# Detection of *HLA-B*5801* by In-House PCR-SSP

# วิธีการตรวจหาเอชแอลเอบี 5801 ด้วยวิธีพีซีอาร์เอสเอสพี

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## ABSTRACT

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 in Asian ethnicity. Another association, *HLA-B*5801* involves with allopurinol hypersensitivity with 26% mortality rate. Since allele frequency of *HLA-B*5801* is quite common in some Asian ethnicity, it is important to develop a rapid and cost effective test for Asian, including our Thai population. To get HLA typing result, commercially available PCR-SSP is too expensive. As a result, in-house PCR-SSP would be the most interesting candidates comparing to other molecular techniques. The results showed that only one set of primer can differentiate *HLA-B*5801* from other alleles specifically in 200 DNA samples with 100% sensitivity and >99.9% specificity. The benefit of these tests would help patients to avoid any life-threatening adverse consequences from allopurinol.

## บทคัดย่อ

ในปัจจุบันสามารถทำนายการเกิดการแพ้ยาอย่างรุนแรงได้โดยการหาอัลลีลของเอชแอลเอยีน โดยเอชแอลเอแรกที่มีการรายงานคือ เอชแอลเอบี 1502 ซึ่งแสดงความสัมพันธ์ในการเกิดการแพ้ยารุนแรงกับยากันชักชื่อคาร์บามาซีพีน อีกยีนหนึ่งคือเอชแอลเอบี 5801 กับยาโรคเกาต์ชื่ออัลโลพูรินอล ยีนเหล่านี้มีความสำคัญมากเนื่องจากเป็นยีนที่พบมากในกลุ่มประชากรเอเชีย ร่วมกับอัตราการตายอยู่ประมาณ 26 เปอร์เซ็นต์ ดังนั้นจึงมีความสำคัญในการหาวิธีหายีนที่ได้ผลถูกต้องราคาเหมาะสม และรวดเร็ว วิธีหนึ่งก็คือ พีซีอาร์เอสเอสพี ในปัจจุบันมีชุดตรวจสำเร็จแต่ราคายังคงแพงเกินไป จึงควรมีการพัฒนาพีซีอาร์เอสเอสพีอย่างง่าย และราคาถูกเพื่อใช้เอง จากผลการทดสอบพบว่าสามารถทำนายเอชแอลเอบี 5801 ในตัวอย่างดีเอนเอจำนวน 200 รายได้ความไว 100 เปอร์เซ็นต์ และแม่นยำ >99.9% ประโยชน์ของผลการทดลองครั้งนี้ทำให้สามรถช่วยคนไข้ไม่ให้เกิดอาการแพ้ที่รุนแรงถึงชีวิตได้ตั้งแต่ก่อนการให้ยา

**Key Words:** *HLA-B*5801*, PCR-SSP

**คำสำคัญ:** เอชแอลเอบี 5801 พีซีอาร์เอสเอสพี

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## 1. Introduction

Drug response in each patient is different. Up to date, there are many evidence on genetic factors which play an important role in these responses, such as cytochrome P450, thiopurine methyltransferase and human leukocyte antigen (HLA) (Ingelman-Sundberg, 2008). Study in inherited predisposing factors which determine drug response is widely known as pharmacogenetics (Wolf et al., 2000). 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 reduce cause of death dramatically. The strongest and the most specific marker for risk assessment so far is HLA gene (Ingelman-Sundberg, 2008).

The poorest prognosis among severe drug-induced skin reactions are Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) (Mockenhaupt, 2009). They are a life-threatening syndrome with high mortality rate. The appearances on mouth, lips, conjunctiva and genital area vary from erythema to blistering second degree burn-like.

In 2004, Chung, *et al.*, first reported HLA gene as a genetic marker for SJS by carbamazepine (Chung et al., 2004). 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 (Chung et al., 2007). Among Asian ethnicity, *HLA-B*5801* involved with severe side effect from antihyperuricemic agents, allopurinol. Its mortality rate is around 26% (Hung et al., 2005). Since allele frequency of this HLA antigens are quite common in some Asian ethnicities, as listed in table 1 (http://www.allelefrequencies.net/default.asp), it is important to develop a rapid test for Asian including our population.

| Ethnicity | *HLA-B*5801* (%) |
|---|---|
| China North Han | 2.9 |
| China South Han | 8.9 |
| Taiwan | 9.8 |
| Japan | 0.5 |
| Korea | 6.5 |
| Singapore | 5.8 |
| Thailand | 7.7 |
| Vietnam | 6.5 |

