DNA TYPING OF HLA-B BY PCR WITH PRIMER MIXES UTILIZING SEQUENCE-SPECIFIC PRIMERS

Thesis

Presented to the Graduate Council of the

University of North Texas in Partial

Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Angela Chen-Yen Chiu, B.S.

Denton, Texas

August, 1997

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The aim of this study was to design a resolution typing system for the HLA-B gene. This technique involves a one-step PCR reaction utilizing genomic DNA and sequence-specific primers to determine the specificity of each allele and to produce a larger primer data base ideal for serological analysis. The application of this technique to serological analysis can improve serology detection which is currently hindered by antibody cross-reactivity and the unavailability of useful typing reagents.

With the increased availability of HLA-B Class I DNA sequence information, it has become feasible to genotype for Class I by polymerase chain reaction using sequence-specific primers. This study was based on available HLA nucleotide sequences which detect all serologically defined antigens in most heterozygous combinations in 36 one-step PCR reaction. This system has been designed for higher resolution than serology, and the same PCR conditions and controls are used allowing a single one-step SSP-PCR for all relevant HLA loci in under 4 hours in a system suitable for the typing of organ transplantation donors and recipients.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

I.1 The HLA System

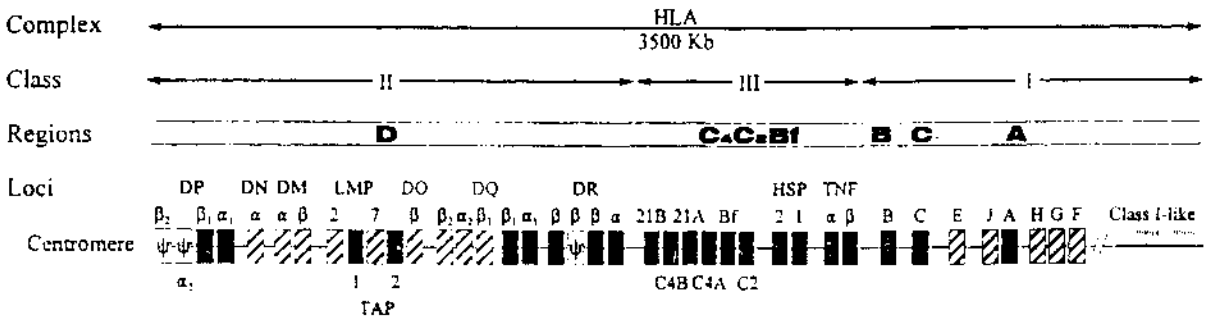
a. Historical Background

The Major Histocompatability Complex (MHC) is a chromosomal region comprising a series of genes that codes proteins involved in the presentation of antigens to thymus-derived (T) lymphocytes. These T-lymphocytic antigens are glycoproteins that are present on the surface of most nucleated cells. The MHC is a genetic locus found in every mammalian species that possess multiple closely linked genes whose products play a primary role in determining the success of allografts. The genes of the MHC are mapped to the H-2 region of the chromosome. The anthropomorphous genetic region called HLA (Human Leukocyte Antigens) is located on the short arm of chromosome 6. (Figure 1)

The HLA system was first described in Dausset's report of 1952 on the leukoaggulutinins in the serum of neutropenic patients. This provided the first clue to the existence of a polymorphic system of histocompatilibility antigens (Dausset,1954). These findings were extended by Payne, who confirmed the resistance of leukoagglutinating antibodies. He also demonstrated that these antibodies could be detected in the serum of multiparous woman (Payne and Rolfs,1958). In 1964, Bach and Hirschhorn introduced

Figure 1: Major Histocompatibility Complex of the Human Chromosome. The nomenclature, relative size of the loci, and general order of the gene devised by Trowsdale and Campbell.

Human chromosome 6



the mixed lymphocyte reaction, revealing HLA differences between individuals with otherwise indistinguishable serological speciicities(Bach and Hirchhorn,1964). Following the discovery of the first leukocyte antigens and the design of a suitable testing system, the number of defined serological specificities increased rapidly.

In 1964, an International Histocompatibility Workshop convened in Washington, D C and at Duke University in Durham, N.C. Cell panels and leukoagglutinating antibodies collected by each investigator were examined for congruence by a variety of serological techniques. During the late 1970's and early 1980's; several HLA genes were cloned employing molecular genetic techniques. The availability of these cDNA clones made it possible to study the restriction fragment length polymorphism (RFLP) of HLA genes (Bidwell and Bignon,1991). The RFLP techniques revealed genetic diversity in the major histocompatibility complex (MHC). This information provided the basis for the first molecular genetic HLA typing method. The development and utilization of the polymerase chain reaction (PCR) led to an explosion in the molecular characterization of HLA polymorphisms.

b. Genetics of HLA Complex (Class I and Class II Antigens)

By mediating the discrimination between "self" and "non-self", HLA molecules play a fundamental role in immunological responses (Shackelford and Strominger,1980). Class I and Class II genes encode cell surface antigens that perform a crucial role in "self"/ "non-self" recognition as well as in acceptance and rejection of allografts. The HLA Class I antigens are composed of a heavy chain coded within the HLA complex of

the Class I gene and a light chain, β-2-Microglobulin, encoded outside the MHC region of chromosome 15. The 43kDa. subunit is noncovalently associated with the 12kDa. β-2-Microglobulin. This region spans approximately 1,600 to 20,00kb.(Hardy *et al.*,1986) The HLA Class I region contains the genes encoding the classical HLA Class I antigens: HLA-A, HLA-B and HLA-C as well as the three non-classical HLA class I genes: HLA-E, HLA-F, and HLA-G.

The expressed Class I genes have similar genomic organization that includes eight exons. The first exon encodes the signal sequence. Exons 2, 3, and 4 encode the three major domains on the extracellular portion of the HLA molecule (α1, α2, and α3). Exon 5 encodes the transmembrane region, and exons 6-8 encode the cytoplasmic tail and 3'-untranslated regions (Rodriguez *et al.*,1989; Fernandez *et al.*,1990). The HLA Class II molecules are heterodimers which consist of two glycosylated polypeptide chains (the 31-33kDa. α polypeptide chain and 26-29kDa.β polypeptide chain) that are noncovalently bonded together when associated with the cell surface. Each of the chains have two external domains composed of a transmembrane component and an intra-cytoplasmic domain. The external domains are referred to as alpha-1 and alpha-2 on the alpha chain and beta 1 and beta 2 on the beta chain. Class II molecules are expressed primarily in cells of the immune system. The primary cells that constituentively express Class II MHC genes are the B and T lymphocytes, cells of the myelo-monocytic lineage, and various antigen-presenting cells found through out the body. Expression of Class II molecules occurs upon stimulation of human T lymphocytes by either antigen-MHC or by super-antigen. Class II alloantigens can induce a humoral immune response, and are

capable of provoking a cellular immune reaction. The cellular response initiated by Class I alloantigens is the mixed lymphocyte culture reaction (MLC). The MLC is an in vitro assay that depicts a correlation in the vivo delayed hypersensitivity type response (Beaty *et al.*,1991). The Class II HLA region of the MHC contains the HLA-DR, -DQ, and -DP genes and several additional Class II genes (Bodmer *et al.*,1991). Most of the Class II HLA genes are composed of six exons. Typically, the exon 1 encodes the signal sequence, exon 2 encodes the membrane-distal domain, exon 3 encodes the immunoglobulin-like domain and exons 4-6 encode the transmembrane domain and 3'-untranslated region.

c. HLA-A, -B, and -C Antigens

The antigens encoded by the three closely linked HLA-A, HLA-B, and HLA-C genes are structurally and functionally congenerous. The first significant contribution to the clarification of the molecular structure was made by Nakamuro *et al.* in 1973. The proteins of HLA-A, HLA-B, and HLA-C are proficient in eliciting strong antibody and cellular responses upon allogenic stimulation. In addition, HLA-A, HLA-B, and HLA-C molecules are known to serve as "restricting" elements for cytolytic T cells. These molecules precisely govern the activity of immune effector cells by presenting foreign peptide antigen bound to the Class I structure (1988) The regulation T cell activity represents the major mechanism for cellular recognition of viral and microbial proteins. The HLA-A, -B, and -C external membrane proteins are composed of three domains of equal length, $\alpha 1$, $\alpha 2$, and $\alpha 3$ (Figure 2). All contain approximately 90 amino acid residues, and all show a high degree of homology to the immunoglobulin domain

(Strominger *et al.*,1977; Orr *et al.*,1979).

d. HLA Polymorphisms

Genetic polymorphism is the occurrence in a population of two or more genetically determined forms in reasonable frequencies such that the rarest of them could not be maintained by mutation alone. An allele is an alternate form of a gene. Multiple alleles have already been discovered for most of the antibody-defined HLA specificities. HLA polymorphisms were initially revealed by the detection of antibodies and T lymphocytes that recognize polymorphic differences between HLA molecules. The number of alleles is likely to expand tremendously during the coming years due to extensive sequencing studies in progress in numerous laboratories (Table 1). The polymorphism of HLA-A, -B, and -C loci located on exons 2 and 3 code for the membrane distal domains. Most molecular typing methods are able to detect polymorphisms in these exons. Current information suggests that HLA-B is the most polymorphic locus, implicating differences in function, mutation rate, or evolutionary pressure. (Bodmer *et al.*1994; Belich *et al.*,1992; Watkins *et al.*;1992)

Figure 2 : Schematic Representation of the Structure of the HLA-A, -B, and -C Antigen

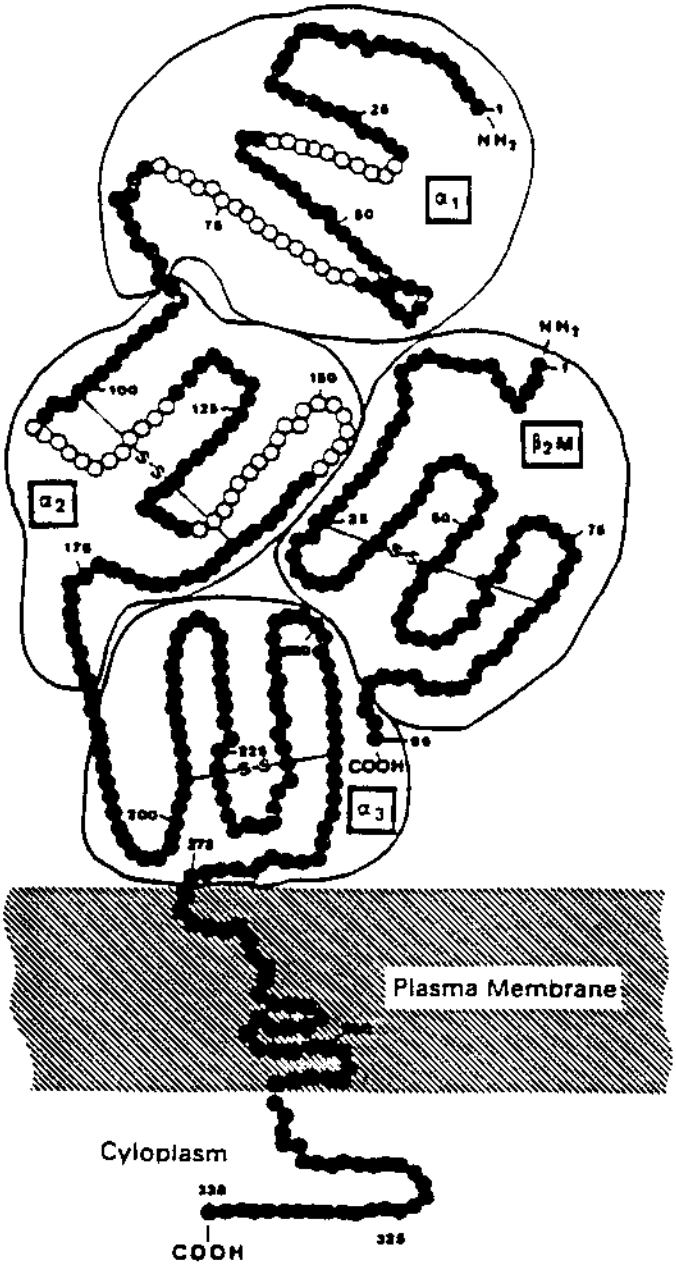

NH2
α1
β2M
α2
α3
COOH
Plasma Membrane
Cytoplasm
COOH

Figure 3: Generic and high resolution types for HLA-B

Locus	Generic Type	Higher Resolution Type(s)
HLA-A	01	0101,0102
	02	0201,0202,0203,0204,0205 0206,0207,0208,0209,0210, 0211,0212,0213,0214,0215N,0216,0217,0218,0219,0220,0221,0222
	03	0301,0302,0303N
	23	2301
	24	2402,2403,2404,2405,2406,2407,2408,2409N,2410,2411N,2413,2414
	25	2501,2502
	26	2601,2602,2603,2604,2605,2606,2607,2608
	34	3401,3402
	66	6601,6602,6603
	11	1101,1102,1103,1104
	29	2901,2902,2903
	30	3001,3002,3003,3004
	31	3101
	32	3201,3202
	33	3301,3302,3303
	74	7401,7402,7403
	68	6801,6802,6803
	69	6901
	36	3601
	43	4301
	80(8001)	8001
HLA-B	51	5101,5102,5103,5104,5105,5106,5107,5108,5109
	52	5201
	07	0702,0703,0704,0705,0706,0707,0708
	08	0801,0802,0803,0804
	44	4402,4403,4404,4405,4406,4407,4408,4409,4410
	45	4501
	13	1301,1302,1303,1304
	14	1401,1402
	15	1501,1502,1503,1504,1505,1506,1507,1508,1509,1510,1511,1512,1513,1514,1515, 1516,1517,1518,1519,1520,1521,1522,1523,1524,1525,1526N,1527,1528,1529,1530, 1531,1532,1533,1534,1535,1536,1537
	38	3801,3802
	39	3901,3902,3903,3904,3905,3906,3907,3908,3909,3910,3911
	57	5701,5702,5703,5704
	58	5801,5802,5803
	18	1801,1802,1803,1804,1805
	49	4901
	50	5001,5002
	54	5401
	55	5501,5502,5503,5504,5505
	56	5601,5602,5603
	27	2701,2702,2703,2704,2705,2706,2707,2708,2709,2710,2711
	35	3501,3502,3503,3504,3505,3506,3507,3508,3509,3510,3511,3512,3513,3514,3515,3516, 3517,3518,3519
	37	3701,3702
	40	4001,4002,4003,4004,4005,4006,4007,4008,4009,4010
	41	4101,4102
	42	4201,4202
	46	4601
	47	4701
	48	4801,4802,4803
	53	5301,5302
	59	5901
	67	6701
	73	7301
	78(7801)	7801,7802
	81(8101)	8101
	82	8201

