









A Novel Allele-Specific PCR Protocol for the Detection of the *HLA-C*03:02* Allele, a Pharmacogenetic Marker, in Vietnamese Kinh People

This article was published in the following Dove Press journal:
The Application of Clinical Genetics

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Background: Allopurinol, a common anti-hyperuricemia drug, is well known as an inducer of severe cutaneous adverse drug reactions (SCARs). One of the most well-defined risk factors of allopurinol-induced SCARs is the presence of polymorphic alleles of human leukocyte antigen (*HLA*) genes, such as *HLA-B*58:01* and *HLA-C*03:02* alleles. There is no commercial test or published in-house protocol for the specific detection of the *HLA-C*03:02* allele. In this article, we established for the first time a simple allele-specific (AS) PCR method to identify *HLA-C*03:02* allele carriers, and at the same time, determine their zygosity.

Methods: A two-step AS-PCR protocol, using four primer sets, was designed to specifically amplify and differentiate the *HLA-C*03:02* allele from 17 other *HLA-C* alleles found in Vietnamese people. The protocol was validated with PCR-sequencing-based typing (SBT) of 100 samples of unknown genotypes.

Results: The PCR protocol can detect the *HLA-C*03:02* allele and determine the zygosity. The results of this protocol were highly consistent with those of the SBT ($\kappa = 0.98$, $p < 0.001$). Regarding the specific detection of the *HLA-C*03:02* allele, the PCR protocol had a sensitivity of 100% (95% CI: 91.61–100%) and specificity of 98.3% (95% CI: 90.9–99.7%). The protocol was used to determine the distribution of the *HLA-C*03:02* allele in 810 unrelated Vietnamese Kinh people, 14.2% of which were *HLA-C*03:02* carriers, the allele frequency was 7.5%.

Conclusion: A novel AS-PCR protocol with a sensitivity of 100% for the detection of the *HLA-C*03:02* allele was established. The protocol can be used for personalized treatment with allopurinol in order to minimize the risk of SCARs in Vietnamese people as well as in other Asian populations with similar genetic characteristics.

Keywords: severe cutaneous adverse reactions, *HLA-C*03:02*, allopurinol, *HLA* genotyping, AS-PCR

Introduction

Allopurinol is a common hyperuricemia drug and one of the top inducers of severe cutaneous drug reactions (SCARs), especially in Asian patients.^{1,2} One of the most well-defined risk factors for allopurinol-induced SCARs is the presence of polymorphic human leukocyte antigen (*HLA*) alleles such as the *HLA-B*58:01* allele,³⁻⁶ and to a lesser extent, the *HLA-C*03:02* allele.^{7,8} The *HLA-C*03:02*

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allele was found at 94% and 92%, respectively, of Han Chinese and Korean patients with allopurinol-induced SCARs. This allele was significantly associated with allopurinol-induced SCARs (OR = 97.7, $p=1.4 \times 10^{-9}$ in Han Chinese patients,⁷ OR = 82.1, $p = 9.39 \times 10^{-11}$ in Korean patients).⁸ These studies implicated that the *HLA-C*03:02* allele might be another pharmacogenomic marker together with the *HLA-B*58:01* allele in allopurinol personalized treatment. Notably, the frequencies of the *HLA-C*03:02* allele in several Asian populations including Vietnamese people are as high as that of the *HLA-B*58:01* allele.⁹

A number of *HLA-B*58:01* allele-specific detection methods have been commercialized to identify the patients at risk, change the prescription and therefore, minimize the SCARs risk. However, there is no protocol for specific detection of the *HLA-C*03:02* allele. *HLA-C*03:02* genotyping methods for research purposes include saturated tiling capture sequencing,¹⁰ next-generation sequencing,¹¹ whole-genome sequencing,¹² multiplex sequencing-based typing (SBT),^{13,14} sequence-specific oligonucleotides (SSO),⁹ and multiplex real-time PCR¹⁵ which uses series of primer sets for analysis of multiple *HLA* loci. All of those methods are very costly and would be difficult to be applied in clinical settings for allopurinol personalized medicine. There is a need for a simple and specific *HLA-C*03:02* detection method for allopurinol personalized therapy, in order to avoid SCAR risk.

