การพัฒนาวิธีการตรวจหาเอชแอลเอบี 1502 และ 5801 ด้วยวิธีเอสเอสพีพีซีอาร์ และแลมพ์โดยใช้พีเอนเอ

นางสาวศิตา วีรกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

DEVELOPMENT IN DETECTION OF HLA-B*1502 AND –B*5801 BY SSP-PCR AND LAMP WITH PNA PROBE

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Microbiology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2009

Thesis Title	DEVELOPMENT IN DETECTION OF HLA-B*1502 AND –B*5801 BY SSP-PCR AND LAMP WITH PNA PROBE
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ศิตา วีรกุล : การพัฒนาวิธีการตรวจหาเอชแอลเอบี 1502 และ 5801 ด้วยวิธีเอสเอสพีพีซีอาร์ และแลมพ์โดยใช้พีเอนเอ. (DEVELOPMENT IN DETECTION OF HLA-B*1502 AND –B*5801 BY SSP-PCR AND LAMP WITH PNA PROBE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.พญ.ดร.ณัฏฐิยา หิรัญกาญจน์,อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.ธีรยุทธ วิไลวัลย์, ผศ.ดร.ปิยะศักดิ์ ชอุ่มพฤกษ์, 77 หน้า.

ในปัจจุบันสามารถทำนายการเกิดการแพ้ยาอย่างรุนแรงได้โดยการหาเอชแอลเอยีน โดยเอชแอลเอตัวแรกที่มีการรายงานคือ เอชแอลเอบี 1502 ซึ่งมีความสัมพันธ์กับการแพ้ยากันชักชื่อคาร์บามาซีพีน อีกยีนหนึ่งคือเอชแอลเอบี 5801 มีสัมพันธ์กับยารักษาโรคเกาต์ชื่ออัลโลพูรินอล ยีนเหล่านี้มีความสำคัญมากเนื่องจากเป็นยีนที่พบมากในกลุ่มประชากรเอเชีย ร่วมกับมีอัตราการตายจากการแพ้ยาอยู่ที่ประมาณ 26 เปอร์เซ็นต์ จึงมีความสำคัญในการหาวิธีตรวจยีนที่ได้ผลถูกต้อง ราคาเหมาะสม และรวดเร็ว วิธีหนึ่งก็คือ เอสเอสพีพีซีอาร์ ในปัจจุบันมีชุดตรวจสำเร็จแต่ราคายังคงแพงเกินไป จึงควรมีการพัฒนาเอสเอสพีพีซีอาร์อย่างง่าย และราคาถูกเพื่อใช้เองภายในประเทศ จากงานวิจัยในครั้งนี้สามารถทำนายเอชแอลเอบี 1502 และเอชแอลเอบี 5801 ได้ความไว 100% และแม่นยำ >99.9% ในขั้นต่อมาได้นำวิธีการทำนายเอชแอลเอบี 1502 และเอชแอลเอบี 5801 ไปใช้กับชุดพีซีอาร์จากเลือดโดยตรง ซึ่งให้ผลที่มีความจำเพาะได้เท่าเทียมชุดพีซีอาร์แบบดั้งเดิม ทำให้สามารถลดขั้นตอนและค่าใช้จ่ายในการแยกสารพันธุกรรมจากเลือดได้ นอกจากนี้แล้วยังมีการใช้วิธีอื่นเข้ามาช่วยการหาเอชแอลเอเช่น วิธีแลมพ์ อันเป็นวิธีการเพิ่มจำนวนสารพันธุกรรมที่อุณหภูมิเดียว โดยอาศัยไพร์เมอร์ 4 เส้นในการทำนายชนิดของเอชแอลเออย่างจำเพาะ จากงานวิจัยในครั้งนี้สามารถทำนายเอชแอลเอบี 5801 ได้ความไว 95.24% และแม่นยำ 83.78% ประโยชน์ของงานวิจัยนี้ทำให้สามรถช่วยคนไข้ไม่ให้เกิดอาการแพ้ที่รุนแรงถึงชีวิตได้ตั้งแต่ก่อนการให้ยา

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....
ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....
ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5187284820 : MAJOR MEDICAL MICROBIOLOGY
KEYWORDS : HLA-B*1502 / HLA-B*5801 / SSP-PCR / LAMP / PNA

SITA VIRAKUL : DEVELOPMENT IN DETECTION OF HLA-B*1502 AND –B*5801 BY SSP-PCR AND LAMP WITH PNA PROBE. THESIS ADVISOR : ASSOC. PROF.NATTIYA HIRANKARN, MD., Ph.D., THESIS CO-ADVISOR : ASSOC. PROF.TIRAYUT VILAIVAN, D.Phil., ASST PROF.PIYASAK CHAUMPLUK, Ph.D., 77 pp.

Pharmacogenetics in HLA allele is very useful for risk assessment in life-threatening drug hypersensitivity. In 2004, HLA-B*1502 first showed strong association with carbamazepine hypersensitivity. Another association, HLA-B*5801 involved with allopurinol hypersensitivity. Since allele frequencies of these HLA-B alleles are quite common in Asian ethnicity and with 26% mortality rate in patients, it is important to develop a rapid and cost effective test for Asian, including our Thai population. To get a HLA type, commercially available SSP-PCR is too expensive. So, in-house SSP-PCR would be the most interesting candidates comparing to other molecular techniques. From this study, HLA-B*1502 can be interpreted specifically by simple nested SSP-PCR, while HLA-B*5801 can be differentiated by only one set of primer with 100% sensitivity and >99.9% specificity. Moreover, blood PCR kit can be applied to both HLA-B*1502 and HLA-B*5801 with the same specificity. Furthermore, LAMP was introduced to HLA typing as an alternative method. LAMP is an isothermal amplification technique which its specificity comes from 4 primers at a time. In this study, HLA-B*5801 can be identified with 95.24% sensitivity and 83.78% specificity. The benefit of these tests would help patients to avoid any life-threatening adverse consequences.

Field of Study : Medical Microbiology Student's Signature .....
Academic Year : 2009 Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

All of these works won't be happened, if I don't have Associate Professor Nattiya Hirankarn as my thesis advisor. From the very beginning, she allowed me to initiate my own research topic. Along the way, she always gives great advice and comment which finally make me get complete research works. Also, I wouldn't have met two other great Professors, my co-advisors. My first co-advisor, Associate Professor Tirayut Vilaivan introduces PNA detection system to my research. He always shares his great experience in Chemistry and also his specialty in PNA. Without his extensive research work, I wouldn't have such a great tool for LAMP detection. My second co-advisor, Assistant Professor Piyasak Chaumpruk is an expert in LAMP. He guides me through all his experience from the past which makes my work goes very fast. These work had been supported by two main grants; the Thailand Research Fund (Senior Research Scholar Grant – RTA5280002) and the 90th Year Anniversary of Chulalongkorn University (Ratchadaphiseksomphot Endowment Fund). Many other people take important parts in this work as well. The main three persons I have to mention are Miss Siraprapa Thongkobpetch, who provided me blind DNA sample in this work, Mr. Jeerawat Nakkuntod, who taught me all basic laboratory techniques and Miss Alisa Lowanitchapat, who did all the PNA probe hybridization assay. Also, without my lab mates that I can't put all the names in here, I wouldn't have such greatest moment in life. Moreover, I'd like to thanks Mr. Dumrong Bunchongsilp who always listens and encourages me to take all important action in life. Last but not least, I'd like to thanks my whole family, especially my mother, for raising me up, giving me cheerfulness in life and all great support, as always.

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CHAPTER I

INTRODUCTION

Background information and rationale

Studies in pharmacogenetics both in finding effective dose and adverse drug reactions have improved treatment dramatically. For adverse drug reactions, there are many FDA recommended of risk factor testing before prescription. In this current study, only risk HLA genes with high frequency in Asian population are interested. The first HLA gene, HLA-B*1502 is associated with carbamazepine, anti-seizure drug. Another allele is HLA-B*5801 which is related with anti-hyperuricemic drug, allopurinol. Drug adverse reactions from these two alleles are life-threatening with about 30% death.

HLA typing can be done by both serological and molecular methods. But these alleles prediction requires subtype identification which serological method cannot provide this information. Thus, molecular technique is more suitable to use for HLA alleles typing for risk prediction. One of the most cost effective methods is SSP-PCR. The technique relies on sequence specific primers which specifically bind and amplify the target DNA. From many previous reports, more than one set of primer is needed. In this study, method was improved with less sets of primers and/or other modified PCR conditions, for example, nested PCR was introduced to eliminate false positive results from heterozygous combination. After PCR reactions were set, whole blood was then added directly to PCR reaction in order to get cheaper, faster method and reliable results.

Another technique rather than PCR was also applied to overcome PCR limitation. LAMP is an isothermal amplification which expensive PCR machine is not required. Moreover, the assay time is only 40 minutes to 1 hour, comparing to time consuming 3 hours for PCR. Also for its detection step, gel electrophoresis is not required. Recently, there was a report on using LAMP for HLA-B*1502 identification by SYBR green detection. In this study, HLA-B*5801 is used as a model. Afterwards, PNA probe is used for sequence specific detection.

Research objectives

1. To develop a simple in-house SSP-PCR for HLA-B*1502 and –B*5801 and validate with SSP-PCR commercial kit and direct sequencing.

2. To develop LAMP system in combination with DNA-PNA strand displacement probes for rapid detection of HLA-B*5801.

Benefits

The benefit of this study would significantly help patients from getting life-threatening adverse reaction before prescription within a very short time and reasonable price.

CHAPTER II

LITERATURE REVIEW

Drug response in each patient is different. Up to date, there are many evidences on genetics factors which play an important role in these responses, such as cytochrome P450, thiopurine methyltransferase (TPMT) and human leukocyte antigen (HLA) (1). Study in inherited predisposing factors which determines drug response is widely known as pharmacogenetics (2). These diverse reactions include not only level of effectiveness in treatment, but also life-threatening severe symptom. For severe drug-induced reaction aspect, although the incident rate is quite low, identification of these genetic markers helps to reduce cause of death dramatically. The strongest and the most specific marker for risk assessment so far is HLA gene, as shown in table 1 (1).

Table 1. Pharmacogenomic biomarkers as predictors of adverse drug reactions.

Gene or Allele	Relevant Drug	Specificity of Biomarker	Percent of Patients with an Adverse Reaction to Drug
TPMT (mutant)	6-Mercaptopurines	Very good	1–10
UGT1A1*28	Irinotecan	Good	30–40
CYP2C9 and VKORC1	Warfarin	Good	5–40
CYP2D6 (mutant)	Tricyclic anti-depressants	Relatively good	5–7
HLA-B*5701	Abacavir	Very good	5–8
HLA-B*1502	Carbamazepine	Very good	10
HLA-DRB1*07 and DQA1*02	Ximelagatran	Good	5–7

The poorest prognosis among severe drug-induced skin reactions are Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (3). They are a life-threatening syndrome with high mortality rate. The appearance on mouth, lips, conjunctiva and genital area vary from erythema to blistering second degree burn-like. These symptoms also involve fever and malaise. For the pathogenesis, they are immune-mediated reactions by delayed-type hypersensitivity (4). Additionally, results from immunohistochemistry suggested that activated T cells and macrophages were found in the lesional skin. A more extensive study on analytical quantification of the inflammatory cell infiltrate revealed that lymphocytes are a major population over macrophages in both dermis and epidermis during the early stage of disease, while in the later phase is vice versa (5). During the active stage in the presence of drugs, blister fluid T cells lyse autologous keratinocytes by granulysin which resulted in the hallmark of SJS and TEN, widespread epidermal necrosis (6).

In 2004, Chung, *et al.*, first reported HLA gene as genetic marker for SJS by carbamazepine with the odd ratio of as high as 2504 (7). Year after, many other studies in pharmacogenetics of HLA alleles and drug hypersensitivity have also been reported. In addition to HLA-drug specific induced SJS, reports on ethnicity shows important role as well (table 2) (8).

Table 2. Association between HLA allele/related loci and drug hypersensitivity is drug specific, phenotype specific and ethnicity specific (8).

Culprit drug	Drug hypersensitivity	HLA association	Population	Reference
Carbamazepine	SJS/TEN	HLA-B*1502 (corrected P=$3.1x10^{-27}$–$1.6x10^{-41}$, OR 1357–2504)	Han Chinese in Taiwan	Hung SI, *et. al.*
Carbamazepine	SJS	Weak association with B44. No association with HLA-B*1502.	Whites in Europe	Lonjou C, *et. al.*
Carbamazepine	SJS/TEN	HLA-B*1502	South-eastern Asians	Lonjou C, *et. al.*
Carbamazepine	HSS	rs2894342 of motilin gene in the MHC region (corrected P=0.0064, OR 7.1)	Han Chinese in Taiwan	Hung SI, *et. al.*
Carbamazepine	MPE	HLA-A*3101 (corrected P=$2.2x10^{-3}$, OR 17.5)	Han Chinese in Taiwan	Hung SI, *et. al.*
Carbamazepine	Severe hypersensitivity reactions (HSS/MPE)	TNF2- DR3-DQ2 haplotypes (P=0.02, OR 3.2)	Whites in Europe	Alfirevic C, *et. al.*
Allopurinol	SJS/TEN/HSS	HLA-B*5801 (corrected P=$4.7x10^{-24}$, OR 580.3)	Han Chinese in Taiwan	Hung SI, *et. al.*
Abacavir	Drug hypersensitivity (HSS/MPE)	HLA-B*5701 (corrected P=$5.2x10^{-20}$, OR 960)	Whites	Mallal S, *et. al.*
Abacavir	Drug hypersensitivity (HSS/MPE)	No association with HLA-B*5701	Hispanics, or African	Hughes AR, *et. al.*
Nevirapine	Drug hypersensitivity	HLA-DRB1*0101	Whites in Australia	Martin AM, *et. al.*
Nevirapine	Drug hypersensitivity	HLA-Cw8-B14 haplotype (corrected P=0.05)	Whites in Italy	Littera R, *et. al.*

HSS, hypersensitivity syndrome; MHC, major histocompatibility complex; MPE, maculopapular eruption; OR, odds ratio; SJS, Stevens–Johnson syndrome; TEN, toxic epidermal necrolysis.

Among Asian ethnicity, HLA-B*1502 associated with carbamazepine (9, 10), while in Caucasian populations, this association was not found (11). This antiepileptic drugs causes death in 30% of all patients. Another association, HLA-B*5801 involved with antihyperuricemic agents, allopurinol. Its mortality rate is around 26% (12, 13). Since allele frequency of these two HLA antigens are quite common in some Asian ethnicity, as listed in table 3 (http://www.allelefrequencies.net/default.asp), it is important to develop a simple rapid test for Asian, including Thai population.

Table 3. HLA-B*1502 and HLA–B*5801 frequencies in Asian ethnicity.

Ethnicity	Frequency (%)	
	HLA-B*1502	HLA-B*5801
China North Han	2.7	2.9
China South Han	7.1	8.9
Taiwan	4.2	9.8
Japan	0.1	0.5
Korea	0.2	6.5
Singapore	11.6	5.8
Thailand	8.5	7.7
Vietnam	13.5	6.5

In immune system, HLA takes place in specific immune response against invading substances. These HLA molecules are on the surface of antigen presenting cell, presenting specific short peptide antigen to T cell. There are two classes of HLA, which are differentiated by their antigen types, antigen processing and their cell targets. First, HLA class I (HLA-A, -B, -C, -E, -F and -G) presents peptide from endogenous antigen to CD8+ T cell (cytotoxic T cell), while HLA class II (HLA-DR, -DP, -DQ, -DM and -DO) presents peptide from exogenous antigen to CD4+ T cell (helper T cell). Resulting of HLA-peptide-TCR complex, protective reaction is activated. Pathogenesis study of drug-induced SJS is explained into two hypotheses (14). First, the hapten hypothesis

suggested that drug or its metabolites bind to some proteins or peptides, following by processing and presenting on MHC molecule which resulting in protein modification (15). Another one is the p-i concept (direct pharmacologic interaction of drugs with immune receptors) pointed that drug can bind directly with MHC molecule, without any protein processing involved. Comparison between the two concepts is shown in table 4.

Table 4. Comparison of hapten hypothesis and p-i concept.

Hapten hypothesis	p-i concept
Chemical (hapten), covalent binding	Structural (fitting into TCR), no covalent binding
Stable protein/peptide modification (covalent)	No covalent binding
MHC-APC directed	TCR-T cell directed
Often processing and metabolism involved	No processing/no metabolism
Very heterogeneous and often combined immune responses (Ig, T-cells)	Only T-cell reactions of different types

Assuming from the two hypotheses that HLA-drug complex and CD8+ T cell mediate cytotoxic response in SJS. More update study from Yang, *et al.* indicated HLA-B*1502-bound peptide in favor of the p-i concept (14). They used liquid chromatography-tandem mass spectrometry for the investigation and found that none of the particular carbamazepine-modified peptides were detected when comparing peptide sequence between with and without drug condition.

Molecular information in both gene and protein profiles of HLA are very important when specific HLA types is needed. Starting with the protein profile, HLA class I heterodimer composes of 1 alpha-chain (45kD) connected with ß2M chain (12 kD) (16). The ß2M is translated from the gene out of the HLA complex, while the alpha-chain is coded from the HLA complex which forming into three major domains;

extracellular, transmembrane and cytoplasmic domain. Another class, HLA class II has 1 alpha-chain (33kD) liked with 1-beta chain (28kD). Both of these protein chains of the HLA class II are coded from the HLA complex and are separated into three parts similarly to the HLA class I. Extracellular membrane of both HLA class I and HLA class II are the very crucial parts for HLA typing, because they are very high polymorphic that numbers of HLA types can be told apart. Typing of these HLA molecules by serological tests, only HLA type is interpreted, while HLA subtype cannot be identified. Using antibody for HLA typing sometimes gives inconclusive results which cause by cross reactivity of monoclonal antibody. Also, misinterpretation can occur when that certain alleles are downregulated when infection takes place (17). As a result, only DNA based HLA typing can give consistent and specific type results.

Beginning with the HLA gene profile, HLA class I alpha-chain has 8 exons, while HLA class II alpha-chain and HLA class II beta-chain has 5 and 6 exons, respectively (16). The first exon of both HLA class I and II is the leading peptide sequence which facilitates the mRNA to go to the ER which will be cleaved later in the ER after full peptide is synthesis. Next, exon 2-4 in HLA class I and exon 2-3 in HLA class II is translated into alpha or beta domain. Finally, other remaining exons are translated into transmembrane and cytoplasmic peptide. All HLA exons and proteins profile of both HLA class I and HLA class II are conclude in figure 1.

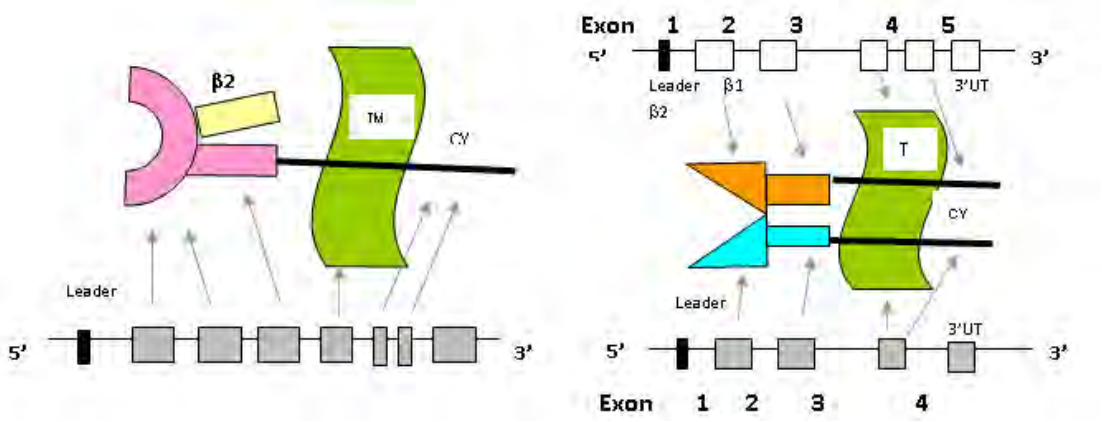


Figure 1. HLA exons and proteins profile of both HLA class I and HLA class II (16). Exon and protein domains alignment of HLA class I (left) and HLA class II (right). TM, transmembrane domain; CY, cytoplasmic domain; 3'UT, 3' untranslated region.

Years after serological methods have been established, molecular technique is introduced to HLA subtype detection, owing to HLA exon sequences data. These techniques become commercially available and are applied to many routine laboratories. They are still expensive and also give lots of unnecessary information for HLA risk prediction, as all alleles are interpreted (18). Testing HLA gene from genomic DNA, laboratories apply either of four different techniques. First of all, polymerase chain reaction-sequence specific oligonucleotide probes (PCR-SSOP). This technique use sets of probes to detect HLA types of multiple samples at the same time. Therefore, PCR-SSOP is suitable for high throughput HLA typing, preferable used in donor center. Secondly, reverse SSOP is a quite expensive method because all probes were designed to bind to target HLA allele at the same temperature. The third technique is PCR-SSOP-luminex® system which applies flow cytometry to detect up to 100 colorimetric beads with different probes in one run (19). The limitations are the cost and requirement of special instrument for analysis. Finally, sequence-specific primer polymerase chain reaction (SSP-PCR) works by 3'mismatch principle with many sets of primer (20). Therefore, these primers would only anneal specifically to the target (figure 2). Comparing to other methods, this technique is quite cheap and very simple which expensive machine and specialist are not required.

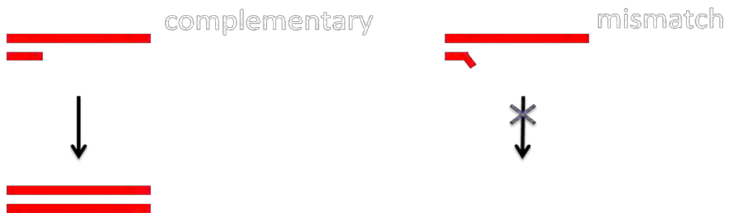


Figure 2. 3'-mismatch principle of SSP-PCR. On the left, primer binds specifically to the target, whereas non specific binding occurs on the right. Consequently, no PCR product is found with 3'-mismatch primer.