1.2 HLA Typing

a. Serological typing

The classical method of performing HLA typing involves the use of a microcytotoxicity assay of human alloantisera containing specific alloantibodies (Teraski *et al.*,1978). Antibodies directed against HLA antigens can be derived from the following sources; sera of organ recipients, sera from volunteers injected with immunizing lymphocytes, and sera of pregnant women. In organ recipients, the antibodies are usually directed against the mismatched HLA antigens present on the transplanted organ. The sera of the volunteers injected with immunizing antibodies is most effective in generating specific antibodies of the best quality. However, there are ethical concerns that preclude this approach. The sera of pregnant women is screened for the antibodies that occur only during gestation.

These antibodies are screened in assays that involve the examination of thousands of samples to identify antibodies that meet standards for use in clinical studies. The criteria for these tests include 1) antibodies that occur at high titers, 2) antibodies that are able to fix rabbit complement, 3) antibodies that demonstrate specificity for a single, a limited number of alleles, and 4) antibodies that are likely to be available from the donor through repeated plasmapheresis.

Difficulties in identifying sufficient sources of high-quality antisera has prompted a number of laboratories to investigate the possibility of developing mouse anti-human HLA monoclonal antibodies. Although a substantial number of such antibodies have been produced, monospecific monoclonal antibodies against every HLA antigen are not

readily available.

The limitations of conventional HLA typing have led to a substantial effort to develop alternative HLA typing methods. The first widely used molecular genetic HLA typing method was based on RFLP of HLA genes. (Bidwell and Bignon,1991) RFLP typing involves the digestion of genomic DNA with a panel of restriction enzymes and the detection of particular gene fragments by hybridization with cDNA. After the discovery of the PCR, this methods was quickly modified to take advantage of substantial improvements that are achieved through the use of selectively amplified target DNA (Hammerling *et al.*,1993; Baxter-Lowe *et al.*,1989).

b. DNA-Based HLA Typing Molecular Typing by Restriction Fragment Length Polymorphisms (RFLP) Analysis

Restriction fragment length polymorphism (RFLP) analysis is a molecular typing approach used to study HLA Class II antigens that was standardized during the 1987 International Histocompatibility Workshop (Parham *et al.*,1979) . The target molecule (e.g.; the second exon of HLA-DRB) is amplified by PCR, and the amplified product is digested with a series of restriction enzymes. Gel electrophoresis is used to separate the DNA fragments according to size. The DNA fragments are visualized with the aid of a stain such as Ethidium Bromide. PCR provides the target specificity that was achieved with cDNA probes in the original versions of RFLP typing (Figure 4). PCR-RFLP typing methods have been reported for HLA-DR, -DQ, and -DP typing, and many of these methods remain in use today (Yunis *et al.*, 1991; Vryu *et al.*, 1990; Maeda *et al.*, 1990).

Molecular typing by hybridization of Sequence-Specific Oligonucleotide Probe Hybridization (SSOP) to polymerase chain reaction (PCR)-amplified DNA is a direct method of detecting sequence polymorphism that is encoded by Class II genes. For this method, DNA is amplified by PCR using oligonucleotide primers designed to be complementary to flanking 5' and 3' sequences (Figure 5). SSOP can be accomplished by using many different formats. Many reports describe HLA typing methods that involve binding selectively amplified DNA to a solid support (e.g., membrane, 96-well tray, nylon strip) followed by detection of polymorphic sequences via hybridization to oligonucleotide probes (Vaughan *et al.*,1990; Molkentin *et al.* 1991).

One limitation of SSOP is a particular hybridization pattern can have more than one interpretation. This is evident when a locus-specific amplification is used to attempt a higher resolution typing. Polymorphic sequences that distinguish one allele from others in a group are often present in several other allele groups as well. SSOP is accurate, and reagents and methods are readily available. The drawback of this method time for panel of probes that can resolve the alleles.

Figure 4: Early RFLP Methods

Early RFLP methods (left) required the isolation of highly purified DNA, digested with restriction endonucleases, electrophoresis, Southern blot transfer of DNA to membranes, and hybridization with radioactiviely labeled cDNA probes. This process typically required several days. PCR-RFLP typing (right) can be completed in less than one day.

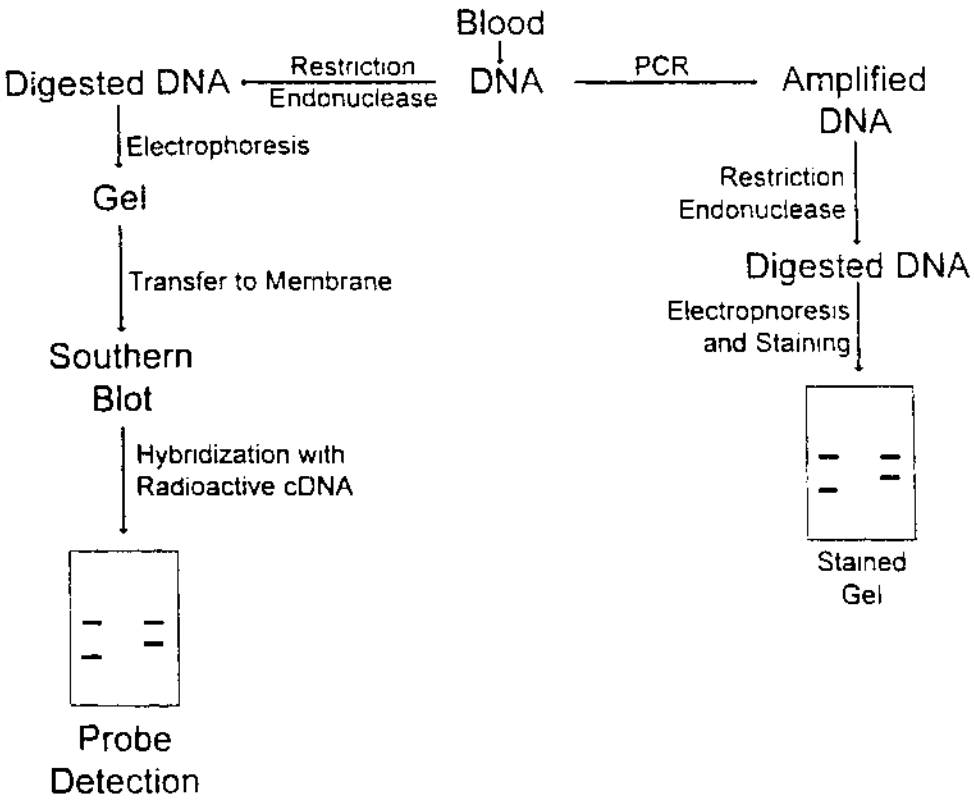
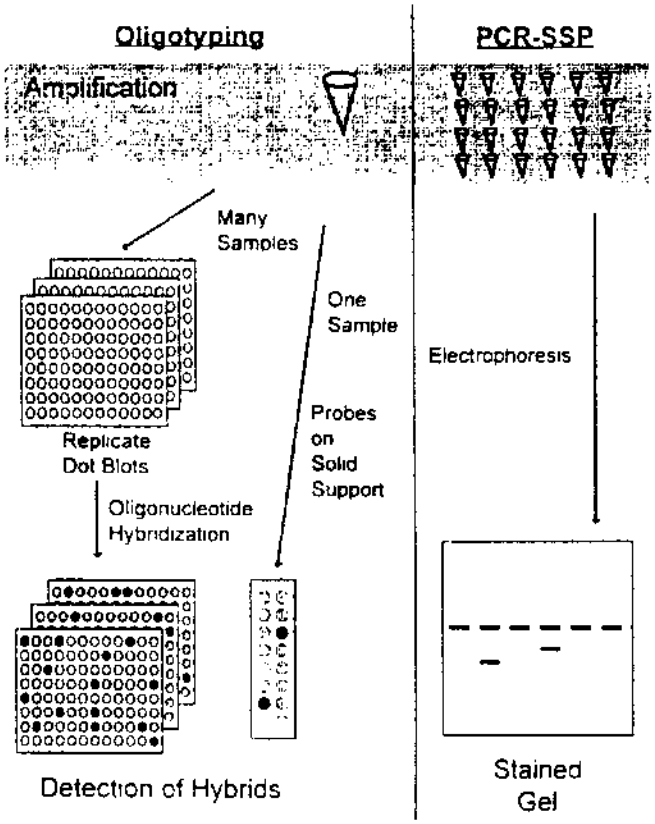

Blood
DNA
Digested DNA
Restriction
Endonuclease
PCR
Amplified
DNA
Electrophoresis
Gel
Transfer to Membrane
Southern
Blot
Hybridization with
Radioactive cDNA
Probe
Detection
Restriction
Endonuclease
Digested DNA
Electrophoresis
and Staining
Stained
Gel

Figure 5: Molecular HLA Typing Methods

Molecular HLA typing method techniques used for HLA typing include sequence-specific oligonucleotide probe hybridization and sequence-specific primers (SSP-PCR)


Oligotyping
PCR-SSP
Amplification
Many
Samples
One
Sample
Electrophoresis
Probes
on
Solid
Support
Replicate
Dot Blots
Oligonucleotide
Hybridization
Detection of Hybrids
Stained
Gel

c.. Polymerase Chain Reaction

The polymerase chain reaction (PCR) permits the selective in vitro amplification of a particular DNA region by mimicking the phenomena of in vivo DNA replication. The PCR technique was devised and named by Mullis and Faloona (1987) at the Cetus Corporation laboratories in Emeryville, California (Figure 6). PCR is an enzymatic process that is carried out in discrete cycles of amplification, each of which can double the amount of target DNA in the sample. Thus, n cycles can produce 2^n times as much target as was present at the beginning of the reaction.

PCR begins by denaturing the double-stranded target DNA, followed by annealing the primers (one for each strand) if added to the sequences flanking the target. Each primer forms a duplex with its flanking sequences so that the 3' hydroxyl end of the primer is base-paired to the template strand. Addition of a thermostable Taq DNA polymerase and deoxynucleoside triphosphates creates a new DNA strand that forms at the beginning at the 3' end of the primer and extending across the target sequence, thereby duplicating the target DNA. These steps -DNA denaturation, primer annealing, and thermostable DNA polymerase extension - represent one cycle of PCR. Each cycle is carried out at the appropriate temperature for the primer (Figure 7).

Amplification by PCR is extremely rapid. Twenty-five cycles can be carried out in just over an hour. Twenty-five cycles of PCR can generate as several micrograms of a specific product. PCR has been used to simplify a number of techniques in molecular biology including cloning, sequencing, and introducing point mutations. PCR applications can be utilized in diagnosis of prenatal genetic disease, the human genome project,

retrospective analysis of human tissue and revelation of evolutionary origin.

d. Specific-Sequence Primer Typing (SSP-PCR)

Sequence-specific primers are employed in the detection of polymorphic sequences used in defining HLA types. The amplification primers detect polymorphic sequences present in the amplification mixture. If both primers are able to anneal to the DNA, amplification will occur. Because the amplification product will not be produced if one primer does not anneal to the target DNA this method detects at least two polymorphic sequences of an HLA type on the same allele.