In this study, we established, for the first time, a simple allele-specific (AS) PCR method to detect *HLA-C*03:02* allele carriers in Vietnamese Kinh people and identify their zygosity. This protocol was applied to determine the frequency of the *HLA-C*03:02* allele in 810 unrelated Vietnamese Kinh people.

Materials and Methods

Human Genomic DNA Samples

For protocol optimization, 10 DNA samples of known *HLA-C* genotype were provided by the Division of Pharmacogenomics and Personalized Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand. The *HLA* genotypes of those samples were determined using the SSO method.¹⁶

For protocol validation, 100 DNA samples were prepared from the whole blood of unrelated Vietnamese Kinh people, including allopurinol-induced SCAR patients (48) and healthy volunteers (52).

For the *HLA-C*03:02* allele frequency identification, 810 DNA samples were prepared from the whole blood of unrelated Vietnamese Kinh people evenly distributed in the North, the Centre and the South of Vietnam.

The study complied with the Declaration of Helsinki and was approved by the Ethics Committee of the Vietnam National Institute of Hygiene and Epidemiology (IRB-VN01057-6/2018). All of the participants provided their informed written consents.

DNA Isolation

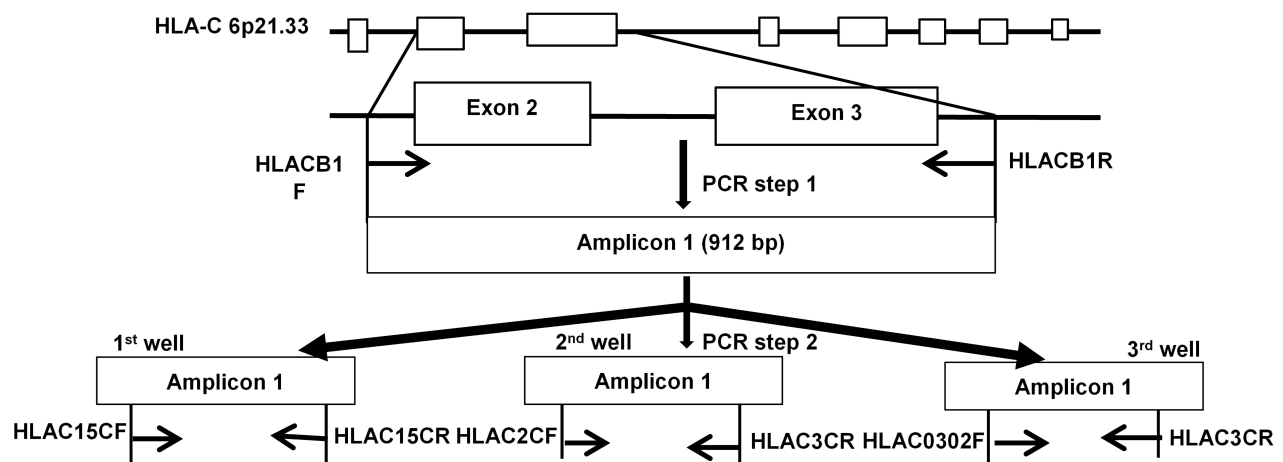
Whole blood was collected into EDTA anticoagulant tubes and stored at -20°C until DNA extraction. Genomic DNA was isolated using the E.Z.N.A.[®] Tissue DNA Kit (Omega Bio-tek, Atlanta, USA). The isolated DNA quantity and quality were assessed using Nanodrop 2000 (Thermo Fisher, Waltham, USA). The samples at the concentration of 35–250 ng/ μL and the A260/280 of 1.65–1.95 were qualified for further experiments.