Although commercial SSP-PCR is quite cheap comparing to other molecular methods, it is still expensive, as several sets of primers are used. Moreover, this technique gives all allele interpretation which is more suitable for tissue matching, but not for only one allele prediction. Previously, HLA-B*1502 and 5801 detection by fewer sets of primers has been reported by Bunce, *et. al.* (21). Firstly, HLA-B*1502 requires four primer sets of primer to specifically identify this allele. The first set of primer amplifies HLA-B*2708, 40, 41, 14, 39, 67, 7, 801, 42, 48, 45, 50, 54, 55, 56, 18, 35, 78, 1501-12, 1514-15, 1518-22, 1525-26, 1528-29, 46, and 8101. Next, the second set of primer detects HLA-B*1501-02, 1504-08, 1511-12, 1514-15, 1519, 1521, 1525, 1526N, and 1528. Then, third set of primer is positive for HLA-B*3501-04, 3506-09, 3511, 5301, 1502, 1513, 5104, 1521, and 4406. Finally, forth set of primer targets HLA-B*1501-02, 1511-16, 1519-21, 1524-26N, 1528, 5701, and 4601. Positive bands from all four sets of primer will be interpreted as HLA-B*1502 without any false positive results from heterozygous combination. All primers for HLA-B*1502 are aligned in figure 3.

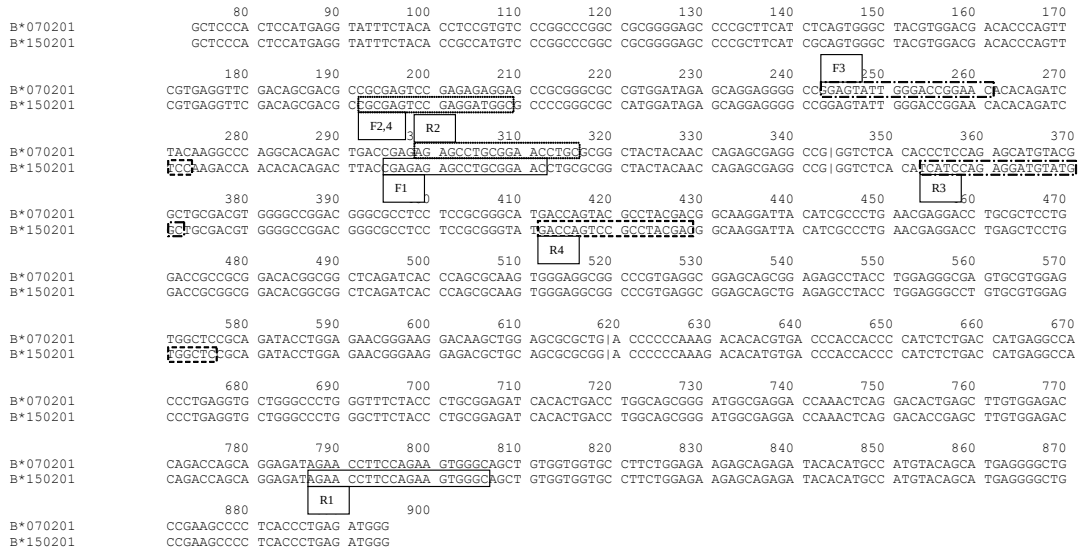


Figure 3. Primers location of HLA-B*1502 by Bunce, *et. al.* (21). HLA-B*1502 requires four separated reactions of primers to specifically identify HLA-B*1502.

For HLA-B*5801, two sets of primer are needed to identify. The first set of primer amplifies HLA-B*5801, 5104, 5301, and 1513, while the second set of primer detects HLA-B*5801-03. By using two sets of primer, false positive results may still occur from heterozygous combination. For example, patient with HLA-B*1513 and 5802 will give two positive bands from these two primers sets and will be misinterpreted as HLA-B*5801 positive. All primers for HLA-B*5801 are aligned in figure 4.

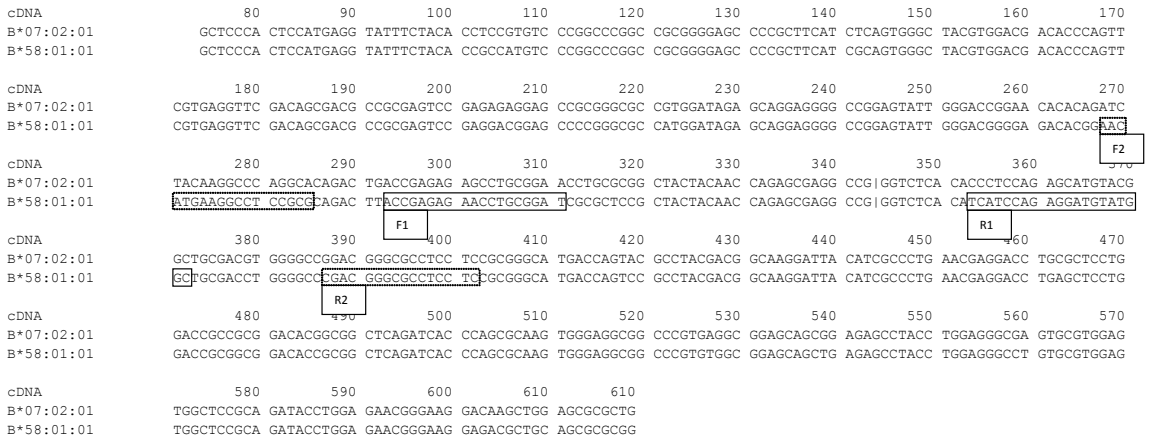


Figure 4. Primers location of HLA-B*5801 by Bunce, *et. al.* (21). HLA-B*5801 requires two separated reactions of primers to specifically identify HLA-B*5801.

All of those techniques mentioned earlier require DNA extraction and purification steps, as whole blood sample contains many inhibitors for PCR reaction. Not only organic substances such as polysaccharides, proteins, lipids, immunoglobulin, bile salts and heme in blood circulation, but also anticoagulating agents of all kinds inhibit the reaction (22). As a result, this additional crucial DNA extraction and purification step reduces turnaround time by conventional salting out method or depends on fast but expensive commercial DNA extraction kit. Many experiments on direct PCR from whole blood have been done extensively for many of years and much improvement has been shown chronologically. The first report in 1990 changed the initial denaturing step to make sure that all cells sample completely break, and DNA are released (23). Two years later, McCusker, *et. al.* used lysate of whole blood dilution as PCR template which resulted in PCR improvement as inhibitors were also diluted (24). Years after, lots of scientists from China, Korea and Japan reported many novel buffer types which enhanced PCR reaction from whole blood by neutralizing the inhibitors (25-27). Recently, another group of scientist successfully created two new mutant Taq DNA polymerase by site direct mutation to make it more resistant to inhibitors (28). In 2010, Zhang, *et. al.* combined both mutant Taq and their novel PCR enhancer cocktails (PEC) to successfully amplified both normal and GC rich amplicons from crude samples,

whole blood and soil samples (22). Even without DNA extraction step, SSP-PCR is still time consuming about 3 - 4 hours and requires PCR and gel electrophoresis machine which make diagnosis at point-of-care impossible. Consequently, it's important to develop the most specific, rapid and cheapest test without any expensive machine for particular HLA alleles of interest to reduce both time and cost comparing to the traditional SSP-PCR. Other amplification methods are interestingly applied to HLA typing area because of their unique advantages over PCR. Some of them require only one temperature to amplify target DNA, which PCR thermal cycler is not needed (29). As a result, applying such isothermal amplification techniques to small hospital or field diagnosis is possible with small and cheap water bath. Some examples are nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA) and loop-mediated isothermal amplification (LAMP).

Beginning with the first isothermal amplification technique, nucleic acid sequence-based amplification (NASBA) amplifies RNA template at one temperature (30). First of all, DNA primer binds to RNA target and following by reverse transcriptase for primer extension. At this point RNaseH will digest original RNA which helps to omit denaturing step at another higher temperature. Finally, another primer with T7 promoter and complementary sequence to the cDNA, will amplify the cDNA. This double stranded product will result in self-sustained template by RNA polymerase. The weak point of this method is its specificity, numbers of enzyme needed and intolerance to biological substances. Some of these limitations are overcome by SDA. Strand displacement amplification (SDA) uses four primers to increase test specificity (30). Like other isothermal amplification, this method uses restriction enzyme to initiate strand separation instead of heat. However, restriction enzyme is limited. As a result, not all DNA targets can be applied to this technique. The loop-mediated isothermal amplification (LAMP) is the latest technique in isothermal amplification which was reported in 2000 (29). This technique takes the advantages of both NASBA and SDA in order to gain specificity and sensitivity. First of all, LAMP contains four primers detecting six regions to get the most specificity comparing to NASBA. Next, LAMP is performed at higher temperature (60-

65°C), so more specificity is gained than NASBA and SDA (37-42°C). Finally, LAMP reaction is resistant to biological substances which crude samples might be applied directly to the reaction. Consequently, between these three techniques, LAMP has overcome NASBA and SDA limitation for HLA typing. NASBA, SDA and LAMP are concluded in table 5.

Table 5. Properties of various isothermal amplification methods and PCR (30).

Property	PCR	NASBA	SDA	LAMP
Temperature(°C)	94, 55–60, 72	37–42	37	60–65
Number of enzyme (s)	1	2-3	2	1
Primer design	Simple	Simple	Complex	Complex
Multiplex amplification	+	+	-	-
Tolerance to biological components	-	-	-	+
Denaturing agent	Heat	RNaseH	Restriction enzyme	Betaine

For the mechanisms of LAMP, four primers, FIP, F3, BIP and B3, detecting 6 regions in target sequence are used to guarantee high specificity and efficiency (figure 5).

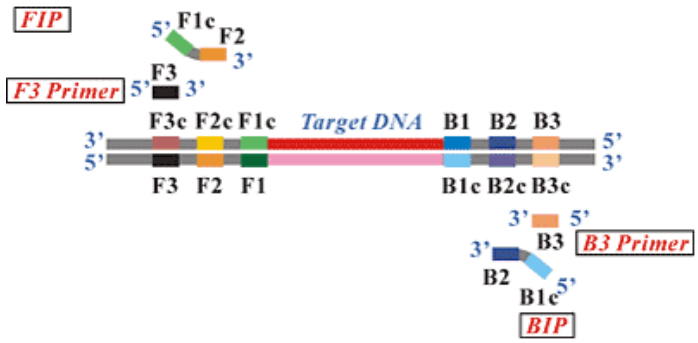
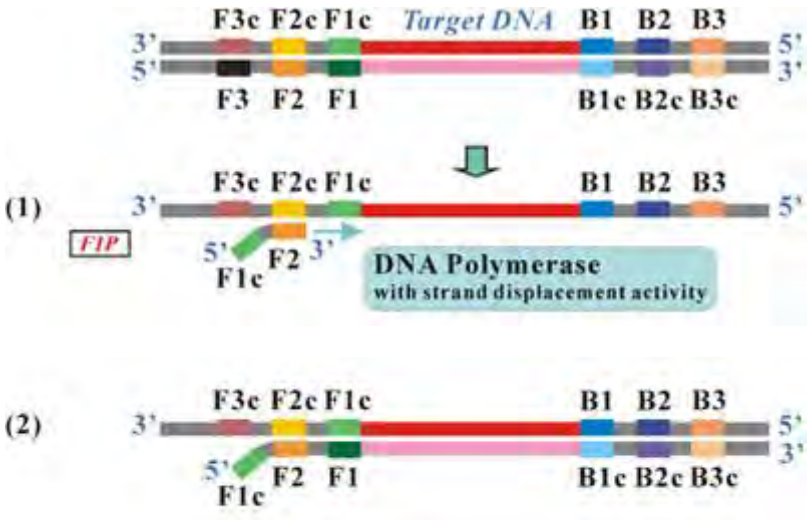
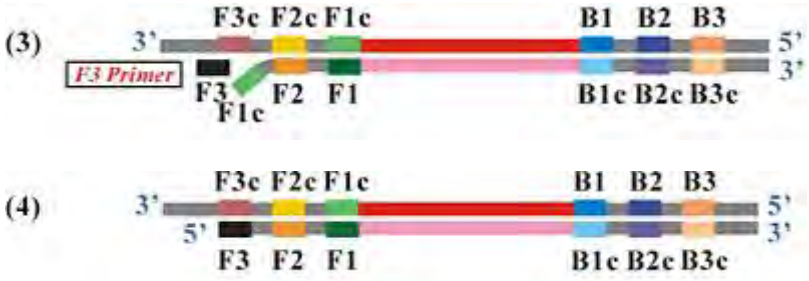


Figure 5. Primer region in LAMP reaction (http://loopamp.eiken.co.jp/e/).

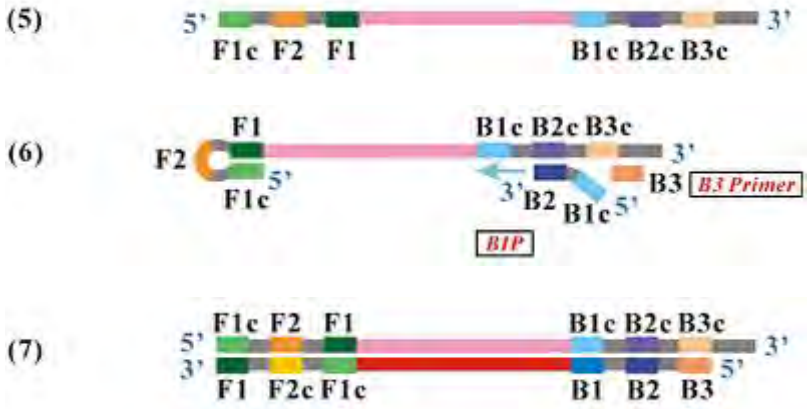
Forward Inner Primer (FIP) consists of two regions F1C and F2, detecting F2C on target (step1), and following by extension step at 3' end of FIP (step 2).



F3 is an outer primer that starts strand displacement activity (step3). So, FIP-linked complementary strand is released from target DNA (step4).

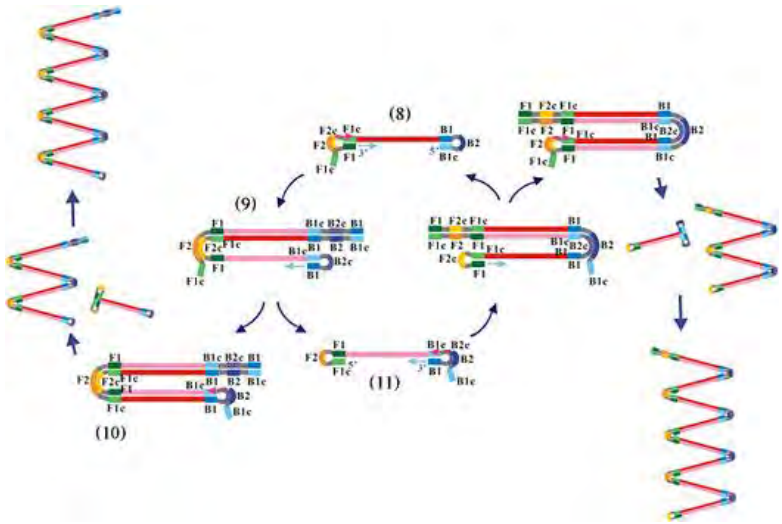


Next, FIP-linked complementary strand acts as template for BIP (step5) and instantly forms self complementary loop (step6). Acting as F3, B3 activates strand displacement and follows by extension step (step7).



FIP and BIP linked template form a dumbbell-like structure at each end (step8). This structure is an initial starter for LAMP cycling. Its free 3' end uses self template for extension step.

In LAMP amplification cycle, FIP starts amplifying at stem loop DNA (step9). Releasing from the template strand, there are 2 forms of DNA as shown in step10 and step 11, respectively. Further on, BIP initiates another strand displacement and so on. From overall reaction, it results in various size structures. As a result, LAMP product can be observed as ladder bands by gel electrophoresis. Moreover, high amount of LAMP products produce lots of pyrophosphate by-product, resulting in turbidity change due to the result of the Magnesium-Pyrophosphate formation can then be seen with bared eyes (31).



Recently, researchers from Hong Kong interestingly demonstrated HLA-B*1502 typing using two LAMP sets directly from whole blood (32). LAMP reactions take only about 15 – 25 minutes and can be interpreted instantly by SYBR Green I. The outcomes compared with sequence-based typing (SBT) and by SSP-PCR gave 100% sensitivity and more than 99.9% specificity from their experiments. Moreover, for its high amplification efficiency, this test can also be used with severe leucopenia patients. This method is one of the most interesting rapid and simplified tests for specific HLA alleles to predict drug allergy which could further applied to bedside testing or point-of-care diagnosis. All HLA typing techniques are summarized in table 6.

Table 6. Comparison between each method in current HLA typing.

Methods	Advantages	Limitations
SSP-PCR	One step detection, no additional probe or enzyme needed, cheaper and easier to interpret than SSOP-PCR as no special devices needed	Cross reactivity with closely related alleles, only one specific allele detection, laborious
SSOP-PCR	High throughput for many alleles detection, applied into many form of assay for selection	Oligonucleotide probe and blotting system needed, expensive, not suitable for small lab service, laborious
LAMP	Isothermal amplification, no special machine needed, more specific in amplification step, visual result, rapid, less laborious, cheap, possible for bedside testing	Complication in primer design, more than 1 set of LAMP primer plus high purity of primer needed

SSP-PCR, sequence-specific primer polymerase chain reaction; **PCR-SSOP**, polymerase chain reaction-sequence specific oligonucleotide probe; **LAMP**, Loop-mediated isothermal amplification.

Although the above results are encouraging, there are still rooms for improvement. For example, the detection system relies on non-specific detection of dsDNA using SYBR green dye. It is possible to combine the high efficiency and specificity of LAMP with other more specific detection platform. One of the most promising detection systems would be peptide nucleic acid (PNA). PNA is a neutral backbone DNA analog first developed by Neilsen, *et al.* in 1991(33). PNA consists of repetitive units of N-(2-aminutesoethyl) glycine pseudo-peptide backbone instead of deoxyribose sugar and phosphodiester bond found in DNA (figure 6). Thus, neutral charge of its pseudo-peptide bond enhances strongly binding to complementary DNA without repulsive force of negative charge in DNA-DNA binding. This property of PNA is highly beneficial for hybridization assay.

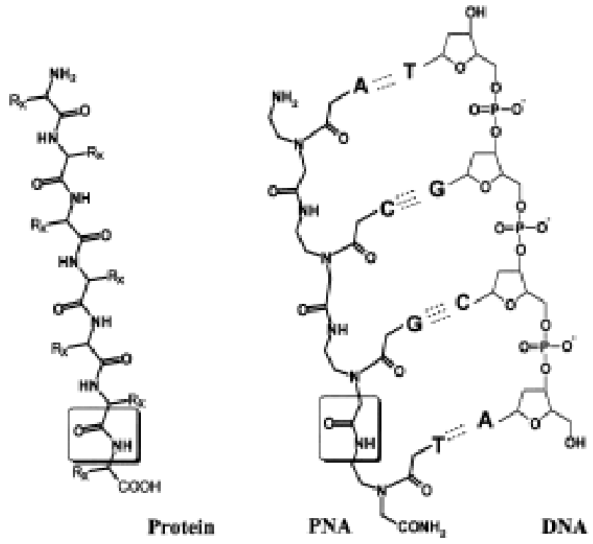


Figure 6. Chemical structures of protein, PNA and DNA. The peptide bond for both peptide and PNA in box (34).

For PNA application, a review in 2008 showed many versatile applications in genetic and cytogenetic analysis, for example, SNP detection, microarrays and biosensors, nucleic acid capture and *in situ* hybridization (35). In 2006, Tirayut Vilaivan, *et. al.* reported a novel pyrrolidinyl PNA (Figure 7), which is very powerful for target DNA detection with high specificity and high affinity in DNA binding (36). An application of

this PNA system in combination with MALDI-TOF mass spectrometry to SNP typing has been demonstrated (37). It is expected that the combination of LAMP with fluorescent-tag PNA probe would enhance the specificity of the detection, and could reduce the number of LAMP reaction to be perform per test sample.

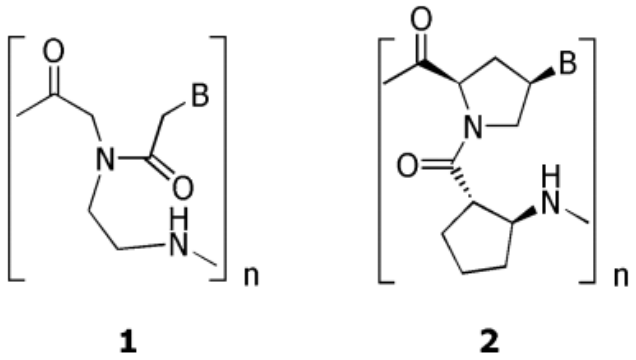


Figure 7. Chemical structures of PNA (1) and new ß-pyrrolidinyl PNA (2) (36). This new ß-pyrrolidinyl PNA has advantage over the neutral PNA charge in DNA binding that its positive charges from nitrogen atom attract phosphate group of DNA.

Using fluorescent nucleic acid hybridization probes has been extensively applied to real-time PCR for instant detection of PCR product at each cycle with higher specificity than SYBR Green detection. Several platforms were created for hybridization probes which depend mainly on two quenching mechanisms, fluorescent resonance energy transfer and contact quenching (38). These two mechanisms consist of two fluorophores, molecules which can absorb, transfer and emit energy.