Many different primer pairs are individually tested in 0.2 ml PCR tubes. Unknown DNA is added to each tube. Amplification products are generated from those primers containing sequences present in the template DNA. Advancement of DNA typing for Class I loci has been expedited by the employment of PCR-SSP methods.

Advantages of PCR-SSP include minimal required equipment (e.g., A thermal cycler and electrophoresis apparatus), the availability of commercial reagents, technical ease and minimal time required to complete tests. An additional advantage of PCR-SSP is the establishment that two polymorphic sequences are present on the same allele.

Figure 6: Schematic procedure of the polymerase chain reaction (from Mullis and Faloona, 1987)

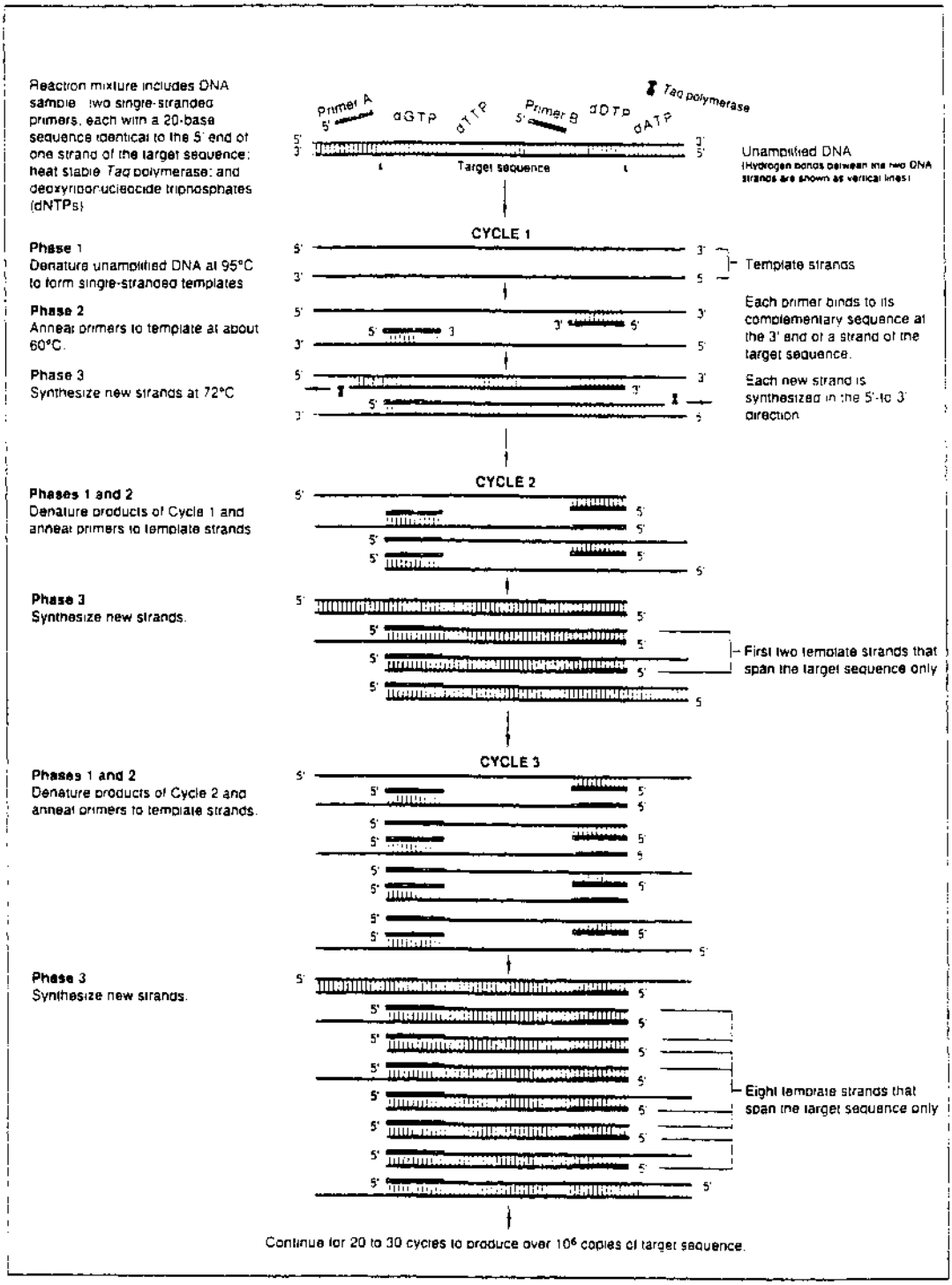
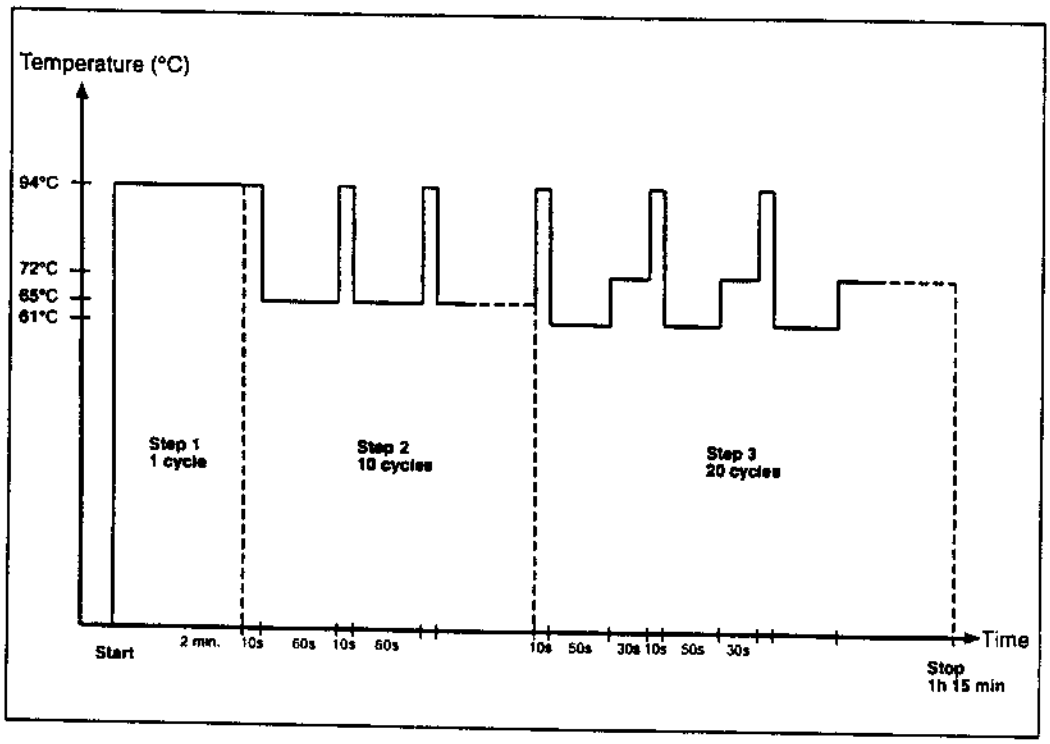

Reaction mixture includes DNA sample, two single-stranded primers, each with a 20-base sequence identical to the 5' end of one strand of the target sequence; heat stable Taq polymerase; and deoxyribonucleocide triphosphates (dNTPs)
Primer A
dGTP
dTTP
Primer B
dCTP
dATP
Taq polymerase
Target sequence
Unamplified DNA
(Hydrogen bonds between the two DNA strands are shown as vertical lines)
CYCLE 1
Phase 1
Denature unamplified DNA at 95°C to form single-stranded templates
Template strands
Phase 2
Anneal primers to template at about 60°C.
Each primer binds to its complementary sequence at the 3' end of a strand of the target sequence.
Phase 3
Synthesize new strands at 72°C
Each new strand is synthesized in the 5'-to-3' direction
CYCLE 2
Phases 1 and 2
Denature products of Cycle 1 and anneal primers to template strands
Phase 3
Synthesize new strands.
First two template strands that span the target sequence only
CYCLE 3
Phases 1 and 2
Denature products of Cycle 2 and anneal primers to template strands.
Phase 3
Synthesize new strands.
Eight template strands that span the target sequence only
Continue for 20 to 30 cycles to produce over 10⁶ copies of target sequence.

Figure 7: The PCR profile of all SSP-PCR amplification


Temperature (°C)
94°C
72°C
65°C
61°C
Step 1
1 cycle
Step 2
10 cycles
Step 3
20 cycles
Start
2 mn.
10s
60s
10s
60s
10s
50s
30s
10s
50s
30s
Time
Stop
1h 15 min

1.3 HLA and Disease Association

Relationship between numerous HLA genes to disease susceptibility (Schlosstein *et al.*, 1973; Hill *et al.*, 1991; Giphart,1992) and immune response (Kruskaij *et al.*,1992) have been established. The most important of these diseases are listed in Table 1. Examination of HLA-B loci has been associated with certain HLA-B antigens and diseases such as HLA-B27 and ankylosing spondylitis. In 1973, two research groups independently observed a remarkably high association of the newly recognized Class I MHC antigen HLA-B27 with ankylosing spondylitis (Brewerton *et al.*, 1973). Ankylosing spondilitis is an arthritic disease in which the spine becomes inflamed and in some cases becomes rigid.

Though mainly found in young adults, it is most prevalent and severe in women. Class I molecules such as B27 perform the specialized function of presenting antigen derived from intracytoplasmic proteins to cytotoxic T cells. Intracytoplasmic proteins must therefore be degraded and transported through the endoplasmic reticulum before binding to HLA-B27 molecules.

The frequency of HLA antigens is markedly diverse among different ethnic groups (Tsai *et al.*,1995). HLA-B35 is a common allotype with frequency variances from 10-28% depending on the ethnic group (Rubinstein *et al.*, 1980). Disease association with HLA-B35 has been noted in several studies. A poor prognosis is given when the HLA-B35 antigen is associated with IgA in patients with nephritis (Berthoux *et al.*, 1983). Similar associations were reported for Italian HIV-infected intravenous drug addicts (Scorza *et al.*, 1989), Latin American Caucasoid patients with chronic hepatitis (Mota *et*

al., 1987), and children with juvenile rheumatic-uveitis (Petty *et al.*, 1987). However, the presence of HLA-B35 was affiliated with decreased frequency of recurrent circumoral hepatitic lesions. An understanding of the specific disease-associated HLA proteins, precise peptide binding motifs, and autoantigenic and/or mimitic protein sequences will permit development of more trenchant methods for disease prevention. The basis for these strategies will include modifications of both environmental and host-specific factors in etiology of HLA-associated diseases (Gorga and Monos,1996).

Table 1: A Listing of HLA Associated Diseases and Their Associated MHC Proteins

- Table 1: A Listing of HLA Associated Diseases and Their Associated MHC Proteins

Disease	Target organ or tissue	Possible target autoantigen	Associated MHC proteins	Proposed precipitating factors
Multiple sclerosis	Nerves	Myelin basic protein, proteolipid protein,MOG	DR2	A number of viruses, Pseudomonas, superantigens
Primary bilary cirrhosis	Liver	Mitochondrial proteins	DRB4*0101	Homologous *E. coli* protein
Reactive arthritis	Peripheral joints, skin,eyes,mouth,spine	urease,heat shock proteins	B27	Sexually transmitted infections, dysentery, samonella
Rheumatoid arthritis	Peripheral joints,widespread inflammation	Self heat shock proteins,cartilage	DR4	*E. coli*,EBV, retroviruses
Systemic lupus erythematosis	Connective tissue, joints,skin, kidneys,lungs,heart, eyes, brain	DNA,nucleosomes,ribonucleoprotein particles	dr2	Chlorpromazine
Systemic sclerosis	Skin,lungs,gut, kidneys,heart	DNA topoismerase I	DR5,DR1,DR4,DQB*0601,0301	Silica dust,silicone implants

CHAPTER II

MATERIALS AND METHODS

2.1 Blood Sample Collection

All positive samples for this study came from the three following sources; 1) Afzal Nikaein Ph.D., Tissue Antigen Lab, Dallas, Texas, 2) UCLA DNA Extraction Program, 3) Einstein Medical School, New York. The whole blood was mixed with EDTA (ethylenediamine tetracetic acid) to prevent coagulation.

2.1 DNA Extraction

Genomic DNA was isolated by the organic solvent.method (need reference).. This involves cell lysis, followed by proteinase K digestion at 55◦C. The DNA yield is approximately 5-15 μg per 0.3ml whole blood, with the average fragment size of 3,000 kb. DNA isolated by this procedure is readily digestible with a variety of restriction enzymes.

The following reagents were used for isolation of genomic DNA from whole human blood;cell lysis buffer [320mM sucrose, 10 mM Tris-HCl (pH 7.5), 5mM MgCl, and 1% (v/v) Triton X-100], protein digestion buffer {10 mM Tris-HCl (pH 7.5), 10mM EDTA, 10mM NaCl, and 0.5% SDS}, Proteinase K, TE buffer {10 mM Tris-HCl (pH 7.5) and 0.1 mM EDTA], 100% ethanol, and 70% ethanol.