Detection of the *HLA-C*03:02* Allele by the AS-PCR Method

The PCR protocol consisted of two steps (Figure 1). The PCR primers were designed based on the alignment of 18 *HLA-C* alleles in the Vietnamese population reported by Hoa et al.⁹ The sequences of the 18 alleles were obtained from the IPD-IMGT/HLA database.¹⁷

In step 1, the primer set (HLACB1F/HLACB1R) was used to amplify specifically the exon 2–3 of the *HLA-C* locus. The first PCR was performed in a reaction mixture of 20 μL containing 40 ng of genomic DNA, 0.5 pM of each primer (Integrated DNA Technologies, Coralville, USA) and 10 μL of GoTaq[®] Green Master Mix 2x (Promega Corporation, Madison, USA). The PCR conditions for the first step were 95°C for 3 minutes, followed by 28 cycles of 95°C for 30 seconds, and 65°C for 30 seconds, 72°C for 60 seconds; and finally 72°C for 7 minutes. The first step PCR products were visualized by ethidium bromide under UV with 1% agarose gel electrophoresis. 1 μL of the first PCR product was diluted 100-fold with distilled sterilized water and used as a template for step 2 PCR.

After the amplification of the exon 2–3 of the *HLA-C* locus, the *HLA-C*03* alleles were amplified specifically in step 2. In step 2, the protocol can be flexibly used for two different purposes – determination of zygosity and screening the allele *HLA-C*03:02*. For the differentiation of homozygous and heterozygous genotypes of the *HLA-C*03:02*



Results of electrophoresis after PCR step 2			Conclusion
HLAC15CF and HLAC15CR	HLAC2CF and HLAC3CR	HLAC0302F and HLAC3CR	
+	+	-	<i>HLA-C*03:02 negative</i>
-	+	-	<i>HLA-C*03:02 negative</i>
+	-	-	<i>HLA-C*03:02 negative</i>
+	-	+	<i>HLA-C*03:02 heterozygote</i>
-	+	+	<i>HLA-C*03:02 heterozygote</i>
-	-	+	<i>HLA-C*03:02 homozygote</i>

Figure 1 Strategy for detecting and distinguishing homozygous/heterozygous genotypes of the *HLA-C*03:02* allele. **(A)** PCR procedures: Step 1. The primer set HLACB1F/HLACB1R specifically amplified the exon 2–3 sequence of the *HLA-C* gene. Step 2. The 912 bp PCR product from step 1 was then used as a template for the step 2 PCR reactions, which used three primer sets. **(B)** Different patterns can be obtained with the three primer sets in the second PCR step according to the *HLA-C*03:02* zygosity. (*): allele number.

alleles, three PCR reactions were performed with three primer sets. Each PCR reaction mixture of 20 μ L contained 1 μ L of the step 1 PCR diluted product, 0.5 pM of each primer (Integrated DNA Technologies, Coralville, USA) and 10 μ L of GoTaq[®] Green Master Mix 2x (Promega Corporation, Madison, USA). For the purpose of *HLA-C*03:02* screening only, one PCR reaction with the primer set (HLAC0302F/HLAC3CR) was needed. Touchdown PCR cycles were used in the second PCR step to increase specificity: 95°C for 3 minutes, followed by 5 cycles of 95°C for 30 seconds, and 70°C for 30 seconds, 72°C for 30 seconds; 5 cycles of 95°C for 30 seconds, 67°C for 30 seconds, 72°C for 30 seconds, 10 cycles of 95°C for 30 seconds, 65°C for 30 seconds, 72°C for 30 seconds, 20 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and finally 72°C for 7 minutes. The PCR products were visualized by ethidium bromide under UV with 1% agarose gel electrophoresis.

PCR-SBT

DNA sequences were determined by the PCR-SBT method using the BigDye[™] Terminator v3.1 Cycle

Sequencing Kit (ThermoFisher Scientific, Waltham, USA) and an ABI[™] 3500 analyzer (Applied Biosystems, Massachusetts, USA). The primer sets and sequencing procedures have been previously described.¹⁴

Data Analysis

The sensitivity and specificity of the AS-PCR protocol were determined using MedCalc v19.2.3 (MedCalc Software, Ostend, Belgium). Cohen's Kappa coefficient for the comparison between the in-house protocol and the PCR-SBT method as well as the allele frequency was determined using SPSS 20 (Chicago, IL, USA). Raw data from direct sequencing were analyzed using Bioedit 7.0.5.3 (Informer Technologies, Inc).

