Separation of *HLA-DRB* Alleles by Denaturing Gradient Gel Electrophoresis Using the DCode™ System

Knapp LA,[1,2] Lehmann E,[2] Hennes L,[3] Eberle ME,[2] and Watkins DI[2,3]
[1] Department of Biological Anthropology, University of Cambridge, U.K.,
[2] Wisconsin Regional Primate Research Center, University of Wisconsin,
[3] University of Wisconsin Histocompatibility Laboratory, University of Wisconsin Hospital and Clinics, Madison, Wisconsin, USA

Introduction

High-resolution *HLA-DRB* typing is required for bone marrow transplantation between unrelated donors and recipients and also for identification of novel *HLA-DRB* alleles. Here we describe a method for the separation of *HLA-DRB* alleles, following PCR amplification of the highly variable second exon of *HLA-DRB* alleles, using denaturing gradient gel electrophoresis (DGGE). When separation of *HLA-DRB* alleles is followed by direct sequencing, this technique provides a reliable, specific, and relatively rapid way of identifying all *HLA-DRB* alleles for high-resolution tissue typing.

Methods

Genomic DNA was extracted from peripheral blood or B-cell lines from four unrelated individuals. Two samples had been typed previously for *HLA-DRB* alleles using both PCR-SSCP and cloning and sequencing, and two samples had been characterized using cloning and sequencing techniques. Genomic DNA (30–40 ng) was amplified in 50 µl of 1x PCR buffer (pH 8.5), 1.5 mM $MgCl_2$, 2.5 mM of each of the four deoxyribonucleotide triphosphates (dGTP, dATP, dTTP, and dCTP), 25 pmol of each of the forward and GC-clamped (Sheffield et al. 1989) reverse primers (Knapp et al. 1997), and 1 U of *Taq* polymerase. Cycling conditions consisted of 30 rounds of 90 sec denaturation at 94°C, 90 sec annealing at 55°C, and 90 sec extension at 74°C.

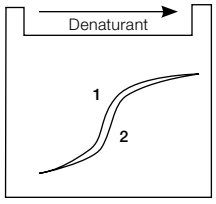
Optimal conditions for separation of *HLA-DRB* alleles using DGGE were established on a perpendicular denaturing gradient as described by Myers et al. (1988). Briefly, 100 µl of the GC-clamped PCR product was electrophoresed in a 12.6% acrylamide (37.5:1 acrylamide:bisacrylamide) gel with an increasing gradient from 0% to 80% denaturant (100% denaturant = 7 M urea and 40% formamide). The perpendicular DGGE was run in 1x TAE buffer at 60°C constant temperature. Samples were electrophoresed for 3.5 hr at a constant 300 V. Following electrophoresis, the perpendicular gel was silver-stained according to a method described by Bassam et al. (1991), and the optimum gradient conditions were identified. To separate *HLA-DRB* alleles, 30 µl of the GC-clamped PCR products were mixed with 15 µl of loading buffer and electrophoresed on a 40–65% parallel denaturing gradient gel according to the method described by Myers et al. (1988). As described for perpendicular DGGE, the 12.6% acrylamide gel was electrophoresed in 1x TAE buffer for 3.5 hr at a constant 300 V and at 60°C constant temperature. Individual bands on the parallel DGGE gels were visualized using SYBR Green stain and UV illumination.

Results and Discussion

A scheme for identifying *HLA-DRB* alleles using DGGE is shown in Figure 1.

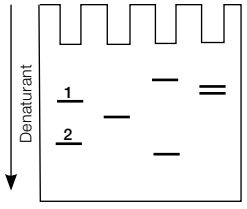
A. PCR with GC-clamped generic DRB primers

B. Separation of PCR products by perpendicular DGGE



Each band corresponds to a different allele

C. Separation of PCR products by parallel DGGE



Each band corresponds to a different allele

D. Reamplify with heminested primers 5' MDRBrM13 and 3' DRB1BM13 and/or 5' HDRBrM13 and 3' DRB1BM13 and sequence DNA eluted from DGGE gel

Fig. 1. Scheme for identifying *HLA-DRB* alleles using DGGE. A, the highly variable exon 2 of all *HLA-DRB* genes was amplified from genomic DNA using a GC-clamped primer; **B**, the optimal denaturing gradient for allele separation was determined using a perpendicular DGGE; **C**, alleles were separated by electrophoresis on the appropriate parallel denaturing gradient; **D**, gel plugs were removed, reamplified, and directly sequenced.