The first mechanism, fluorescent resonance energy transfer or FRET works by electron transfer from excited donor fluorophore to receptor fluorophore. The example of this probe type is adjacent probes (figure 8A) and 5'-nuclease probes or TaqMan probes (figure 8B). For the adjacent probe, there are two probe strands with different labeling, donor fluorophore at 3'end on one strand, and acceptor fluorophore at 5' end on another probe. Without binding to the target, there's no energy transfer between the two fluorophores, resulting in no fluorescence released. Once these two probes bind closely at their target site, FRET takes place from donor to acceptor fluorophore,

resulting in fluorescence released (figure 8A). Whereas in 5'-nuclease probes or TaqMan probes, they are a one strand probe with two fluorophore binding at both ends. They emit only slightly amount of fluorescence. When the probe attaches to the target and Taq DNA polymerase encounters the probe, the two fluorophores are free by 5'-nuclease activity. This results in fluorescent signal (figure 8B).

Another mechanism, contact quenching works by forming hydrogen bond and transferring photon-electron of the two fluorophores simultaneously. Once this complex is activated by energy absorption, ground state instantly remains by quencher. As a result, no fluorescent emits. This mechanism is applied to molecular beacon probes (figure 8C) and strand-displacement probes (figure 8D). When molecular beacon probes and strand-displacement probes bind to the target, quencher separates from fluorescence and can no longer absorb the energy, resulting in fluorescent emission. Lately, there is a report on replacing PNA-DNA complex with DNA by strand displacement mechanism with fluorescent labeling (39, 40). Therefore, PNA-DNA strand displacement probe was applied for LAMP detection in order to skip gel electrophoresis step. This would prevent cross contamination and reduce total cost and assay time.

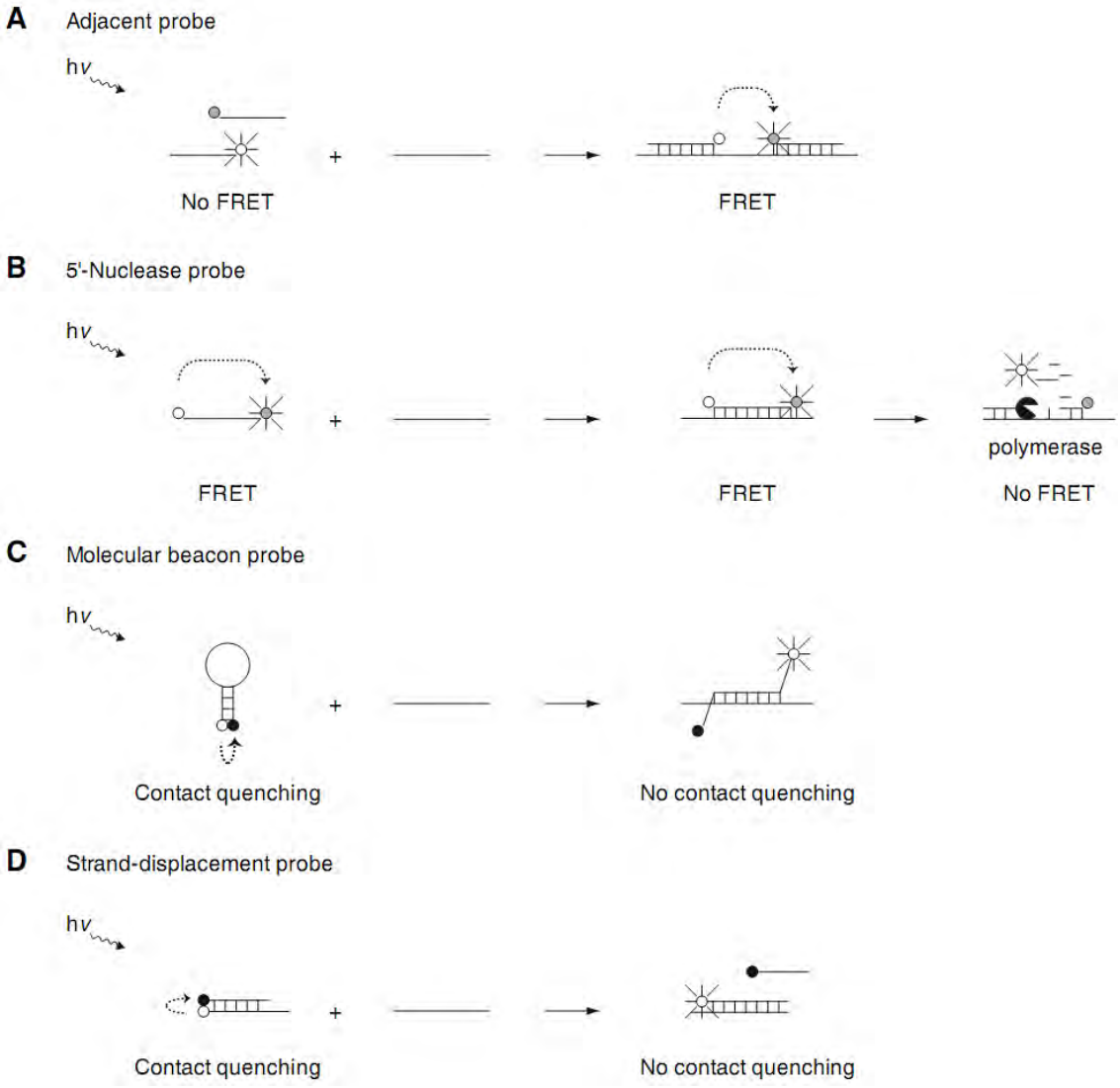


Figure 8. Summary of fluorescent hybridization probes. **A:** adjacent probe, **B:** 5'-nuclease probe, **C:** molecular beacon probe, **D:** strand-displacement probe (38). PNA-DNA strand displacement probe was applied for LAMP detection.

Choosing the fluorophore pairs has to match the assay types, the detectors and the quenching mechanisms. From a literature review in 2008, there are many fluorophores (table 7) and quenchers (table 8) commercially available, which are compatible with all spectrofluorometric thermal cycler platform that provides the different energy source (38). For FRET fluorophore pair's selection, their spectrums have to be overlapped, while fluorophore could be absorbed by any quencher in quenching mechanism. Some example of the fluorophore-quencher pairs are FAM, TET and HEX

with BHQ-1, TMR, ROX, Texas Red, Cy3 and Cy5 with BHQ-2 or Blackberry Quencher 650 (38). In general, FAM, TET and HEX are widely used in various applications due to the fact that they can be excited and detected with all machines. Moreover, it's relatively cheap and easier to synthesis, comparing to other fluorophore (38).

Table 7. Fluorophore labels for fluorescent hybridization probe (38).

Fluorophore	Excitation (nm)	Emission (nm)
Coumarin	430	475
FAM	495	515
TET	525	540
HEX	535	555
Cy3	550	570
TMR	555	575
ROX	575	605
Texas Red	585	605
LC Red 640	625	640
Cy5	650	670
LC Red 705	680	710

Table 8. Quencher labels for fluorescent hybridization probe (38).

Quencher	Absorption maximum (nm)
Deep Dark Quencher I	430
Dabcyl	475
Eclipse	530
Iowa Black FQ	532
Black Hole Quencher 1	534
QSY-7	571
Black Hole Quencher 2	580
Deep Dark Quencher II	630
Iowa Black RQ	645
Blackberry Quencher 650	650
QSY-21	660
Black Hole Quencher 3	670

Not only the right pairs of fluorophore are important, but some other factors also have to be considered in designing the nucleic acid probe. False interpretation can occur by unintentional lower fluorescence signal. One report mentioned that nucleotides can absorb fluorescent (41). Orderly, guanosine, adenosine, cytidine and thymidine have more quenching effect respectively (38). So, guanosine shouldn't be located at fluorophore labeled end. Another report showed that higher temperature reduces fluorescence signal of FAM, TMR, Cy5 and Cy3 dramatically (38). The solution to this problem is to increase the probe concentration in the assay. Additional consideration on PNA probe design, probe length and GC content are important. PNA shouldn't be longer than 10 bases, while GC content shouldn't have more than two molecules in the strand.

In conclusion, in-house SSP-PCR for HLA-B*1502 and HLA-B*5801 with fewer primers, and LAMP for rapid detection of HLA-B*5801 with PNA-DNA strand displacement probe were developed.

CHAPTER III

MATERIALS AND METHODS

Reagents

All primers were synthesized from the 1st Base Company. Three enzymes were used for PCR in this study. There were *Taq* DNA polymerase recombinant (Fermentas), Platinum® *Taq* DNA polymerase (Invitrogen), and FastStart *Taq* DNA polymerase (Roach). dNTP mix (Fermentas) were used as substrate for both PCR and LAMP reaction. Whole blood sample were applied to whole blood kit (KAPA Blood PCR Kit, Kapa Biosystems). Loopamp DNA Amplification Kit™ (Eiken Chemical) was used for LAMP reaction together with *Bst* DNA polymerase large fragment (New England Biolabs) and 5M PCR grade Betaine solution (Sigma). Detection step by gel electrophoresis, GeneRuler™ 50bp and 100bp (Fermentas) were used for amplified product size prediction. For SDS-PAGE, 30%/0.8% w/v Acrylamide/Bis-Acrylamide (Biorad), 10% Ammonium persulfate (APS) (GE), *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) (GE), and Coomassie Brilliant Blue R-250 stain (Biorad) were used in this study.

DNA Sample

In a setup step, known HLA-B*1502, -B*5801 and 6 HLA-B alleles (1301, 1513, 1517, 1520, 1521, 1525 and 5701) that have similar sequences were used.

In a validation step, 105 genomic DNA samples of blood or bone marrow donors at the Thai Red Cross were analyzed by gold standard methods using SSP-PCR commercial kit (Micro SSP™ HLA DNA Typing Trays, One Lambda, Inc.) and direct sequencing. These genomic DNA samples were sent to us blindly to test for HLA-B*1502 and HLA–B*5801. Within these 105 samples, all HLA-B alleles present in Thai population with a frequency more than one percent, including HLA-B*0705, 1301, 1501, 1511/12/13, 1517, 1525, 1801, 3501, 3801, 3901, 4001/02, 4402, 4601, 4801, 5101/02, 5201 and 5701, were tested.

Sequence-specific Primer design

HLA*B is the most polymorphic gene in human genome. Around 1,249 variants are reported in 2009 from the IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla). In order to design specific primer, all HLA-B sequences were as well taken from IMGT/HLA database with HLA-B*070201 as consensus sequence. From the alignment analysis, high polymorphisms are in exon 2 and 3. Consequently, these two regions were used as primer region. All HLA-B alleles with more than one percent frequency in Thai population, including interested HLA-B*1502 and 5801 were lined up in figure 9.

```
cDNA                  10         20         30         40         50         60         70         80         90        100
B*07:02:01        ATGCTGGTCA TGGCGCCCCG AACCGTCCTC CTGCTGCTCT CGGCGGCCCT GGCCCTGACC GAGACCTGGG CCG|GCTCCCA CTCCATGAGG TATTTCTACA
B*07:05:01        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---|------- ---------- ----------
B*13:01:01        ----G----- C--------- ----C----- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------
B*15:01:01:01     ----G----- C--------- ---------- ---------- ---GA----- ---------- ---------- ---|------- ---------- ----------
B*15:02:01        ----G----- C--------- ---------- ---------- ---GA----- ---------- ---------- ---|------- ---------- ----------
B*15:11:01        ----G----- C--------- ---------- ---------- ---GA----- ---------- ---------- ---|------- ---------- ----------
B*15:17:01:01     ----G----- C--------- ---------- ---------- ---GA----- ---------- ---------- ---|------- ---------- ----------
B*15:25:01        ----G----- C--------- ---------- ---------- ---GA----- ---------- ---------- ---|------- ---------- ----------
B*15:120          ********** ********** ********** ********** ********** ********** ********** ***|------- ---------- ----------
B*18:01:01        ----G----- C--------- ----C----- ---------- G--G---AG- ---------- ---------- -T-|------- ---------- ------C---
B*35:01:01        ----G----- C--------- ---------- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------
B*38:01:01        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---|------- ---------- ----------
B*39:01:01:01     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---|------- ---------- ----------
B*44:02:01:01     ----G----- C--------- ----C----- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------
B*46:01:01        ----G----- C--------- ---------- ---------- ---GA----- ---------- ---------- ---|------- ---------- ----------
B*48:01:01        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---|------- ---------- ----------
B*51:01:01        ----G----- C--------- ---------- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------
B*51:02:01        ----G----- C--------- ---------- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------
B*52:01:01        ----G----- C--------- ---------- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------
B*57:01:01        ----G----- C---A----- ---------- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------
B*58:01:01        ----G----- C--------- ---------- ---------- G--G---AG- ---------- ---------- ---|------- ---------- ----------


cDNA                 110        120        130        140        150        160        170        180        190        200
B*07:02:01        CCTCCGTGTC CCGGCCCGGC CGCGGGGAGC CCCGCTTCAT CTCAGTGGGC TACGTGGACG ACACCCAGTT CGTGAGGTTC GACAGCGACG CCGCGAGTCC
B*07:05:01        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*13:01:01        --G--A---- ---------- ---------- ---------- -A-C------ ---------- ---------- ---------- ---------- --A-------
B*15:01:01:01     --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*15:02:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*15:11:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*15:17:01:01     --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*15:25:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*15:120          --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*18:01:01        ---------- ---------- ---------- ---------- ---------- ---------- G--------- ---------- ---------- ----------
B*35:01:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*38:01:01        ---------- ---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ----------
B*39:01:01:01     ---------- ---------- ---------- ---------- ---------- ---------- ----G----- ---------- ---------- ----------
B*44:02:01:01     --G--A---- ---------- ---------- ---------- -A-C------ ---------- ----G-T--- ---------- ---------- --A-------
B*46:01:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*48:01:01        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*51:01:01        --G--A---- ---------- ---------- ---------- TG-------- ---------- ---------- ---------- ---------- ----------
B*51:02:01        --G--A---- ---------- ---------- ---------- TG-------- ---------- ---------- ---------- ---------- ----------
B*52:01:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*57:01:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
B*58:01:01        --G--A---- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- ---------- ----------
```

```
cDNA                 210        220        230        240        250        260        270        280        290        300
B*07:02:01       GAGAGAGGAG CCGCGGGCGC CGTGGATAGA GCAGGAGGGG CCGGAGTATT GGGACCGGAA CACACAGATC TACAAGGCCC AGGCACAGAC TGACCGAGAG
B*07:05:01       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*13:01:01       ---GAT--C- --C------- -A-------- ---------- ---------- --------G- G--------- -C----A--A -CA------- -T--------
B*15:01:01:01    ---GAT--C- --C------- -A-------- ---------- ---------- --------G- G--------- -C----A--A -CA------- -T--------
B*15:02:01       ---GAT--C- --C------- -A-------- ---------- ---------- ---------- ---------- -C----A--A -CA------- -T--------
B*15:11:01       ---GAT--C- --C------- -A-------- ---------- ---------- ---------- ---------- ------A--A -CA------- -T--------
B*15:17:01:01    ---GAT--C- --C------- -A-------- ---------- ---------- --------G- G----G--A- ATG------T CC--G----- -T--------
B*15:25:01       ---GAT--C- --C------- -A-------- ---------- ---------- --------G- G--------- -C----A--A -CA------- -T--------
B*15:120         ---GAT--C- --C------- -A-------- ---------- ---------- --------G- T--------- -C----A--A -CA------- -T--------
B*18:01:01       ---GAC---- --C------- ---A------ ---------- ---------- ---------- ---------- -C----A--A -CA------- -T--------
B*35:01:01       ---GAC---- --C------- -A-------- ---------- ---------- ---------- ---------- -T----A--A -CA------- -T--------
B*38:01:01       ---------- ---------- ---------- ---------- -----A---- ---------- ---------- -G----A--A -CA------- -T--------
B*39:01:01:01    ---------- ---------- ---------- ---------- -----A---- ---------- ---------- -G----A--A -CA------- ----------
B*44:02:01:01    ---GA----- ---------- -A-------- ---------- ---------- --------G- G--------- -C----A--A -CA------- -T--------
B*46:01:01       ---GAT--C- --C------- -A-------- ---------- ---------- --------G- G------AG ------CG-- ---------- --------T-
B*48:01:01       ---------- ---------- ---------- ---------- ---------- --------G- G--------- -C----A--A -CA------- -T--------
B*51:01:01       ---GAC---- --C------- -A-------- ---------- ---------- ---------- ---------- -T----A--A -CA------- -T--------
B*51:02:01       ---GAC---- --C------- -A-------- ---------- ---------- ---------- ---------- -T----A--A -CA------- -T--------
B*52:01:01       ---GAC---- --C------- -A-------- ---------- ---------- --------G- G--------- -C----A--A -CA------- -T--------
B*57:01:01       ---GAT--C- --C------- -A-------- ---------- ---------- -----G--G- G----G--A- ATG------T CC--G----- -T--------
B*58:01:01       ---GAC---- --C------- -A-------- ---------- ---------- -----G--G- G----G--A- ATG------T CC--G----- -T--------


cDNA                 310        320        330        340        350        360        370        380        390        400
B*07:02:01       AGCCTGCGGA ACCTGCGCGG CTACTACAAC CAGAGCGAGG CCG|GGTCTCA CACCCTCCAG AGCATGTACG GCTGCGACGT GGGGCCGGAC GGGCGCCTCC
B*07:05:01       ---------- ---------- ---------- ---------- ---|------- ---------- ---------- ---------- ---------- ----------
B*13:01:01       -A------C- C-GC--T-C- ---------- ---------- ---|------- --T-A----- --G-----T- --------C- ---------- ----------
B*15:01:01:01    ---------- ---------- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*15:02:01       ---------- ---------- ---------- ---------- ---|------- --T-A----- --G-----T- ---------- ---------- ----------
B*15:11:01       ---------- ---------- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*15:17:01:01    -A-------- T-GC--T-C- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*15:25:01       ---------- ---------- ---------- ---------- ---|------- --T-A----- --G-----T- ---------- ---------- ----------
B*15:120         ---------- ---------- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*18:01:01       ---------- ---------- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*35:01:01       ---------- ---------- ---------- ---------- ---|------- --T-A----- --G-----T- --------C- ------C--- ----------
B*38:01:01       -A-------- T-GC--T-C- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*39:01:01:01    ---------- ---------- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*44:02:01:01    -A------C- C-GC--T-C- ---------- ---------- ---|------- --T-A----- --G------- ---------- ---------- ----------
B*46:01:01       ---------- ---------- ---------- ---------- ---|------- ---------- --G------- ---------- ---------- ----------
B*48:01:01       ---------- ---------- ---------- ---------- ---|------- ---------- ---------- ---------- ---------- ----------
B*51:01:01       -A-------- T-GC--T-C- ---------- ---------- ---|------- ---TTGG--- -CG-----T- ---------- ---------- ----------
B*51:02:01       -A-------- T-GC--T-C- ---------- ---------- ---|------- ---TTGG--- -CG-----T- ---------- ---------- ----------
B*52:01:01       -A-------- T-GC--T-C- ---------- ---------- ---|------- ---TTGG--- -CG-----T- ---------- ---------- ----------
B*57:01:01       -A-------- T-GC--T-C- ---------- ---------- ---|------- --T-A----- GTG-----T- ---------- ---------- ----------
B*58:01:01       -A-------- T-GC--T-C- ---------- ---------- ---|------- --T-A----- --G-----T- --------C- ------C--- ----------


cDNA                 410        420        430        440        450        460        470        480        490        500
B*07:02:01       TCCGCGGGCA TGACCAGTAC GCCTACGACG GCAAGGATTA CATCGCCCTG AACGAGGACC TGCGCTCCTG GACCGCCGCG GACACGGCGG CTCAGATCAC
B*07:05:01       ---------- -A-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*13:01:01       ---------- -A------TA ---------- ---------- ---------- ---------- --A------- ------G--- -----C---- ----------
B*15:01:01:01    ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*15:02:01       --------T- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*15:11:01       ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*15:17:01:01    --------T- CC-----G-- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*15:25:01       --------T- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*15:120         ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*18:01:01       ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- -----C---- ----------
B*35:01:01       ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- -----C---- ----------
B*38:01:01       ---------- -A------T- ---------- ---------- ---------- ---------- --A------- ------G--- -----C---- ----------
B*39:01:01:01    ---------- -A------T- ---------- ---------- ---------- ---------- --A------- ------G--- -----C---- ----------
B*44:02:01:01    --------T- -------G-- ---------- ---------- ---------- ---------- --A------- ------G--- -----C---- ----------
B*46:01:01       ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*48:01:01       ---------- -A-------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --------T-
B*51:01:01       ---------- -A-------- ---------- ----A----- ---------- ---------- --A------- ------G--- -----C---- ----------
B*51:02:01       ---------- -A-------- ---------- ----A----- ---------- ---------- --A------- ------G--- -----C---- ----------
B*52:01:01       ---------- -A-------- ---------- ----A----- ---------- ---------- --A------- ------G--- -----C---- ----------
B*57:01:01       ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- ---------- ----------
B*58:01:01       ---------- --------C- ---------- ---------- ---------- ---------- --A------- ------G--- -----C---- ----------


cDNA                 510        520        530        540        550        560        570        580        590        600
B*07:02:01       CCAGCGCAAG TGGGAGGCGG CCCGTGAGGC GGAGCAGCGG AGAGCCTACC TGGAGGGCGA GTGCGTGGAG TGGCTCCGCA GATACCTGGA GAACGGGAAG
B*07:05:01       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*13:01:01       -----T---- ---------- ------T--- --------T- ---------- ---------- ---------- ---------- ---------- ----------
B*15:01:01:01    ---------- ---------- ---------- -------T-- ---------- --------CT ---------- ---------- ---------- ----------
B*15:02:01       ---------- ---------- ---------- --------T- ---------- --------CT ---------- ---------- ---------- ----------
B*15:11:01       ---------- ---------- ---------- -------T-- ---------- --------CT ---------- ---------- ---------- ----------
B*15:17:01:01    ---------- ---------- ---------- --------T- ---------- --------CT ---------- ---------- ---------- ----------
B*15:25:01       ---------- ---------- ---------- --------T- ---------- --------CT ---------- ---------- ---------- ----------
B*15:120         ---------- ---------- ---------- -------T-- ---------- --------CT ---------- ---------- ---------- ----------
B*18:01:01       ---------- ---------- ------T--- --------T- ---------- --------AC ---------- ---------- --C------- ----------
B*35:01:01       ---------- ---------- ------T--- --------T- ---------- --------CT ---------- ---------- ---------- ----------
B*38:01:01       ---------- ---------- ------T--- --------T- ---A------ --------AC ---------- ---------- ---------- ----------
B*39:01:01:01    ---------- ---------- ------T--- --------T- ---A------ --------AC ---------- ---------- ---------- ----------
B*44:02:01:01    ---------- ---------- ------T--- -------GAC ---------- --------CT ---------- -C-------- ---------- ----------
B*46:01:01       ---------- ---------- ---------- -------T-- ---------- --------CT ---------- ---------- ---------- ----------
B*48:01:01       ---------- -T-------- ------T--- --------T- ---------- ---------- ---------- ---------- ---------- ----------
B*51:01:01       ---------- ---------- ---------- --------T- ---------- --------CT ---------- ---------- --C------- ----------
B*51:02:01       ---------- ---------- ---------- --------T- ---------- --------CT ---------- ---------- ---------- ----------
B*52:01:01       ---------- ---------- ---------- --------T- ---------- --------CT ---------- ---------- --C------- ----------
B*57:01:01       ---------- ---------- ------T--- --------T- ---------- --------CT ---------- ---------- ---------- ----------
B*58:01:01       ---------- ---------- ------T--- --------T- ---------- --------CT ---------- ---------- ---------- ----------
```

```
cDNA                 610        620        630        640        650        660        670        680        690        700
B*07:02:01 GACAAGCTGG AGCGCGCTG|A CCCCCCAAAG ACACACGTGA CCCACCACCC CATCTCTGAC CATGAGGCCA CCCTGAGGTG CTGGGCCCTG GGTTTCTACC
B*07:05:01 ---------- ---------|- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*13:01:01 --G-C----C -------G-|- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --C-------
B*15:01:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*15:02:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*15:11:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*15:17:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*15:25:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*15:120   --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*18:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*35:01:01 --G-C----C -------G-|- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- --C-------
B*38:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*39:01:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*44:02:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- -------T-- ---------- ---------- --C-------
B*46:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*48:01:01 ---------- ---------|- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*51:01:01 --G-C----C -------G-|- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- --C-------
B*51:02:01 --G-C----C -------G-|- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- --C-------
B*52:01:01 --G-C----C -------G-|- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- --C-------
B*57:01:01 --G-C----C -------G-|- ---------- -----T---- ---------- ---------- ---------- ---------- ---------- --C-------
B*58:01:01 --G-C----C -------G-|- ---------- ---------- ---------- -G-------- ---------- ---------- ---------- --C-------


cDNA                 710        720        730        740        750        760        770        780        790        800
B*07:02:01 CTGCGGAGAT CACACTGACC TGGCAGCGGG ATGGCGAGGA CCAAACTCAG GACACTGAGC TTGTGGAGAC CAGACCAGCA GGAGATAGAA CCTTCCAGAA
B*07:05:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*13:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*15:01:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*15:02:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*15:11:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*15:17:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*15:25:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*15:120   ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*18:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*35:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*38:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- -----C---- ----------
B*39:01:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- -----C---- ----------
B*44:02:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*46:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*48:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*51:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*51:02:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*52:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*57:01:01 ---------- ---------- ---------- ---------- ---------- -----C---- ---------- ---------- ---------- ----------
B*58:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------


cDNA                 810        820        830        840        850        860        870        880        890        900
B*07:02:01 GTGGGCAGCT GTGGTGGTGC CTTCTGGAGA AGAGCAGAGA TACACATGCC ATGTACAGCA TGAGGGGCTG CCGAAGCCCC TCACCCTGAG ATGGG|AGCCG
B*07:05:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|-----
B*13:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*15:01:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*15:02:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*15:11:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*15:17:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*15:25:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*15:120   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|*****
B*18:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*35:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*38:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*39:01:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*44:02:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|-----
B*46:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*48:01:01 ----A----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|-----
B*51:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*51:02:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*52:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A
B*57:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- --A------- ---------- -----|----A
B*58:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----|----A


cDNA                 910        920        930        940        950        960        970        980        990       1000
B*07:02:01 TCTTCCCAGT CCACCGTCCC CATCGTGGGC ATTGTTGCTG GCCTGGCTGT CCTAGCAGTT GTGGTCATCG GAGCTGTGGT CGCTGCTGTG ATGTGTAGGA
B*07:05:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*13:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*15:01:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*15:02:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*15:11:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*15:17:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*15:25:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*15:120   ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
B*18:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*35:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*38:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*39:01:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*44:02:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*46:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*48:01:01 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*51:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*51:02:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*52:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
B*57:01:01 --------A- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
B*58:01:01 ---------- -----A---- ---------- ---------- ---------- ---------- ---------- ---------- ----A----- ----------
```

```
cDNA                    1010       1020       1030       1040       1050       1060       1070       1080       1080
B*07:02:01        GGAAGAGTTC AG|GTGGAAAA GGAGGGAGCT ACTCTCAGGC TGCGT|GCAGC GACAGTGCCC AGGGCTCTGA TGTGTCTCTC ACAGCTTGA
B*07:05:01        ---------- --|-------- ---------- ---------- -----|----- ---------- ---------- ---------- ---------
B*13:01:01        -------C-- --|-------- ---------- ---------- -----|----- ---------- ---------- ---------- ---------
B*15:01:01:01     -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*15:02:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*15:11:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*15:17:01:01     -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*15:25:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*15:120          ********** **|******** ********** ********** *****|***** ********** ********** ********** *********
B*18:01:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*35:01:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*38:01:01        ---------- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*39:01:01:01     ---------- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*44:02:01:01     -------C-- --|-------- ---------- ---------- -----|----- ---------- ---------- ---------- ---------
B*46:01:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*48:01:01        ---------- --|-------- ---------- ---------- -----|----- ---------- ---------- ---------- ---------
B*51:01:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*51:02:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*52:01:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
B*57:01:01        -------C-- --|-------- ---------- ---------- -----|----- ---------- ---------- ---------- ---------
B*58:01:01        -------C-- --|-------- ---------- ---------- -----|C---- ---------- ---------- ---------- ---------
```