Results

The strategy for the *HLA-C*03:02* allele detection is described in Figure 1. The first PCR step with the primer set HLACB1F/HLACB1R was performed to selectively amplify the exon 2–3 which is the most polymorphic region containing most of the SNPs of the *HLA-C* locus.

Table 1 Sequences of Primer Sets Used for the Two PCR Steps

Primer	Sequence (5' – 3')	Amplicon	Tm (°C)
Step 1 PCR			
HLACB1F	GCGAGGTGCCCGCCCGGCTA	912	72
HLACB1R	GAGATGGGGAAGGCTCCCCACT		72
Step 2 PCR			
HLAC15CF	GGAGACACAGAAGTACAAGC	569	60
HLAC15CR	GAGCCACTCCACGCACGT		60
HLAC2CF	GGCCAGGGTCTCACACCA	241	60
HLAC3CR	GAGCCACTCCACGCACAG		60
HLAC0302F	GGCCAGGGTCTCACATTC	241	58
HLAC3CR	GAGCCACTCCACGCACAG		60

Three primer sets were used in the second PCR to differentiate the *HLA-C*03:02* allele from the other known *HLA-C* alleles in the Vietnamese population, especially the two highly homologous alleles *HLA-C*03:03* and *HLA-C*03:04*⁹. Results of the three parallel PCR reactions enabled the conclusion of either homozygous or heterozygous genotypes of the *HLA-C*03:02* allele. Alternatively, only one PCR reaction with the primer set HLAC0302F/HLAC3CR is needed for the detection of *HLA-C*03:02* carriers. The sequences of the primer sets designed for these purposes are shown in Table 1. Their binding sites are described in Figures 2 and 3.

First, the AS-PCR protocol was tested on 10 samples of known genotypes. After the first PCR, a single band of 912 bp was obtained in all of the 10 samples (Figure 4A). After the second PCR with the primer set HLAC15CF/HLC15CR,

a single band of 569 bp was obtained with 4 samples (numbered 4, 8, 9, 10) (Figure 4B). The PCR with the primer set HLAC2CF/HLAC3CR resulted in a single band of 241 bp with samples numbered 2, 3, 5, 6, 7, 8, 9 (Figure 4C). The specific amplification of the *HLA-C*03:02* allele with the primer set HLAC0302F/HLAC3CR resulted in a single band of 241 bp with samples numbered 1, 2, 3, and 4 (Figure 4D). The comparison in Table 2 shows a hundred-percent agreement.

This protocol was used to genotype 100 samples of unknown *HLA-C* genotype, of which, we detected seven samples of homozygous *HLA-C*03:02* carriers, 36 heterozygous *HLA-C*03:02* carriers, and 57 *HLA-C*03:02*-negative samples. For validation, we used PCR-SBT with all the 100 samples. The results of the protocol highly agreed with the SBT results ($\kappa=0.98$, $p < 0.001$). For