Each blood samples was mixed to throughly homogonize prior to extraction. A 0.5 ml aliquot of the whole blood was placed into appropriately labeled microcentrifuge tube along with 1 ml of the prechilled cell lysis buffer. The tube was vortexed to ensure complete cell lysis. The tubes were centrifugated at 4500rpm for 5 minutes in a microcentrifuge with a fixed-angle rotor. The supernatant was carefully decanted into the biologic waste hazard disposal unit. An additional lysis step was performed as described above. A Master Mix was prepared for protein digestion using the following formulas: (n +2) X395ul protein lysis buffer and (n +2)X5 ul proteinase K. To ensure that an adequate amount of the master mix is available for use, a slight excess of the mix is prepared. Add 400ul of the master mix to each microcentrifuge tube. Pipet sample up and down with pipetter to break up nuclear pellet, and vortex for 15 seconds. Place samples in 55 degree heat block. Incubate samples at 55 degree for 2 hours. After the 2 hours incubation., centrifuge tubes for 5 minutes at 14,000 rpm (maximum speed) to pellet cellular debris. Add 200 ul of 7.5 M Lithium chloride to each sample. Vortex for 5 seconds and place the samples on wet ice for 10 minutes. Centrifuge the samples for 15 minutes at 14,000 rpm to pellet proteins and other contaminants.

For each sample, carefully transfer the supernatant to a new microcentrifuge tube. Add 1ml of cold 100% ethanol to each tube. Invert 50 times or until white flocules of DNA are visible. Spin tubes at 14,000 rpm for 10 minutes to precipitate the DNA. Pour off the ethanol in one smooth motion. Add 1 ml 70% ethanol to each tube and vortex samples to break up DNA pellet. Spin at 14,000 rpm for 5 minutes to precipitate the DNA. Pour off the ethanol in one smooth motion. Dry the samples in a speed vac for 5

minutes. Dissolve the DNA pellet in low TE buffer, and measure the DNA concentration with the UV-100 Spectro-phometer at 260nm and 280nm wavelengths.

2.3 Amplification of primers

Primers for PCR-SSP amplification are designed by positioning the nucleotides exclusively defining an allele or group of alleles at the extreme 3' end of the target sequence. This 3' location is the site where Taq DNA polymerase initiates elongation of the annealed primer. The melting temperature (T_m) of each primer in the SSP assay must fall between 65◦C-75◦C to allow amplification of different alleles in the same thermal cycling profile. The annealing temperature used in the profile is designed to accommodate the T_m of all primers used.

Amplification primers were designed using the known published nucleotide sequences. (Arrnett and Parham, 1995; Bodmer *et al.*, 1995) (Table 2). This study of HLA-B allelic classification followed the classifications in Table 1. All primer mixes designed for this study were purchased as crude oligonucleotides from Bio-Synthesis,Inc. (Lewisville,Texas), and the primer sequences are shown in Table 2. All amplification primers were designed to have a T_m of 65◦C and 75◦C with a specificity for the terminal 3' nucleotide. The Class I HLA-B components can be typed in one reaction by using a 36-well reaction plate and the appropriate primer mixes

Table 2: HLA-B low-resolution nucleotide sequence coding primers

Coding Primers	Final 5' nt	Primer Length	Positive control	Negative control
B7-5	GT GA	17	#0116, A17	#0126, #0122
B7-3	TT GT	17	AN21	#0133
B8-5	TTC G	22	A18, #0121	#0122, #0126
B8-3	TGT T	22		#0133
B13-5	GTT A	19	#0123, #0131	#0126, #0122
B13-3	TT GA	17		
B14-5	T TGG	18	#0126, #0122	#0123, #0115
B14-3	GGT G	19		
B15-5A	GG GA	17	AN14, AN20	#0118, A13
B15-3A	CT GA	20	AN21, AN24	
B15-5B1	AG TC	17	A7, AN4, AN8	#0129, A2, A9
B15-5B2	AGT C	19	AN19, AN24	A15
B15-3B1	G CCT	18		
B15-3B2	G CCT	18		
B18-5	A CGG	18	#0115, #0119	AN16, A13
B18-3	C TCT	18		
B27-5	CGC T	19	AN16	#0115, #0116
B27-3	GC CA	20		
B35-5	GG AC	17	#0118, #0119	AN4, AN15
B35-3	GC CA	20		
B37-5	TG TC	20	A1	#0117, #0133
B37-3	T GTC	18		
B38-5	GG AA	17	AN10, A13	#0126, #0122
B39-5	AG TT	17	A13, HP	#0130. AN23
B39-3	A GGT	18		
B40-5	ACC T	22	#0127, AN18	#0118, #0119
B40-3	TCG T	19		

B60-5	AGT T	19	#0115, #0116	A17, AN21
B60-3	T TGT	18		
B41-5	G GAC	18	A11,A18	#0121
B41-3	CG TC	17	AN26	
B42-5	CA TA	17	AN26, #0121	AN18, #0115
B42-3	TG TC	20	A11	#0127
B44-5	ATC A	19	#0117, #0130	#0118, #0119
B44-3	A GCG	18	#0135	#0131
B45-5	G TTA	18	AN19	#0123, #0137
B45-3	T GTC	18		A11, A18
B46-5	TG GC	17	AN11, AN12	#0131, AN8
B46-3	CTCA	17	AN22	A15
B47-5	TG TT	23	#0129, #0130	#0120
B47-3	C GTC	18		
B48-5	C GGC	18	AN13, AN21	#0126, A13, A6
B48-3	CT GG	20	#0127, AN17	
B49-5	CGC T	19	AN29, #0136	AN28
B49-3	GCG A	19		
B50-5	GT TA	20	#0133, #0136	#0131
B50-3	G CCT	18	AN29	
B51-5	A GAC	18	#0121, A15	AN17
B51-3	A TCT	21		
B52-5	GG AC	17	A9	#0121
B52-3	TTG G	22		
B53-5	GG AC	17	AN26, AN28, A13	A9, AN23
B53-3	GT CG	17	#0125, #0129	
B54-5	GTGC	18	#0120, #0130	A17,#0117
B54-3	CCAC	17	AN6	AN29
B55-5	GGCA	15	AN6,	#0116, #0137

			AN7	
B55-3	TTGT	21	#0122	
B56-5	GTTA	18	AN8, AN15	A2, AN5, AN10
B56-3	ACAG	18		AN30
B57-5	AGGT	20	#0125	A11,A18
B57-3	ACAG	20		
B58-5	GGAC	19	#0123, AN5	#0132, #0134
B58-3	CCCC	21	AN23	
B59-5	GAGA	17	A11, #0121	#0116, #0126
B59-3	TTGA	21		#0138, #0125
B67-5	ACCT	22	HP, #0116, AN26	A9, A13, A6
B67-3	TTGT	19	AN21	
B73-5	GCCT	17	AN10	#0130, #0122
B73-3	TGCC	20		AN7
B78-5	AGAC	18	#0121,A 15	AN7
B78-3	ATCT	21		
B81-5	GAGC	19	AN13, AN17	AN23, #-137
B81-3	GGGA	17		#0116

***QC Control: AN: from Afzal Nikaein PhD, Tissue Antigen Lab
#: from UCLA DNA Exchange Program
A: from Einstein Medical School

Table 3: Serological Types and Allele Specificity

Mix #	Serology Types	Allele-Specificity	~ PCR product size	Fwd. primer 3' start codon	Rev primer 3' start codon
1	B7	B*0702-*0705	115 bp	176(TGA)***	201(TGT)***
2	B8	B*0801-*0802	200 bp	33(TCG)**	87(GTT)**
3	B13	B*1301-*1303	125 bp	140(TTA)***	169(TGA)***
4	B14	B*1401-*1402	260 bp	121(AGT)***	195(GTG)***
5	B15	B*1503,*1509,*1510,*1518,*1523 ,B*1529	270 bp	15(GGA)*	48(TGA)**
6	N15	B*1501-*1529, *3511,*4601	160 bp	140(GTC)***	176(CCT)***
7	B18	B*1801-*1802	110 bp	54(CGG)**	78(CTT)**
8	B27	B*2701-*2709	155 bp	56(GCT)**	95(CCT)**
9	B35	B*3501-*3510,*7801, *1801-*1802,*1522	135 bp	69(GAC)**	111(GGC)**
10	B37	B*3701	210 bp	123(GTC)***	180(GTC)***
11	B38	B*3801-*3802	105 bp	82(GAA)**	105(CGC)**
12	B39	B*3901-*3908, *3801-*3802,*6701	165 bp	140(GTT)***	182(GGT)***
13	B40	B*4002-*4006	130 bp	35(CCT)**	65(CGT)**
14	B60	B*40011-*40012, *4801,*8101	130 bp	171(GTT)***	201(TGT)***
15	B41	B*4101-*4102, B*40011-*40012, B*4002-*4007, B*45,B*49,B*50	135 bp	33(TCC)*	65(CGT)**
16	B42	B*4201, B*4101-*4102,B8	170 bp	138(ATA)***	180(GTC)***
17	B44	B*4402-*4406	255 bp	119(TCA)**	191(GCG)***
18	B45	B*4501	160 bp	140(TTA)***	180(GTC)***
19	B46	B*4601	135 bp	70(GGC)**	100(TCA)***
20	B47	B*4701	90 bp	123(GTT)***	140(GTC)***
21	B48	B*4801-03,B*702-5, B*40011-12,B*7301	190 bp	15(GGC)*	55(TGG)**
22	B49	B*4901,B*2702	185 bp	56(GCT)**	104(CGA)**
23	B50	B*5001,*B4901	160 bp	140(AAA)***	187(CAG)***
24	B51	B*5101-05, *B5201,*B7801	165 bp	145(GAC)***	145(TCT)***
25	B52	B*5201,B*1801-02, B*2701-09	105 bp	69(GAC)**	91(TGG)**
26	B53	B*5301,B*35,B*51, B*78,B*1522,B*4406	105 bp	69(GAC)**	91(TGA)**
27	B54	B*5401	135 bp	59(TGC)**	91(TGT)**
28	B55	B*5501-*5502, B*5401,B*5901	180 bp	140(TTA)***	187(CGT)***
29	B56	B*5601-*5602, B*1303	160 bp	140(TTA)***	187(CAG)***
30	B57	B*5701-*5703	250 bp	121(GGT)***	190(CAG)***
31	B58	B*5801-*5803	90 bp	69(GAC)**	86(CCC)**
32	B59	B*5901,B*0801-02	105 bp	69(AGA)**	91(TGA)**
33	B67	B*67011-*67012, B*702-05, B*4201,B*8101	209 bp	35(CCT)**	91(TGT)**
34	B73	B*7301	205 bp	39(GGT)**	95(GCC)**
35	B78	B*7801,B*5101-05, B*5201	115 bp	121(GAC)***	145(TCT)***
36	B81	B*8101, B*4801	175 bp	121(AGC)***	167(GGA)***

* Primer site located on exon 1
** Primer site located on exon 2
*** Primer site located on exon 3

2.4 PCR-Reaction Primer Mixes of HLA-B

The HLA-B serological types have been divided into 36 groups in this study (Browning *et al.*). The low resolution typing mixes are listed in Table 3. Each typing mix contains the allele and group specific 5' and 3' primer pairs. The panel of HLA-B primer pairs were employed in the identification of HLA-B specificities. Both primers in a primer pair are designed with homology to the 3'-ends of a single allele. Each group of alleles corresponds to a serological specificity. The internal control incorporated into the mixes was purchased from Bio-Synthesis,Inc., Lewisville,Texas. The internal control amplifies a 400bp non-allelic portion of the human β-actin gene. All primer mixes and internal control concentrations were between 1.25μM and 5.0μM. The primer stock solutions were stored at -20∘C.

2.5 Reaction Mixes

A PCR solution containing the PCR buffer, glycerol, nucleotides and cresol red is prepared in advance. Each 10mM stock solution of dATP (deoxyadenine triphosphate), dCTP (deoxycytosine triphosphate), dGTP (deoxyguanosine triphosphate), and dTTP (deoxythymide triphosphate) was prepared as a stock solution in ddH_2O. The final concentration of the nucleotide solution was 200μM. The final concentration of glycerol was 5%. Glycerol is a co-solvent in the PCR reaction and it facilitates the loading of the PCR product on to an agarose gel. The final concentration of cresol red was 100μg/ml. The PCR buffer was composed of 500mM KCl, 15mM $MgCl_2$, 100mM Tris-HCl,pH 8.3,

and 0.01% (w/v) gelatin in ddH_2O. These solutions were made as 10X stock solutions and dispensed into 1ml tubes and stored at -20◦C for future use. The PCR reactions were prepared so that the reaction buffer was 1X after addition of all components for the final reaction.

