Brief communication

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Novel associations among HLA-DQA1 and -DQB1 alleles, revealed by high-resolution sequence-based typing (SBT)

Key words:
linkage disequilibrium; HLA-DQA1 and DQB1; PCR-SBT; PCR-SSP

Acknowledgments:
We thank Nadine Botta and Thomas Wiley for their help with manuscript preparation. We also thank Dr. Marco Colonna, Basel Institute for Immunology, for helpful suggestions. This study was supported by funds from PF "C.N.R. Target Project on Biotechnology" 1998, Ministero della Sanita' R.F. 1997 e 1998, and from MURST National Research Programs 1998.

Abstract: Althought it is a valuable tool for the identification of HLA alleles, sequence-based typing (SBT) presents difficulties when used to determine HLA-DQA1 and -DQB1 alleles. Specifically, some HLA-DQA1 alleles have a three-base deletion at codon 56 of exon 2 that interferes with the sequencing read. Moreover, the frequently used primers for HLA-DQB1 may co-amplify the HLA-DQB2 pseudogene. To overcome these problems, we amplified DQA1 exon 2 using five group-specific polymerase chain reactions (PCRs) which allowed separation of deleted from non-deleted DQA1 alleles. DQB1 exon 2 was amplified using two group-specific amplifications. To increase typing resolution, we also analyzed DQA1 exons 1, 3 and 4 and DQB1 exon 3 by PCR using sequence-specific primers (PCR-SSP) or SBT analysis. Using this method we found some important associations between DQA1 and DQB1 alleles: DQA1*05011 and DQB1*0201, DQA1*0505 and DQB1*03011, DQA1*01021 and DQB1*06, DQA1*01022 and DQB1*0502.

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HLA-DQA1 and DQB1 molecules are involved in a number of pathological conditions, chief of which are susceptibility to autoimmune diseases, graft rejection and graft vs. host reaction after bone marrow transplantation (BMT) (1). As a consequence, it is imperative to obtain a complete and accurate typing of HLA-DQA1 and HLA-DQB1 gene polymorphisms. Sequence-based typing (SBT) would provide the ideal high-resolution approach for this purpose, but the method is hampered by several technical problems. First, the absence of a trinucleotide segment at codon 56 of exon 2 in a subset of 10 DQA1 alleles (deleted alleles), compared to the 10 other alleles (non-deleted alleles), causes strong interference in the sequence read (2). Second, the primers commonly used for HLA-DQB1 amplification frequently coamplify the HLA-DQB2 pseudogene (3). Third, typing of each HLA-DQ exon requires a separate amplification, since HLA-DQ exons are separated by large introns (4) which preclude the amplification of several exons with one primer pair. In an attempt to improve the SBT of DQA, we amplified exon 2 of DQA1 with five group-specific polymerase chain reaction (PCR) primer