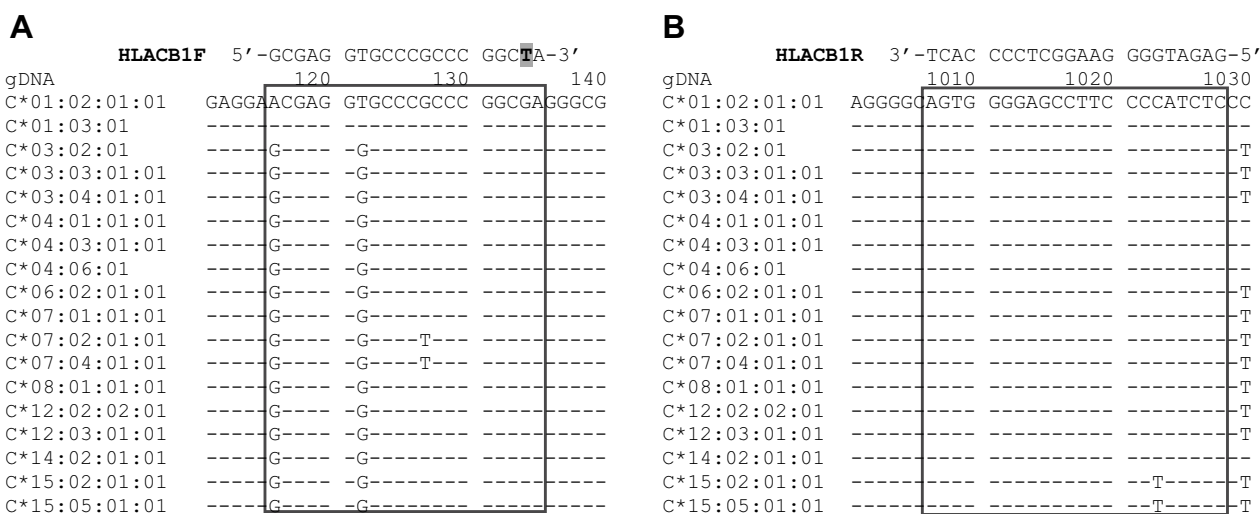


Figure 2 Binding sites of the primer set used in the step I PCR. (A) Forward primer HLACB1F: a mismatch (replacement of G with T) at the penultimate position of the 3' terminus is shown in grey. (B) Reverse primer HLACB1R. The reference sequences were obtained from <https://www.ebi.ac.uk/ipd/imgt/hla/>.¹⁷