The thermostable enzyme Taq polymerase (Gibco BRL, Gaithersburg,Md.) was isolated from *Thermophillus aquaticus* strain YT1. The enzyme is derived from a recombinant, thermostable, 94 kDa. DNA polymerase. (Figure 6)

2.6 PCR Cycling Parameters

PCR amplification depends on PCR cycling parameters. The parameters are composed of the appropriate number of cycles to amplify products, a suitable annealing temperature for the primers and the proper cycling times for each step of the reaction to proceed. The Perkin-Elmer Gene Amp 9600 was chosen as the thermal cycler for this project. The following PCR cycling parameters were used for Class I HLA-B. The initial denaturation of 1 minutes is critical to the progression of the reaction because this region is exceptional G/C rich.

1.	Denaturation	94◦C	2 minutes	1 cycle
2.	Denaturation	94◦C	20 seconds	10 cycles
	Annealing	60◦C	60 seconds	10 cycles
3.	Denaturation	94◦C	20 seconds	20 cycles
	Annealing	61◦C	50 seconds	20 cycles
	Extension	72◦C	30 seconds	20 cycles

4. Sweep cycle 72◦C 5 minutes 1 cycle

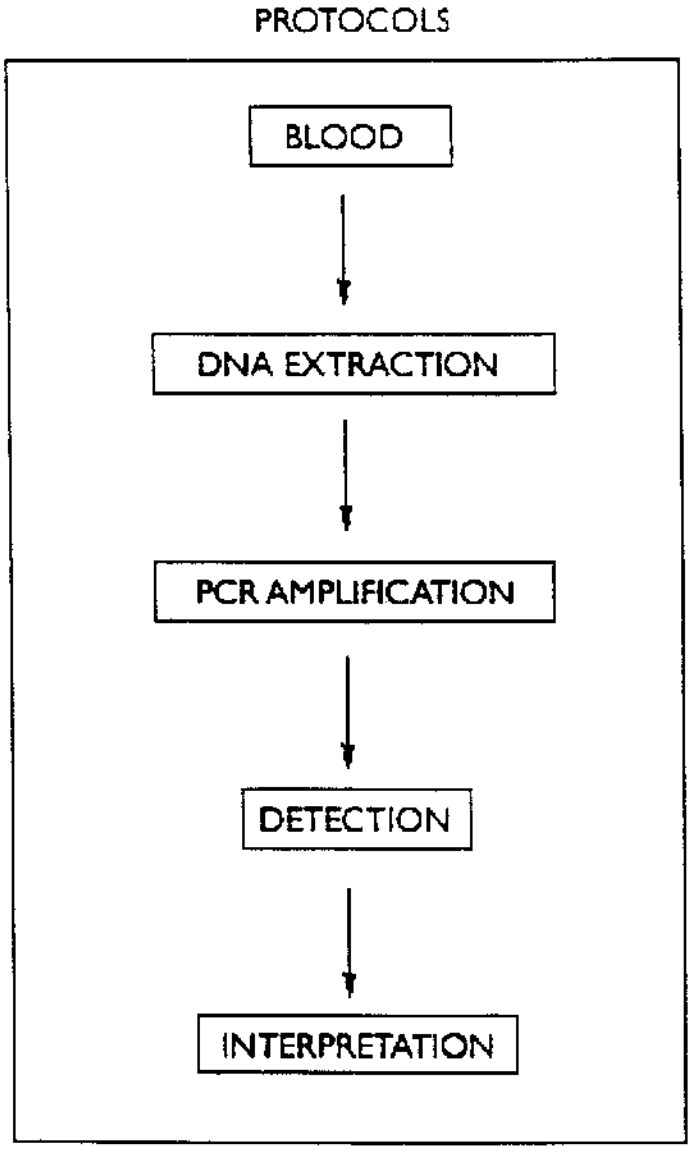
2.7 Agarose Gel Electrophoresis

PCR products were visualized by gel electrophoresis. The PCR products (10µl) were loaded onto a 2% agarose gel containing 0.5µg/ml ethidium bromide. Agarose gels were run in a DNX-2 unit (Bio-Synthesis, Inc., Lewisville,Tx.) for 30 minutes at 120 Volts in 0.5X TBE buffer(89mM Tris base, 89mM boric acid, and 2mM EDTA at pH 8.0). PCR product bands were visualized under an ultra-violet transilluminator and documented with UV sensitive photographic film (Polaroid, Cambridge,Ma.).

2.8 PCR Protocol of HLA-B Typing

The PCR-SSP typing procedure is comprised of four steps; extraction, amplification, detection, and documentation and interpretation (Figure 8). To begin this procedure, extraction of chromosomal DNA from whole blood is necessary. Next, PCR amplification is performed on the extracted DNA (50ng/µl) and HLA-B set of primers, (See Table 2 5' and 3' Primer Sequences) including all components for the reaction. Thirdly, the results are detected by electrophoretic seperation of the PCR amplification products on an agarose gel. Finally, the results are documented on film and interpretation is performed.

Figure 8: The Four Steps in DNA Typing Using HLA-B SSP-PCR Technique


PROTOCOLS
BLOOD
DNA EXTRACTION
PCR AMPLIFICATION
DETECTION
INTERPRETATION

CHAPTER III

RESULTS

3.1 Primer Design for all HLA-B Allele Groups

The HLA-B locus is highly polymorphic based on DNA sequence data identifying 110 separated sequence specific alleles (Bodmer *et al.*, 1995). The HLA-B low-resolution SSP typing panel was designed to examine all known serologically defined antigens for the 36 individual allele or group-specific reactions described in Table 4. The upstream primers possess homology to sequences of either exon 1 or exon 2 of HLA-B. The downstream primers were designed to be complimentary to sequences in exon 2 or exon 3. Suitable primers for the positive control are shown in Figure 9. All positive and negative control DNA were obtained from the following HLA laboratories; 1) UCLA Tissue Typing DNA Laboratory, 2) Afzal Nikaein, Ph.D., Tissue Antigen Lab, Dallas, Texas, and 3) Einstein Medical School, New York.

The negative control is homologous to the 3' start of exon 1 or exon 2, or the 3' start codon of exon 2 or exon 3. The results for the HLA-B negative control are shown in Figure 10.

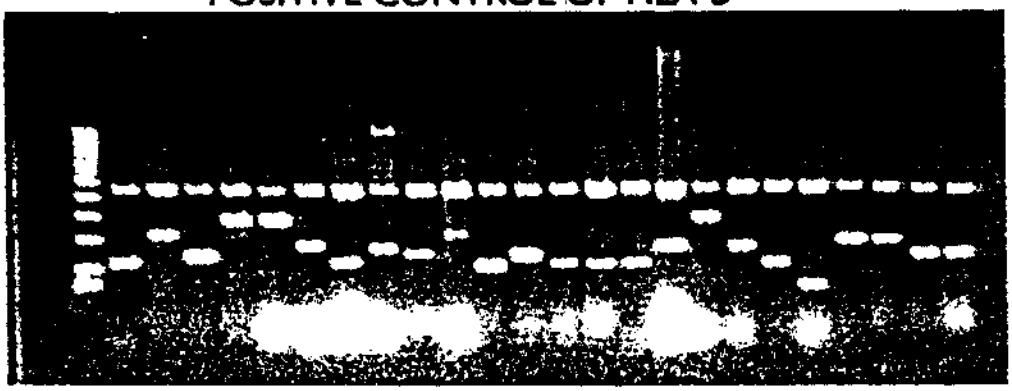
Each PCR reaction contained a pair of positive internal control primers that amplified a 400 bp fragment. All HLA-B low-resolution SSP typing primer pairs were designed to amplify fragments smaller than the internal control fragment.

Table 4: HLA-B Primer Mixes

Generic Type	PCR product in bp	Resolution Type	P.C.	N.C.	Number
B7	116	B0702-05	#0116,A17, AN21	#0126,#0122. #0133	1
B8	202	B801-02	A11,#0121	#0122,#0126,#0133,AN6	2
B13	126	B1301-03	#0123,#0131	#0126,#0122	3
B14	262	B1401-02	#0126,#0122	#0123,#0115	4
B15	267	B1503,09,10, 18,23,29	AN14,AN20,AN21, AN24,AN25	#0118,A13	5
B15	158	B15,B3511, B4601	A7,AN4,AN8,AN19, AN24	#0129,A2,A9,A15	6
B18	112	B1801-02	#0115,#0119	AN16,A13	7
B27	156	B2702-09	AN16	#0115,#0116	8
B35	136	B35,B78,B18, B1522	#0118,#0119,A16	AN4,AN15	9
B37	210	B3701	A1	#0117,#0133	10
B38	107	B3801-02	AN10,A13	#0126,#0122, #0125	11
B39	165	B3901-08, B38,B67	A13,HP	#0130,AN23, #0126	12
B40	130	B4002-06	#0127,AN18	#0118,#0119, HP,#0117	13
B60	130	B40011-12, B4801, B8101	#0115,#0116	A17,AN2	14
B41	136	B40011-12, B4002-7, B4101-2,B45,B49.B50	#0116,#0127,#0137,AN18,A11,#0133,#0136	#0123,#0127,AN10	15
B42	168	B4201, B4101-02, B081-02	A11,A18, AN26, #0121	#0123,#0127, AN10	16
B44	257	B4402-06	#117#130,#135	#118,#119.#131	17
B45	158	B4501	AN19	#123,#137,A11,A18	18
B46	138	B4601	AN11,AN12,AN22	#131,AN8,A15	19
B47	90	B4701	#129,#130	#120	20
B48	290	B4801-2 , B0702-5, B40011-12, B7301	AN13,AN17,#116,#127	#115,#116	21
B49	184	B4901,B2702	AN29,#136	AN28	22
B50	158	B5001,B4901	#133,#136,#AN29	#131	23
B51	164	B5101-05, B52,B78,B1509	#125,#121,A9	#131	24
B52	106	B52,B18,B37	A9	#121	25
B53	106	B5301,B1522, B35,B51,B4406, B78	AN26,AN28,A13,#125, #129	A9,AN23	26
B54	137	B5401	#120,#130,AN6	A17,#117,AN29	27
B55	180	B55,B5401, B5901	#120,#122,AN7,AN13	#123,#119,#121	28
B56	140	B5601-02, B1303	AN8,AN15	A2,AN5,AN10.AN30	29
B57	248	B5701-03	#125	A11,A8	30
B58	90	B5801-03	#123,AN5,AN23	#132,#134	31
B59	90	B5901,B0801-2	A11,#121	#116,#126,#138,#125	32
B67	209	B67011-12,B7, B4201,B8101	HP,#0116,AN21,AN26	A9,A13,A6	33
B73	206	B7301	AN10	#0130,#0122	34
B78	113	B7801-02, B51,B52	#0121,A15	AN7	35
B81	176	B8101,B4801	AN13,AN17	AN23,#0137,#0116	36

Figure 9: The 36 HLA-B Specific Groups Tested as Positive Control

POSITIVE CONTROL OF HLA-B



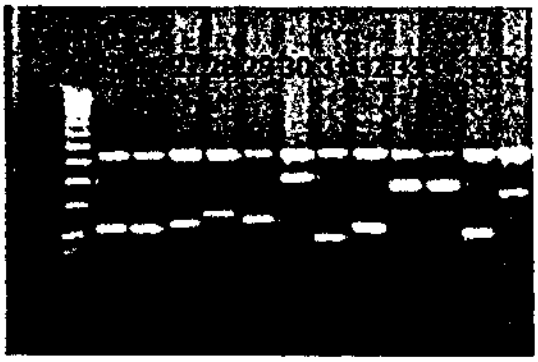
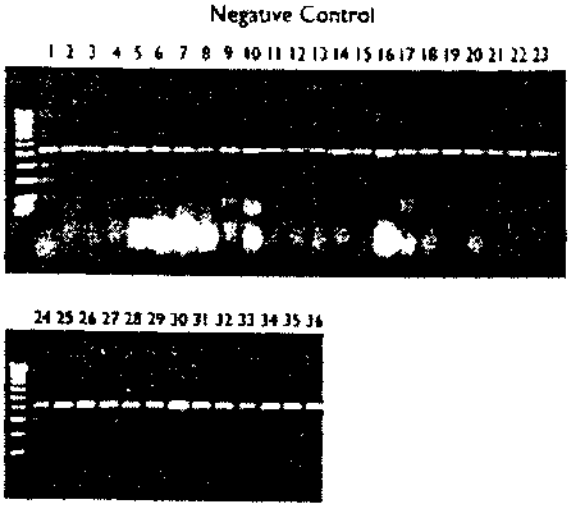


Figure 10: The 36 HLA-B Specific Groups Tested as a Negative Control


Negative Control
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
24 25 26 27 28 29 30 31 32 33 34 35 36

3.2 Extrapolation of SSP Typing Data to Serological Type

The HLA-B locus is highly polymorphic and is difficult to differentiate with a single PCR reaction. The primer pairs for HLA-B low-resolution SSP typing are comprised of 5' and 3' primers used to identify alleles B7 through B81. PCR typing of the HLA-B alleles was grouped according to the corresponding serological groups with respect to all defined splits. Thirty-six HLA-B low-resolution typing PCR reactions were prepared in a 36-well tray and, the tray was placed on a thermal cycler. After HLA-B alleles were amplified by low-resolution SSP and resolved on an agarose gel, the results were recorded in the HLA-B low-resolution typing data sheet for interpretation (Table 5).

