

Fig. 2 MHC class II. MHC class II molecules are heterodimeric and consist of two peptides— α and β chains, which include α_1 , β_1 domains encoding binding cleft, and α_2 , β_2 —membrane bound domain

The class I region, encoding HLA A, B, and C molecules, is expressed on the cell surface of nucleated cells that are involved in the presentation of endogenous antigens to CD8+ cytotoxic T cells (Tc) and contribute to risk of T1D [11] and the HLA A*24 allele is associated with rapid progression to T1D [12].

The HLA class II region encodes membrane bound proteins expressed on the cell surface of antigen-presenting cells (APCs): B-lymphocytes, macrophages, and dendritic cells that are involved in the processing and presentation of exogenous antigens to CD4+ T helper cells (Th) (Fig. 3) leading to T cell activation. The numerous subsets of T cells are derived from a single T cell precursor and some subsets have the capacity to regulate one another (Fig. 4).

Studies of the pathogenesis of type 1 diabetes proving roles for the HLA as well as effector and regulator T cell population mechanistically have utilized the predominant animal model of type 1 diabetes, the nonobese diabetic (NOD) mouse transgenic for HLA Class II [13].

HLA class II genes contribute to both susceptibility and resistance to T1D; risk is associated with the HLA class II haplotypes *DRB1*04-DQB1*0302* and *DRB1*03-DQB1*02* while the haplotype *DRB1*15-DQB1*0602* is dominantly protective. The risk of

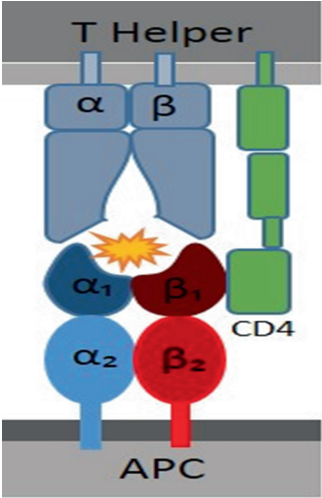


Fig. 3 Antigen presentation. During antigen presentation CD4 receptors of T helper lymphocytes bind to β domain of the HLA Class II molecule that activates the T cell

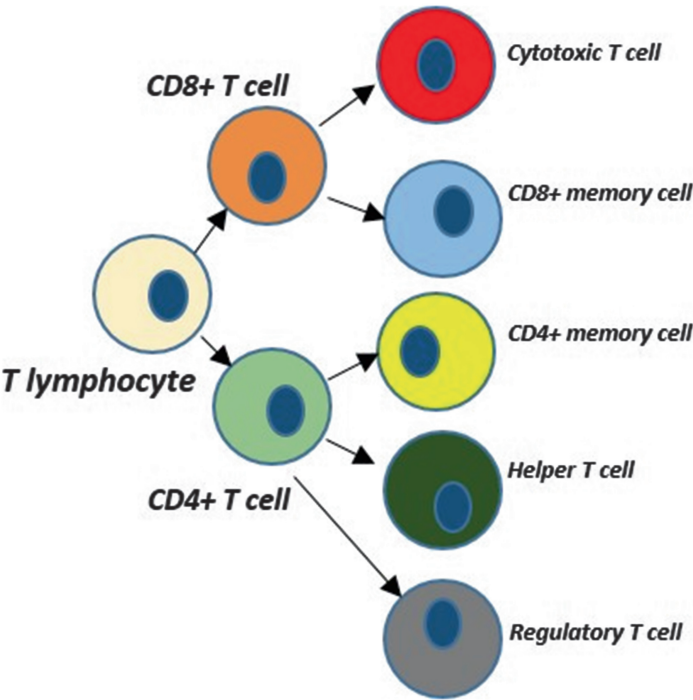


Fig. 4 T cell subsets. CD8+ T cells mature into cytotoxic T cells, CD8+ memory cells; CD4+ T cells mature into helper T cells, regulatory T cells, CD4+ memory cells