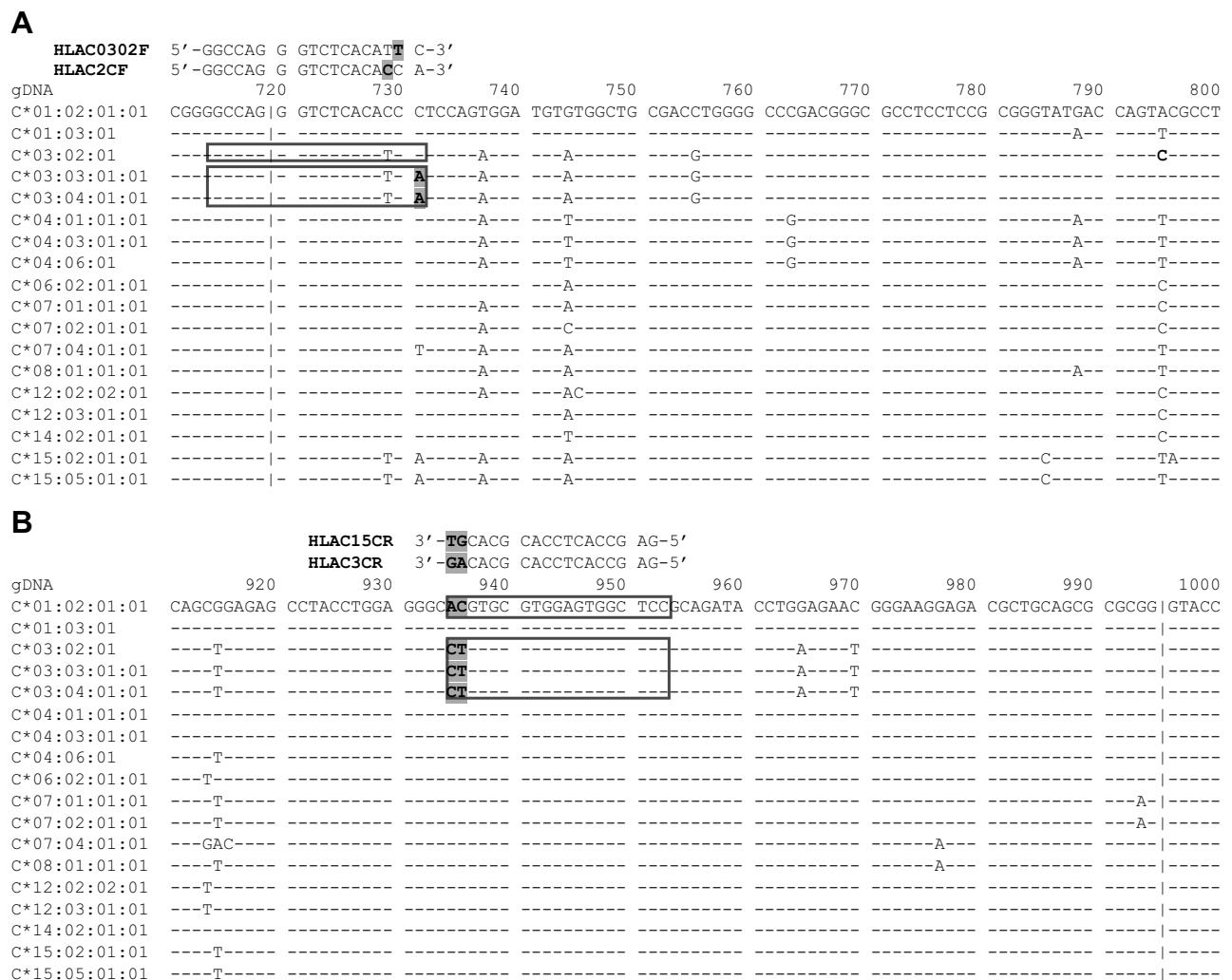


Figure 3 Binding sites of the primer sets used in the step 2 PCR. **(A)** HLAC0302F has one mismatch (replacement of C with T) at the penultimate position of the 3' terminus; HLAC2CF has one mismatch (replacement of T with C) at the third position from the 3' terminus. The mismatches are highlighted in grey; **(B)** HLAC3CR and HLAC15CR have two different nucleotides (highlighted in grey) at the 3' terminus that ensure the specificity of the primers. The reference sequences were obtained from <https://www.ebi.ac.uk/ipd/imgt/hla/>.¹⁷

specific detection of the *HLA-C*03:02* allele, the PCR protocol had a sensitivity of 100% (95% CI: 91.6–100%) and specificity of 98.3% (95% CI: 90.9–99.7%) (Table 3).

This protocol was applied to determine the frequency of the *HLA-C*03:02* allele in 810 unrelated Vietnamese Kinh people, 14.2% of which were *HLA-C*03:02* carriers, the allele frequency was 7.5% (Table 4).

Discussion

The *HLA-C* gene is a polymorphic region of the human genome. According to the IPD-IMGT/HLA database, 6223 *HLA-C* alleles and 1540 distinct variant positions had been discovered.¹⁸ These SNPs are located mostly in the exon 2–3 region, and approximately one SNP is present every 20–30 nucleotides.¹⁹ To date, few PCR-based methods for the

specific detection of *HLA-A* or *HLA-B* alleles at the two-field classification have been published^{20,21} and there have been no reports on the detection protocol of *HLA-C* alleles in general or the specific detection of the *HLA-C*03:02* allele.

Due to the polymorphic characteristic of the *HLA-C* gene, it is difficult to design specific primers for direct amplification of each allele of this locus, it is necessary to cluster the alleles before a specific detection of each target allele. The primer set in the first step PCR was designed for specific amplification of the exon 2–3 region of the *HLA-C* gene. A mismatch (replacement of G with T) was placed at the second nucleotide from the 3' terminus of the forward primer (HLACB1F) (Figure 2A) in order to avoid non-specific amplification of other class I *HLA* loci such as *HLA-A*, *B*, *E*, *F*, *G*.