Received 2 July, revised,
accepted for publication 6 December 1999

Tissue Antigens . ISSN 0001-2815

Tissue Antigens 2000: **55**: 275–279
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pairs (QA1 to QA5), to separate the deleted alleles from the non-deleted alleles, and subsequently performed sequencing reactions in both orientations. This method was tested on 30 Epstein-Barr virus (EBV) cell lines, 88 samples from the "Centro Ricerche Immunoematologiche AVIS" (Bergamo, Italy) and 32 samples from UCLA (Los Angeles, CA, USA), that allows the identification of all known 20 HLA-DQA1 alleles, with the exception of the following groups: DQA1*0101, DQA1*0104 and DQA1*0105 alleles that are distinguished in exon 1 and 4; DQA1*03011, DQA1*0302 and DQA1*0303 alleles that require typing of exons 1 and 3; DQA1*01021 and DQA1*01022, distinguished in exon 3; DQA1*05012 that differs from DQA1*05011/0502/0503/0505 group at codon 8 of exon 2, before our 5′ primer; DQA1*0505, previously reported as DQA1*05013, differing from DQA1*0501 alleles in exon 1 and 3. Typing of these alleles also required analysis of exons 1, 3 and 4 (see Figs. 1 and 2) by one PCR-sequence-specific primer (PCR-SSP) reaction or one PCR amplification followed by two sequencing reactions. It is noteworthy that while all 94 DQA1*05 alleles in our panel were previously typed as DQA1*0501 by PCR-SSP kit (Dynal, Oslo, Norway), our approach revealed that 65 of these were DQA1*0505, and that only 29 were true DQA1*0501 (27 of which were DQA1*05011 positives and two showed the DQA1*05012 allele). The analysis of exon 3, by additional PCR-SSP (Fig. 3) or by SBT, revealed that out of 44 DQA1*0102 samples, 28 were DQA1*01021, 17 DQA1*01022 and one DQA1*01021/01022, while these samples were previously typed as DQA1*0102 by Dynal kit. No ambiguous heterozygous combinations of DQA1 alleles were detected in our samples. To improve the SBT of DQB, exon 2 of the abovementioned samples, previously typed by PCR-SSP (Dynal kits) and serology, was amplified with two pairs of group-specific primers, that divide the DQB1 alleles into two groups, designated as QB1 (DQB1*05/06) and QB2 (DQB1*02/03/04) (Fig. 4). The SBT approach to HLA-DQB1 exon 2 was combined with either PCR-SSP subtyping or with the SBT of exon 3 (Fig. 5) in order to distinguish between DQB1*0201 vs. 0202, DQB1*06011 vs. 06013 and DQB1*03011 vs. 0309. Our findings showed that all DQB1*0503 allele in our population were DQB1*05031, all DQB1*0601 were DQB1*06011 and that DQB1*0301 was only DQB1*03011. Furthermore, distinction of DQB1*03011 from DQB1*0309 confirmed the presence of only the DQB1*03011 in our population. Last generation SSP kits for DQB1 typing cannot distinguish the DQB1*0302 from the DQB1*0308 allele. Utilizing the method described here, we confirmed the DQB1*0302 positivity of our samples. Finally, the DQB1*03033 allele was not found in our samples, confirming that our individuals were DQB1*03032.

Only one heterozygous combination found in our panel, the DQB1*0301/0302=DQB1*0303/0304, was then resolved by a PCR-SSP. Importantly, our results revealed a strong association between the DQA1*05011 and DQA1*05012 alleles with the DQB1*0201, in DRB1*03011-positive samples. In addition, the DQA1*0505 allele displayed a strong association with the DQB1*03011 allele in Drw52-positive, especially DRB1*11011, samples. Although the

Primer MixN°	Name	Primer location	Nucleotide Sequence	Name	Primer location	Nucleotide Sequence
QA1	DQA1O1/02/04F*#	Ex 2 aa. 4_11	5'-G.TGT.AAA.CTT.GTA.CCA.GT(C/T).TTA.C-3'	DQA1 01R *#	Ex 2 aa. 76_83	5'- G.TAG.AGT.TGT.AGC.GTT.TAA.TCA.T -3'
QA 2	DQA1O1/02/04F*#	Ex 2 aa. 4_11	5'-G.TGT.AAA.CTT.GTA.CCA.GT(C/T).TTA.C-3'	DQA1del R *#	Ex 2 aa. 76_83	5'- GT.GAG.TTG.GAG.CGT.TTA.ATC.AG -3'
QA 3	DQA1 03F*#	Ex 2 aa. 4_11	5'- G.TGT.AAA.CTT.GTA.CCA.GTC.TTA.T-3'	DQA103R *#	Ex 2 aa. 76_83	5'- GT.GAG.TTG.GAG.CGT.TTA.ATC.AC -3'
QA 4	DQA1O1/02/04F*	Ex 2 aa. 4_11	5'-G.TGT.AAA.CTT.GTA.CCA.GT(C/T).TTAC-3'	DQA10401R *	Ex2aa. 83_intr2	5'- ACA.TAC.CAT.TGG.TAG.CAG.CA -3'
QA 5	DQA1O1/02/04F*	Ex 2 aa. 4_11	5'-G.TGT.AAA.CTT.GTA.CCA.GT(C/T).TTAC-3'	DQA1 no04R *	Ex2aa. 83_intr2	5'- CAT.ACC.ATT.GGT.AGC.AGC.G -3'
EX 1	1DQA1 F*#	Ex 1 aa.-26_-20	5'- GGG.AAG.AGG.ATG.ATC.CTA.AAC-3'	1DQA1 R *#	Ex 1aa. 3_intr 1	5'- TCA.TGC.ACT.CAC.CCA.CAA.T -3'
EX 3	3DQA1 F*#	Ex 3 aa.93_99	5'- ACA.GTG.TTT.TCC.AAG.TCT.CCC-3'	3DQA1 R *#	Ex3aa.177_intr3	5'- TCC.TTA.CCC.CAG.TGT.TTC.AGA -3'
EX 4	4DQA1 F*#	Ex4aa.intr3_186	5'- CCA.CAG.AGC.CTG.AGA.TTC.CA-3'	4DQA1 R *#	Ex 4aa.218_224	5'- ACC.AAC.TGA.ACG.CAG.GCC.T -3'