To determine the locus-specificity of these sequences, primers were used in varying combinations. Table 5 reflects the primer combinations. A positive response for allele B7 in serological typing corresponds to visible amplification products from primer pairs 1,21, and 33. The serological type for allele B8 is analogous to a positive response for primer pairs 2,16, and 32. The primer mixes yielded products that corresponded to the serological typing data and the accuracy is confirmed by the primers aligning with the target sequences and producing fragments of appropriate length.

3.3 Comparative Analysis of Results Obtained by Molecular and Serological Typing of the HLA-B Locus

This study was conducted over a period of 5 months resulting in primer mixes resolving low resolution for HLA-B DNA typing. The results of the PCR-SSP typing were compatible with UCLA's DNA reference panel for serological typing. A phototype

is considered successful when the positive control amplification product and amplification products for one allele or allelic group are visible for each locus. If a phototype appears to be non-specific, the vibrance of the amplicons is used to determine if the phototype is successful. Results of the PCR-SSP are presented in Figures 11-18.

The sample designated UCLA DNA #125 produced positive signals at alleles B51 and B57. For this PCR-SSP study to correspond to the serological typing data amplicons must be observed for primer mixes 24,26,35, and 30. (Please see Table 5: HLA-B low-resolution typing data sheet) Using the data obtained with HLA-B low-resolution SSP typing, it can be stated with a confidence level of 100% that sample UCLA DNA #125 is analogous to the UCLA DNA reference panel. (Figures 11 and 12) An additional sample, UCLA DNA #126, serologically typed to alleles B14 and B44. SSP primer mixes gave amplicons for mixes 4 and 44. The results for HLA-B PCR-SSP typing are in concordance with the UCLA DNA reference panel.

All 9 samples were successfully typed using the low-resolution HLA-B typing panel. The SSP typing data was completely congruent to the UCLA serological typing data. All samples chosen were heterozygous for the alleles typed.

Table 5: HLA-B Low-Resolution Typing Data Sheet

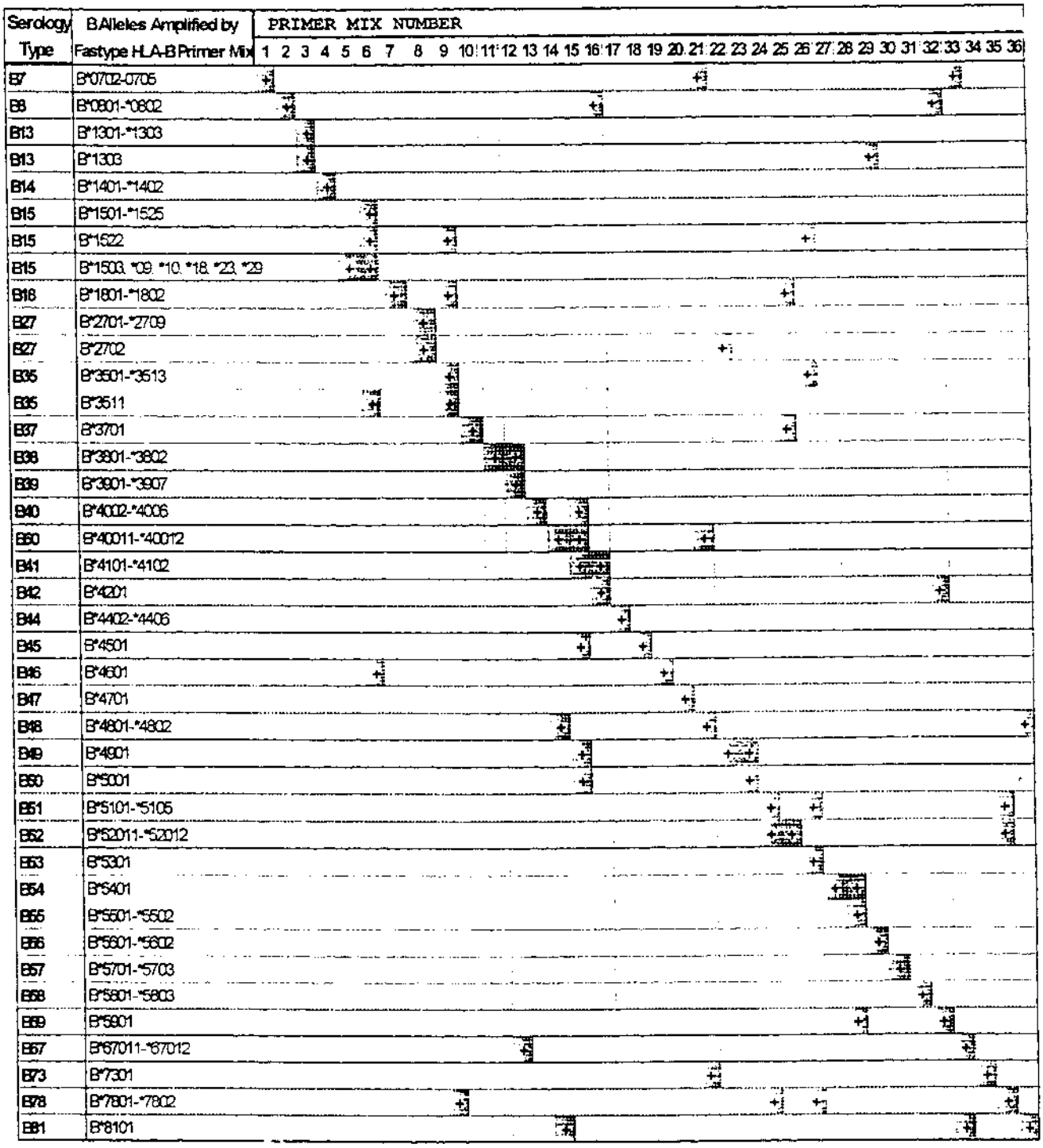
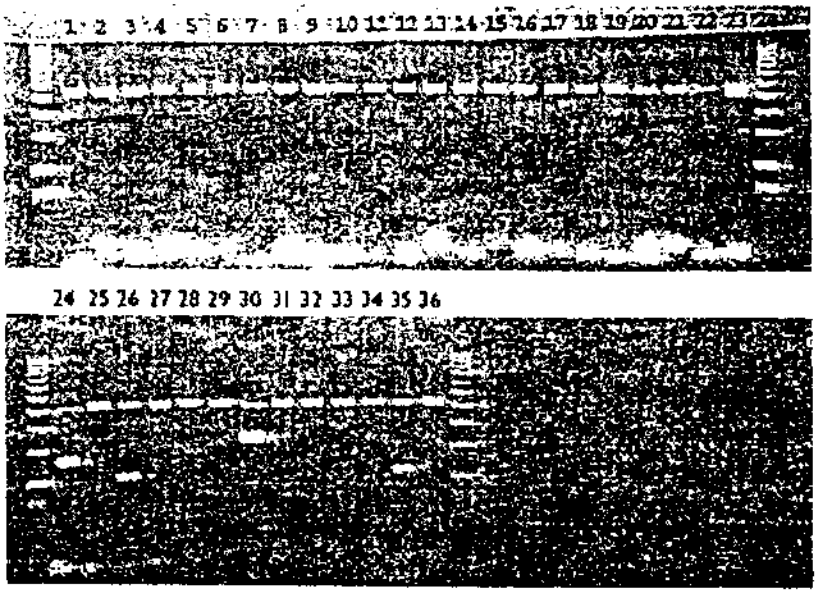


Figure 11: Comparison of the HLA-B SSP-PCR Typing of the UCLA Typing of #125 for Alleles B51 and B57 Reference Panel

UCLA # 125



DNA #0125

CENTER	A-LOCUS	A-LOCUS	B-LOCUS	B-LOCUS	C-LOCUS	C-LOCUS
0010	*24	*30	0051	0057		
0178	blank	*30	0051	0057		
0805	*24	*30	51/52	0057	0004	0014
1466	*24	3002	0051	0057		
2004	*24	*30	51/52	0057	0004	0014
2019	2402	3002				
2347	2402	3002	0051	0057	0004	0014
2549	*24	*30				
2685	*24	*30	0005	0057	0004	0014
3625	2402	*30	0051	0057	0004	0014
3631	2402	3002				
4337	*24	*30	0051	5701/2	0401/2	0014
4359	*24	*30				
4585	*24	*30	0051	0057	0003	0014
4628	*24	*30	0051	0057	0004	blank

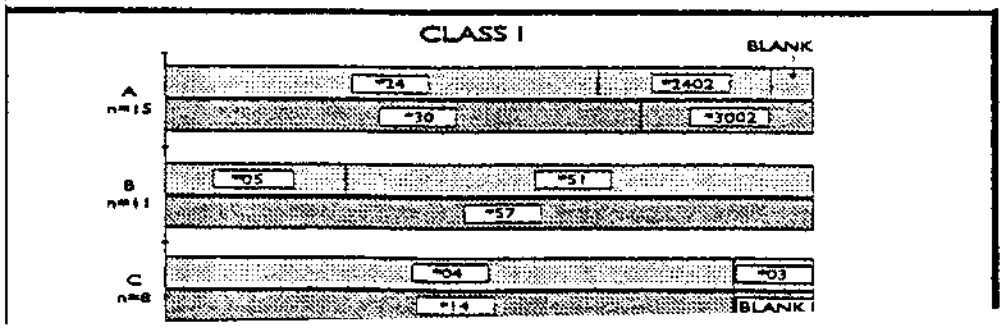
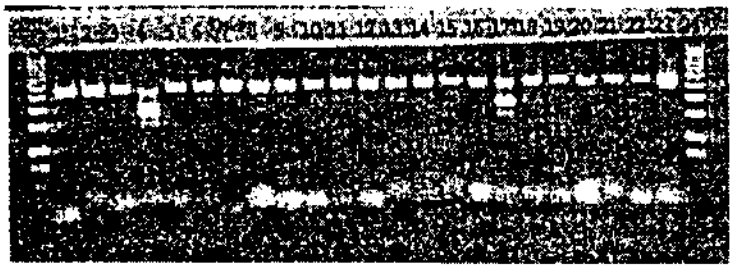
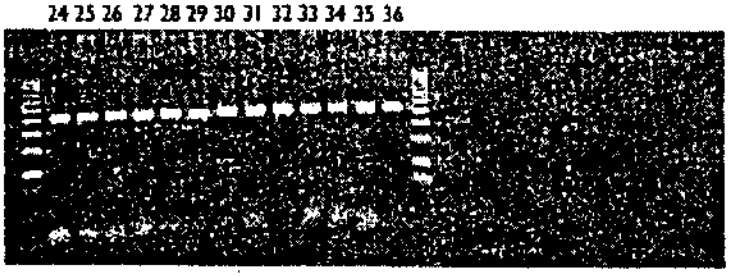


Figure 12: Comparison of the HLA-B SSP-PCR Typing of the UCLA Typing of # 126 for Alleles B14 and B44 Reference Panel

UCLA # 126





DNA #0126

CENTER	A-LOCUS	A-LOCUS	B-LOCUS	B-LOCUS	C-LOCUS	C-LOCUS
0010	*02	*80	0014	0044		
0178	*02		0014	0044		
0805	*02	8001	0014	0044	0004	0008
1466	0201	8001	0014	0044		
2004	*02	8001	0014	0044	0004	0008
2019	*02	8001				
2347	*02	8001	1401	0044	0004	0008
2549	*02	*80				
2685	*02	8001	0014	0044	0004	0008
3625	*02	8001	1401	0044	0004	0008
3631	0201	8001				
4337	*02	8001	0044	1401/2	0401/2	0008
4359	0216	*24				
4585	*02	*80	0014	0044	0008	
4628	*02	8001	0014	0044	0004	0008

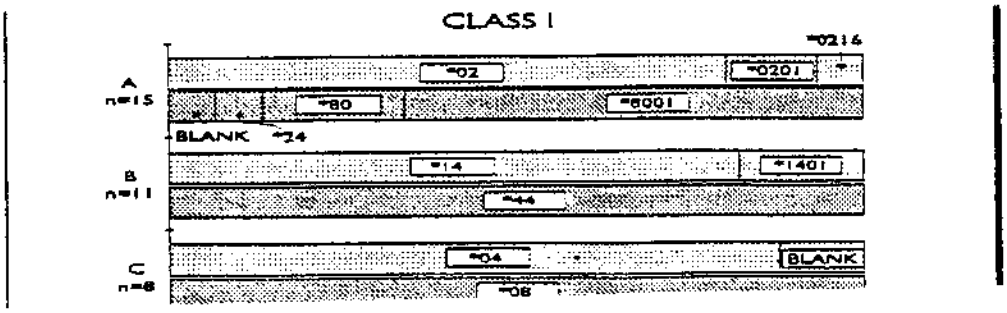
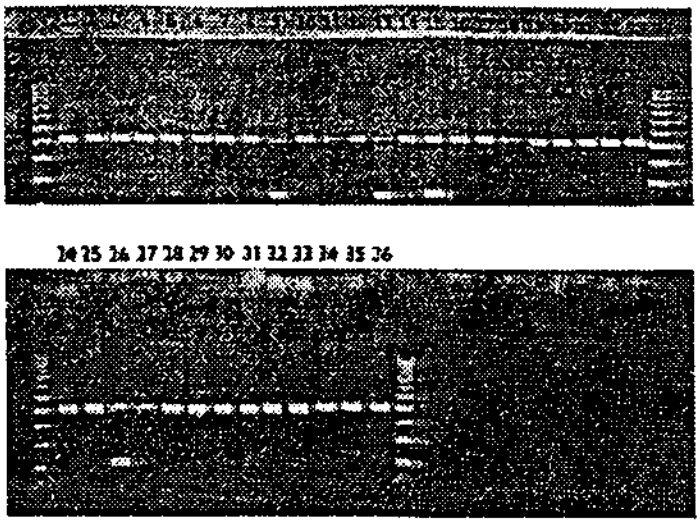


