCACCTGTGACAGAAACAG	5		
2DS2	2DS2_A	F1 TTCTGCACAGAGGGGAAAGTA	4	175	
		R1 GGGTCACTGGGAGCTGACAA	4		
2DS2	2DS2_B	F2 CGGGCCCCACGGTTT	5	240	Misses*00104
		R2 GGTCACCTCGAGTTTGACCACTCA	5		
2DS3	2DS3_A	F1 TGGCCCCACCCAGGTCTG	4	242	
		R1 TGA AAAA CTGATAGGGGGAGTGAGG	4		
2DS3	2DS3_B	F2 CTATGACATGTACCACTCTATCCAC	5	190	
		R2 AAGCAGTGGGTCACTTGAC	5		
2DS4	2DS4_A	F1 CTGGCCCCCTCCAGGTCA	4	204	
		R1 TCTGTAGGTTCTGTAAAGGACAG	4		

Gene	Primers	Sequences	Exon	Size (bp)	Comments
	2DS4_B F2	GTTCAGGCAGGAGAGAAT	5	197/219	
	R2	GTTTGACCACTCTGTAGGGGAC	5		
<i>2DS5</i>	2DS5_A F1	TGATGGGTCTCCAAGGG	4	126	Misses *003
	R1	TCCAGAGGGTCACTGGGC	4		
	2DS5_B F2	ACAGAGAGGGGACGTTTAACC	4	178	
	R2	ATGTCCAGAGGGTCACTGGG	4		
<i>3DL1</i>	3DL1_A F1	CGCTGTGGTGCCCTCGA	3	191	Misses *009
	R1	GGTGTGAACCCCGACATG	3		
	3DL1_B F2	CCCTGGTGAAATCAGGAGAGAG	4	186	
	R2	TGTAGTCCCTGCAAGGGCAA	4		
<i>3DL2</i>	3DL2_A F1	CAAAACCTTCTCTGTCTGCC	3	242	Misses *013, *014
	R1	GTGCCGACCAACCAGTGA	3		
	3DL2_B F2	CCCATGAACGTAGGCTCCG	5	130	
	R2	CACACGACGGGCAGGG	5		
<i>3DS1</i>	3DS1_A F1	AGCTGCAGGGAACAGAAAG	8	~300	
	R1	GCCTGACTGTGTGCTCG	3'UTR		
	3DS1_B F2	CCTGTGAAATCAGGAGAGAG	4	180	
	R2	GTCCCTGCAAGGGCAC	4		
<i>3DL3</i>	3DL3_A F1	GTCAGGACAAAGCCCTTCTC	3	232	
	R1	GAGTGTGGGTGTGAACTGCA	3		
	3DL3_B F2	TTCTGCACAGAGAGGGGATCA	4	165	
	R2	GAGCCGACAACTCATAAGGTA	4		
<i>2DL5</i>	2DL5_A F1	GCGCTGTGTGCTCTCG	3	214	
	R1	GACCACTCAATGGGGAGC	3		
	2DL5_B F2	TGCAGCTCCAGGAGCTCA	5	191	
	R2	GGGTCTGACCACTCATAGGGT	5		
<i>2DPI</i>	2DPI_A F1	GTCCTGCTGGCCAGCT	3	205	
	R1	GTGTGAACCCCGACATCTGTAC	3		
	2DPI_B F2	CCATCGGTCCCATGATGG	4	89	

Gene	Primers	Sequences	Exon	Size (bp)	Comments
<i>2DS1</i>	R2	CACTGGGAGCTGACAACTGATG	4		
	F1	CTTCTCCATCAGTCGCATGAA	4	102	
	F2	CTTCTCCATCAGTCGCATGAG	4		
	R1	AGAGGGTCACTGGGAGCTGAC	4		
<i>DRB1</i>	F1	TGCCAAGTGGAGCACCCAA	intron 3	796	Internal control
	R1	GCATCTTGCTCTGTGCAGAT	Intron 3		

Table 25.2

Primer mixes for multiplex PCR

Mix #	Primers	Product size (bp)	Primer concentrations (μM)
1	2DL1_B	330	5
	2DL2_A	173	5
	2DL2_B	151	4
2	2DS3_B	190	5
	2DP1B	89	5
3	3DL1_A	191	5
	3DS1_A	300	5
	DRB1	800	4
4	2DL5_A	214	5
	2DL5_B	191	5
	2DS1	102	5
5	2DL3_B	800	5
	2DL4_A	254	5
6	2DL3_A	550	5
	2DS3_A	242	5
	3DS1_B	180	5
7	2DS4_A	204	4
	3DL1_B	186	5
	2DL1_A	146	5
8	2DS4_B	197/219	5
	2DS5_A	126	5
9	3DL3_A	232	5
	2DS5_B	178	5
	3DL2_B	130	5
10	2DP1_A	205	5
	2DS2_A	175	5
11	2DS2_A	240	5
12	2DL4_B	288	5
	3DL2_A	242	10
	3DL3_B	165	5