Fig. 1. **Samples underwent five PCRs: four PCRs were performed with the DQA1*01/02/04/05/06 forward primer and one of the following group-specific 3′ primers: DQA1*O1+ reverse (QA1 group), DQA1*02/0401/05/06+ reverse (QA2 group), DQA1*0401+ reverse (QA4 group), DQA1*not 04 reverse (QA5 group). A fifth amplification was performed with the DQA1*03+ forward primer and the DQA1*03+ reverse primer (QA3 group).** The choice of the primers that allow amplification of deleted and not-deleted DQA1 alleles is limited to particular regions at the beginning and at the end of exon 2. Primers were designed to be completely matched with the common nucleotide sequences of the exon 2 regions specific to each group. In fact, the QA4 mix (which reveals the DQA1*0401 allele), combined with the QA2 mix (positive for the DQA1*02,0401,05,06 alleles) gave us the final typing for the relative allele; the QA5 mix was performed only to check the good functionality of the two specific reverse primers (DQA1*0401 rev and DQA1*no04 rev) and to distinguish homozygous (DQA1*0401=QA2+QA4) from heterozygous (DQA1*0401/02,05,06=QA2+QA4+QA5) individuals. Exon 1, 3 and 4 SBT primers were selected to completely sequence the HLA-DQA1 locus. The PCR cycle profile for each locus (HLA-DQA1 and -DQB1) and exon (exons 1–4) was the same: 30 amplification cycles were carried out in a GeneAmp 9600 with 10 min at 95°C, followed by 30 cycles at 96°C for 20 s, 60°C for 30 s and 72°C for 1 min. * HLA-DQA1 PCR primers, # HLA-DQA1 sequencing primers.