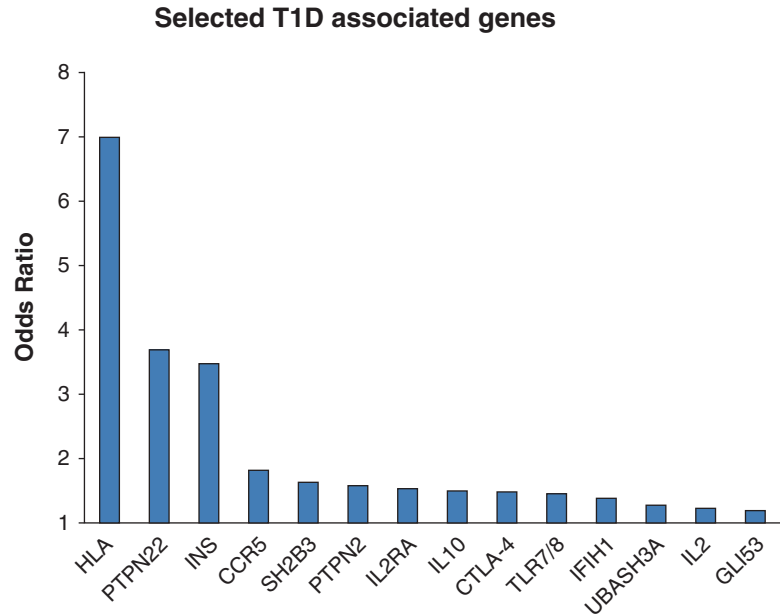


Fig. 5 The relative effects of selected T1D associated genes on susceptibility to T1D (adapted from Todd, 2010) [15]

developing T1D in siblings of affected children varies from 0.3 to 30 % depending on their HLA class II genotype [14].

Additional genetic risk markers were identified in the 1980s and 1990s but the advent of genome-wide association studies (GWAS), conducted since 2007, has allowed identification of approximately 40 additional genes that contribute to susceptibility to T1D (Fig. 5) [15]. Ongoing research now focuses on the biological pathways in immune and beta cells where these T1D associated genes function [16]. Despite these huge advances, all the genes identified to date do not account for the sum of genetic susceptibility. The concept of “missing heritability” has led to a focus on rare variants.

The increasing role of a “diabetogenic” environment was suggested following reports of the rising incidence and decreasing age at diagnosis of T1D while the frequency of the high risk HLA DR3/DR4 genotype is decreasing [17, 18]. A variety of environmental factors such as infections in early life, diet, and early development of the gut microbiome have been implicated in promoting the rising incidence of T1D [19] but none yet unequivocally proven. It is likely that one mechanism by which the environment influences risk of autoimmune diabetes is through epigenetic changes.

3 The Natural History of Type 1 Diabetes

Over the last 20 years, a series of birth cohort studies [20–23] have contributed hugely to our understanding of the natural history of the condition. Islet antibodies are markers of ongoing autoimmune destruction [24] and the best characterized are specific to the islet proteins insulin [25], glutamic acid decarboxylase (GAD) [26], IA-2 [27], and the zinc transporter ZnT8 [28, 29]. The autoimmune process begins very early in life: studies of neonatal diabetes suggest that most cases of diabetes diagnosed before 6 months are unlikely to be autoimmune, but those diagnosed after the age of 6 months have the genetic characteristics of T1D [30]. Antibodies to insulin (generally the first to appear) have been detected as early as 6–12 months of age [31]. Longer term follow-up of birth cohorts in Finland and Germany suggests that there is an explosion of islet autoimmunity in at risk children between the ages of 6 months and 3 years [21, 32]. The techniques to detect islet autoantibodies with high sensitivity and specificity have resulted from decades of collaborative workshops where blinded reference samples are tested in participating laboratories [33] resulting in high quality radioimmunoassays [34] and more recently ELISAs and chemiluminescence assays [35].

