

# Development of PCR-ELISA technique for determination of HLA DRB1\*01 group alleles

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## Abstract

We have developed an allotyping assay for detection of four HLA DRB1\*01 group alleles based on polymerase chain reaction and solution hybridization in a microtiter plate. Using group specific primers a region within exon 2 of HLA DRB1 gene was amplified by PCR. Labeling of PCR product was achieved by adding small amount of Dig-dUTP in place of dTTP. Labeled PCR product was hybridized to allele (HLA DRB1\*0101, \*0102, \*0103 and \*0104) specific and a group (HLA DRB1\*01) specific oligonucleotide probes in separate wells of the plate. Hybridized amplicones were detected by an enzymatic procedure. Ninety DNA samples were tested in parallel with PCR-SSP typing. The results were found to be well correlated by two methods. These results further suggest that, PCR-ELISA would be a rapid, specific and simple method that can be used for high resolution HLA typing before bone marrow and stem cell transplantation.

**Keywords:** HLA DRB1, PCR-ELISA, Hybridization, Group specific amplification

## INTRODUCTION

A number of PCR based methods for HLA class II typing has been developed in the past few years (Erlich *et al.*, 2001). PCR-sequence specific priming (Feolo *et al.*, 1999, Antony *et al.*, 1999, Davidson and Poulton, 2001) and sequence specific oligonucleotide probe hybridization are the most prevalent techniques that are being used for molecular HLA typing (Saiki *et al.*, 1986, Inamdar *et al.*, 1996, Williams *et al.*, 2002). In

situations such as bone marrow transplantation from unrelated individuals, a sensitive method with high resolution should be used (Begovich and Erlich, 1995 and Gerlach, 2002). In order to obtain such a high resolution, methods such as PCR-SSP or PCR-SSOP can be used but these procedures require large number of primers and probes. Designing such a large number of oligonucleotides with equivalent thermoregulation of annealing or hybridization is a challenge that is not easily overcome unless using toxic compounds such as tetramethylammonium chloride (TMAC) (Chen *et al.*, 1994). In this study attempts were made to benefit from the advantages of both SSP and SSOP procedures (Middleton *et al.*, 2000). For this purpose, HLA DRB1 alleles were divided to several allelic groups and pairs of primers were designed for amplification of each group alleles (Kawai *et al.*, 1996; Doxiadis and Claas, 2003). After amplification of each allelic group, we used oligonucleotide probes for determination of each allele. In this study the first allelic group (HLA DRB1\*01) was selected for group specific amplification and each of the alleles (\*0101, \*0102, \*0103, \*0104) were detected by corresponding allele specific probes.

## MATERIALS AND METHODS

**Isolation of DNA:** A modified salting out procedure using commercially available laundry detergent instead of proteinase K (Bahl and Pfeninger, 1996; Drabek and Petrek, 2002) used to isolate DNA from peripheral blood samples. This procedure is rapid, simple and inexpensive and can be used for DNA preparation in case of high-sample throughput such as HLA

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typing for epidemiologic studies. Briefly: 8 ml of lysis buffer (0.3 M sucrose, 0.01M Tris-HCl pH 7.5, 5 mM MgCl<sub>2</sub>, 1% Triton X100) was added to each tube containing 2 ml of whole blood. Tubes were centrifuged for 5 min at 2500×g. Supernatant was discarded and 300 µl of 10 mM Tris-HCl pH 8.0, was added to nuclear pellets. The pellet was released from the bottom of the tube and transferred quickly to a fresh microfuge tube. The sediment was resuspended by vigorously vortexing and tubes were centrifuged for 1 min at 700×g. After discarding of supernatant 330 µl of 10 mM Tris-HCl pH 8.0, 330 µl of laundry detergent (30 µg/ml) and a glass bead was added to each tube. Samples were vortexed for 1 min at maximum speed. 250 µl of 6M NaCl was added and tubes were vortexed again for 20 sec and then spun down for 5 min at 15000×g. The supernatant was transferred to fresh tubes (about 750 µl) and DNA precipitated by adding 75 µl of 96% ethanol. DNA precipitate was retrieved using a heat-sealed thin end glass pipette, washed twice in 0.5 ml 70% ethanol and finally dissolved in 100 µl of 10 mM Tris-HCl pH 8.0 The DNA was further dissolved by incubation at 70°C for 5 min.