Name	Primer location	Nucleotide Sequence	Name	Primer location	Nucleotide Sequence
A500(DRA1Ex 2F)	Int.Control	5'ACC.TGT.CAC.CAC.AGG.AGT.GTC-3'	CTR2(DRA1Ex2R)	Int Control	5'-CAG.ACC.CAC.AGT.CAG.GCC.C-3'
DQA1:					
0104/0105 F	Ex1aa.-13_-7	5'-GCC.CTC.GCT.CTG.ACC.ACC.A -3'	DQA1IntRev	Intron1	5'-AGT.GGT.TGG.GGC.TCT.GGT.TT -3'
0101 F	Ex1aa.-13_-7	5'-CCC.TCG.CTC.TGA.CCA.CCG -3'	DQA1IntRev	Intron1	5'-AGT.GGT.TGG.GGC.TCT.GGT.TT -3'
DQA1 0104 F	Ex4aa. 193_197	5'-CAC.AGA.GAC.TGT.GGT.CTG.CA -3'	DQA1 0104/5R	Ex4aa.219_224	5'-ACC.AAC.TGA.ACG.CAG.GCC.TT -3'
DQA1 0105 F	Ex4aa.193_197	5'-ACA.GAG.ACT.GTG.GTC.TGC.G-3'	DQA1 0104/5R	Ex4aa.219_224	5'-ACC.AAC.TGA.ACG.CAG.GCC.TT -3'
0302 F	Ex1aa.-12_- 6	5'-TCG.CCC.TGA.CCA.CCG.TGA.T -3'	DQA1 Int Rev	Intron1	5'-AGT.GGT.TGG.GGC.TCT.GGT.TT -3'
0301/03F	Ex1aa.-12_- 6	5'-CGC.CCT.GAC.CAC.CGT.GAC-3'	DQA1 Int Rev	Intron1	5'-AGT.GGT.TGG.GGC.TCT.GGT.TT -3'
3DQA1 F	Ex3aa.93_99	5'-ACA.GTG.TTT.TCC.AAG.TCT.CCC-3'	0301 R	Ex3 aa.160_166	5'-TTG.CTG.TCA.TAA.ATC.TCA.TCA.G -3'
3DQA1 F	Ex3aa.93_99	5'-ACA.GTG.TTT.TCC.AAG.TCT.CCC-3'	0302 R	Ex3 aa.160_166	5'-CTT.GCT.GTC.ATA.AAT.CTC.ATC.AT -3'
DQA1 0505 F	Ex1aa- 20_-13	5'-ACA.AAG.CTC.TGA.TGC.TGG.GGA -3'	DQA1 Int Rev	Intron1	5'-AGT.GGT.TGG.GGC.TCT.GGT.TT -3'
DQA1 05011F	Ex1aa- 20_-13	5'-ACA.AAG.CTC.TGA.TGC.TGG.GGG-3'	DQA1 Int Rev	Intron1	5'-AGT.GGT.TGG.GGC.TCT.GGT.TT -3'
3DQA1 F	Ex3aa.93_99	5'-ACA.GTG.TTT.TCC.AAG.TCT.CCC-3'	05011 Ex3 R	Ex3aa.172_177	5'-TCA.GAA.GAG.GCT.TGT.CCA.GGG -3'
3DQA1 F	Ex3aa.93_99	5'-ACA.GTG.TTT.TCC.AAG.TCT.CCC-3'	0505 Ex3 R	Ex3aa.172_177	5'-TTC.AGA.AGA.GGC.TTG.TCC.AGT -3'
DQA1*05011	Ex2intr1_8	5'-CTC.TTT.CAG.CTG.ACC.ACG.TC-3'	DQA1*del R	Ex 2 aa. 76_83	5'-GTG.AGT.TGG.AGC.GTT.TAA.TCA.G-3'
DQA1*05012	Ex2intr1_8	5'-ACT.CTT.TCA.GCT.GAC.CAC.GTT -3'	DQA1*del R	Ex 2 aa. 76_83	5'-GTG.AGT.TGG.AGC.GTT.TAA.TCA.G-3'
01021 F	Ex3aa.103_107	5'-GTC.AGC.CCA.ACA.CCC.TCA.TT -3'	3DQA1 R	Ex3aa.177_intr3	5'-TCC.TTA.CCC.CAG.TGT.TTC.AGA -3'
01022 F	Ex3aa.103_107	5'-GTC.AGC.CCA.ACA.CCC.TCA.TC-3'	3DQA1 R	Ex3aa.177_intr3	5'-TCC.TTA.CCC.CAG.TGT.TTC.AGA -3'
3DQA1 F	Ex3aa.93_99	5'-ACA.GTG.TTT.TCC.AAG.TCT.CCC-3'	0503 R	Ex3aa.160_167	5'-TGC.AGT.CAT.AAC.TCT.CCT.CAG.A-3'
3DQA1 F	Ex3aa.93_99	5'-ACA.GTG.TTT.TCC.AAG.TCT.CCC-3'	No0503 R	Ex3aa.160_167	5'-GCA.GTC.ATA.ACT.CTC.CTC.AGC-3'

Fig. 2. **The additional PCR-SSP subtyping of HLA-DQA1 exon 1, 3 and 4 analysis (alternative to the SBT analysis of these exons).** The PCR sequence-specific-primers for HLA-DQA1 locus are listed. Our SSP approach was performed as follows: each primer mix contained the specific primers and a pair of positive internal control primers, selected in the conservative regions of HLA-DRA1. The forward primer A500, matching codons 126–133, and the reverse primer CTR2, matching codons 197–203, amplified a 560-bp product. PCR reactions were performed following Dynal kit procedures. The PCR-SSP mixes gave rise to PCR band ranging from 90 to 300 bp, and the internal control, of 560 bp in length, was adopted to obviate misinterpretation of results.