Islet autoantibody studies have demonstrated differing rates of progression in individuals positive for multiple islet autoantibodies; many progress rapidly [27] but there is also accumulating evidence for “slow burning” autoimmunity. For instance, within the Bart’s Oxford study of type 1 diabetes (<http://www.bristol.ac.uk/clinical-sciences/research/diabetes/research/box/>), ongoing since 1985, some “at risk” individuals with two or more islet autoantibodies remain diabetes free after 20 years. A relapsing/remitting process of beta cell destruction has been postulated that could help explain differences in rates of progression but as yet there is no widely available marker of beta cell death although an assay to detect demethylated insulin for this purpose has been described [36, 37].

Although the pancreas in type 1 diabetes has been described previously [38, 39], recent histological analysis revealed new insights into the immune cell subsets comprising insulinitis with the observation that B cells are present in greater frequency than expected [40]. Further analysis of T1D pancreas has been made possible by the Network for pancreatic organ donors with diabetes (nPOD) initiative (www.jdrfnpod.org). Improving techniques raise the possibility of analysis of single laser captured islet beta cells.

Once diagnosed, the insulin-free “honeymoon period” is variable and there is increasing evidence that some individuals with long-standing diabetes can continue to make low levels of insulin [41]. Large scale testing has been made possible through a straightforward test to detect c-peptide in urine [42].

Future perspectives in type 1 diabetes include improved bio-marker identification to support a number of ongoing clinical trials orchestrated internationally by the TrialNet consortium (www.diabetestrialnet.org).

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Part I

Genes

Type 1 Diabetes High-Risk HLA Class II Determination by Polymerase Chain Reaction Sequence-Specific Primers

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Abstract

The only strategy to select individuals at increased risk for type 1 diabetes for primary prevention trials is through genetic risk assessment. While genome-wide association studies have identified more than 40 loci associated with type 1 diabetes, the single most important genetic determinants lie within the human leucocyte antigen gene family on chromosome 6.

In this chapter we describe a protocol for a straightforward, cheap strategy to determine HLA class II mediated risk of type 1 diabetes. This method has proved robust for genotyping whole-genome-amplified DNA as well as DNA extracted directly from human tissues.

Keywords: HLA class II, HLA class I, Genetic risk

1 Introduction

HLA class II presents self-peptides to the immune system and autoimmunity results from impaired tolerance to self-antigens. A fundamental role for HLA class II in susceptibility to type 1 diabetes (T1D) is therefore not surprising. An association between HLA and T1D was originally demonstrated in the 1970s [1, 2] with several major steps forward in the ensuing decades when the importance of HLA class II alleles was described [3] the relationship between age at onset of diabetes and HLA class II-mediated risk [4–6], and the hierarchy of HLA class II-mediated risk in type 1 diabetes [7].

The method described here has been adapted from the original “Phototyping” method for discrimination of HLA genotype by Bunce and colleagues [8]. It allows identification of all alleles of HLA *DRB1* facilitating identification of homozygosity and heterozygosity as well as selected alleles from HLA *DQA1* and *DQB1*. Overall the highest risk diplotype is HLA *DRB1**03-*DQB1**0201/*DRB1**04-*DQB1**0302.

2 Materials

2.1 DNA

DNA extracted from human tissues: This includes tissues extracted from fixed tissues using appropriate extraction kits and whole-genome-amplified DNA (**Note 1**).