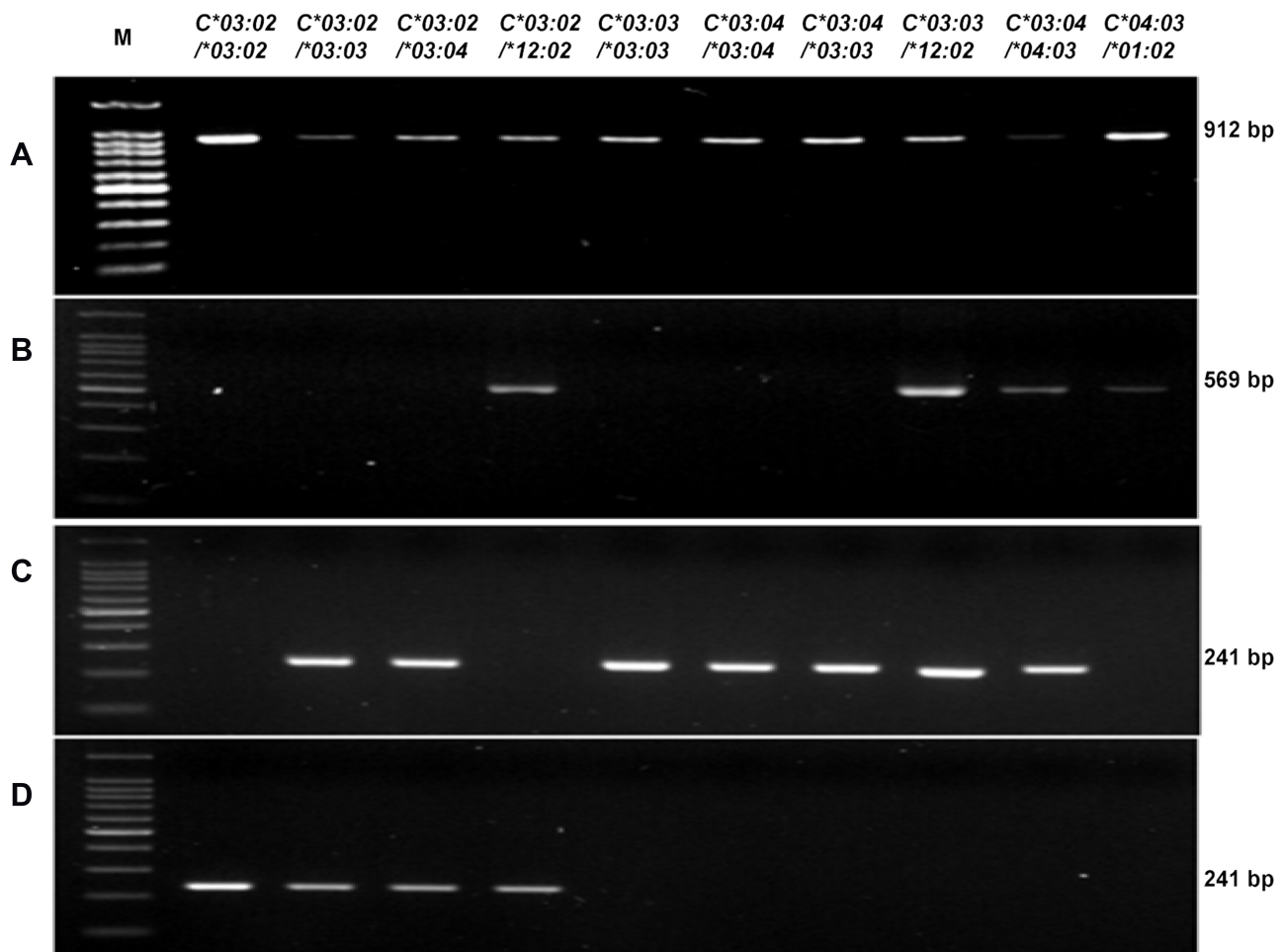


Figure 4 The detection of the *HLA-C*03:02* allele in 10 samples of known genotype. **(A)** Step 1: *HLA-C* exon 2–3 amplicon, 912 bp; **(B)** Step 2: amplicon from the primer set HLAC15CF and HLAC15CR, 569 bp; **(C)** Step 2 amplicon from the primer set HLAC2CF and HLAC3CR, 241 bp; **(D)** Step 2: amplicon from the primer set HLAC0302F and HLAC3CR, 241 bp. (*): allele number.

This PCR protocol was customized for the Vietnamese population, with the 18 known *HLA-C* alleles.⁹ Therefore, an approach to differentiate the *HLA-C*03:02* allele (presenting in 6.8% of the Vietnamese population⁹) from the other 17 alleles was designed. Three primer sets were used in the second PCR for differentiation purposes. The exon 2–3 sequences of *HLA-C*03:02*, **03:03*, and **03:04* alleles are highly homologous. Moreover, they all have dinucleotide polymorphisms (at position 935–936) that are different from those of the other 15 *HLA-C* alleles reported by Hoa et al.⁹ This is the favorable position for designing the reverse primer (HLAC3CR) which is specific for the three homologous alleles, and the reverse primer (HLAC15CR), which is specific for the other 15 *HLA-C* alleles (Figure 3B).