Figure 9. Alignment of all HLA-B exons of HLA-B*1502, 5801 and other alleles with more than 1% frequency in Thai population. There are seven exons for each HLA class I alleles which are nucleotide coding sequence. Comparing to HLA-B*070201 as consensus sequences, exon2 and 3 are the most polymorphic region in HLA sequence. Thus, primers were chosen from these two regions. Each exons are separated by a straight line (|). A dash (-) refers to a similar sequence, while a star (*) represents unknown sequence.

SSP-PCR for HLA-B*1502 Setup

All primers sets were tested for specificity by initial $MgCl_2$ final concentration and annealing temperature gradient. Final concentration of initial $MgCl_2$ is 2.5 mM, while the temperature was varied from 50.0 - 66.5^{o}C. Reaction mix in the total volume of 10 µl contains the final concentration of 1x *Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 µM primer for forward and reverse primer, 0.5 U *Taq* DNA polymerase (Fermentas), and 50 ng of genomic DNA. PCR was performed at 95^{o}C 2 minutes for 1 cycle; 95^{o}C 30s, 58 ± 8^{o}C 30s and 72^{o}C 30s for 35 cycles; 72^{o}C 5 minutes for 1 cycle and holding at 4^{o}C. Electrophoresis used 2% agarose to determine PCR product size by running with 50 or 100bp DNA Ladder at 100 V for 40 minutes. Afterwards, internal control primer (IFN-γ) was multiplexed with specific HLA-B*1502 primer with touchdown thermal cycle in order to get the most specific results with good PCR efficiency.

SSP-PCR for HLA-B*1502 from Genomic DNA

Typing of HLA-B*1502, nested PCR was performed to exclude heterozygous alleles combination from multiplex PCR with several sets of HLA-B*1502 primers in one reaction. In the first PCR, HLA-B*1502 SSP primers are F1: 5'-GCGAGTCCGAGGATGGC-3' and R1: 5'- AGCCATACATCCTCTGGATGA-3'. Reaction mix in the total volume of 20 µl contains final concentration of 1x *Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 µM primer, 0.25 µM internal control primer and 0.5 U *Taq* DNA polymerase (Fermentas). For the second PCR, there are two separated reactions. Two forward primers are F2.1: 5'-GGAACACACAGATCTCCAAGA-3' and F2.2: 5'-GGAGTATTGGGACCGGAAC-3' with the same reverse primer R2: 5'-TTGTAGTAGCCGCGCAGGT-3'. Reaction mix in the total volume of 20 µl is similar to the first reaction with 2 µl of PCR product from F1 and R1, except that internal control primer is not added. All primers from the first and second PCR are located in figure 10 and concluded in table 9.

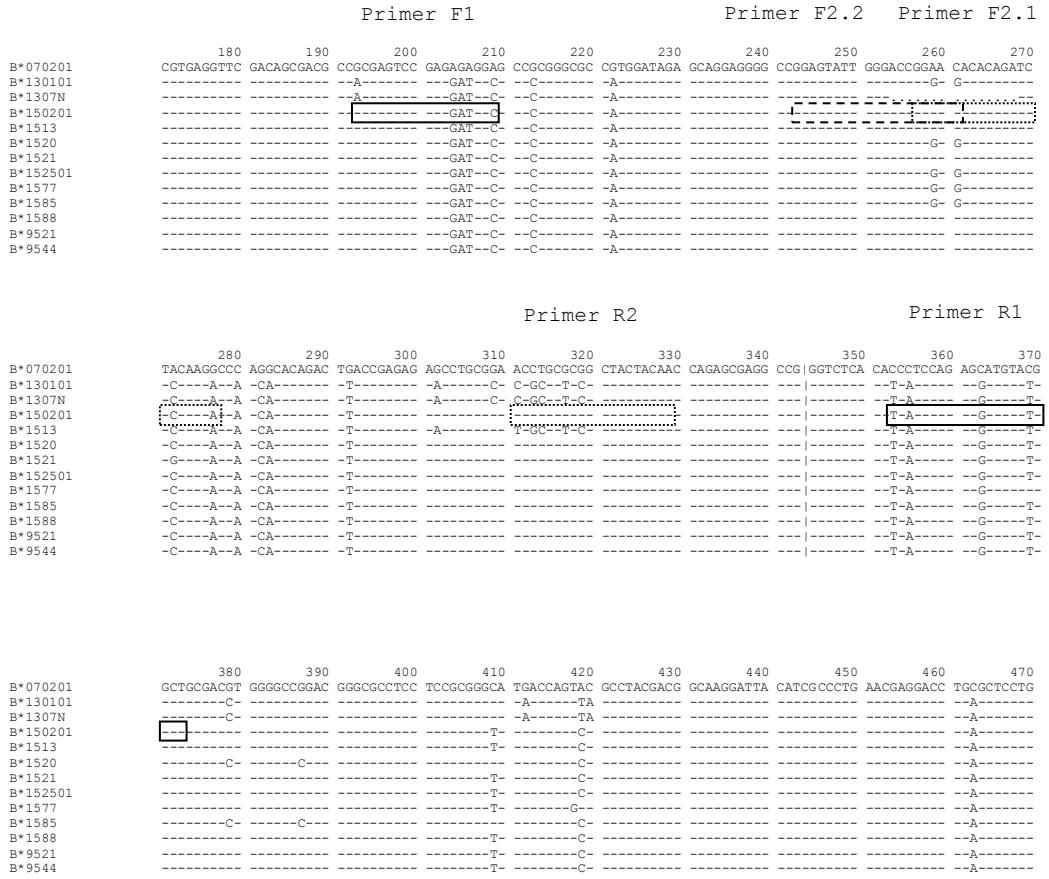


Figure 10. Primer region within HLA-B*1502 exon2 and 3 sequences. All of these sequences were taken from IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla). HLA-B*070201 is used as consensus sequences. Two outer primers, F1 and R1, are used in the first PCR, while F2.1 and R2 and F2.2 and R2 are used in separated second PCR as inner primers to eliminate heterozygous combination. A dash refers to similar sequences, while a dot represents unknown sequences.

SSP-PCR reaction for both HLA-B*1502 and HLA–B*5801 use the same thermal cycles and housekeeping primers (IFN-γ), IF: 5'-CCTCACATGATATGACTTTGACAT-3' and IR: 5'-AACATCAGAAGCATTGACCTTG-3' (table 9). Touchdown PCR cycles with the annealing temperature from 70 – 55°C were used to get the specific amplification with good PCR efficiency. The cycles composed of 95°C 2 minutes for 1 cycle; 95°C 30s, 70°C 30s and 72°C 30s for 5 cycles; 95°C 30s, 68°C 30s and 72°C 30s for 5 cycles; 95°C 30s, 67.1°C 30s and 72°C 30s for 5 cycles; 95°C 30s, 65°C 30s and 72°C 30s for 10

cycles; 95°C 30s, 55°C 30s and 72°C 30s for 20 cycles; 72°C 5 minutes for 1 cycle and holding at 4°C. Total genomic DNA in every reaction is 200 ng. For the second PCR, the cycles are composed of 95°C 2 minutes for 1 cycle; 95°C 30s, 66°C 30s and 72°C 30s for 10 cycles; 72°C 5 minutes for 1 cycle and holding at 4°C. Electrophoresis used 2% agarose to determine PCR product size by running with 100bp DNA Ladder at 100 V for 40 minutes.

SSP-PCR for HLA-B*5801 Setup

Primers F1 and R1 were tested for specificity by initial $MgCl_2$ final concentration and annealing temperature gradient. The initial recommended concentration of $MgCl_2$ is 1.5 mM, while the temperature range was from 56.1 – 64.0°C. Reaction mix in the total volume of 10 µl contains the final concentration of 1x *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 µM primer for forward and reverse primer, 0.5 U Platinum® *Taq* DNA polymerase (Invitrogen), and 50 ng of genomic DNA. PCR was performed at 95°C 2 minutes for 1 cycle; 95°C 30s, 58±8°C 30s and 72°C 30s for 35 cycles; 72°C 5 minutes for 1 cycle and holding at 4°C. Electrophoresis used 2% agarose to determine PCR product size by running with 100bp DNA Ladder at 100 V for 40 minutes. Afterwards, internal control primer (IFN-γ) was added in the same reaction with specific HLA-B*5801 primer with touchdown thermal cycle in order to get the most specific results with good PCR efficiency.

SSP-PCR for HLA-B*5801 from Genomic DNA

HLA-B*5801 only need one set of primer. The forward primer is 5'-ACGGAACATGAAGGCCTCC-3' and reverse primer is 5'-CAGCCATACATCCTCTGGATGA-3'. Reaction mix in the total volume of 20 µl contains final concentration of 1x *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 µM primer, 0.25 µM internal control primer and 0.5 U Platinum® *Taq* DNA polymerase (Invitrogen). Total genomic DNA in every reaction is 200 ng. Both forward and reverse primers are located as in figure 11 and concluded in table 9.

Figure 11. Primer region within HLA-B*5801 exon2 and 3 sequences. All of these sequences were taken from IMGT/HLA database (http://www.ebi.ac.uk/imgt/hla). A dash refers to similar sequences, while a dot represents unknown sequences. F1 and R1 were used to differentiate HLA-B*5801 from other alleles.

Sensitivity Testing for HLA-B*1502 and HLA-B*5801 SSP-PCR

Genomic DNA from 0.0001 – 0.1 ng/µl was tested for detection limit. The condition was the same as SSP-PCR for HLA-B*1502 and HLA-B*5801 from genomic DNA.

SSP-PCR for HLA-B*1502 and HLA-B*5801 from Whole Blood

In this part, only the internal control (IFN-γ) was amplified in order to find the most suitable condition to apply to the HLA-B*1502 and HLA-B*5801 SSP-PCR. According to the first article, modified initial denaturing step was applied with 94°C 3 minutes, 55°C 3 minutes for 3 cycles to completely lyse blood cell before starting PCR cycles (23). In the total volume of 10 µl contains final concentration of 1x *Taq* buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM primer, 0.25 U *Taq* DNA polymerase (Fermentas) and 0.2, 0.6 and 1.0 µl of whole blood. This mix is then incubated at 94°C 3 minutes, 55°C 3 minutes for 3 cycles; 95°C 30s, 55°C 30s and 72°C 30s for 30 cycles; 72°C 5 minutes for

1 cycle and holding at 4°C. The PCR product is detected by gel electrophoresis, using 2% agarose at 100 V for 40 minutes.

For the second report, whole blood serial dilution was used as template instead of directly adding whole blood to PCR mix. As a result, PCR activity is increased, as inhibitors are also diluted (24). Preparing the series of whole blood dilution, whole blood from 1, 2, 5, 10, 20, 30, 40, 45 and 50 µl were used and the volume were adjusted to 50 µl with sterile water. Then, this mixture was incubated at 95°C for 15 minutes before using as PCR template. The remaining crude whole blood was centrifuge at maximum speed for 2 minutes and the supernatant was also used as PCR template. PCR reaction was performed as mentioned in the first experiment. Results from crude and supernatant from whole blood were compared. Nested SSP-PCR for HLA-B*1502 was also done by this method. Moreover, traditional *Taq* Polymerase was also changed from Fermentas to FastStart *Taq* from Roach with the same PCR condition to observe the *Taq* activity in the present of inhibitors.

Finally, nine blind whole blood samples were applied to current in-house nested SSP-PCR for HLA-B*1502 and HLA-B*5801 condition to direct PCR from whole blood kit (KAPA Blood PCR Kit, Kapa Biosystems). In the total volume of 10 µl contains final concentration of 1x KAPA Blood PCR Mix B, 1.0 µM primer, 0.25 µM internal control primer and 1.0 µl of whole blood or purified DNA (50 ng) as template for the first PCR for HLA-B*1502 and for HLA-B*5801. The second PCR for HLA-B*1502 uses the same component as the first one, but internal control primers are not added. Then, the PCR product is detected by gel electrophoresis, using 2% agarose at 100 V for 40 minutes.

LAMP Primer Design for HLA-B*5801

Primers were designed by Primer Explorer V4 (http://primerexplorer.jp/e/). Exon 2 was chosen for primer template as polymorphisms in exon 2 are higher than in exon 3. Two outer primers are F3: 5'-TTTCTACACCGCCATGTCC-3' and B3: 5'-AAGTCTGCGCGGAGGC-3' and another two inner primers are FIP (F2 and F1C: 5'-TCGCTGTCGAACCTCACGAACAGCCCCGCTTCATCGC-3') and BIP (B2 and B1c: 5'-CGGGCGCCATGGATAGAGCATGTTCCGTGTCTCCCCGT-3'). All primers are located in figure 12 and concluded in table 9. From the primer search tools (http://www.ebi.ac.uk/imgt/hla), HLA-B*5701 and 5801 are positive.

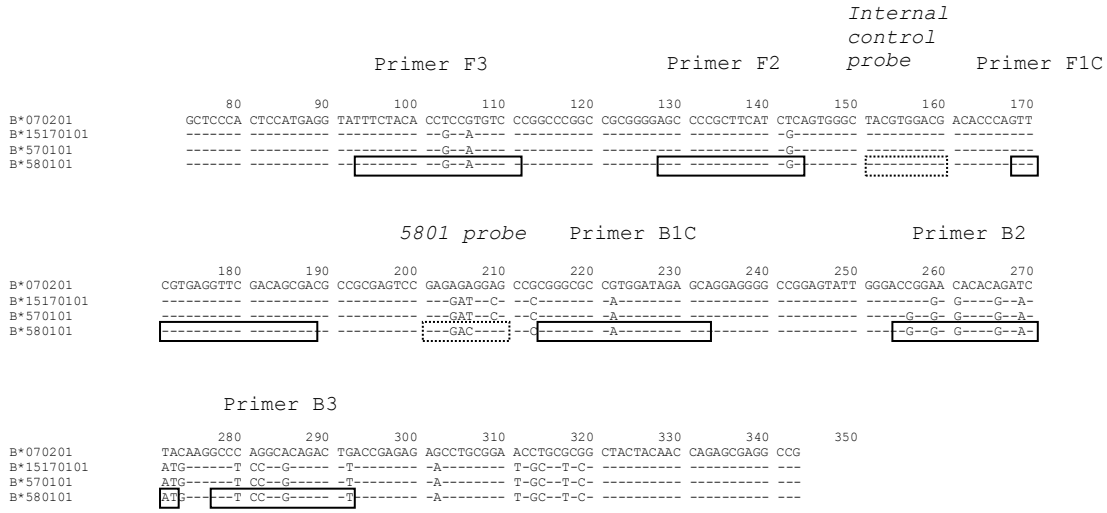


Figure 12. LAMP primer/probe region within HLA-B*5801 exon2 sequences. There are 4 primers for LAMP reaction; F3, FIP (F2 & F1C), BIP (B2 & B1C), B3. It shows that HLA-B*5701 and HLA-B*5801 cannot be told apart by LAMP reaction. In order to differentiate these two alleles, PNA-DNA strand displacement probes were used. Internal control probe is used to detect all LAMP product, while 5801 probe is used for specifically detecting HLA-B*5801 allele.

LAMP Reaction Setup

In order to find the most specific condition for LAMP reaction, series of $MgSO_4$ concentration and thermal gradient were applied to LAMP reaction. $MgSO_4$ concentration is diluted from commercial LAMP kit (Eiken Chemical) by conventional LAMP reaction (New England Biolabs). LAMP reaction in the total volume of 6 - 9 µl mixture (Eiken buffer: New England Biolabs buffer = 5:1, 5:2, 5:3, 5:4) consists of 0.8 µM of each FIP and BIP, 0.2 µM of each F3 and B3, 400 µM of each dNTPs, 4.44, 5.00, 5,71 and 6.77 mM $MgSO_4$, 1.0 M betaine (Sigma), 1x ThermoPol Reaction Buffer and 50 ng DNA template. These LAMP reactions were heated at 95°C for 5 minutes as initial denaturing step and chilled on ice immediately. Following by 8U of *Bst* DNA polymerase large fragment (New England Biolabs) was added to the mixture. Next, the reaction mixture is incubated at 66.0 – 74.1°C for 40 minutes. Using 2.5% agarose with 100bp DNA Ladder, 1 µl LAMP product was run at 100 V for 40 minutes.