2.2 Reagents and Supplies

PCR reaction

1. Oligonucleotide primers specific for HLA *DRB1*, *DQA1*, and *DQB1* as described in Tables 1 and 2 (Fig. 1). Make up to a standardized concentration of 100 pmol/μl and store in aliquots at −20 °C. A control primer set (any robust set will suffice) must be added to each allele specific primer set.
2. Taq polymerase and PCR Buffer, MgCl₂ (usually supplied together). Go Taq and buffer from Promega are used in the method described (**Note 2**).
3. dNTPs commercially available
4. 96-Well PCR plates
5. 96-Well thermocycler
6. 10× TBE buffer: 108 g Tris and 55 g boric acid in 800 ml dH₂O. Add 40 ml 0.5 M Na₂EDTA (pH 8.0). Adjust volume to 1 l. Store at room temperature.
7. Agarose gel electrophoresis tank, gel-forming tray, and powerpack

Agarose Gel Electrophoresis

1. 2 % agarose gel: 4 g Ultrapure Agarose, 200 ml 1× TBE buffer
2. Ethidium bromide/Midori green or alternative

Table 1

HLA DRB1 primer sequences. DR2 has been split to DR15 and 16, DR5 to DR11 and 12, and DR6 to DR13 and 14

Mix No.	Gene	Sequence (5'–3')
1	DR1F DR1R1 DR1R2	TTG TGG CAG CTT AAG TTT GAA T CTG CAC TGT GAA GCT CTC AC CTG CAC TGT GAA GCT CTC CA
2	DR15F DR15R	TCC TGT GGC AGC CTA AGA G CCG CGC CTG CTC CAG GAT
3	DR16F DR16R	TCC TGT GGC AGC CTA AGA G AGG TGT CCA CCG CGG CG
4	DR3F DR3R	GTT TCT TGG AGT ACT CTA CGT C TGC AGT AGT TGT CCA CCC G

(continued)

Table 1
(continued)

Mix No.	Gene	Sequence (5'–3')
5	DR4F DR4R1 DR4R2	GTT TCT TGG AGC AGG TTA AAC A CTG CAC TGT GAA GCT CTC AC CTG CAC TGT GAA GCT CTC CA
6	DR11F DR11R2	GTT TCT TGG AGT ACT CTA CGT C CTG GCT GTT CCA GTA CTC CT
7	DR12F DR12R	AGT ACT CTA CGG GTG AGT GTT CAC TGT GAA GCT CTC CAC AG
8	DR13F1 DR13F2 DR13R1 DR13R2 DR13R3	TAC TTC CAT AAC CAG GAG GAG A GTT TCT TGG AGT ACT CTA CGT C CCC GCT CGT CTT CCA GGA T TGT TCC AGT ACT CGG CGC T CCC GCC TGT CTT CCA GGA A
9	DR14F1 DR14F2 DR14R	GTT TCT TGG AGT ACT CTA CGT C AGT ACT CTA CGG GTG AGT GTT TCT GCA ATA GGT GTC CAC CT
10	DR7F DR7R	CCT GTG GCA GGG TAA GTA TA CCC GTA GTT GTG TCT GCA CAC
11	DR8F DR8R DR8R	AGT ACT CTA CGG GTG AGT GTT TGT TCC AGT ACT CGG CGC T GCT GTT CCA GTA CTC GGC AT
12	DR9F DR9R	GTT TCT TGA AGC AGG ATA AGT TT CCC GTA GTT GTG TCT GCA CAC
13	DR10F DR10R	CGG TTG CTG GAA AGA CGC G CTG CAC TGT GAA GCT CTC AC

Table 2
HLA DQB1 primer sequences

Mix No.	Gene	Sequence (5'–3')
1	DQB1*02 F DQB1*02 R	GTGCGTCTTGTGAGCAGAAG GTAGTTGTGTCTGCACACCC
2	DQB1*0201 F DQB1*0201 R	GTCCGGTGGTTTCGGAATGA TGCTCTGGGCAGATTCAGAT
3	DQB1*0301/0304 F DQB1*0301/0304 R	GACGGAGCGCGTGCGTTA CGTGCGGAGCTCCAACCTG
4	DQB1*0304 F DQB1*0304 R	TTTCGTGCTCCAGTTTAAGGC TGGCTGTTCCAGTACTCGGCGG

(continued)

Table 2
(continued)