**HLA DRB1 typing by PCR-SSP:** SSP typing of HLA DRB1 was performed using two commercially available kits (One Lambda, USA) one of which was generic for DRB1 locus and the other was allele specific for DRB1\*01 group. These kits could resolve HLA DRB1\*01 group alleles at allelic level.

**HLA DRB1\*01 typing by PCR-ELISA: PCR reaction:** Group-specific amplification of DRB1\*01 allelic group was performed for both known and unknown samples using primers specific for a region within exon2 of HLA DRB1 gene (Allen *et al.*, 1998). Forward primer (5'-TTTCTTGTGGCAGCT-

TAAGTTTGAA) at 3' terminal base is specific for this allelic group. Therefore, there is no amplification product from other alleles of DRB1 or other loci. In contrast, reverse primer (5'-ACTCGCCGCTGCACTGTGAAGCT) was common to all allelic groups and could be used in combination with specific forward primers for amplification of other allelic groups. For PCR amplification 0.1-0.25 µg genomic DNA, 0.5 µm of each primers, 0.2 mM each of the nucleotides, 1.5 mM MgCl<sub>2</sub>, 5 mM KCl, 10 mM Tris-HCl pH 8.3, 0.01 % 1 gelatin and 1 unit *Taq* polymerase was used in a final volume of 50 µl. The PCR was performed in a thermocycler for 35 cycles. The DNA was denatured at 94°C for 5 min prior to cycling. Each cycle consisted of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec. The last cycle was followed by an extension at 72°C for 5 min. To introduce a reporter molecule randomly at different positions of amplified fragments for subsequent detection digoxigenin-11-deoxyuridine-5-triphosphate (DIG-11-dUTP; Roche Applied Science) was used at proportion of 1 to 20 of dTTP nucleotides. Finally, PCR products were detected by 1 % agarose gel electrophoresis.

**Hybridization and ELISA detection:** Following hybridization of the probes with PCR products, the resultant assembly was detected by Dig detection kit (Roche Applied Science). For this purpose those samples that had PCR product in electrophoresis and confirmed to be included in HLA DRB1\*01 allelic group, were selected for ELISA detection. Hence, five allele specific oligonucleotide probes were designed, the sequence of one of which was complementary to a common region in all of this group alleles and other four were specific to \*0101, \*0102, \*0103 and \*0104 alleles (Table 1). For every assay, 5 µl of amplification

**Table 1.** Sequences of oligonucleotide probes used for detection of different alleles.

Probe name	Sequence	Specificity
HNM1	5'-Biotin-GGGTTGGTGAGAGCTTCA	*0101, *0103
HNS1	5'-Biotin-ACGGGGCTGTGGAGAG	*0102
HNN2	5'-Biotin-CTGGAAGACGAGCGGG	*0103
HNT1	5'-Biotin-GGGGTTGTGGAGAGCTT	*0104
HNG1	5'-Biotin-TGGAACAGCCAGAAGGAC	*0101, *0102, *0103, *0104

**Table 2.** Reactivity pattern for the standard alleles with specific probes used in this study. (Results are expressed as OD±SD).

Probe name	Alleles			
	*0101	*0102	*0103	*0104
HNMI	1.29±0.18	0.12±0.02	1.31±0.16	0.13±0.02
HNS1	0.11±0.2	1.32±0.19	0.13±0.03	0.12±0.02
HNN2	0.12±0.2	0.11±0.02	1.37±0.21	0.12±0.02
HNT1	0.12±0.2	0.12±0.02	0.13±0.02	1.33±0.22
HNG1	1.35±0.21	1.37±0.2	1.36±0.22	1.34±0.19

product was denatured and hybridized with biotin labeled capture probes (7.5 pmol/ml) in 200 µl hybridization buffer. Then aliquots were pipetted into streptavidin-coated microtiter plates, and subjected to hybridization. After hybridization for 1h, with three times washing at 37°C, specifically bound sequences were identified using horse radish peroxidase-labeled anti-digoxigenen antibody and 2,2'-azino-di-(3-ethyl)benzthiazoline-6-sulfonic acid (ABTS) solution as a substrate and the OD was measured at 405 nm.