LAMP Reaction for HLA-B*5801 from Genomic DNA

LAMP reaction for HLA-B*5801, 6 µl mixture consists of 0.8 µM of each FIP and BIP, 0.2 µM of each F3 and B3, 400 µM of each dNTPs, 6.67 mM $MgSO_4$, 1.0 M betaine, 1x ThermoPol Reaction Buffer and 50 ng DNA template. This mixture is heated at 95°C for 5 minutes as initial denaturing step and chilled on ice immediately, following by 8 U of *Bst* DNA polymerase large fragment (New England Biolabs). Next, the reaction mixture is incubated at 70°C for 40 minutes.

In detection step, there are two approaches. First, LAMP product was detected by gel electrophoresis as smear band. Using 2.5% agarose with 100bp DNA Ladder, 1 µl of LAMP product was run at 100 V for 40 minutes. To develop another instantly visual detection, strand-displacement probe consisting of a fluorescently-labeled PNA and a short quencher-labeled DNA were used for detection. For the PNA probe, HLA-B*5801 PNA probe sequence is Fluorescein-*N*-CTCCGTCCTC-*C*, while internal control probe Fluorescein-*N*-CGTCCACGTA-*C* is used (Figure 12). These PNA probes were synthesized from Associate Professor Tirayut Vilaivan's laboratory, Department of Chemistry, Faculty of Science, Chulalongkorn University. The complementary short

quencher DNA probes for HLA-B*5801 and internal control are 5'-ACGGAG-3'-(BHQ1) and 5'-TGGACG-3'-BHQ1, respectively. These PNA-DNA complexes were used to detect LAMP product by strand displacement mechanism. All probes were concluded in table 9.

Table 9. Primers and probes from both SSP-PCR and LAMP reaction.

SSP-PCR Primers for HLA-B*1502
F1: 5'-GCGAGTCCGAGGATGGC-3'
R1: 5'- AGCCATACATCCTCTGGATGA-3'
F2.1: 5'-GGAACACACAGATCTCCAAGA-3'
F2.2: 5'-GGAGTATTGGGACCGGAAC-3'
R2: 5'-TTGTAGTAGCCGCGCAGGT-3'
SSP-PCR Primers for HLA-B*5801
F1: 5'-ACGGAACATGAAGGCCTCC-3'
R1: 5'-CAGCCATACATCCTCTGGATGA-3'
Internal Control Primers for SSP-PCR (IFN-γ)
IF: 5'-CCTCACATGATATGACTTTGACAT-3'
IR: 5'-AACATCAGAAGCATTGACCTTG-3'
LAMP Primers for HLA-B*5801
F3: 5'-TTTCTACACCGCCATGTCC-3'
B3: 5'-AAGTCTGCGCGGAGGC-3'
FIP (F2 and F1C: 5'-TCGCTGTCGAACCTCACGAACAGCCCCGCTTCATCGC-3')
BIP (B2 and B1c: 5'-CGGGCGCCATGGATAGAGCATGTTCCGTGTCTCCCCGT-3')
HLA-B*5801 Probes for HLA-B*5801 LAMP
Fluorescein-*N*-CTCCGTCCTC-*C*
5'-ACGGAG-3'-(BHQ1)
Internal Control Probes for HLA-B*5801 LAMP
Fluorescein-*N*-CGTCCACGTA-*C*
5'-TGGACG-3'-BHQ1

SDS-PAGE

In order to compare the proteins difference in conventional (New England Biolabs) and commercial LAMP buffer (Eiken Chemical), SDS-PAGE was performed as a screening technique. In this study, 10 µl of each buffers were loaded in 12% SDS-PAGE. Commassie Blue was used for gel staining.

PNA-DNA Complex for LAMP Detection

First of all, 1 µl of LAMP product was added to PNA-DNA complex solution. Fixed 3 µl of 10 µM fluorescently-labeled PNA probe was added with 1 - 10 µl of 10 µM quencher-labeled DNA probe. Next, NaCl and heat were introduced as required in order to find the specific condition. 1.5 µM of NaCl ranging from 0.5 to 4.5 µl and heating up to 90^{o}C were introduced to the solution. Finally, fluorescence is observed under ultraviolet light.

Assay Analysis

To compare the efficiency between the new developed method with the traditional one, sensitivity and specificity of the assay is determined (42).

		Commercial SSP-PCR		
		Positive	Negative	
Test	Positive	True positive	False positive	PPV = TP/(TP+FP)
	Negative	False negative	True negative	NPV = TN/(TN+FN)
		Sensitivity = TP/(TP+FN)	Specificity = TN/(FP+TN)	

CHAPTER IV

RESULTS

1. SSP-PCR for HLA-B*1502 and HLA-B*5801 Setup

1.1. SSP-PCR for HLA-B*1502 Setup

Starting with the initial suggested $MgCl_2$ final concentration at 2.5 mM (Fermentas), specific bands were achieved from F1+R1 and F2.2+R2 with gradient temperature of 58 ± 8°C. From F1 and R1, specific band was shown as 430bp product as predicted at as high as 65.8°C, while 87bp of F2.2 and R2 was also amplified specifically with strong efficiency at 66.5°C (figure 13). As F2.1 primer used the same reverse primer (R2) and attached to the target intercepted with F2.2 region (figure 10), specificity in amplification was predicted to be similar to F2.2 and R2.

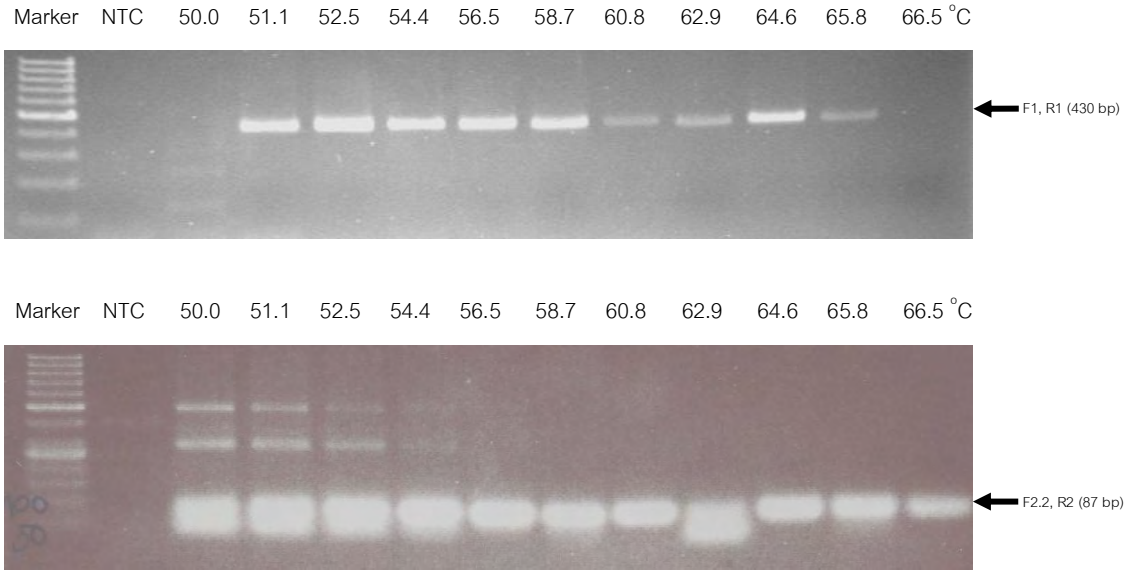


Figure 13. PCR product from different temperature of F1 and R1 and F2.2 and R2 at a constant 2.5 mM $MgCl_2$ concentration. F1 and R1 gave 430bp specific band at as high as 65.8°C, whereas 87bp product of F2.2 and R2 could be strongly amplified at as high as 66.5°C.

1.2. SSP-PCR for HLA-B*5801 Setup

Beginning with the recommended initial $MgCl_2$ concentration at 1.5 mM (Invitrogen) and gradient temperature of 60 ± 4°C, specific band of F1 and R1 was successfully amplified with 360bp PCR products at 64°C (figure 14).

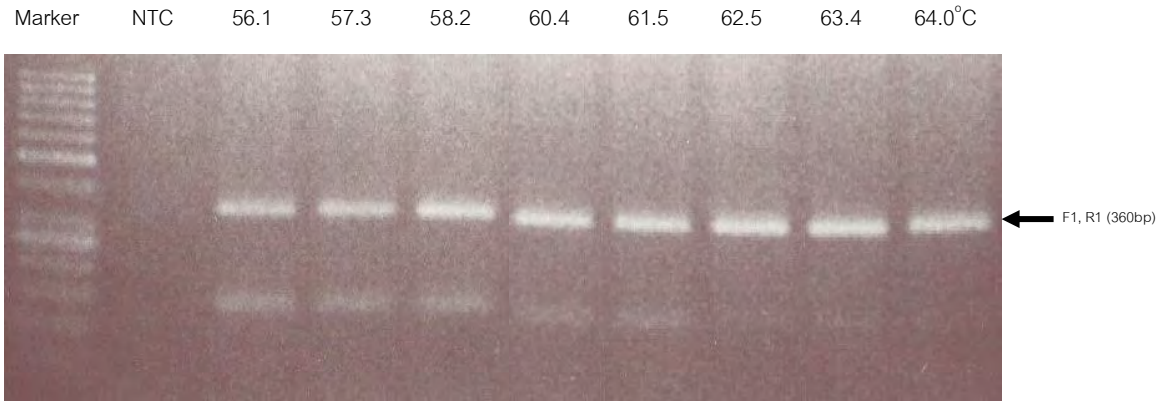


Figure 14. PCR products from different temperature of F1 and R1 at a constant 1.5 mM $MgCl_2$ concentration. F1 and R1 gave 360bp specific band as predicted at as high as 64.0°C.

2. SSP-PCR for HLA-B*1502 and HLA-B*5801 from Genomic DNA

2.1. SSP-PCR for HLA-B*1502 from Genomic DNA

Using 10 bases from 3' end of each primer to predict amplified alleles, only HLA class B is expected to be amplified with 430bp product in the first PCR (F1 and R1), including HLA-B*130101-02, 1306, 1307N, 1312-13, 1317, 1320-21, 132201-02, 1323, 1325-26, 1328-29, 150201-02, 1513, 1520-21, 152501-02, 1536, 1544, 1577, 1585, 1588-89, 3546, 4408, 4457, 4460, 5705, 9521, 9539, 9544 and 9554. Following by the second PCR (F2.1 and R2), most of other rare unrelated HLA-B alleles will be eliminated, resulting in HLA-B*1502, 1520, 1525, 1577, 1585, 1588, 9521 and 9544 positive. Another set of second PCR (F2.2 and R2) gives HLA-B*1502, 1521, 1544, 1588, 3546, 9521, 9539 and 9544 positive. All Alleles predicted to be amplified are concluded in table 10 with its frequency in Thai population (http://www.allelefrequencies.net/default.asp). This prediction showed that in combination of three positive bands from

the first and the two second PCR will be determined as HLA-B*1502. Still, HLA-B*1588, 9521 and 9544 are false positive alleles from this system. According to the frequency record of these three alleles in Thai and world population (0%), this simple nested SSP-PCR can be used to determine HLA-B*1502 specifically.

Table 10. Predicted amplified alleles from 430bp F1 and R1, 74bp F2.1 and R2 and 87bp F2.2 and R2. HLA-B*1502, 1588, 9521 and 9544 are expected to be interpreted as HLA-B*1502. From the frequency data, HLA-B*1588, 9521 and 9544 are not found in Thai population, therefore this assay was predicted to identify HLA-B*1502 specifically.

Alleles	First PCR	Second PCR		Frequency (%)
	F1 and R1 (430bp)	F2.1 and R2 (74bp)	F2.2 and R2 (87bp)	
B*130101-02	✓			2.1
B*1307N	✓			8.5
B*150201-02	✓	✓	✓	8.5
B*1513	✓			1.8
B*1521	✓		✓	0.7
B*152501-02	✓	✓		2.5
B*1588	✓	✓	✓	0.0
B*9521	✓	✓	✓	0.0
B*9544	✓	✓	✓	0.0

Using DNA with known HLA-B alleles which are predicted to be amplified at each PCR step (HLA-B*1301, 1513, 1521 and 1525) and HLA-B alleles which are found more than 1% among Thai population to test for predicted positive alleles and assay specificity, migration patterns from different alleles are shown in figure 15. For the first PCR, HLA-B*1301, 1513, 1521 and 1525 were amplified as predicted, but no other alleles with more than 1% present in Thai population were amplified. Next in the second PCR, HLA-B*1502 was amplified, but HLA-B*1525 was slightly amplified by F2.1 and R2, resulting in strong band for HLA-B*1502, but faint band for HLA-B*1525. According to HLA-B*1502 and 1525 sequences within F2.1 and R2 regions, HLA-B*1525 has two mismatch bases from F2.1, but no mismatch with R2 (figure 10). Thus, HLA-B*1525 could be slightly amplified from F2.1 and R2. Finally, HLA-B*1502 and 1521 was

amplified from F2.2 and R2, resulting in strong band for both HLA-B*1502 and 1521. According to HLA-B*1502 and 1521 sequences within these primer regions, HLA-B*1521 has no mismatch in F2.2 nor R2 (figure 10). This perfect complementary explained why HLA-B*1521 was amplified stronger by F2.2 and R2 than HLA-B*1525 from F2.1 and R2.

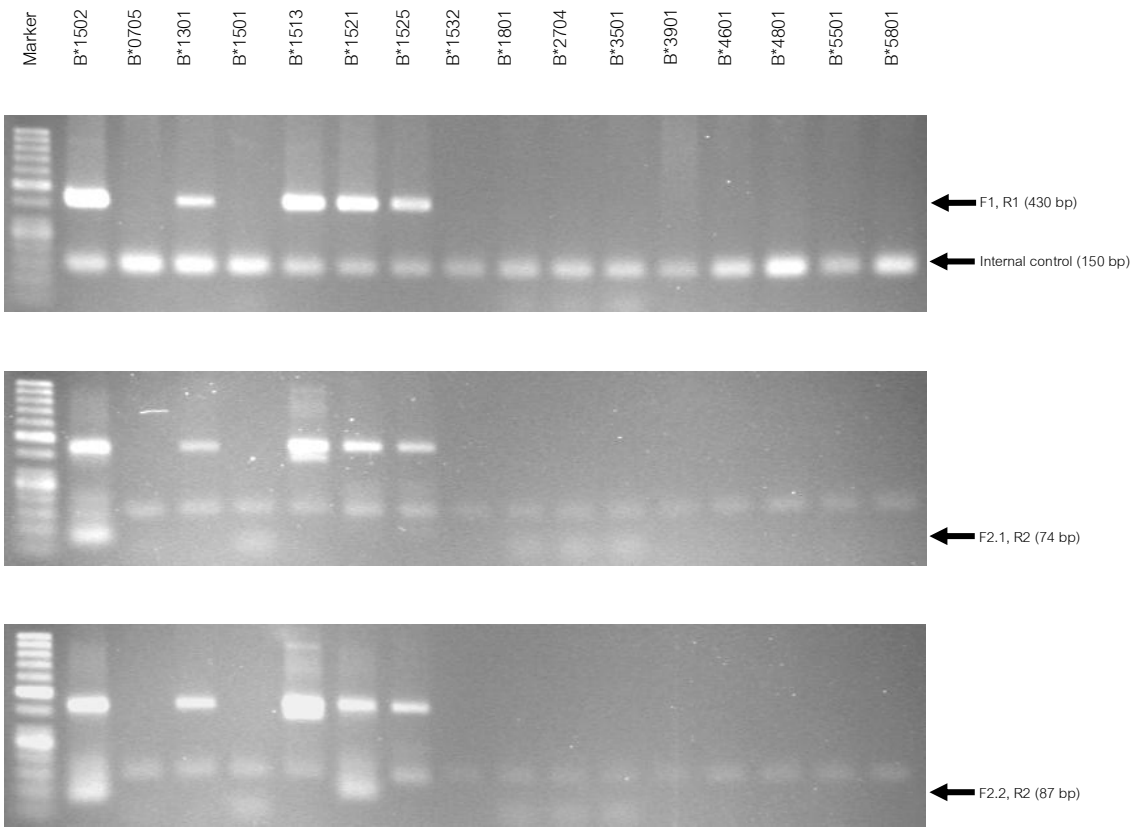


Figure 15. Migration patterns to differentiate HLA-B*1502 from other alleles. These products were done by nested SSP-PCR. Three positive bands from both first (430bp from F1 and R1) and two second PCR (74bp F2.1 and R2, and 87bp F2.2 and R2) will be reported as HLA-B*1502. Negative results only show the internal control band (150bp) from the first PCR.

From 104 blind DNA samples from the Thai Red Cross Society with commercial SSP-PCR and SBT determined alleles, all HLA-B*1502 were detected correctly (n=24) and did not amplify any other HLA-B alleles (n=80). Therefore, this assay could achieve

100% sensitivity and very close to 100% specificity, since rare alleles that did not found in this study (HLA-B*1588, 9521 and 9544) were predicted to be amplified (table 11). All blind samples were concluded in table15.

Table 11. Sensitivity and specificity calculation of in-house SSP-PCR for HLA-B*1502.

		Commercial SSP-PCR		
		Positive	Negative	
In-house SSP-PCR HLA-B*1502	Positive	24	0	PPV = 24/24 = 100.0%
	Negative	0	80	NPV = 80/80 = 100.0%
		Sensitivity = 24/24 = 100.0%	Specificity = 80/80 = 100.0%	

Comparing to the previous report, HLA-B*1502 typing used 4 sets of primer in 4 reaction, therefore the benefit of this study would be reducing both number of reactions and cost. Moreover, problem of false positives from heterozygous combination in multiplex PCR were not found in this assay, as nested PCR was used to narrow down unrelated alleles at each step. Another advantage of nested PCR is sensitivity, because DNA template at very little amount can be detected. Detection limit of this simple nested SSP-PCR showed that DNA concentration as least as 0.005 ng/µl could be amplified (figure 16).

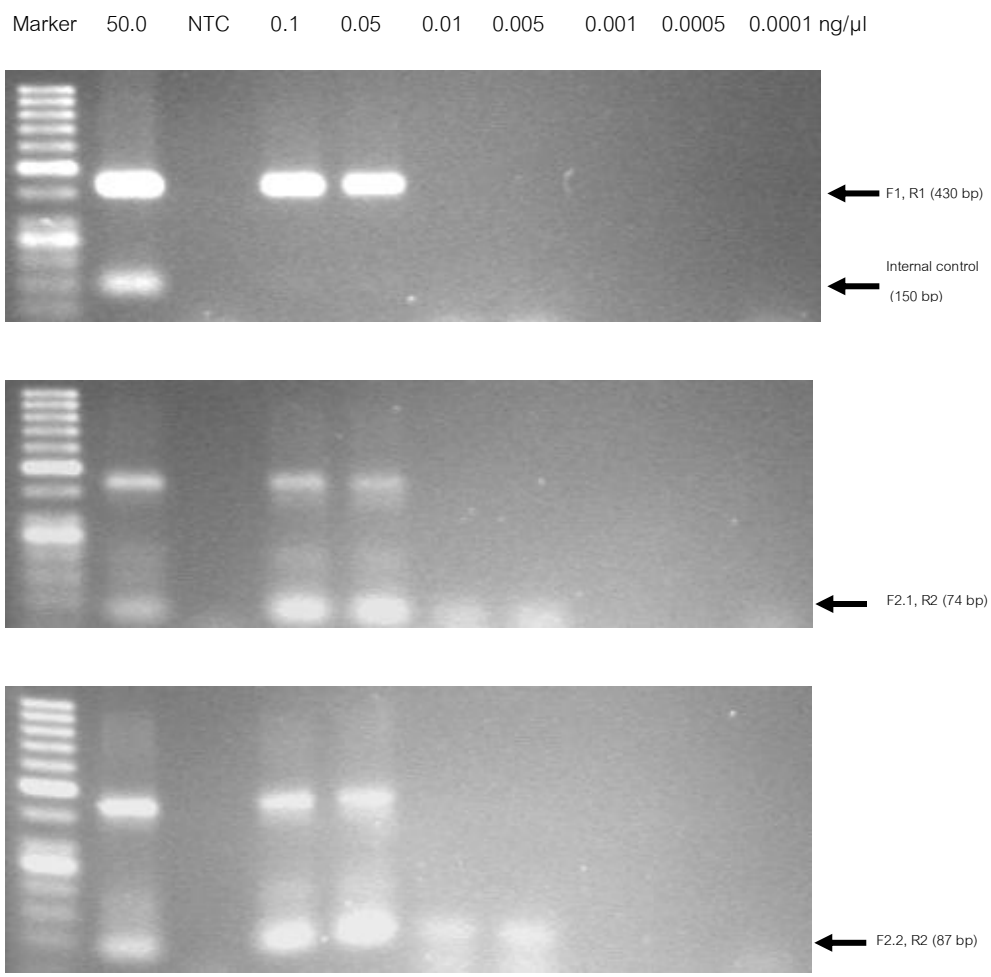


Figure 16. Testing detection limit at 0.0001 – 0.1 ng/µl DNA template for HLA-B*1502. For the first PCR, the maximum DNA concentration for 430bp F1 and R1 amplification was at 0.05 ng/µl, while the second PCR, 74bp F2.1 and R2 and 87bp F2.2 and R2 were amplified at as high as 0.005 ng/µl. Consequently, the detection limit of this test was 0.005 ng/µl.

In conclusion, this assay could amplified with DNA template as least as 0.005 ng at 100% sensitivity and >99.9% specificity. The benefit of these tests would help patients to avoid any adverse consequences from carbamazepine in advance by reliable standard SSP-PCR.

2.2. SSP-PCR for HLA-B*5801 from Genomic DNA

Using 10 bases from 3' end of each primer to predict amplified alleles, HLA-B*5705, 580101-04, 5804-05, 5809, 5810N, 5811-13, 5817N, 5819, 5821-24 and 5828 are expected to be amplified. According to the allele frequency of world population database (http://www.allelefrequencies.net/default.asp), HLA-B*5801 is the only allele that was reported to be found in Thai and world population, whereas HLA-B*5705 is not found in Thai nor world population. Moreover, polymorphisms among HLA-B*58 group, both in intron and exon, are scatter around the sequences. Thus HLA-B*5801 cannot be differentiated from other subtype of HLA-B*58. As a result, HLA-B*5801 was predicted to be amplified specifically by only one set of primer.