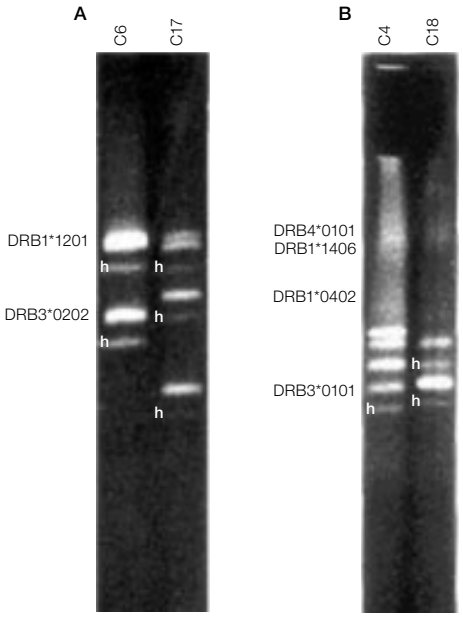


Fig. 2. Validation of the DGGE-based *HLA-DRB* typing technique (A) and DGGE separation of *HLA-DRB* alleles (B) in two unrelated individuals. **A**, 40–65% parallel denaturing gradient gel showing *HLA-DRB* typing results from two unrelated individuals (C6 and C17) previously characterized with PCR-SSCP and cloning and sequencing (see Table 1 for comparison of *HLA-DRB* typing results); **B**, 40–65% parallel denaturing gradient gel showing separation of two unrelated individuals (C4 and C18) previously typed by cloning and sequencing. A small white "h" is positioned to the left of each heteroduplex band.

Analysis of PCR-amplified *HLA-DRB* alleles by perpendicular DGGE revealed a single-domain melting structure when the GC clamp was positioned at the 3' side of the PCR product and suggested that the optimal denaturing gradient for allele separation ranged from 40–65%. Thus, our PCR products were electrophoresed on a 40–65% parallel denaturing gradient. Figures 2A and 2B demonstrate separation of *HLA-DRB* alleles on 40–65% parallel denaturing gradient gels.

To validate our DGGE separation method, we first analyzed samples from two unrelated individuals, for which PCR-SSCP and cloning and sequencing data were available. DGGE separated 4–6 bands from each individual (Figure 2A). Subsequent analysis of the two remaining individuals revealed up to 5 bands per individual (Figure 2B). Interestingly, each individual had a unique DGGE banding pattern. Moreover, when DGGE was combined with direct sequencing, we were able to identify all *HLA-DRB* alleles previously described using PCR-SSCP or cloning and sequencing. A comparison of results from three different typing methods is shown in Table 1.

High-resolution typing of *HLA-DRB* alleles generally relies upon labor-intensive cloning and sequencing techniques or PCR-SSCP; however, we rapidly and unambiguously identified 11 different class II *HLA-DRB* alleles in four unrelated individuals. Thus, separation of *HLA-DRB* alleles using DGGE followed by direct sequencing represents a significant improvement over traditional class II typing methods. Incorporation of a GC-clamped PCR primer theoretically allows separation of alleles that differ by a single nucleotide substitution. In the present study, DGGE resulted in physical separation of HLA-DRB1, -DRB3, and -DRB4 alleles. Notably, our results suggest that DGGE could also be used for rapid screening of HLA-DR subtype-identical unrelated bone marrow donor/recipient pairs. Specifically, when two individuals share the same combination of *HLA-DRB* alleles, they should also share the same banding patterns on gradient gels. Thus, the DGGE approach can be used for both *HLA-DRB* typing and screening.

Table 1. Comparison of *HLA-DRB* typing results.

ID	PCR-SSCP	Sequencing	DGGE[1]
C6	DR12	1*1201	1*1201
		3*0202	3*0202
C17	DR14	1*1406	4*0101
	DR4	1*0402	1*1406
			1*0402
			3*0101
C4	ND[2]	1*0301	6*0101
		1*1001	1*03011
		3*0202	1*1001
			3*0202
C18	ND	1*1302	1*1302
		3*0301	3*0301

[1] Bands reported according to position on DGGE gels in Figure 2A–B.
[2] ND, not done.

References

Bassam BJ et al., Fast and sensitive silver staining of DNA in polyacrylamide gels, Anal Biochem 196, 80–83 (1991)

Knapp LA et al., High-resolution HLA-DRB typing using denaturing gradient gel electrophoresis and direct sequencing, Tissue Antigens 50, 170–177 (1997)

Myers RM and Maniatis T, Recent advances in the development of methods for detecting single-base substitutions associated with human genetic diseases, Cold Spring Harb Symp Quant Biol 51, 275–84 (1986)

Myers RM et al., Detecting changes in DNA: Ribonuclease cleavage and denaturing gradient gel electrophoresis, pp 95–139 in Davies KE (ed), Genomic Analysis: A Practical Approach, IRL Press Ltd, Oxford (1988)

Sheffield VC et al., Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes, Proc Natl Acad Sci USA 86, 232–236 (1989)

Information in this tech note was current as of the date of writing (1998) and not necessarily the date this version (rev B, 2008) was published.

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *800 4BIORAD* ***Australia*** *61 02 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 21 3237 9400* ***Canada*** *905 364 3435* ***China*** *86 21 6426 0808* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84 0* ***Greece*** *30 210 777 4396* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 455 8800* ***India*** *91 124 4029300* ***Israel*** *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666* ***New Zealand*** *0508 805 500* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *061 717 95 55* ***Taiwan*** *886 2 2578 7189* ***United Kingdom*** *020 8328 2000*