**Improvement of hybridization and detection efficiency:** Although results obtained from early studies were satisfactory, we tried to increase the signals, detectable without using photometric instruments. Hence, another set of allele specific probes with sequences entirely similar to those of previous probes, but only had a polyT (10 thymidilate nucleotides) spacer between first nucleotide and biotin molecule at the 5'end were designed and used.

## RESULTS

Results of PCR-ELISA detection system for known standard samples of \*0101, \*0102, \*0103, and \*0104 alleles are shown in table 2. Each assay was repeated three times and the standard deviation was calculated. The result obtained from hybridization of these known alleles with the corresponding probes confirmed the validity of this assay system.

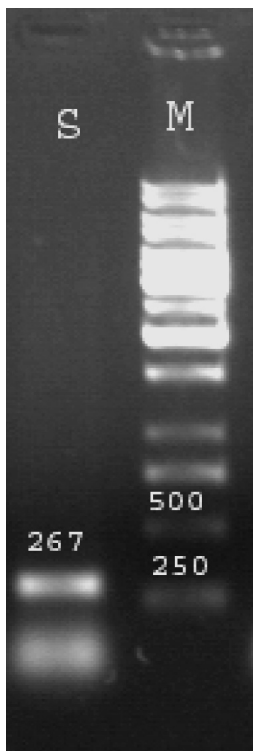
HLA DRB1 allelic type was determined in 90 blood samples by SSP method. Out of 90 samples, 9 samples were typed as HLA DRB1\*01 group, out of which 8 samples were \*0101 type and 1 case was \*0103 type.

PCR amplification with HLA DRB1\*01 group spe-

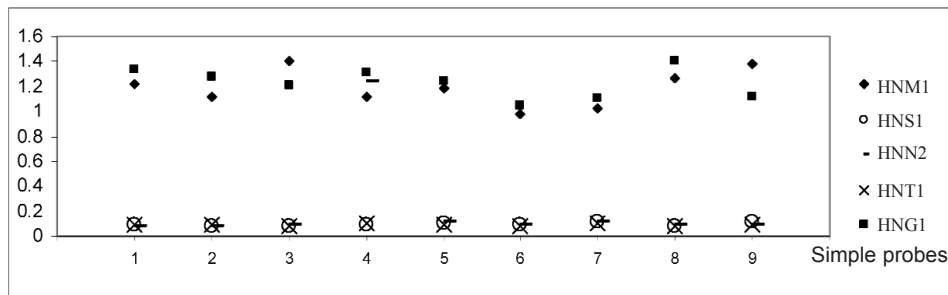
cific primers was performed on all samples and only those samples which were determined to be one of the DRB1\*01 group alleles by SSP, could be detected by gel electrophoresis (Fig. 1). PCR products from nine PCR positive samples were typed by hybridization and ELISA reaction using both sets of probes. The probes without spacer gave ODs ranging from 0.98 to 1.41 while with probes having polyT spacer ODs from 2.12 to 3.03 was recorded (Fig. 2 and 3). All negative reactions had ODs under 0.20. A clear differentiation between negative and positive reactions was possible. The mean OD for positive reactions by probes having spacer was 2.54 and for negative reactions it was 0.15. Control hybridization by group specific probe was clearly positive considering optical density as the cut off criteria.