Using DNA with known HLA-B alleles, HLA-B*1517 and 5701 which their sequences are closely to HLA-B*5801 and HLA-B alleles which are found more than 1% among Thai population to test for predicted positive alleles and assay specificity, migration patterns from different alleles are shown in figure 17. Only HLA-B*5801 can be amplified by this system.

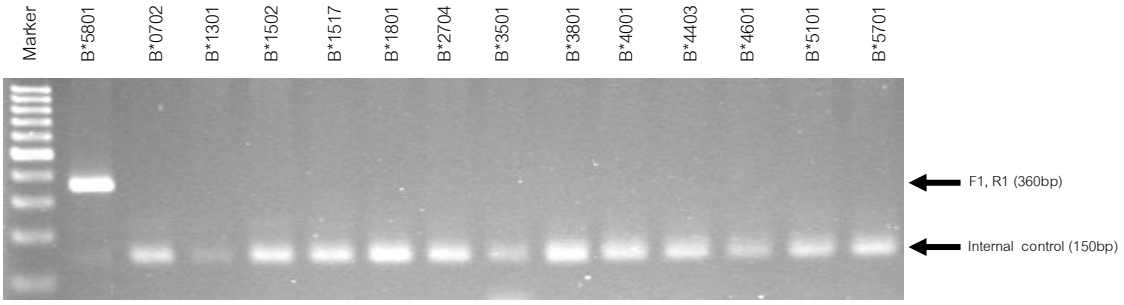


Figure 17. Migration patterns to differentiate HLA-B*5801 from other alleles. These products are done by simple SSP-PCR with one set of primer (360bp F1 and R1). Positive bands will be reported as HLA-B*5801.

Within 95 samples, all B*5801 samples were detected correctly (n=13), while all HLA-B alleles presented more than 1% in Thai population including HLA-B*0705, 1301, 1501, 1511/12/13, 1525, 1801, 3501, 3801, 3901, 4001/02, 4402, 4601, 4801, 5101/02,

and 5201 gave negative result with our primer (n=82). Thus, this assay gives 100% sensitivity and very close to 100% specificity, as extremely rare alleles that did not found in this study were predicted to be amplified (table 12). All blind sample results were shown in table 15.

Table 12. Sensitivity and specificity calculation of in-house SSP-PCR for HLA-B*5801.

		Commercial SSP-PCR		
		Positive	Negative	
In-house SSP-PCR HLA-B*5801	Positive	13	0	PPV = 13/13 = 100.0%
	Negative	0	82	NPV = 82/82 = 100.0%
		Sensitivity = 13/13 = 100.0%	Specificity = 82/82 = 100.0%	

Comparing to the previous report, HLA-B*5801 detection composed of two sets of primer. From the reference, first primer set amplifies HLA-B*5801, 5104, 5301 and 1513, while another set amplifies HLA-B*5801-3. A successful typing for HLA-B*5801 will give two positive bands from both sets of primer in separated reactions. The limitation of this earlier method could occur with the combination of non HLA-B*5801 heterozygote, for example, patient with HLA-B*1513 and HLA-B*5802 will result as two positive bands and misinterpret as HLA-B*5801. Here, only one set of primer is used, therefore assay number is reduced to only one reaction and false positive from heterozygote is eliminated. Also, as least as 0.005 ng/µl of genomic DNA concentration was detected from this in-house SSP-PCR for HLA-B*5801 (figure 18).

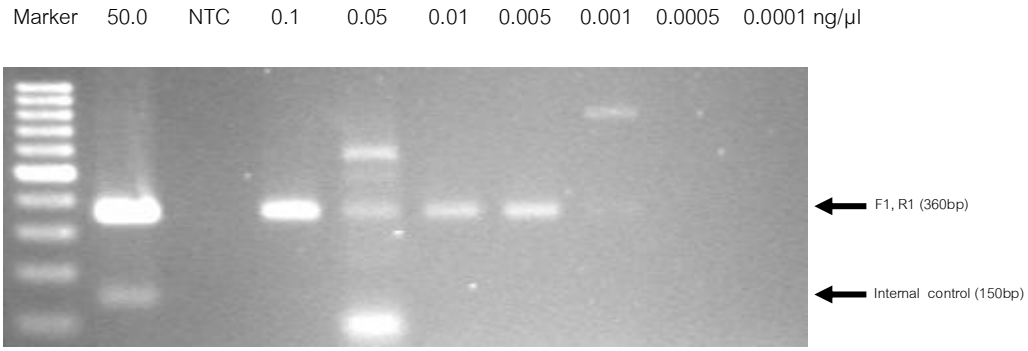


Figure 18. Testing detection limit at 0.0001 – 0.1 ng/µl DNA template for HLA-B*5801. The maximum DNA concentration for 360bp F1 and R1 amplification was at 0.005 ng/µl. Consequently, the detection limit of this test was 0.005 ng/µl.

In conclusion, this assay could amplified HLA-B*5801 with DNA template as least as 0.005 ng/µl at 100% sensitivity and >99.9% specificity. The benefit of these tests would help patients to avoid any life-threatening adverse consequences from allopurinol by reliable standard SSP-PCR.

3. SSP-PCR for HLA-B*1502 and HLA-B*5801 from Whole Blood

Starting with internal control amplification by modified initial denaturing step, IFN-γ was detected from whole blood sample at all amount of whole blood volume (figure 19). But the PCR efficiency by this method is extremely low. Thus, it is not suitable for nested SSP-PCR for HLA-B*1502. As discussed in the previous report, this is due to the increase of whole blood PCR inhibitor. As a result, the whole blood dilution was used as template to further test with nested SSP-PCR of HLA-B*1502.

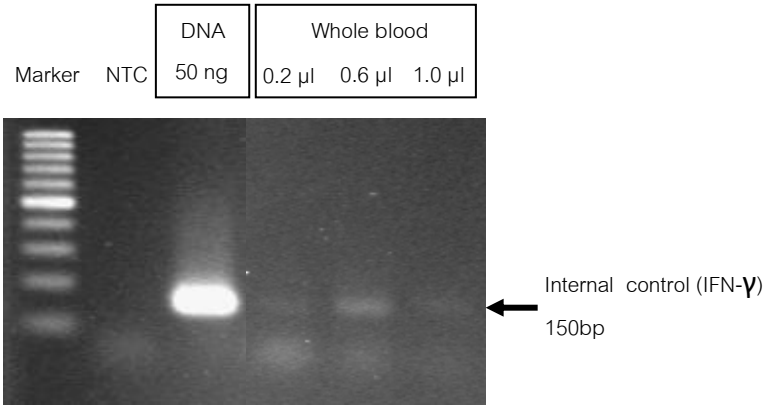


Figure 19. PCR from whole blood, comparing 50ng genomic DNA to whole blood sample at 0.2, 0.6 and 1.0 µl as template. Using 0.6 µl of whole blood gave the strongest band comparing to 0.2 and 1.0 µl whole blood. However, adding whole blood directly to the PCR reaction gave very low PCR efficiency.

Next, the blood dilution was used as the whole crude and the supernatant from whole blood was used as template. For the crude whole blood sample, IFN-γ was detected from 2-30 µl, while at 5-20 µl of supernatant from the whole blood dilution (figure 20). When looking at the PCR efficiency, supernatant gave better results than that of crude whole blood sample, although at lower whole blood concentration. Thus, supernatant were used from the whole blood dilution for nested SSP-PCR of HLA-B*1502 from whole blood dilution. Moreover, the absorbance was checked at A260 and A280 (table 13). Showing that while DNA concentration was higher (A260), protein and other substances (A280) also increased. This explains why PCR activity is lower at higher whole blood content in the dilution.

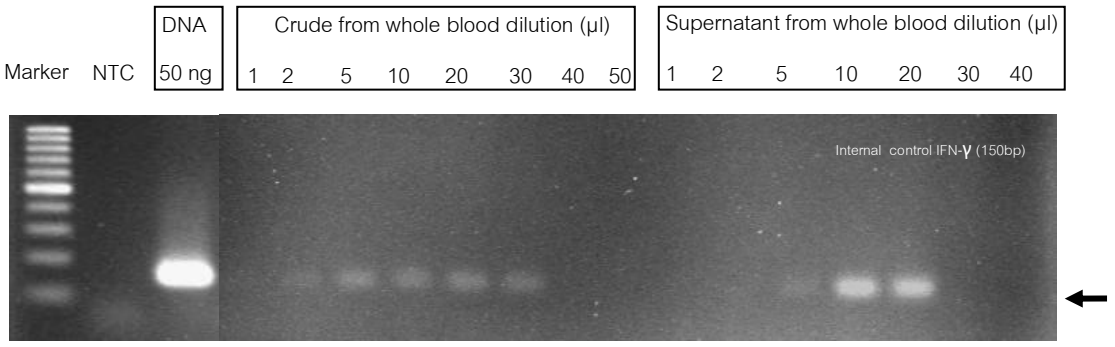


Figure 20. PCR with crude and supernatant from whole blood dilution. Crude whole blood can detect as high as 30 µl of whole blood dilution, while supernatant can detect as high as 20 µl of whole blood dilution, with stronger PCR activity.

Table 13. Absorption of supernatant from series of whole blood dilution.

Sample	ng/µl	A260	A280	260/280
1	272.2	5.444	3.538	1.54
2	424.47	8.489	5.523	1.54
5	244.65	4.893	3.103	1.58
10	281.72	5.634	3.391	1.66
20	519.56	10.391	5.364	1.94
30	703.85	14.077	7.454	1.89
40	1052.49	21.05	10.472	2.01
45	1214.67	24.293	12.043	2.02

When supernatant was used from the whole blood dilution for nested SSP-PCR for HLA-B*1502, only the internal control band could be detected from the reaction at 10, 20 µl of supernatant whole blood with *Taq* polymerase from Fermentas (figure 21). This failure in HLA-B*1502 might be caused by multiplex primer of HLA-B*1502 and internal control IFN-γ.

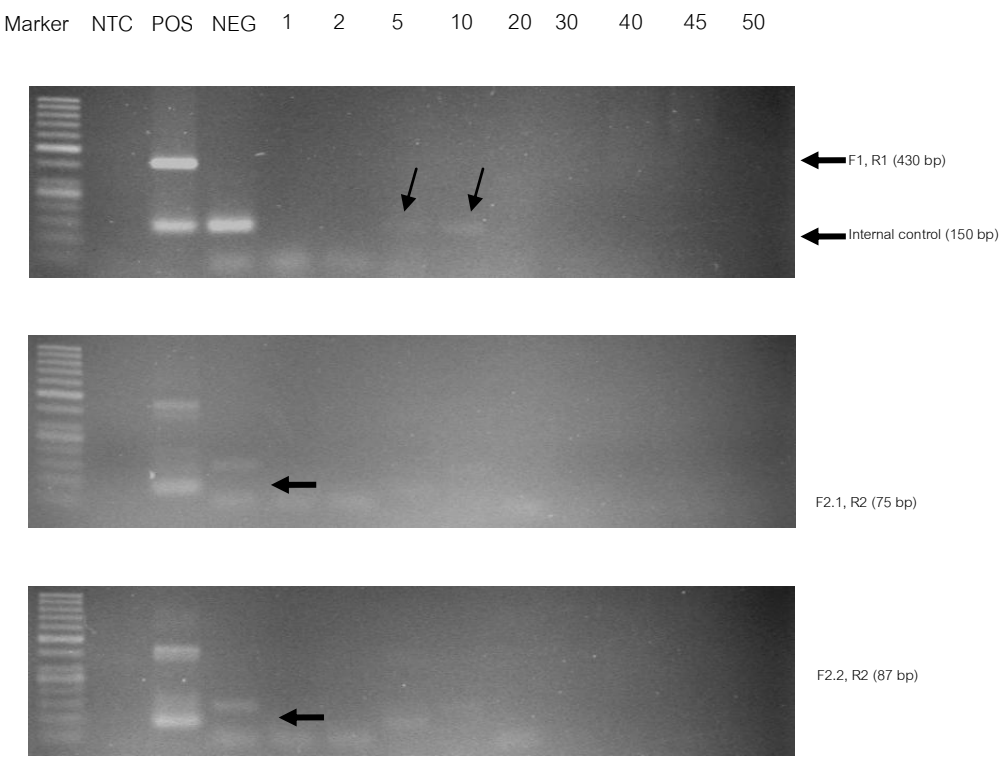


Figure 21. Nested SSP-PCR of HLA-B*1502 from whole blood dilution supernatant with *Taq* polymerase (Fermentas). Only the internal control band at 5 and 10 µl of the supernatant from whole blood dilution was found, whereas bands were not found in both second PCR.

Moreover, FastStart *Taq* Polymerase™ from Roach was also used to see the effect of Hot Start *Taq* polymerase. It shown that HLA-B*1502 still cannot be amplified (figure 22). This results can be concluded that Hot Start *Taq* polymerase did not help in multiplex PCR, and was not more resistant to PCR inhibitors than normal *Taq* polymerase.

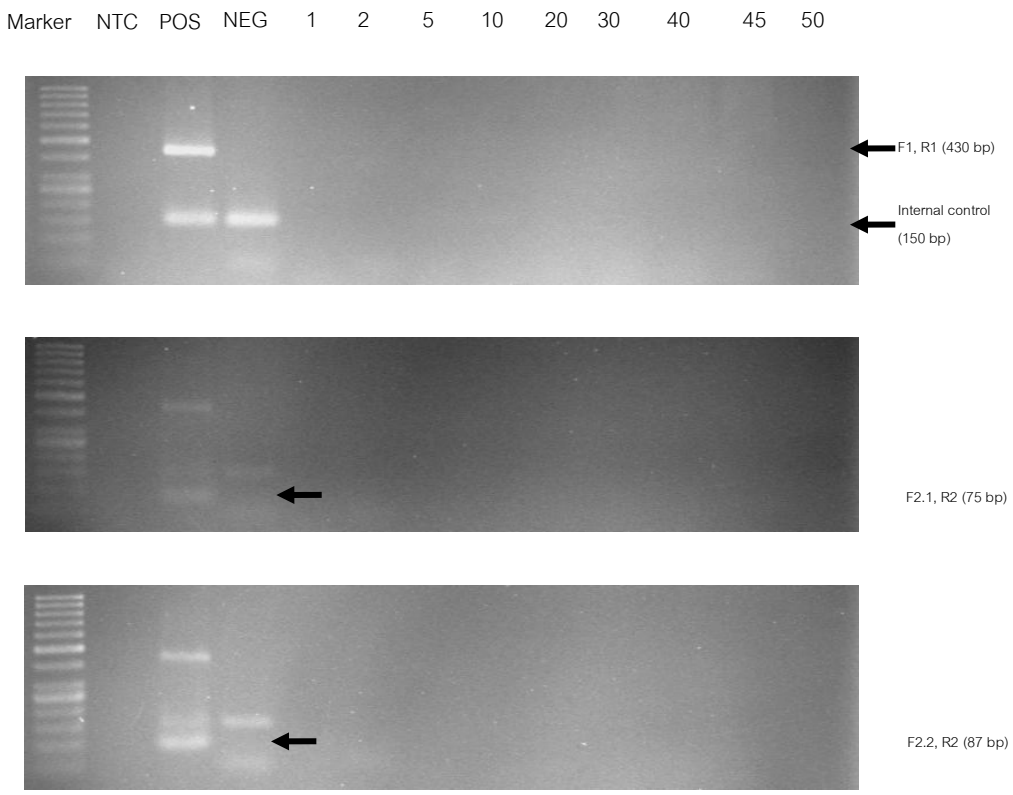


Figure 22. Nested SSP-PCR of HLA-B*1502 from whole blood dilution supernatant with FastStart *Taq* Polymerase (Roach). Neither HLA-B*1502 nor internal control band were found from first and second PCR.

Finally, commercial blood PCR kit (Kapa Biosystems) was used with whole blood sample. With the same touchdown PCR cycles, HLA-B*1502 can be amplified the whole blood sample specifically with this commercial reagent (figure 23). But when using purified DNA as template, there were lots of non specific bands. This might cause by high amplification efficiency of the blood PCR kit.

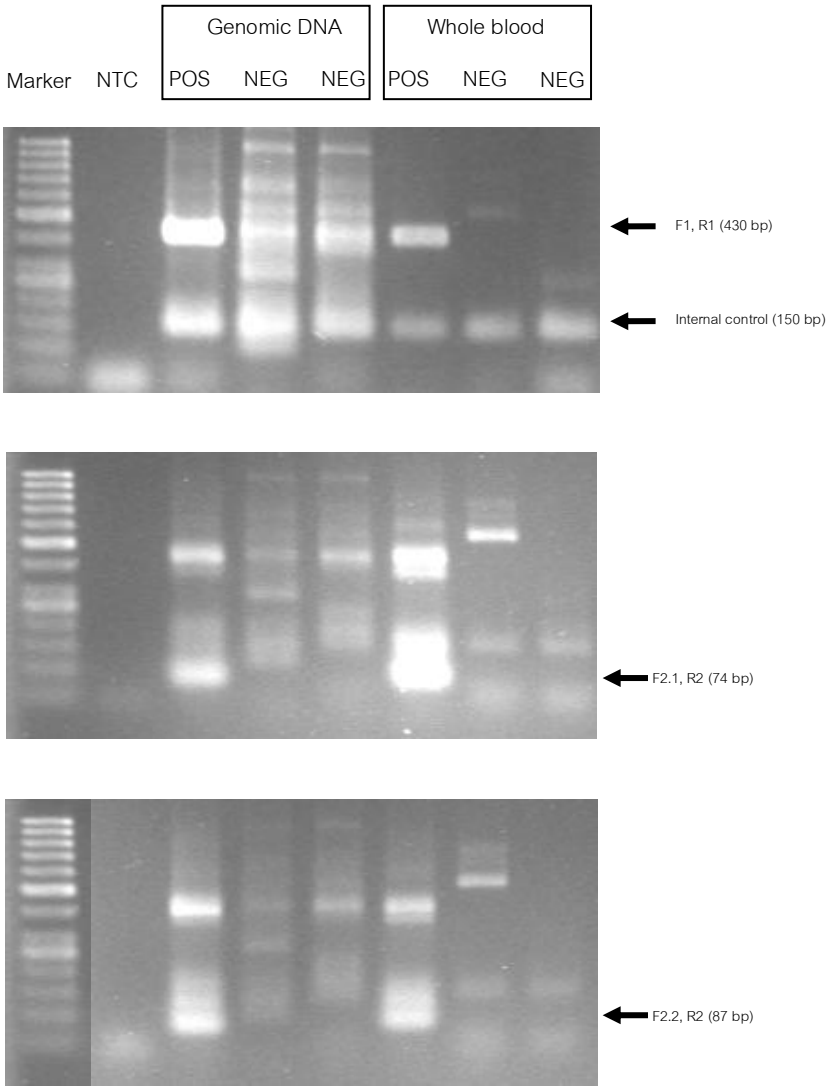


Figure 23. Nested SSP-PCR of HLA-B*1502 from whole blood with commercial blood PCR Kit (Kapa Biosystems). HLA-B*1502 could be amplified directly from whole blood as specific as from purified genomic DNA.

From nine blind whole blood samples, two HLA-B*1502 (figure 24) and two HLA-B*5801 (figure 25) were correctly amplified by this blood PCR kit. However, more samples are needed to be recruited before applying this system for service.

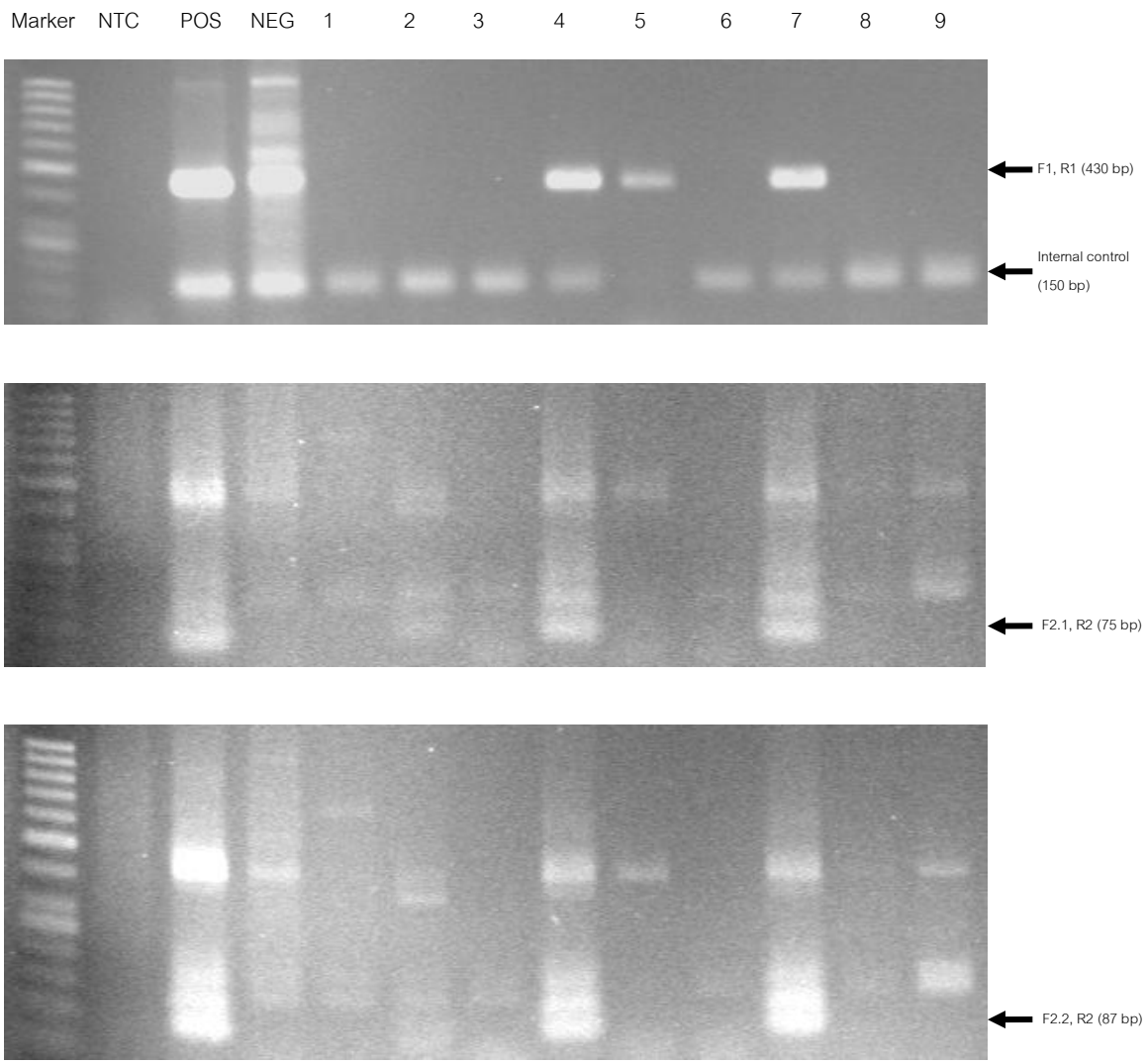


Figure 24. Blind whole blood results of HLA-B*1502 from whole blood with Commercial Blood PCR Kit (Kapa Biosystems). Sample 4 and 9 were positive for HLA-B*1502.