**Table1.** *HLA–B*5801* frequencies in Asian ethnicity.

For HLA typing, standard serological tests give inconclusive results which were caused by cross reactivity of monoclonal antibodies. As a result, HLA subtypes cannot be identified accurately. Years after, molecular techniques become commercially available and are applied to many routine laboratories. It's still

expensive and also gives lots of unnecessary information, as all alleles are interpreted (Martin et al., 2005). Testing HLA gene from genomic DNA, most laboratories may apply either of four different techniques. First, polymerase chain reaction-sequence specific oligonucleotide probe (PCR-SSOP). This technique use sets of probes to detect HLA types of multiple samples at the same time. This is suitable for high throughput HLA typing, preferable used in donor center. Second, reverse SSOP is quite expensive due to the development of high efficiency probes that were designed to bind target allele at the same temperature. The third technique is PCR-SSOP-luminex® system which applies flow cytometry to detect different alleles with up to 100 DNA probe attached colorimetric beads (Itoh et al., 2005). Its limitations are cost and requirement of special instrument. Finally, sequence-specific primer polymerase chain reaction (PCR-SSP) technique works by amplification HLA gene with many sets of primers to specifically differentiate closely related alleles. This technique is available in many forms of commercial sets of primers, which cost is still quite expensive. In order to get the most specific, rapid and cost effective test for *HLA-B*5801*, it's challenging to develop an in-house PCR-SSP to predict drug allergy for each patients before prescription.

## 2. Objective

To develop an in-house PCR-SSP for *HLA-B*5801* and validate with PCR-SSP commercial kit and direct sequencing.

## 3. Materials and methods

### Sample

In a setup step, known *HLA-B*5801*, *B*1517* and *B*5701*, which have the closest sequences to *HLA-B*5801* and are present in Thai population, along with other alleles (*HLA-B*0705, 1301, 1501, 1511/12/13, 1525, 1801, 3501, 3801, 3901, 4001/02, 4402, 4601, 4801, 5101/02, 5201* and *5701*) that have high frequency in Thai population (http://www.allelefrequencies.net/default.asp), were used. In a validation step, two hundred genomic DNA samples of blood or bone marrow donors at the Thai Red Cross were analyzed by gold standard methods using PCR-SSP 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*5801*, with 9.5% of *HLA-B*5801*.

### 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, HLA-B sequence alignment was

taken from the database with *HLA-B*070201* as consensus sequence (Figure 1). It's noted that polymorphism among *HLA-B*58* group is scattered around the whole sequence, so rare *HLA-B*5705* and other rare subtypes of *HLA-B*58* cannot be differentiated. Reaction mix at 20 µl contains final concentration of 1x Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 µM primer (F1: 5'-ACGGAACATGAAGGCCTCC-3' and R1: 5'-CAGCCATACATCCTCTGGATGA-3'), 0.25 µM internal control primer (IF: 5'-CCTCACATGATATGACTTTGACAT-3' and IR: 5'-AACATCAGAAGCATTGACCTTG-3') and 0.25 U Platinum® Taq DNA polymerase (Invitrogen). Touchdown PCR cycles are used to increase specificity, set up by gradient of temperature and cycles that results in both positive band for *HLA-B*5801* and housekeeping gene. They are composed of 95°C 2 min 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 min for 1 cycle and holding at 4°C. Total genomic DNA is 100 ng. Electrophoresis uses 2% agarose gel to determine PCR product size by running with 100bp DNA Ladder at 100 V for 40 min.