Figure 13: Comparison of the HLA-B SSP-PCR Typing to the UCLA Typing of #127 for Alleles B35 and B40 Reference Panel

UCLA # 127



DNA #0127

DIRECTOR	A - LOCUS		B - LOCUS		C - LOCUS	
Adams, Demenco	10**		35**	40**	0303	15**
Alesse, Sheron	34**	blank	35**	4002		
Chen, Rita	nt	nt				
Curtoni & Amarose	10**	blank	35**	40**	0303	15**
Darke, Chris	34**	blank	35**	40**	0303	15**
Dunckley, Heather	3401	blank	35**	40**	0303	15**
Eastwese, Stuart	3401	6602	35**	40**	0303	15**
Faggia, Susan	34**	blank	35**	40**	0303	15**
Hammens, M.G.	10**	03**	blank	60**	nt	nt
Han, Hoon	3401	66**	35**	40**	03**	1501
Harman, Leigh	34**	blank	35**	22**	03**	15**
Kukurase, Debra	34**	66**				
Meahulia, Helmut	34**	blank				
Meimmy, Hidejet	3401	blank				
Olenus, Ollo	3401	blank				
Saji, Hiren	66**	blank				
Scheus, Betty	3401	blank			03**	15**
Siauw, Poe-An	34**	blank	35**	61**		
Takeda, Alan	34**	blank	35**	61**		
Tilanus, Marcel	FTA	FTA				

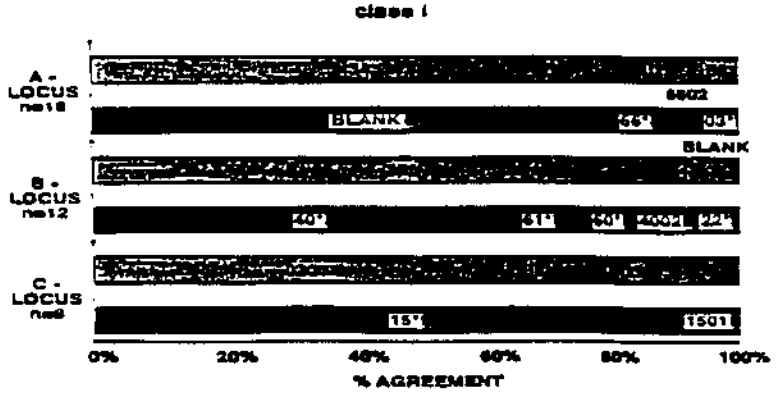
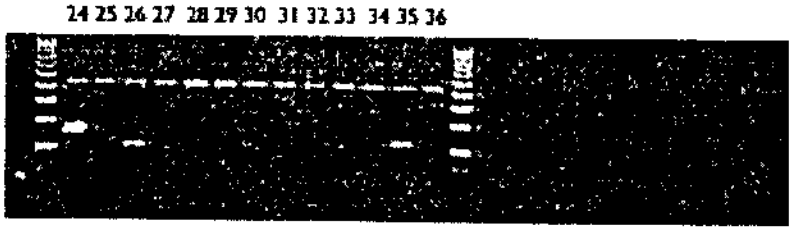


Figure 14: Comparison of the HLA-B SSP-PCR Typing to the UCLA Typing of #128 for Alleles B47 and B51 Reference Panel.

UCLA # 128



DNA #0128

DIRECTOR	A - LOCUS		B - LOCUS		C - LOCUS	
Adorno, Domenico	26**	6802	4701	05**	07**	1601
Alesce, Sharon	26**	6802	4701	51**		
Chen, Rita	26**	02**				
Curtoni & Amorose	26**	6802	47**	05**	07**	1601
Darke, Chris	26**	68**	4701	51**	07**	1601
Dunckley, Heather	2601	6802	4701	51**	07**	1601
Eastwood, Stuart	26**	6801	blank	51**	07**	1601
Faggie, Susan	26**	68**	4701	51**	07**	16**
Hammond, M.G.	26**	03**	14**	51**	07**	1601
Han, Hoon	26**	6802	4701	05**	0701	1601
Herman, Leigh	26**	6802	4701	05**	07**	1601
Kukuraga, Debra	26**	68**				
Machulla, Helmut	26**	6802				
Melanny, Hideist	2601	68**				
Olerup, Olle	2601	6802				
Saji, Hiroh	26**	6802				
Schaub, Betty	2601	6801			07**	11**
Sleuw, Poo-An	26**	68**	47**	51**		
Takeda, Alan	26**	68**	47**	51**		
Tilanus, Marcel	2601	6802				

class I

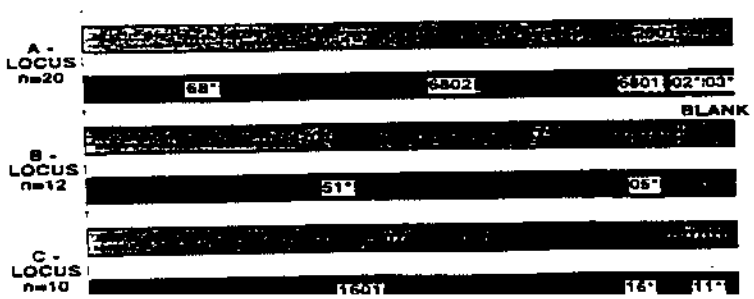
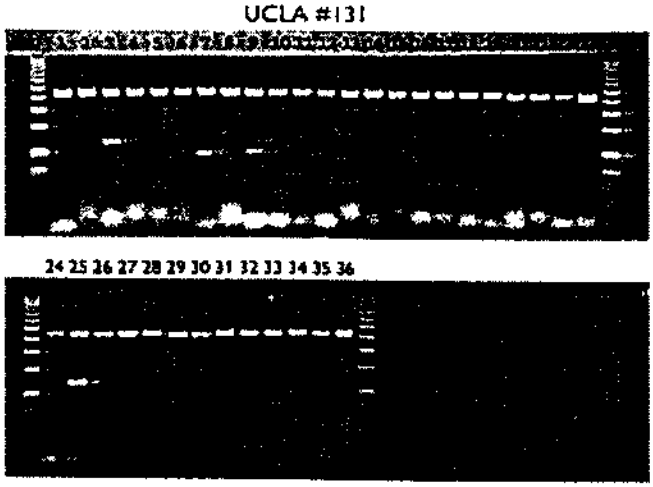


Figure 15: Comparison of the HLA-B SSP-PCR Typing to the UCLA Typing of #131 for Alleles B13 and B18 Reference Panel.



DNA #0131

DIRECTOR	A - LOCUS		B - LOCUS		C - LOCUS	
Adamo, Domenico	02**	03**	13**	18**	0602	07**
Alosco, Sharon	0201	0301	1302	1801		
Chan, Rita	02**	03**				
Curtoni & Amoroso	02**	03**	13**	18**	0602	04**
Darke, Chris	02**	03**	13**	18**	0602	07**
Dunckley, Heather	02**	03**	13**	18**	0602	07**
Eastwood, Stuart	02**	03**	57**	blank	06**	blank
Faggie, Susan	02**	03**	13**	18**	06**	07**
Hammond, M.G.	02**	03**	13**	18**	06**	blank
Han, Hoon	02**	03**	13**	18**	0602	07**
Harman, Leigh	02**	03**	18**	blank	0602	07**
Kukuraga, Debra	02**	03**				
Mechulla, Helmut	02**	03**				
Melenny, Hidalet	02**	03**				
Olerup, Olle	0201	0301				
Saji, Hiroh	02**	03**				
Schaub, Betty	02**	03**			06**	07**
Siauw, Poo-An	02**	03**	13**	18**		
Takeda, Alan	02**	03**	13**	18**		
Tilanus, Marcel	0201	0301				

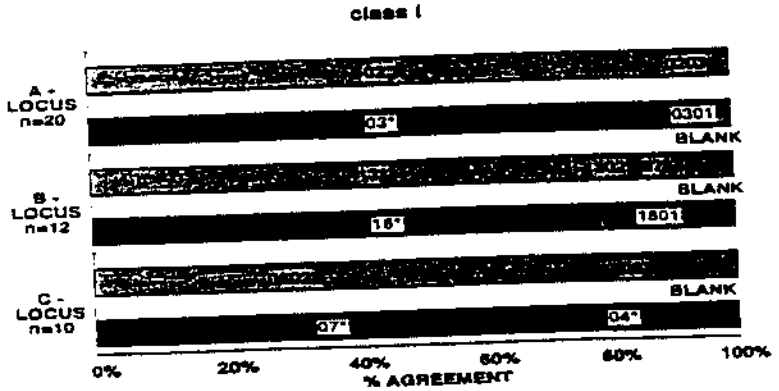
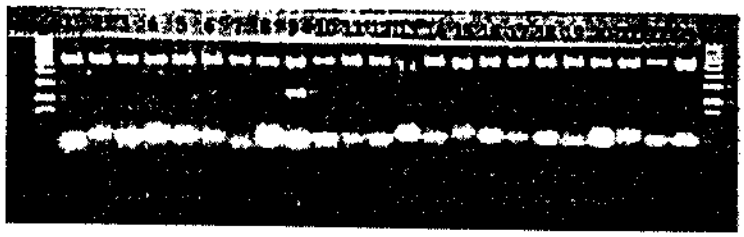
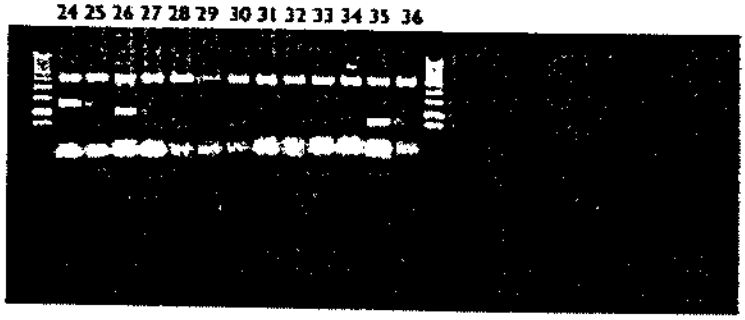


Figure 16: Comparison of the HLA-B SSP-PCR Typing to the UCLA Typing of #132 for Alleles B35 and B51 Reference Panel.

UCLA # 132





DNA #0132

DIRECTOR	A - LOCUS		B - LOCUS		C - LOCUS	
Adorno, Domenico	0202	24**	35/5301*	05**	04**	0501
Alosco, Sharon	0202	24**	0035	51**		
Chen, Rita	02**	24**				
Curtoni & Amoroso	0202	24**	35**	05**	04**	05**
Darke, Chris	02**	2402	35**	51**	04**	0501
Dunckley, Heather	0202	24**	35**	51**	04**	0501
Eastwood, Stuart	02**	2402	35**	blank	04**	blank
Feggle, Susan	02**	24**	35**	51**	04**	05**
Hammond, M.G.	0202	24**	35**	5104	nt	nt
Han, Hoon	02**	24**	3501	5301	0401	0501
Harman, Leigh	0205	24**	35**	05**	04**	0501
Kukuruga, Debra	02**	24**				
Machulla, Helmut	02**	24**				
Melanny, Hidajat	02**	2402				
Olerup, Olle	0202	2402				
Saji, Hiroh	02**	24**				
Schaub, Betty	02**	2402			04**	05**
Sisuw, Poo-An	02**	24**	35**	51**		
Takeda, Alan	02**	24**	35**	51**		
Tilanus, Marcel	FTA	FTA				

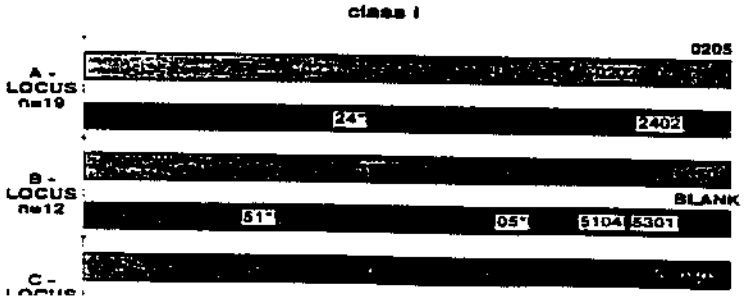
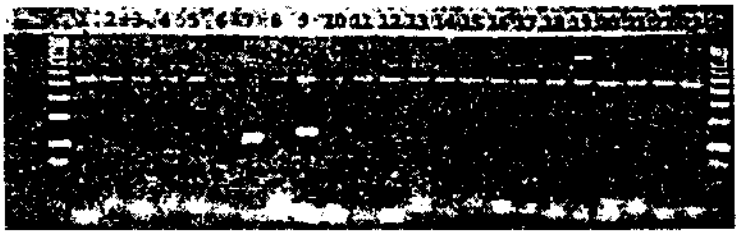
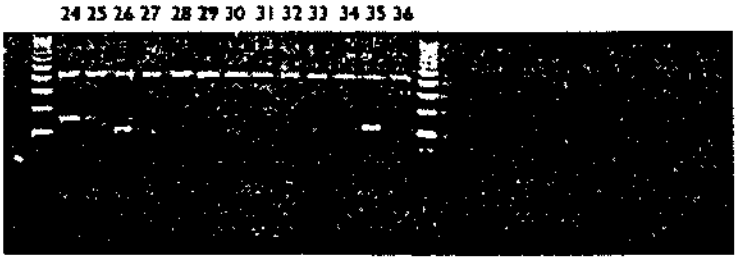


Figure 17: Comparison of the HLA-B SSP-PCR Typing to the UCLA Typing of #134 for Alleles B18 and B51 Reference Panel.