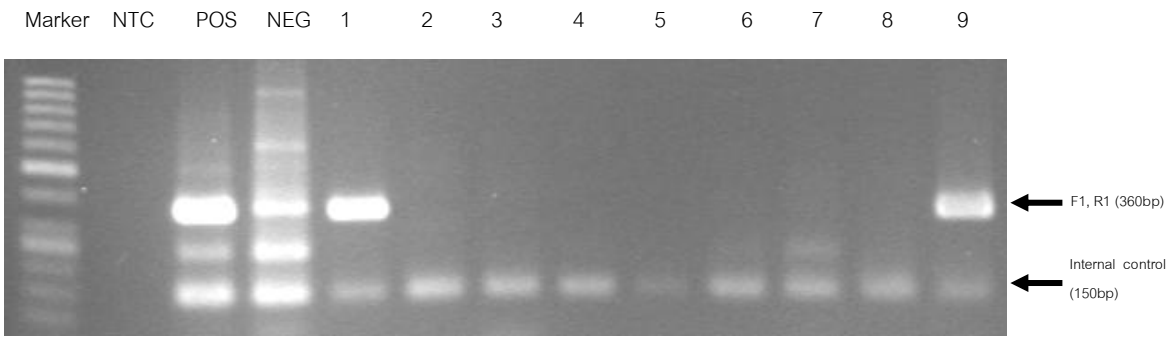


Figure 25. Blind whole blood results of HLA-B*5801 from whole blood with Commercial Blood PCR Kit (Kapa Biosystems). Sample 1 and 9 were positive for HLA-B*5801.

4. LAMP for HLA-B*5801 setup

HLA-B*5801 has 100% sequence similarity to HLA-B*5701 among all 6 primer regions in LAMP reaction. Thus, these two alleles were predicted to be positive in LAMP reaction as smear bands when detecting with gel electrophoresis. As a result, this assay can be used to predict two drug-induced skin reactions, HLA-B*5801 for allopurinol, anti-hyperuricemic agent and HLA-B*5701 for abacavir, anti-HIV drug. In order to get the most specific condition for both HLA-B*5801 and 5701, gradient temperature of 70±4^{o}C was performed with LAMP reaction using HLA-B*5801 genomic DNA as template. From figure 26, LAMP product was successfully produced with the highest temperature at 70.5^{o}C. From this result, 70^{o}C was chosen for LAMP reaction of HLA-B*5801 and 5701.

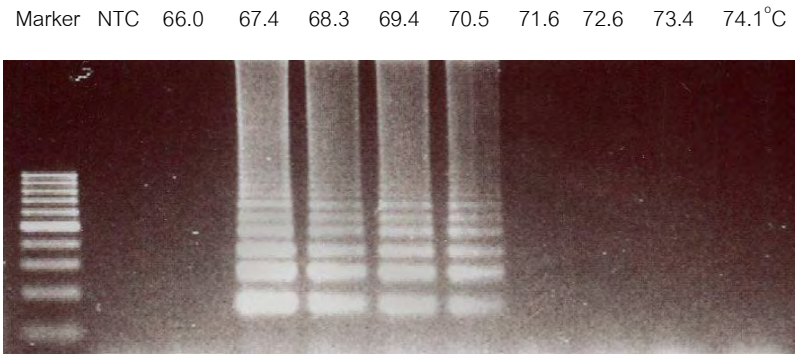


Figure 26. LAMP reaction at different temperature. HLA-B*5801 could be amplified by LAMP primer at as high as 70.5°C.

Also, gradient of $MgSO_4$ concentration was done from 4.44 – 6.67 mM with HLA-B*5801 and 5701 as template. Concentration at 6.67 mM of $MgSO_4$ was chosen, as both HLA-B*5801 and 5701 were amplified specifically at 70°C (figure 27). These temperature and $MgSO_4$ conditions were later used for specificity testing.

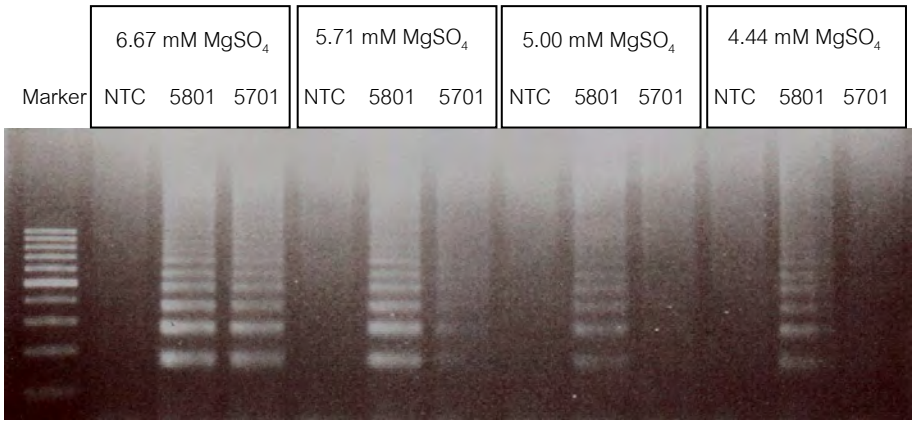


Figure 27. LAMP reaction at different $MgSO_4$ concentration. HLA-B*5801 and 5701 could be amplified by LAMP primer at 6.67 mM $MgSO_4$.

5. LAMP Reaction

Using DNA with known HLA-B alleles, HLA-B*1517 and 5701, which their sequences were close to HLA-B*5801, and other HLA-B alleles, which were found more than 1% among Thai population, were used to test for predicted positive alleles and assay specificity. Migration patterns of LAMP product from different alleles are shown in figure 28.

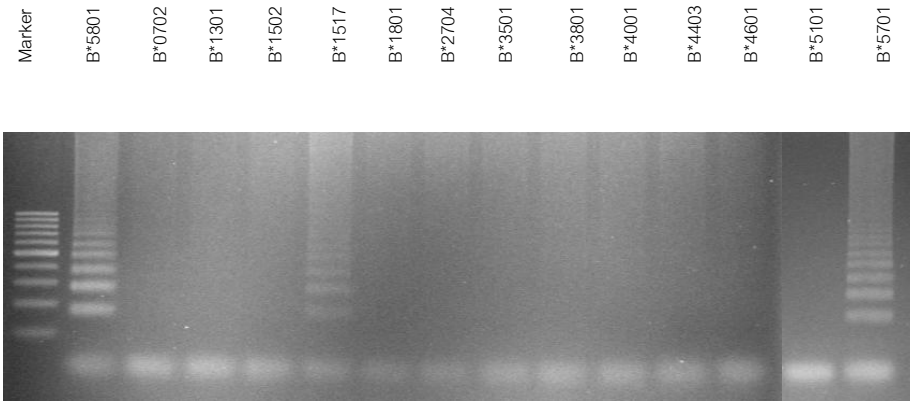


Figure 28. LAMP reaction with HLA-B*5801 and other alleles. LAMP products could be observed in 40 minutes as smear bands when detecting with gel electrophoresis from both HLA-B*5801 and 5701. HLA-B*1517 was the only false positive allele from this system.

From LAMP reaction, HLA-B*1517 was a false positive allele due to their close sequence (figure 12). From all primer regions, HLA-B*1517 shows only one mismatch at B2 region, therefore HLA-B*1517 can be amplified with lighter bands than HLA-B*5801 and 5701 by this system. According to the frequency database of HLA-B*1517 (http://www.allelefrequencies.net/default.asp), this allele can be found at 0.7% among Thai population and not more than 1.0% among world population, except Bulgaria, Mongolia, India, Kenya and Saudi Arabia. Thus, this assay is still quite specific for HLA-B*5801 and 5701 prediction.

Within 95 blind samples, 20 HLA-B*5801 and 5701 were detected as true positive results, 12 false positive results, 62 true negative results and 2 false negative

result. From the results, 12 false positive results were shown from LAMP reaction, which none of these false positive results were occurred in SSP-PCR. Moreover, alleles among false positive samples were not related nor have similar sequence to LAMP primers. This problem might be explained by LAMP high sensitivity and other unknown contaminant, for example, dust, glove powder and contaminated DNA. After sensitivity and specificity calculation, this method can achieve 83.78% sensitivity and 95.24% sensitivity (table 14). All blind sample results were summarized in table15.

Table 14. Sensitivity and specificity calculation of LAMP for HLA-B*5801 and 5701.

		Commercial SSP-PCR		
		Positive	Negative	
LAMP	Positive	20	12	PPV = 20/(20+12) = 62.50%
	Negative	1	62	NPV = 62/(62+1) = 98.41%
		Sensitivity = 20/(20+1) = 95.24%	Specificity = 62/(62+12) = 83.78%	

Table 15. All blind DNA samples with results from both SSP-PCR and LAMP.

Code	Sample	HLA Alleles	SSP-PCR		LAMP
			HLA-B*1502	HLA-B*5801	HLA-B*5701/5801
B1	CB 57	B*1502, B*39	POS	neg	neg
B2	CB 66	B*1502, B*38	POS	neg	neg
B3	CB 70	B*1502, B*3801-02/08+	POS	neg	neg
B4	CB 71	B*1502, B*46	POS	neg	*POS*
B5	CB 72	B*4002/08+ (B61), B*51	neg	neg	neg
B6	CB 73	B*1502, B*4201-02/06	POS	neg	neg
B7	CB 75	B*1502, B*1801	POS	neg	*POS*

B8	CB 76	B*4001, B*4601	neg	neg	neg
B9	CB 77	B*2707, B*3501	neg	neg	neg
B10	CB 78	B*1502, B*4001/07+	POS	neg	neg
B11	CB 79	B*1502, B*1801/03+	N/A	neg	*POS*
B12	CB 146	B*5801, B*1501	neg	POS	POS
B13	CB 147	B*1502, B*4001	POS	neg	neg
B14	CB 149	B*4402, B*4601	neg	neg	neg
B15	CB 150	B*1502, B*5701	POS	neg	*POS*
B16	CB 151	B*1525, B*3801	neg	neg	neg
B17	CB 152	B*44, B*57	neg	neg	*neg*
B18	CB 155	B*4001, B*5801	neg	POS	POS
B19	CB 156	B*1502, B*3701	POS	neg	neg
B20	CB 157	B*1502, B4001	POS	neg	neg
B21	CB 158	B*4402, B*4601	neg	neg	neg
B22	CB 159	B*2704, B*5801	neg	POS	POS
B23	G 2476	B*1301-03/08+, B*4601-03	neg	neg	neg
B25	G 2486	B*13, B*46	neg	neg	neg
B26	G 2487	B*07	neg	neg	neg
B27	G 2488	B*5201/04*, B*1525	neg	neg	neg
B28	G 2489	B*1502	POS	neg	neg
B29	G 2492	B*5701/06, B*4001/07	neg	neg	POS
B30	G 2496	B*1501/06, B*1513	neg	neg	neg
B31	CKP 67	B*4601/05+, B*5801/06+	POS	neg	POS
B32	CKP 68	B*4801/03+, B*4402/04+	neg	neg	neg
B33	SKM 4	B*4001/07+, B*4601/05+	neg	neg	neg
B34	SKM 6	B*1801/08+, B*5101/03+	neg	neg	POS
B35	SKM 11	B*1502, B*4601/03+	POS	neg	neg

B36	SKM 18	B*1508/1101, B*4402/03+	neg	neg	neg
B37	SKM 20	B*1301/0201(1306/10/12), B*4048	neg	neg	neg
B38	SKM 22	B*5701/06+(5702/03, 5708), B*5801/06+	neg	POS	POS
B39	SKD 22	B*5201-05+, B*3901/05+	neg	neg	neg
B40	SKM 31	B*0734, B*5615	neg	neg	neg
B41	SKD 31	B*1301/02-03+, B*5701-10	neg	neg	POS
B42	SKM 32	B*4444, B*3901-04/05+	neg	neg	neg
B43	SKD 32	B*4601/05+, B*1501-05/06+	neg	neg	neg
B44	SKM 35	B*4601/05+	neg	neg	neg
B45	SKD 36	B*1517, B*1801/03+	neg	neg	*POS*
B46	SKM 40	B*3901/05+, B*5801/06+	neg	POS	POS
B47	SKM 41	B*2704/06+, B*4601/05+	neg	neg	neg
B48	SKM 45	B*1801/03+, B*1525	neg	neg	neg
B49	SKM 47	B*1501/06+, B*4601/05+	neg	neg	neg
B50	CB44	B*58	neg	POS	POS
B51	CB45	B*35, B*1801/03+	neg	neg	neg
B52	CB46	B*4001/07+, B*5041/04/08N	neg	neg	neg
B53	CB54	B*13, B*52	neg	neg	neg
B54	CB56	B*1502, B*5102/34	POS	neg	neg
B55	CB58	B*07, B*46	neg	neg	neg
B56	CB60	B*13, B*46	neg	neg	neg
B57	CB63	B*40(B60), B*52	neg	neg	neg
B58	CB67	B*13, B*75	neg	neg	neg
B59	CB68	B*18, B*40(B60)	neg	neg	neg
B60	CB69	B*1502, B*5201	POS	neg	neg
B61	CB80	B*3801/02+, B*5801/06+	neg	POS	POS
B62	CB81	B*4601/05+, B*8102	neg	neg	neg

B63	CB82	B*4001/07+, B*4601/05+	neg	neg	neg
B64	CB83	B*1502, B*4601/05+	POS	neg	neg
B65	CB85	B*4402/05+, B*4601/05+	neg	neg	neg
B66	CB86	B*2704/06+, B*2718	neg	neg	*POS*
B67	CB87	B*5101/03+, B*4001/07+	neg	neg	neg
B68	CB88	B*5101/03+, B*1301/08+	neg	neg	POS
B69	CB91	B*5102, B*3801	neg	neg	neg
B70	CB105	B*1801, B*0705	neg	neg	POS
B71	CB109	B*1301, B*4801	neg	neg	neg
B72	CB111	B*1508, B*1510	neg	neg	neg
B73	CB114	B*1502, B*1525	POS	neg	neg
B74	CB116	B*1512, B*5101	neg	neg	neg
B75	CB118	B*1801, B*4001	neg	neg	neg
B76	CB122	B*1301, B*4201	neg	neg	neg
B77	CB123	B*1525, B*1501	neg	neg	neg
B78	CB124	B*3501, B*5102	neg	neg	neg
B79	CB126	B*4001, B*4601	neg	neg	*POS*
B80	CB127	B*5801, B*4601	neg	POS	POS
B81	CB128	B*1502, B*4402	neg	neg	neg
B82	CB133	B*1301, B*1501	neg	neg	*POS*
B83	CB138	B*1801, B*3801	neg	neg	*POS*
B84	CB153	B*4001, B*4601	neg	neg	*POS*
B85	CB160	B*2704, B*4601	neg	neg	*POS*
B86	CB162	B*4601	neg	neg	*POS*
B87	CB163	B*5201, B*5801	neg	POS	POS
B88	CBZ-003	B*1502, B*1502	POS	N/A	N/A
B89	CBZ-012	B*1301, B*4001	neg	N/A	N/A

B90	CBZ-015	B*1802, B*5502	neg	N/A	N/A
B91	CBZ-017	B*2704, B*1525	neg	N/A	N/A
B92	CBZ-057	B*1521, B*4801	neg	N/A	N/A
B93	CBZ-300	B*5201, B*1502	POS	N/A	N/A
B94	CCB-026	B*1301, B*1502	POS	N/A	N/A
B95	CCB-028	B*0705, B*1525	neg	N/A	N/A
B96	All-002	B*3909, B*5801	N/A	POS	POS
B97	All-009	B*5801, B*1502	N/A	POS	POS
B98	All-011	B*1301, B*5801	N/A	POS	POS
B99	CAL-089	B*5101, B*1502	N/A	neg	neg
B100	CAL-096	B*1502, B*5801	N/A	POS	POS
B101	CAL-113	B*4801, B*5801	N/A	neg	neg
B102	CAL-140	B*1502, B*4001	N/A	neg	neg
B104	N2	B*1502	POS	N/A	N/A
B105	N3	B*1502/9512, B*4001	POS	N/A	N/A
B107	N5	B*1532, B*1801/20/23N/24	neg	N/A	N/A
B108	N6	B*1525, B*4601/07N/08/10	neg	N/A	N/A
B109	N7	B*1502, B*1801/23N/24	POS	N/A	N/A
B110	N8	B*2704, B*5101	neg	N/A	N/A
B111	N9	B*4601	neg	N/A	N/A
B112	N10	B*1301, B*1801	neg	N/A	N/A
		Total samples	104	95	95
True positive			24	13	20
False positive			0	0	12
True negative			80	82	62
False negative			0	0	1

Specificity (%)	100.00	100.00	83.78
Sensitivity (%)	100.00	100.00	95.24

*POS, true positive; *POS*, false positive; neg, true negative; *neg*, false negative; N/A, not Avialable.

6. PNA Probe Hybridization

In order to gain more specificity to current LAMP system, a strand displacement PNA-DNA probe consisting of a fluorescently-labeled PNA probe and a quencher-labeled short DNA strand was specifically designed for HLA-B*5801 (figure 12). In theory, if these were LAMP products, the PNA probe should bind to its DNA target with concomitant displacement of the quencher strand. This will separate the fluorescent PNA probe from the quencher DNA, resulting in fluorescent enhancement of the PNA probe (figure 29). As a result, if LAMP product with probe target region is observed under UV light, green fluorescent will be shown (figure 30, right). In a contrast, if LAMP product with non-target region is observed under UV light, green fluorescent will not be shown (figure 30, left).

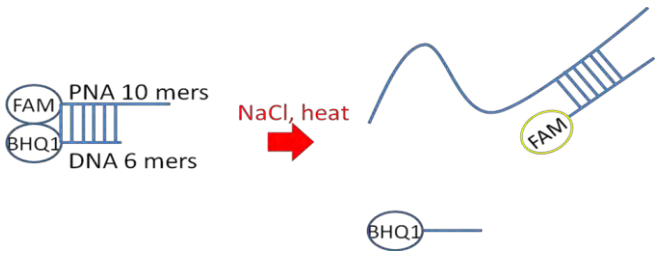


Figure 29. Strand displacement probe mechanism. When fluorescently-labeled PNA probe binds to the target, a quencher-labeled short DNA strand will be free. As a result, fluorescent on the PNA probe is no longer quenched by the quencher from the DNA probe and released from the fluorescently-labeled PNA probe.

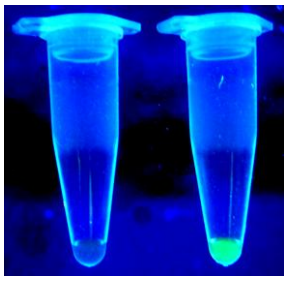


Figure 30. Fluorescent from LAMP product with non-probe target (left) and probe target (right) under UV light. When applying stand displacement probe to the LAMP product, fluorescent can be detected with probe target region, whereas in non-target LAMP product, it's vice versa.

Not only the HLA-B*5801 fluorescently-labeled PNA probe was designed in this study, an internal control quenched fluorescent PNA probe was also designed for the purpose of confirmation. This internal control probe was firstly applied for LAMP product detection. Beginning with preparing PNA-DNA strand displacement probe, fixed 3 µl of 10 µM fluorescently-labeled PNA probe was added with series of 1 - 8 µl of 10 µM quencher-labeled DNA probe. Afterwards, 1 µl of LAMP product was added to PNA-DNA complex solution. Next, 1.5 µM NaCl ranging from 0.5 – 1.5 µl were introduced to find the specific condition. Finally, fluorescence is observed under ultraviolet light. Comparing between non-template control and HLA-B*5801 LAMP product, no difference in fluorescent signals was observed. Once the fluorescent signals in non-template control were quenched, the fluorescent signals in positive LAMP product were quenched. Also, when the fluorescent signals in non-template control were fluorescent, the fluorescent signals in HLA-B*5801 positive product were fluorescent. It was hypothesized that some molecules including proteins in the Loopamp DNA Amplification (Eiken Chemical) interfered the hybridization assay. Thus, SDS-PAGE was performed between the two buffers, no unique protein was found (figure 31). Also, when purified LAMP product was used, the results were the same.

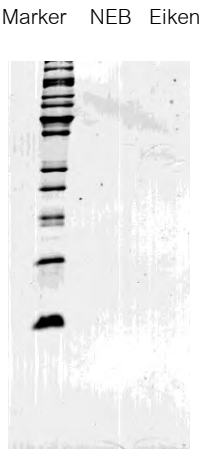


Figure 31. SDS-PAGE from conventional (New England Biolabs) and commercial LAMP buffer (Eiken Chemical). No unique proteins were observed between the two buffers.