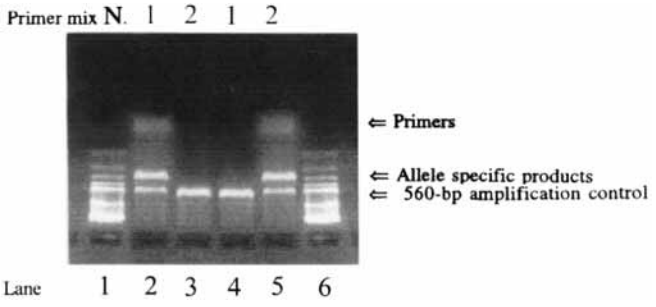

Fig. 3. **Example of additional PCR-SSP subtyping.** HLA-DQA1*01021 and -DQA1*01022 subtyping by PCR-SSP. The length of the allele-specific PCR products was 280 bp. Each PCR reaction included the positive control primer pair of A500 and CTR2, which gave rise to a 560-bp amplified fragment. The number of each lane that the primer mix corresponds to is the primer mix of interest (no. 1 corresponds to primer mix DQA1*01021F+3DQA1R and no. 2 corresponds to primer mix DQA1*01022F+3DQA1R). Amplified DNA samples were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide. PCR primer combinations and reaction specificities were as follows: two PCR reactions for these combinations of alleles were performed per sample (lanes 2 and 3 for the first sample; lanes 4 and 5 for the second sample). A molecular weight marker (100-bp ladder) was loaded in lanes 1 and 6. The first sample was DQA1*01021-positive, and the second was DQA1*01022-positive.

samples studied did not represent a random population, this last as yet undescribed, association (DQA1*0505/DQB1*03011), was extremely significant (*P*-value<0.0001) (Fig. 6) and well characterized in our population. Furthermore, our typings confirmed the previously reported (5) strong association between DQA1*0201/DQB1*0202 in DRB1*0701-positive samples, an association that has not been evaluated by many typing laboratories (6–9), but that have been found to be correlated with some important diseases (10). Our subtyping of DQA1*0102 also proved important, since the DQA1*01021 in our panel was found in association with DQB1*06(02, 04, 09), while the DQA1*01022 samples were found in association with the DQB1*0502 (Fig. 6). Finally, a number of important results emerged from the DQA1 and DQB1 sequence-based typing on homozygous cell panel: 1) the cell line 9047 resulted to

Mix N°	Name		Primer location	Nucleotide Sequence	Name or Definition		Primer location	Nucleotide Sequence
QB 1	DQB1PF2	@/#	Ex 2 aa.intr1_8	5'-CCC.CGC.AGA.GGA.TTT.CGT.G-3'	DQB5/6R2noc	@	Ex 2aa.89_94	5'-CTC.TCC.TCT.GCA.(A/G)GA.TCC.C-3'
QB 2	DQB1 PF2	@/#	Ex 2 aa.intr1_8	5'-CCC.CGC.AGA.GGA.TTT.CGT.G-3'	DQB2/3/4R2noc	@	Ex 2aa.89_94	5'-CTC.GCC.GCT.GCA.AGG.TCG.T -3'
	DQB 5/6R	#	Ex 2aa. 89_94	5'-TCT.CCT.CTG.CA(A/G).GAT.CCC3'	Sequencing primers			
	DQB 02/03/04R	#	Ex 2aa.86_91	5'-GTG.CGG.AGC.TCC.AAC.TG-3'	Sequencing primers			
EX 3	3DBQ1F	@/#	Ex 3. aa 95_ 101	5'-GTG.GAG.CCC.ACA.GTG.ACC.AT -3'	3DQB1R	@/#	Ex3aa.173_181	5'-GGT.GCT.CCA.CGT.GGC.AGG.T -3'

***Fig. 4.* The generic DQB1-Primer-Forward-Exon 2 (DQB1 PF2) was combined, alternatively, with the DQB1*05/06-specific reverse primer (QB1 group) and with the DQB1*02/03/04-specific reverse primer (QB2) in two distinct PCR reactions for the same sample, one for the identification of DQB1*05, 06 alleles and another for the identification of DQB1*02,03 and 04 alleles.** The heterozygous individuals DQB1*group1 and group2 can be easily identified by the two positive PCRs. PCR and sequencing primers for exon 3 are included with the same cycle profiles described in the legend to Fig. 1 @ HLA-DQB1 PCR primers, # HLA-DQB1 sequencing primers..