UCLA # 134





CLASS I RESULTS

DIRECTOR	DNA #	A-LOCUS		B-LOCUS		C-LOCUS	
ALCALAY, D.	134	2501	6801	18*	51*	1203	14*
BARTOVA, ADELA	134	25*	68*			1203	14*
CHEN, RITA	134					1203	1402
CURTONI/AMOROSO	134	2501	6801	18*	05*	1203	14*
DARKE, CHRISTOPHER	134	2501	68*	18*	51*	1203	14*
DUNCKLEY, HEATHER	134	*25	*68	*18	*51	1203	*14
DUNN, SUSAN	134					nt	nt
EASTWOOD, STUART	134	2501	BLANK	18*	51*	BLANK	14*
FAGGLE, SUSAN	134	25*	68*	18*	51*	BLANK	14*
FISCHER, HARALD	134	2501	68*				
HAMMOND, M.G.	134	25*	6801	18*	51*	NT	NT
HAN, HOON	134	2501	3402	18*	05*	1203	14*
KUKURAGA, DEBRA	134	25*	68*				
MACHULLA, HELMUT	134	2501	6801	BLANK	51*		
MELLANY, HIDAJAT	134	2501	6801				
OLERUP, OLLE	134	2501	6801	18*	51*	12*	14*
RODRIGUEZ, SUSANA	134			18*	51*		
SAJI, HIROH	134	0216	*29				
SCHAUB, BETTY	134	2501	BLANK			12*	14*
SIAUW, POO-AN	134	2501	68*	18*	51*		
TAKEDA, ALAN	134	25*	28*	18*	51*		
TILANUS, MARCEL	134	2501	68012				

CLASS I

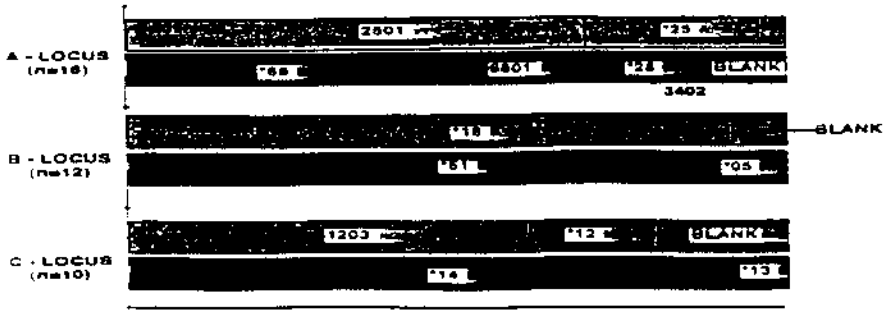
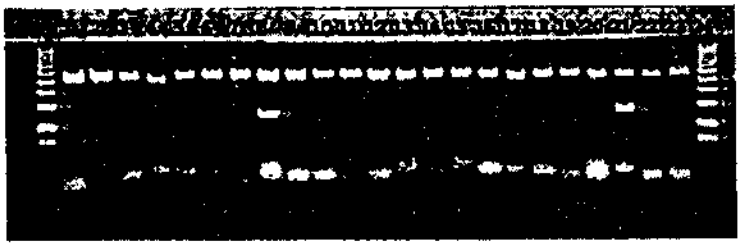


Figure 18: Comparison of the HLA-B SSP-PCR Typing to the UCLA Typing of #139 for Alleles B27 and B73 Reference Panel.

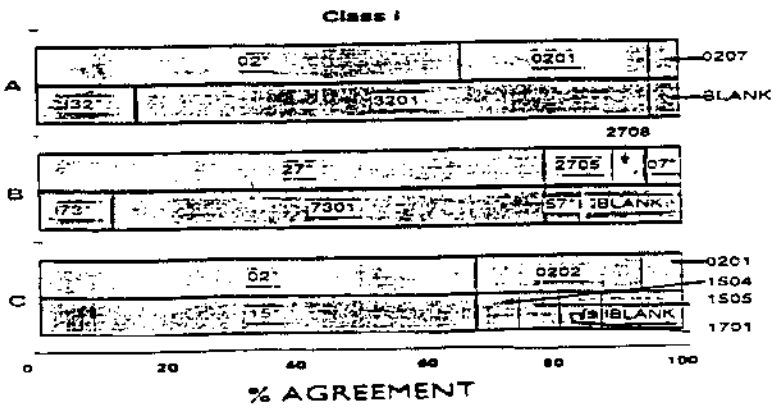
UCLA # 139



24 25 26 27 28 29 30 31 32 33 34 35 36

DNA #0139

DIRECTOR	A-LOCUS		B-LOCUS		C-LOCUS	
ADORNO, DOMENICO	0207	3201	27*	7301	02*	15*
ALBERT, EKKEHARD			27052	7301		
ALCALAY, D.	02*	3201	27*	57*	02*	15*
BARTOVA, ADELA	02*	BLANK	27*	73*	02*	1701
CHEN, RITA					02*	15*
DARKE, CHRIS	02*	3201	27*	7301	0202	15*
DUNCKLEY, HEATHER	02*	32*	27*	7301	02*	15*
DUNN, SUSAN					0202	BLANK
EASTWOOD, STUART	0201	3201	27*	BLANK	0202	15*
FISCHER, HARALD	02*	3201				
FUGGLE, SUSAN	0201	3201	27*	7301	02022	1505
GROSSE-WILDE, HANS			27*	7301		
HAMMOND, MG	02*	3201	07*	BLANK	02*	BLANK
HAN, HOON	02*	3201	2708	7301	0201	1504
HARMAN, LEIGH	02*	3201	27*	7301	02*	15*
KUKURAGA, DEBRA	02*	32*				
MACHULLA, HELMUT	02*	3201	27*	7301		
MELANNY, HIDAJAT	0201	3201			02*	15*
OLERUP, OLLE	0201	3201	2705	7301	02*	15*
RODRIGUEZ, SUSANA	0201	3201	27*	BLANK		
SAITOU, SATOSHI	0201	3201				
SCHAUB, BETTY	02*	3201	27*	7301	02*	15*
SIAUW, POO-AN	02*	3201	27*	7301		
TAKEDA, ALAN	02*	32*	27*	73*		
TILANUS, MARCEL	NT	NT				



3.4 Positive Internal Control

The presence of the positive internal control primer pair, included in each typing reaction, permits one to discern that the absence of an amplicon was due to the absence of a target for the HLA-B primer pair tested. The positive control primers amplify a 400bp fragment of the human growth hormone. It was designed to amplify a larger product than the HLA-B SSP primer mixes. The positive control is easily distinguishable from the amplicons of the HLA-B SSP primers.

CHAPTER IV

DISCUSSION

The objective for developing the HLA-B low-resolution DNA typing primer panel was to demonstrate in a comprehensive SSP-PCR system that would amplify HLA-B alleles in a single series of typing reactions. The primer panel was based on the Class I nucleotide allels sequences and encompasses all the HLA-B serological specificities. The procedure take advantage of a single PCR reaction to determine HLA-B specificity using genomic DNA and sequence-specific primers in particular combinations to determine allele specificity. The foundation for the HLA-B low-resolution primer panel was published genomic sequences from UCLA's HLA-B serological data. The panel consists of 72 sequence-specific primers (SSP) table #2 in 36 PCR reactions that incorporate all known HLA-B alleles. Advantages to this system include speed completed typing results can be obtained in under 4 hours including DNA extraction and the results are ease of interpretation.

To perform SSP-PCR typing; it is necessary to obtain purified chromosomal DNA as the template for the SSP primers to amplify. If the quality of the DNA is inferior, yields of product tend to be less than adequate; the resulting amplicon may disappear or appear as a false positive. Due to a nearly 70% G/C content of the HLA-B sequence from exon 2 to exon 3, the denaturation temperature at the beginning of the first amplification cycle, as well as within each PCR cycling profile, was raised to generate functional PCR template

free of secondary structure. In addition, the annealing temperature is decreased after the first 10 cycles to maximize the efficiency of the PCR amplification. The number of amplification cycles and annealing temperatures should be adjusted to obtain similar DNA amplification yields for different alleles.

Design of sequence-specific primers should meet minimal inter or intra-complementary binding sites, particularly at the 3' ends. Certain nucleotide sequences are predisposed to the formation of intramolecular bonds creating hairpins. This secondary structure can prevent primers from annealing, therefore impeding amplification. For example, HLA-B15 is highly polymorphic and cross reactions have been obtained for the following subgroups; B15, B62, B63, B70, B71, B72, B75, B76, AND B77. For HLA-B15, two primer pairs of the panel were utilized in typing HLA-B15 subgroups. Two different primer pairs were used to deter primer-dimer formation. Primer-dimers are an amplification artifact caused when one primer is extended by the Taq DNA polymerase using the other primer as a template. An additional type of artifact can occur if the 3' end of one primer can fold back and self-anneal serving as a self-template for extension. Both types of artifacts (hairpins and primer-dimers) result in the formation of a single band or ladder of short incorrect products. Furthermore, when using a single pair of generic primers to amplify sequence information of interest, co-amplification of other HLA Class I genes may occur. Sharing of sequence motifs can exist between different alleles at one locus or between different genes. Prevention of cross-reactivity necessitates strategic designing for some primer pairs for HLA-B to prevent cross reaction of HLA-C groups which result in false amplicons. False amplicons lead to false positive results or incorrect

typing bands.

The number of PCR cycles was varied in the beginning of this study to determine the correct number of cycles necessary to produce sufficient amplification products. Thirty cycles gave optimum results for the detection of HLA Class I alleles. Cycling temperatures and duration are crucial for PCR cycling parameters to produce accurately amplified target DNA. The initial denaturation of 1 minute was significant in the HLA Class I typing since its composition is G/C rich. This initial denaturation step allowed reduction of subsequent denaturation times of the PCR amplification cycle to 10 seconds. Decreasing the annealing temperature after the first 10 cycles (from 65°-75°C) increases the efficiency of the PCR amplification. The primer template complex is sturdier at lower temperatures and the yield is increased. A decrease in annealing time or increasing the final elongation step can diminish the final amplification product.

Agarose gel electrophoresis is a traditional method for separating DNA fragments by size and, it is advantageous to SSP-PCR. For example, it allows visualization of internal control fragments, furnishes relative fragment size information, and permits distinction of non-specific amplification and primer oligomer artifacts from true positive amplicons. The cause weaker amplification signals can be trace to aged ethidium bromide agarose gel staining solution probably due to degradation of the ethidium bromide from exposure to light and air.

The following is a summarization of the conclusions drawn from this study:

The typing of HLA-B specificity has greatly benefited from the introduction of polymerase chain reaction (PCR) based on sequence-specific primers (SSP). Reliability

and accuracy have increased and, since viable cells are no longer a necessity, the work in clinical laboratories can be solidly structured.

A system was devised for typing exon 2 and exon 3 of the HLA-B locus based on known genomic DNA sequences. This approach can be expanded to include other polymorphic exons of HLA-B (high resolution) and aid in developing a model with similar strategies for typing other Class I loci.

This system is complementary to previous published SSP-PCR systems for HLA Class I in that similar PCR conditions, protocols, and controls are used for all alleles which permitting one step SSP-PCR for all relevant HLA loci in under 4 hours in a system suitable for the typing of organ transplantation and bone marrow transplantation.

Serology is a standard and conventional method of HLA Class I detection but it is hindered by serological cross reactivity and lack of useful typing reagents. SSP-PCR offers an alternative to serological tissue typing which is restricted by the requirement for viable cells and the limited available of specific alloantisera.

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