Mix No.	Gene	Sequence (5'–3')
5	DQB1*0302 F DQB1*0302 R	GTGCGTCTTGTGACCAGATA CTGTTCCAGTACTCGGCGG
6	DQB1*0307 F DQB1*0307 R	CCCGCAGAGGATTTTCGTGTA CCCCAGCGGCGTCACCA
7	DQB1*0303 F DQB1*0303 R	GACCGAGCGCGTGCGTCT CTGTTCCAGTACTCGGCGT
8	DQB1*0305 F DQB1*0305 R	GCTACTTCACCAACGGGACC TGCACACCGTGTCCAATC
9	DQB1*0401/0402 F DQB1*0401/0402 R	CTACTTCACCAACGGGACC TGGTAGTTGTGTCTGCATACG
10	DQB1*0501 DQB1*0501	ACGGAGCGCGTGCGGGG GCTGTTCCAGTACTCGGCAA
11	DQB1*0502 DQB1*0502	TGCGGGGTGTGACCAGAC TGTTCCAGTACTCGGCGCT
12	DQB1*0503 DQB1*0503	TGCGGGGTGTGACCAGAC GCGGCGTCACCGCCCGA
13	DQB1*0601 DQB1*0601	TTTCGTGCTCCAGTTTAAGGC CCGCGGAACGCCAGCTC
14	DQB1*0602/10/13 DQB1*0602/10/13	CCCGCAGAGGATTTTCGTGTT CCTGCGGCGTCACCGCG
15	DQB1*0603/7 DQB1*0603/7	GGAGCGCGTGCGTCTTGTA GCTGTTCCAGTACTCGGCAT
16	DQB1*0603/8/12 DQB1*0603/8/12	GGAGCGCGTGCGTCTTGTA AACTCCGCCCGGGTCCC
17	DQB1*0603-05 0607-09 0612 DQB1*0603-05 0607-09 0612	GGAGCGCGTGCGTCTTGTA TGCACACCGTGTCCAATC

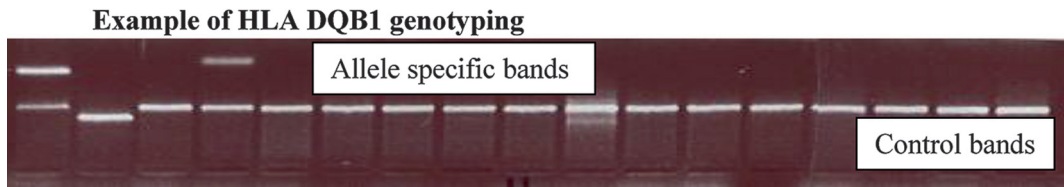


Fig. 1 A typical example of an HLA DQB1 genotyping result. In this case the sample is positive for *HLA DQB1*02* (**0201*) and *DQB1*0302*. Note that in lane 2 the target *HLA DQB1*0201* allele is the larger band observed. The control band set used in this experiment is Human growth hormone; F5'CCAGCTCAAGGATCCCAA and R5' CACCCATTACCCAAGAGCTTA

3 Methods

3.1 Set Up PCR Reaction

1. Label a 96-well plate for the number of reactions to be carried out; for instance a full HLA *DRB1* type requires 14 reactions per sample.
2. Following the worksheet provided in Table 3; briefly add the DNA (**Note 1**) to the appropriate well. Ensure that positive and negative (dH₂O) controls are included.
3. Make up a “cocktail” of all other reagents for the required number of reactions