Name		Primer location	Nucleotide Sequence	Name or definition	Primer location	Nucleotide Sequence
A500(DRA1Ex 2F)		Int. Control	5'-ACC.TGT.CAC.CAC.AGG.AGT.GTC-3'	CTR2(DRA1Ex2R)	Int.Control	5'-CAG.ACC.CAC.AGT.CAG.GCC.C-3'
DQB1:						
0301/0304 F	A%	Ex2aa.7_13	5'-TTC.GTG.TAC.CAG.TTT.AAG.GC-3'	For ambiguous typing		
0302/03032 F	B	Ex2aa.7_13	5'-TTC.GTG.TAC.CAG.TTT.AAG.GG-3'	For ambiguous typing		
0302/0304 R	C	Ex2aa.57_63	5'-TGT.TCC.AGT.ACT.CGG.CGG-3'	For ambiguous typing		
0301/03032R	D	Ex2aa.57_63	5'-CTG.TTC.CAG.TAC.TCG.GCG.T -3'	For ambiguous typing		
DQB1* 02genF		Ex3aa.96_102	5'-AGC.CCA.CAG.TGA.CCA.TCT.C-3'	DQB1*0201 R	Ex3aa.135_141	5'-C.CAG.CTG.TCT.CCT.CCT.GGT -3'
DQB1* 02genF		Ex3aa.96_102	5'-AGC.CCA.CAG.TGA.CCA.TCT.C-3'	DQB1*0202 R	Ex3aa.135_141	5'-C.AGC.TGT.CTC.CTC.CTG.GC-3'
3DQB1F		Ex3aa 95_ 101	5'-GTG.GAG.CCC.ACA.GTG.ACC.AT -3'	DQB1*06011R	Ex3aa.137_144	5'-ACA.CAA.CGC.CAG.CTG.TCT.CC-3'
3DQB1F		Ex3aa 95_ 101	5'-GTG.GAG.CCC.ACA.GTG.ACC.AT -3'	DQB1*06013R	Ex3aa.137_143	5'-CAC.ACA.ACG.CCA.GCT.GTC.TCT-3'
3DQB1F		Ex3aa 95_ 101	5'-GTG.GAG.CCC.ACA.GTG.ACC.AT -3'	DQB1*03011	Ex3aa.169_175	5'-CAC.GTG.GCA.GGT.GTA.GAC.GT -3'
3DQB1F		Ex3aa 95_ 101	5'-GTG.GAG.CCC.ACA.GTG.ACC.AT -3'	DQB1*0309 ✒	Ex3aa.169_175	5'-AAC.GTG.GCA.GGT.GTA.GAC.GG-3'

***Fig. 5.* Additional PCR-SSP of HLA-DQB1 exon 3, as in the legend to Fig. 2.** % The forward and the reverse primers were used in four different primer mixes in order to amplify each allele of the ambiguous heterozygous combination. For example, A+C was used for the DQB1*0304 allele amplification , B+C for DQB1*0302, A+D for DQB1*0301 and finally B+D for DQB1*03032. ✒ The sequence of this reverse primer was modified at the 5′ end by adding an "A", instead of a "C" according to the consensus sequence, in order to arrange the TM of the primer with the TM of the DQB1*03011 PCR-SSP primer. The final typing results were not affected by this intentional modification.

DQA1	DQB1	% inclusion	p – value
01021	06 (02,04,09)	100	<0,0001
01022	0502	100	<0,0001
0201	0202	79	<0.0001
05011,05012	0201	100	<0,0001
0505	03011	100	<0,0001

***Fig. 6.* The strong association between some DQA1 and DQB1 alleles in our population.** Statistical analysis was performed using a Fisher's two-tailed exact probability test for 2×2 contingency tables, and introducing a 0.5 Yates' correction value.

be DQA1*0201/DQB1*0202 instead of DQB1*0201 as reported in previous literature (10); and 2) the cell lines 9016, 9038, 9043, 9178, 9200, 9201 and 9202, previously typed as either DQA1*05013 or DQA1*0501, were all DQA1*0505, in keeping with recent literature (11). The protocol for HLA-DQA1 and -DQB1 sequence-based typing described here provides a rapid and reliable method for the molecular characterization of the 20 HLA-DQA1 and 42 DQB1 variants thus far described (12). It allowed the high-resolution typing of our DQA1*05 samples, the majority of which, while previously included in the DQA1*0501 group, were re-typed as DQA1*0505,. Most importantly, the development of our SBT method allows the identification of previously undetected strong locus associations that could be relevant in correlating a given haplotype with HLA-associated diseases (13), and in finding potential unrelated donors for bone marrow registries.

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