Another assay on HLA-B*5801 probe, PNA-DNA strand displacement probes were prepared, fixed 3 µl of 10 µM fluorescently-labeled PNA probe was added with series of 1 - 10 µl of 10 µM quencher-labeled DNA probe. Afterwards, 1 µl of LAMP product or purified LAMP products were added to PNA-DNA complex solution. Next, 1.5 µM NaCl ranging from 0.5 – 4.5 µl were introduced to find the specific condition. Finally, fluorescence is observed under ultraviolet light. Comparing between non-template control and HLA-B*5801 LAMP product, quenching phenomenon was observed in the non-template control and the HLA-B*5801 positive product. In addition, heating was introduced to facilitate hybridization, but at as high as 90^{o}C, fluorescent still could not be detected. When looking at the probe sequences, Fluorescein-*N*-CTCCGTCCTC-*C*, it seemed that several C might increase high stability binding and affect fluorescent released by nucleotide quenching mechanisms from their complementary G in the target DNA. Thus, only fluorescently-labeled PNA probe was used without quencher-labeled DNA probe to proof this assumption. It turned out to be true, as more fluorescent was observed in that of non-template control than was in HLA-B*5801 and 5701 LAMP products. Although, all the reasons for the absence of fluorescent enhancement is not clear at the present, more experiments are required to clarify this point. Alternative

explanation could be that the PNA probe is too short to allow efficient invasion to the dsDNA.

In conclusion, the LAMP assay could amplified HLA-B*5801 and 5701, both HLA alleles that associated with drug reaction at 95.24% sensitivity and 83.78% specificity. The benefit of LAMP could reduce the assay time to only 40 minutes, comparing to around 4 hours by SSP-PCR. As a result, this would help patients to avoid any life-threatening adverse consequences from allopurinol and abacavir with shorter assay time.

CHAPTER V

DISCUSSION

From the previous typing of HLA-B*1502 SSP-PCR, four sets of primers were used in four separated reaction within 30 touchdown PCR cycles. The detection limit of this previous report was 10 ng/13µl PCR reaction (21). The aim of this study was to reduce both number of reaction and cost. Most importantly, our design will overcome the problem of false positive from heterozygous combination in multiplex PCR, as nested PCR was used to narrow down unrelated alleles at each step. In summary, one set of primers were successfully amplified HLA-B*1502 by 45 cycles of the first SSP-PCR and the PCR product was amplified by another 2 primer sets as nested SSP-PCR with additional 10 cycles. Interpretation of HLA-B*1502 requires positive bands from both F2.1 and R2, and F2.2 and R2 reactions. Another advantage of nested PCR is the high sensitivity. Detection limit of this simple nested SSP-PCR showed that DNA concentration as least as 0.005 ng/µl could be amplified. However, the nested PCR was subjected to contamination by the PCR product and the PCR reaction needed to be performed with extra precaution. However, in the validation step using 104 blind samples, we proved that this method has 100% sensitivity and very close to 100% specificity (since rare alleles that didn't found in this study (HLA-B*1588, 9521 and 9544) were predicted to be amplified). It should be noted that one HLA-B*1502 positive sample failed to show positive band from the first reaction of F1 and R1. But when the second PCR was completed, 430bp band of F1 and R1 could be seen by gel electrophoresis. It was most likely that the first PCR failed due to lower amount of DNA or poor quality of DNA (The concentration is 50 ng/µl and OD260/280 ratio = 1.86). This case helps strengthen the advantage of high sensitivity of nested PCR which gave positive result with very small amount of PCR product.

Another allele typing from the previous report, HLA-B*5801 required two sets of primers used in two separated reaction within 30 touchdown PCR cycles (21). The first primer set amplifies HLA-B*5801, 5104, 5301 and 1513, while another set amplifies HLA-B*5801-3. A successful typing for HLA-B*5801 will give two positive bands from

both sets of primer in separated reactions. The major weak point of this technique was the possibility of false positive results from heterozygous combination. For example, patient with HLA-B*1513 and HLA-B*5802 will result in two positive bands and misinterpret as HLA-B*5801. Another limitation was the detection limit. This previous report would successfully amplify DNA of more than 10 ng/13µl PCR reaction. Therefore, similar to HLA-B*1502 typing above, the aim of this study was to reduce both primer sets and also numbers of PCR reaction, and most importantly to eliminate false positive results from heterozygous combination. In summary, only one set of primer was successfully amplified HLA-B*5801 by 45 cycles SSP-PCR with the detection limit of as little as 0.02 ng/20µl PCR reaction. It should be noted that in HLA-B*5801 SSP-PCR, we used HotStart *Taq* DNA polymerase (Invitrogen), and this might be one reason why we could obtain a high sensitivity comparable to nested PCR for HLA-B*1502. Again in the validation step using 104 blind samples, we could also show that this method has 100% sensitivity and very close to 100% specificity.

In summary, these in-house SSP-PCRs for both HLA-B*1502 and 5801 from this study were very specific and high sensitive, as they both gave 100% sensitivity and very close to 100% specificity. They can be readily applied to any routine hospital laboratories which already have the PCR machine. These new techniques will considerably reduce patient expense from expensive commercial SSP-PCR.

It should be noted that for the result interpretation of both SSP-PCR of HLA-B*1502 and HLA–B*5801, the internal control band in positive alleles may be faint or absent. This event was significantly observed when DNA concentration was low. This might be explained by substrates lacking, as all substrates were preferable used for HLA allele amplification.

Although SSP-PCR is the most cost effective method, its DNA extraction step still depends on costly DNA extraction kit. Optional DNA extraction method by salting out is very cheap, but it is time consuming (~6 hours). A number of attempt have been tried to amplify DNA target directly from whole blood to reduce total assay time as much as possible. In the current study, we tested different protocols suggested in the literatures using the internal control gene (IFN-γ). First using the modified initial denaturing step

(23), the PCR product was very difficult to be seen by gel electrophoresis. Author proposed that the modification of initial denaturing step could help lyse the cell more completely and thus releasing more DNA into the reaction (23). Another approach was to use supernatant instead of whole blood to reduce the PCR inhibitor (24). Using serial blood dilution in two forms, crude extract and supernatant (24), PCR efficiency was much better when using supernatant as template. Therefore, the supernatant of blood dilution was used for SSP-PCR system of HLA-B*1502 that have already been set up using purified genomic DNA as template. However, the results were quite disappointed, as only the band of internal control was shown with very low intensity. After FastStart *Taq* had been used, the problem is still persisting. This result suggested that FastStart *Taq* was not more resistant to inhibitor than original *Taq* polymerase. We hypothesized that the failure in applying the whole blood amplification method to HLA typing was mainly due to the HLA gene nature, as IFN-γ was successfully amplified directly from whole blood. Finally, the commercial blood PCR kit (Kapa Biosystems) was used to conduct the SSP-PCR for both HLA-B*1502 and HLA-B*5801 successfully. Therefore, for HLA gene amplification, special *Taq* polymerase enzyme with specific buffer that could better tolerate PCR inhibitor was needed. Comparing between purified DNA and whole blood in non-target sample, purified DNA gave non-specific bands from this blood PCR kit. This might be explained by high amplification efficiency of commercial blood PCR kit. However, more experiment has to be done in the future to proof this assumption. This in-house SSP-PCR directly from whole blood sample could be finished within 4 hours including PCR reaction and gel electrophoresis. However, more whole blood samples are needed to be tested comparing to purified DNA results, before applying this system to the service laboratory.

As Cheng, *et. al.*, reported a novel HLA-B*1502 typing by LAMP within 40 minutes, this new isothermal amplification technique was quite promising for other risk HLA typing comparing to ~4 hours standard SSP-PCR. Moreover, LAMP depends on amplification of target DNA at an instant temperature; thus, PCR thermocycler is not required. This would cut investment cost for small hospital or could be adapt to field experiment with cheaper water bath machine. From their report, two sets of primers in

two separated reactions were used to amplify HLA-B*1502. Positive results from the two reactions would be interpreted as HLA-B*1502. In this study, HLA-B*5801 was used as target for LAMP detection with only one LAMP reaction. This would reduce half of the assay price. From their sequence alignment, all four primers specifically amplified two alleles, HLA-B*5801 and HLA–B*5701 in order to predict adverse reaction from allopurinol and abacavir, respectively. However, HLA-B*1517 was the only false positive alleles from this current system because of just one mismatch from the four primers. From 90 blind DNA samples, 95.24% sensitivity and 83.78% specificity were achieved. It was noted that all 12 false positive results were not occurred in in-house SSP-PCR for HLA-B*5801. All of these alleles among false positive samples did not overlap nor had similar sequence to LAMP primers. This problem might be explained by the high sensitivity of LAMP to other unknown contaminant such as dust, glove powder and contaminated DNA. Moreover, one positive HLA-B*5701 DNA failed to be amplify by LAMP primers. This could be explained by LAMP high stringent condition, as all four primers have to work at the same time to successfully amplify DNA target.

In order to increase the specificity to LAMP, the PNA probes with fluorescent labeling were used with sequence specific detection. Unfortunately, these PNA probes failed to bind to their target. This might be explained by its high stability in short quencher-labeled DNA probe binding due to the high GC component in PNA probe. Alternatively, the quencher phenomenon might result from nucleotides from the target DNA. However, more experiments are required to clarify this point.

Further development to increase specificity might be to develop additional LAMP reaction in exon 3 which can differentiate closely related sequences such as HLA-B*1517 and HLA-B*5701. Although HLA-B*1517 and –B*5701 will not be amplified by primers from exon 3, this extra reaction might increase false positive results from heterozygous combination from two LAMP reactions. Other target amplification systems that do not required PCR machine such as NASBA, SDA and HDA or the signal amplification system such as DNA hybridization in a lateral flow system might be the alternative approach in the development of rapid kit for specific HLA allele and should be further explored. The comparison and the advantages and disadvantages of

standard SSP-PCR, whole blood SSP-PCR and LAMP are concluded in table16 and table17, respectively.

Table16. Comparison of standard SSP-PCR, whole blood SSP-PCR and LAMP from this current study.

Aspect/Technique	Standard SSP-PCR	Blood SSP-PCR	LAMP
DNA extraction	+	-	+
Specimen requirement	0.005 ng/µl DNA	1 µl whole blood	50 ng/µl DNA
PCR machine	+	+	-
Assay time	~10 hours	~4 hours	40 minutes
Price (per reaction)	4.0 Baht	25.5 Baht	185.5 Baht
DNA extraction cost	50.0 Baht by conventional method or 180 Baht by commercial kit	-	50.0 Baht by conventional method or 180 Baht by commercial kit
PCR machine cost (Eppendorf)	~150,000 Baht	~150,000 Baht	-
Water bath (Memmert)	-	-	~23,000 Baht

Table17. Advantages and disadvantages of standard SSP-PCR, whole blood SSP-PCR and LAMP from this current study.

Aspect/Technique	Standard SSP-PCR	Blood SSP-PCR	LAMP
Advantages	Standard method with high sensitivity (100%) and specificity (>99.99%) Easy primer design	Faster and cheaper than standard PCR Less laborious Easy primer design	Very fast (40 min) No expensive thermocycler required Possible for single visit or field diagnosis
Disadvantages	Time consuming plus DNA extraction step (~10 hours) Laborious Expensive thermocycler required Impossible for single visit or field diagnosis	Time consuming (~4 hours) Expensive thermocycler required Impossible for single visit or field diagnosis	Complicated primer design only possible with program Lower specificity (83.78%) and sensitivity (95.24%) comparing to SSP-PCR Sequence restricted for probe design

CHAPTER VI

CONCLUSION

From this study, two simple SSP-PCR for HLA-B*1502 and -B*5801 were successfully set up. Comparing to the previous report, these two assays can reduce both number of reaction tubes and cost, and also reduce false positive results from heterozygous combination. Moreover, this in-house SSP-PCR can be used with high specificity and sensitivity, in place of expensive commercial SSP-PCR. Currently, there are many direct PCR commercial kits from whole blood. This in-house SSP-PCR of HLA-B*1502 and HLA-B*5801 can also be applied with the same specificity by using only 1µl of whole blood. This could reduce turnaround time from conventional salting out DNA extraction technique. However, other optional methods are also interesting because of their exceptional advantages. For example, LAMP is an isothermal amplification technique, which expensive PCR machine is not need. This technique is quite promising for laboratories which are not well equipped. In addition, its detection technique does not need time consuming and does not require as many steps as gel electrophoresis. This current study can successfully amplified HLA-B*5801 by LAMP technique with 95.24% sensitivity and 83.78% specificity. In conclusion, all these new methods could help patients from getting life threatening drug-induced skin reaction with cost effective and reliable techniques.

REFERENCES

[1] Ingelman-Sundberg M. Pharmacogenomic biomarkers for prediction of severe adverse drug reactions. N Engl J Med. 6 (Feb 7 2008): 637-9.

[2] Wolf CR, Smith G, Smith RL. Science, medicine, and the future: Pharmacogenetics. BMJ. 7240 (Apr 8 2000): 987-90.

[3] Mockenhaupt M. Severe drug-induced skin reactions: clinical pattern, diagnostics and therapy. J Dtsch Dermatol Ges. 2 (Feb 2009): 142-60; quiz 61-2.

[4] Borchers AT, Lee JL, Naguwa SM, Cheema GS, Gershwin ME. Stevens-Johnson syndrome and toxic epidermal necrolysis. Autoimmun Rev. 8 (Sep 2008): 598-605.

[5] Paquet P, Jacob E, Damas P, Pirson J, Pierard G. Analytical quantification of the inflammatory cell infiltrate and CD95R expression during treatment of drug-induced toxic epidermal necrolysis. Arch Dermatol Res. 6 (Dec 2005): 266-73.

[6] Chung WH, Hung SI, Yang JY, Su SC, Huang SP, Wei CY, et al. Granulysin is a key mediator for disseminated keratinocyte death in Stevens-Johnson syndrome and toxic epidermal necrolysis. Nat Med. 12 (Dec 2008): 1343-50.

[7] Chung WH, Hung SI, Hong HS, Hsih MS, Yang LC, Ho HC, et al. Medical genetics: a marker for Stevens-Johnson syndrome. Nature. 6982 (Apr 1 2004): 486.

[8] Chung WH, Hung SI, Chen YT. Human leukocyte antigens and drug hypersensitivity. Curr Opin Allergy Clin Immunol. 4 (Aug 2007): 317-23.

[9] Man CB, Kwan P, Baum L, Yu E, Lau KM, Cheng AS, et al. Association between HLA-B*1502 allele and antiepileptic drug-induced cutaneous reactions in Han Chinese. Epilepsia. 5 (May 2007): 1015-8.

[10] Locharernkul C, Loplumlert J, Limotai C, Korkij W, Desudchit T, Tongkobpetch S, et al. Carbamazepine and phenytoin induced Stevens-Johnson syndrome is associated with HLA-B*1502 allele in Thai population. Epilepsia. 12 (Dec 2008): 2087-91.

[11] Alfirevic A, Jorgensen AL, Williamson PR, Chadwick DW, Park BK, Pirmohamed

M. HLA-B locus in Caucasian patients with carbamazepine hypersensitivity. Pharmacogenomics. 6 (Sep 2006): 813-8.

[12] Hung SI, Chung WH, Liou LB, Chu CC, Lin M, Huang HP, et al. HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. Proc Natl Acad Sci U S A. 11 (Mar 15 2005): 4134-9.

[13] Tassaneeyakul W, Jantararoungtong T, Chen P, Lin PY, Tiamkao S, Khunarkornsiri U, et al. Strong association between HLA-B*5801 and allopurinol-induced Stevens-Johnson syndrome and toxic epidermal necrolysis in a Thai population. Pharmacogenet Genomics. 9 (Sep 2009): 704-9.

[14] Yang CW, Hung SI, Juo CG, Lin YP, Fang WH, Lu IH, et al. HLA-B*1502-bound peptides: implications for the pathogenesis of carbamazepine-induced Stevens Johnson syndrome. J Allergy Clin Immunol. 4 (Oct 2007): 870-7.

[15] Pichler WJ, Beeler A, Keller M, Lerch M, Posadas S, Schmid D, et al. Pharmacological interaction of drugs with immune receptors: the p-i concept. Allergol Int. 1 (Mar 2006): 17-25.

[16] Hanvivatvong O. Basic and Clinical Immunology. Bangkok: Faculty of Medicine, Chulalongkorn University, 2008.

[17] Sayer DC, Cassell HS, Christiansen FT. HLA-B*27 typing by sequence specific amplification without DNA extraction. Mol Pathol. 5 (Oct 1999): 300-1.

[18] Martin AM, Nolan D, Mallal S. HLA-B*5701 typing by sequence-specific amplification: validation and comparison with sequence-based typing. Tissue Antigens. 6 (Jun 2005): 571-4.

[19] Itoh Y, Mizuki N, Shimada T, Azuma F, Itakura M, Kashiwase K, et al. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. Immunogenetics. 10 (Nov 2005): 717-29.

[20] Welsh K, Bunce M. Molecular typing for the MHC with PCR-SSP. Rev Immunogenet. 21999): 157-76.

[21] Bunce M, O'Neill CM, Barnardo MC, Krausa P, Browning MJ, Morris PJ, et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4,

DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). Tissue Antigens. 5 (Nov 1995): 355-67.

[22] Zhang Z, Kermekchiev MB, Barnes WM. Direct DNA amplification from crude clinical samples using a PCR enhancer cocktail and novel mutants of taq. J Mol Diagn. 2 (Mar 2010): 152-61.

[23] Mercier B, Gaucher C, Feugeas O, Mazurier C. Direct PCR from whole blood, without DNA extraction. Nucleic acids research. 19 (Oct 11 1990): 5908.

[24] McCusker J, Dawson MT, Noone D, Gannon F, Smith T. Improved method for direct PCR amplification from whole blood. Nucleic acids research. 24 (Dec 25 1992): 6747.

[25] Bu Y, Huang H, Zhou G. Direct polymerase chain reaction (PCR) from human whole blood and filter-paper-dried blood by using a PCR buffer with a higher pH. Anal Biochem. 2 (Apr 15 2008): 370-2.

[26] Yang YG, Kim JY, Song YH, Kim DS. A novel buffer system, AnyDirect, can improve polymerase chain reaction from whole blood without DNA isolation. Clin Chim Acta. 1-2 (May 1 2007): 112-7.

[27] Nishimura N, Nakayama T, Tonoike H, Kojima K, Kato S. Direct polymerase chain reaction from whole blood without DNA isolation. Ann Clin Biochem. (Sep 2000): 674-80.

[28] Kermekchiev MB, Kirilova LI, Vail EE, Barnes WM. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. Nucleic acids research. 5 (Apr 2009): e40.

[29] Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, et al. Loop-mediated isothermal amplification of DNA. Nucleic acids research. 12 (Jun 15 2000): E63.

[30] Gill P, Ghaemi A. Nucleic acid isothermal amplification technologies: a review. Nucleosides Nucleotides Nucleic Acids. 3 (Mar 2008): 224-43.

[31] Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun. 1 (Nov 23 2001): 150-4.

[32] Cheng SH, Kwan P, Ng HK, Ng MH. New testing approach in HLA genotyping helps overcome barriers in effective clinical practice. Clin Chem. 8 (Aug 2009): 1568-72.

[33] Nielsen PE, Egholm M, Berg RH, Buchardt O. Science. 1991): 1497-500.

[34] Nielsen PE, Egholm M. An introduction to peptide nucleic acid. Curr Issues Mol Biol. 1-21999): 89-104.

[35] Pellestor F, Paulasova P, Hamamah S. Peptide nucleic acids (PNAs) as diagnostic devices for genetic and cytogenetic analysis. Curr Pharm Des. 242008): 2439-44.

[36] Vilaivan T, Srisuwannaket C. Hybridization of pyrrolidinyl peptide nucleic acids and DNA: selectivity, base-pairing specificity, and direction of binding. Org Lett. 9 (Apr 27 2006): 1897-900.

[37] Boontha B, Nakkuntod J, Hirankarn N, Chaumpluk P, Vilaivan T. Multiplex mass spectrometric genotyping of single nucleotide polymorphisms employing pyrrolidinyl peptide nucleic acid in combination with ion-exchange capture. Analytical chemistry. 21 (Nov 1 2008): 8178-86.

[38] Marras SA. Interactive fluorophore and quencher pairs for labeling fluorescent nucleic acid hybridization probes. Mol Biotechnol. 3 (Mar 2008): 247-55.

[39] Grossmann TN, Sasaki S, Ritzefeld M, Choi SW, Maruyama A, Seitz O. Inducing the replacement of PNA in DNA.PNA duplexes by DNA. Bioorg Med Chem. 1 (Jan 1 2008): 34-9.

[40] Sawada S, Imada I, Kato N, Kaihatsu K. Regulation of duplex DNA strand displacement by visible light sensitive bis-peptide nucleic acid. Nucleic Acids Symp Ser (Oxf). 532009): 191-2.

[41] Seidel CAM, Schulz A, Sauer MHM. Nucleobase-specific quenching of fluorescent dyes .1. Nucleobase one-electron redox potentials and their correlation with static and dynamic quenching efficiencies. Journal of Physical Chemistry. 13 (Mar 28 1996): 5541-53.

[42] Altman DG, Bland JM. Diagnostic tests. 1: Sensitivity and specificity. BMJ. 6943 (Jun 11 1994): 1552.

BIOGRAPHY

Miss Sita Virakul was born in September, 22nd 1985 in Bangkok. She received her Bachelor Degree from the Department of Microbiology, Faculty of Science, Chulalongkorn University (second class honor) in the academic year of 2007. Moreover, she received a certification for excellent student from the Professor Dr. Tab Nilanidhi Foundation in 2008. For her research work, she received the 90th Year Anniversary of Chulalongkorn University (Ratchadaphiseksomphot Endowment Fund) in 2010 to perform her thesis. In August, 22th to 27th 2010, she has been awarded a Travel Bursary to attend the 14th International Congress of Immunology (ICI 2010) in Kobe, Japan. The topic of her presentation will be 'A simple nested sequence specific primer-polymerase chain reaction for HLA-B*1502'.