Table 3
Work sheet for typing HLA class II DRB1 and DQB1

PCR mix	DNA (20 ng/μl)/whole-genome-amplified DNA		
	Per reaction (10 μl)	Per x reactions	
		DR (×16 to include controls)	DQB (×20 to include controls)
GoTaq G2 Green buffer	2	32	40
dNTP's 1 mM	0.3	4.8	6
25 mM MgCl ₂	0.6	9.6	12
DNA	0.6	9.6	12
dH ₂ O	1.45	23.2	29
GoTaq G2 5 U/μl HLA	0.05	0.8	1
Primer (F&R) + control (FdR)	5		
<i>Samples to type</i>	<i>Comments</i>	<i>Samples to type</i>	<i>Comments</i>
1		13	
2		14	
3		15	
4		16	
5		17	
6		18	
7		19	
8		20	
9		21	
10		22	
11		23	
12		24	

- 4. Vortex to mix
- 5. Add the appropriate volume of “cocktail” to each well.
- 6. Seal plate
- 7. Spin down in an appropriate centrifuge or gently tap the plate to ensure that all reagents are mixed.

3.2 Thermal Cycler Program

Select the following touchdown PCR program on the thermal cycler

Steps	Temperature (°C)	Time (sec.)	Action
1 cycle	96	60	Denaturation
5 cycles	96	25	Denaturation
	70	50	Annealing
	72	45	Extension
21 cycles	96	25	Denaturation
	65	50	Annealing
	72	45	Extension
4 cycles	96	25	Denaturation
	55	60	Annealing
	72	120	Extension
Hold	4	Specify time	

3.3 Agarose Gel Electrophoresis

- 1. Weigh out 4 g of agarose into a conical flask. Add 200 mL of 1× TBE, and swirl to mix (**Notes 3 and 4**).
- 2. Microwave for about 2 min to completely dissolve the agarose. Stop after 45 s to give agarose mix a swirl taking precautions not to allow agarose to boil over causing a burn.
- 3. Ensure the agarose is completely dissolved. If not replace in the microwave until dissolved monitoring carefully.
- 4. Cool the agarose by swirling the conical flask under a running cold tap until the gel is about 60 °C.
- 5. Add 2 µL of ethidium bromide (10 mg/mL) or alternative DNA stain for instance Midori green and swirl to mix (**Note 5**).
- 6. Pour the gel into a pre-prepared gel tank with appropriate sized gel combs on a level surface.
- 7. Allow to set for 1 h.
- 8. Pour 1× TBE buffer into the gel tank to submerge the gel (**Note 6**).
- 9. Remove combs ensuring that wells are well formed
- 10. Pipette 10 µl of PCR product into the gel and run at 100–115 V for approximately an hour (**Note 7**).
- 11. Visualise the gel on an appropriate gel documentation system.

4 Notes

1. This method works well on DNA from tissues or whole-genome-amplified DNA but ensure that whole-genome-amplified DNA is appropriately diluted.
2. Go Taq (Promega) has been used in this protocol but other enzymes should also work. This enzyme comes with a coloured buffer with sufficient density that a gel loading dye is not required. An alternative to this addition of sucrose creosol red which acts as a density agent for gel electrophoresis but can be added to the PCR reaction without any negative affect. This saves time adding a loading buffer for electrophoresis. The recipe is as follows: 100 mM Creosol Red dye: dissolve 0.4 g creosol red in 10 ml ddH₂O, vortex to mix. Before setting up the PCR reaction a loading dye can be generated as follows: 60 % Sucrose/1 mM Creosol Red (50 ml): Dissolve 30 g sucrose in 50 ml autoclaved deionized H₂O, add 500 µl of 100 mM creosol red, vortex to mix. Aliquot 1 ml into 1.5 ml Eppendorfs, and store at −20 °C. Add 1.5 µl during the setup of a 10 µl reaction
3. Use a large container, as long as it fits in the microwave, because the agarose boils over easily.
4. The volume of gel can be scaled up depending on the number of samples to be analyzed and the gel equipment available.
5. Ethidium bromide is mutagenic and should be handled with caution. Contaminated tips can be disposed of in a dedicated ethidium bromide waste container. Alternative DNA stains are increasingly used.
6. The gel must be run in the same buffer as used to make up the gel.
7. DNA is negatively charged and will run towards the anode.

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