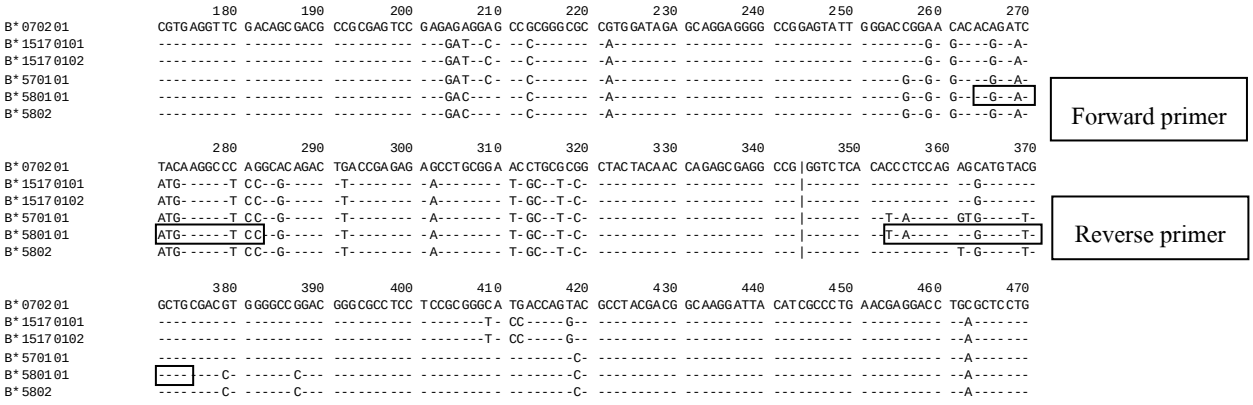


**Figure 1** Related *HLA-B* alleles sequence alignment with primer regions. *HLA-B*5801* can be differentiated specifically within those closely related alleles, *HLA-B*1517*, *5701* and *5802*.

**Assay Analysis**

To compare the efficiency between the new developed method with traditional one, sensitivity and specificity of the assay is determined (Altman and Bland, 1994).

Sensitivity = number of true positive / number of true positive + number of false negative

Specificity = number of true negative

number of true negative + number of false positive

## 4. Results and discussion

Previous report on *HLA-B*5801* detection composed of two sets of primer (Bunce et al., 1995). 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 method can 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, we used only one set of primer to reduce assay number to one reaction and to also exclude false positive from heterozygote. Of 200 samples, we can detect 19 *B*5801* samples correctly. Moreover, this current test could reduce the HLA typing cost from 3,000 baht/test by commercial PCR-SSP to 1,000 baht/test.

In addition, within these 200 samples, 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, 5201 and 5701* gave negative result with our primer. A representative gel picture is shown in Fig 2.

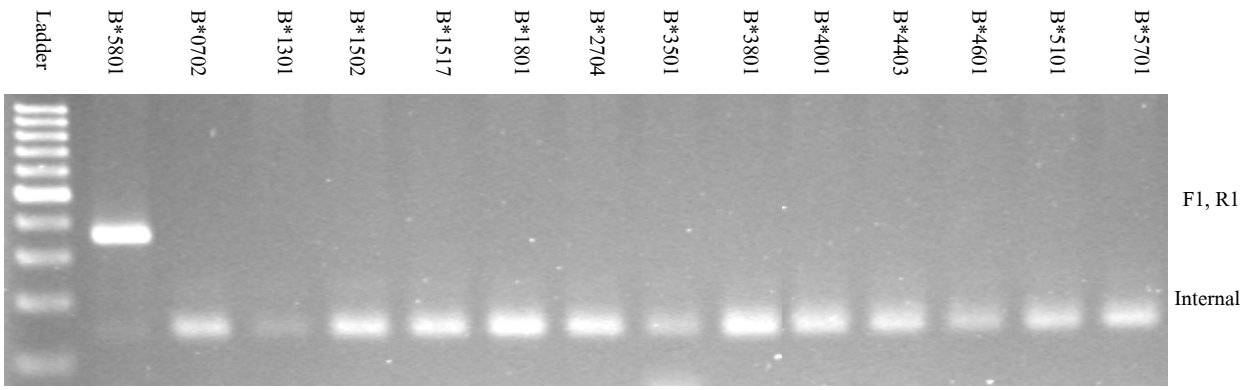


**Figure 2** Migration patterns from both *HLA-B*5801* (F1, R1) and housekeeping primer.

As mentioned earlier, polymorphisms among other related alleles, both in intron and exon, are scattered around the sequences, so *HLA-B*5705* and other subtype of *HLA-B*58* cannot be differentiated. Predicted amplified alleles are *HLA-B*5705, 5801, 5804-5, 5809, 5810N, 5811-13, 5815, 5817N, 5819* and *5821-24*. However, from the allele frequencies database

(http://www.allelefrequencies.net/default.asp) of world populations, those cross reactive alleles are very

rare, close to 0%. From 200 blind samples, this assay gives 100% sensitivity and >99.9% specificity, as extremely rare alleles mentioned above can still be amplified. According to our samples, we didn't found those extremely rare alleles, positive predictive value and negative predictive value are 100%. Therefore, this SSP assay with one set of primer pairs can be used with high specificity in our Thai population.

## 5. Conclusion

This PCR-SSP system can be used to define *HLA-B*5801*. The benefit of these tests would help patients to avoid any life-threatening adverse consequences from allopurinol with less cost.

## 6. Acknowledgement

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