## DISCUSSION

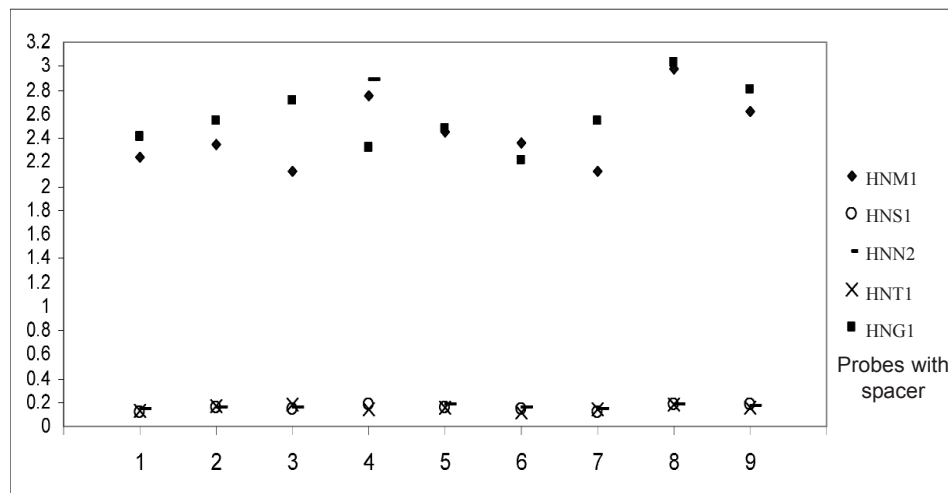
Different methods are currently being used for genetic typing of class II HLA. Some of these procedures are based on specific amplification of a fragment of the gene, so are called sequence specific priming. Based on this procedure, for detecting all alleles of a specific locus such as DRB1 for which more than 300 alleles have been identified, we require a large number of primers. Another procedure for detection of genetic polymorphism is based on sequence specific oligonucleotide probes (Kennedy *et al.*, 1995). High resolution HLA typing by SSOP method requires large number of probes and development of a common condition for hybridization of such a large number of probes makes it very difficult. However, combination of SSP and SSOP methods could overcome this problem. In this respect, we used group specific primers for ampli-



**Figure 1.** Gel electrophoresis of group specific PCR product. (M, Size marker S, PCR product of sample).



**Figure 2.** Test results and distribution of final OD values obtained from testing by simple probes (1 to 9: Samples that had PCR product in group-specific PCR reaction).



**Figure 3.** Test results and distribution of final OD values obtained from testing by probes having spacer (1 to 9: Samples which had PCR product in group specific PCR reaction).

fication of an allelic group and specific probes for identification of related alleles in that particular group. Thus we only used limited number of probes that were specific for the allelic group amplified by PCR.

Different materials are being used as a solid support for SSOP assay, but membranes are more commonly used (Bugawan *et al.*, 1994). Using membranes, there are several commercially available kits based on reverse hybridization. But no standard robotic equipment for handling and interpretation of results are available (Wagner *et al.*, 1998). It appears that, methods based on PCR-ELISA format are better suited for automated high sample throughput (Moribe *et al.*, 1999 and 2002).

Establishment of PCR-ELISA is usually associated with limitations. One of the major problems is designing suitable probes for detection of polymorphisms, which is due to the lack of freedom in the selection of a location within a gene for hybridization, specifically when it is considered to hybridize a large

number of probes simultaneously under the same condition. Although the assay used is fairly sensitive, however each probe must be individually hybridized to the amplified DNA, where many different polymorphisms occur. This process may become cumbersome since all probes must be hybridized specifically under identical condition. Thus different methods were used to increase sensitivity of detection to an extent that the results could be detected with unaided eyes. The major change that was introduced for this purpose was to design special probes that had a polynucleotide spacer linked to a solid support (Chou *et al.*, 2004 and Mike 2003). The specific sequences of these probes were identical to probes used previously but there were ten thymidilate nucleotides between the most 5' nucleotide of specific sequence and the biotin molecule which is attached to microtiter plate. Using these probes, results of the assay was simply detected by unaided eye (Fig. 3.). Therefore it is concluded that in cases of single nucleotide polymorphisms (SNPs) detection, especial-



ly molecular HLA typing, addition of a spacer at the attachment sites of probes, can be used to overcome problems related to positional limitation of probe designing which otherwise could lead to a very weak results.

In this study all of the data obtained from PCR-ELISA procedure were in accordance with results obtained by standard SSP method, suggesting that this assay is suitable for the molecular HLA typing.

In conclusion PCR-ELISA that enables allotyping of HLA DRB1\*01, can be applied for high resolution typing of all other allelic groups of this locus and other loci. This procedure is rapid, sensitive, specific, and simple to perform, and applicable for automation (Knight *et al.*, 1999). It can be used for “first round” typing of HLA genes on large number of samples in bone marrow registry centers (Hurley *et al.*, 1999, Anasetti *et al.*, 2001). This assay can also be used for high resolution HLA typing before transplantation from unrelated donors.

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