For the cluster of the three homologous alleles including *HLA-C*03:02*, **03:03* and **03:04*, there are only two SNPs (at position 731 and 795) within the exon 2–3 sequence, that can

be used to distinguish the *HLA-C*03:02* allele from the other two alleles (Figure 3A). The SNP at position 731 was used to design the forward primer (HLAC2CF) which was specific to the *HLA-C*03:03* and *HLA-C*03:04* alleles and the forward primer (HLAC0302F) which was specific to the *HLA-C*03:02* allele. The PCR reaction using these primers resulted in a longer PCR product which is more favorable for detection by electrophoresis. Additionally, a mismatch (replacement of C with T) was placed at the penultimate position of the 3' terminus of the HLAC0302F primer and another mismatch (replacement of T with C) was placed at the third nucleotide from the 3' terminus of the HLAC2CF primer (Figure 3A). The protocol was tested on 10 samples of known genotypes, resulting in a hundred-percent agreement, indicating the efficacy of the PCR strategy mentioned above. The validation by PCR-SBT of 100 samples of unknown genotypes showed a sensitivity of 100%, assuring no false negatives, which

Table 2 AS-PCR Results of 10 Samples of Known Genotypes

Sample No.	HLA-C Genotype	Result After Step 2 PCR			Conclusion of HLA-C*03:02 Genotype with the AS-PCR Protocol
		HLAC15CF and HLAC15CR Primers	HLAC2CF and HLAC3CR Primers	HLAC0302F and HLAC3CR Primers	
1	C*03:02/03:02	–	–	+	C*03:02 homozygote
2	C*03:02/03:03	–	+	+	C*03:02 heterozygote
3	C*03:02/03:04	–	+	+	C*03:02 heterozygote
4	C*03:02/12:02	+	–	+	C*03:02 heterozygote
5	C*03:03/03:03	–	+	–	C*03:02 negative
6	C*03:03/03:04	–	+	–	C*03:02 negative
7	C*03:04/03:04	–	+	–	C*03:02 negative
8	C*03:03/12:02	+	+	–	C*03:02 negative
9	C*03:04/04:03	+	+	–	C*03:02 negative
10	C*04:03/01:02	+	–	–	C*03:02 negative

means that no patients at risk for SCARs due to the *HLA-C*03:02* genotype would be missed if this test is applied in clinical settings.

The *HLA-B*58:01* allele has been reported to be a predominant allele,²² for this reason, most of *HLA-B*58*

:01 specific genotyping methods only aim to determine the presence or absence of the allele in the genotype. Meanwhile, there has not been any report on the difference between homozygous and heterozygous genotypes of the *HLA-C*03:02* allele in the association with allopurinol-induced SCARs. Therefore, we established a flexible protocol that can either determine the presence or absence of the *HLA-C*03:02* allele in the genotype or determine zygosity. For the first aim, only one primer set (HLAC0302F/HLAC3CR) is needed in

Table 3 Comparison of the AS-PCR Method with PCR-SBT

AS-PCR Results for HLA-C*03:02	PCR-SBT Results for HLA-C*03:02		
	Positive	Negative	Total
Positive	42	1	43
Negative	0	57	57
Total	42	58	100
Sensitivity	100% (95% CI: 91.6–100%)		
Specificity	98.3% (95% CI: 90.9–99.7%)		
Cohen's Kappa	$\kappa = 0.98, p < 0.001$		

Table 4 HLA-C*03:02 Distributions in Vietnamese Kinh People

Allele frequency (%)	7.5
HLA-C*03:02 carriers (n, %)	115 (14.2)
Homozygous HLA-C*03:02 genotype (n, %)	7 (8.6)
Heterozygous HLA-C*03:02 genotypes (n, %)	108 (13.3)
Non-HLA-C*03:02 carriers (n, %)	695 (85.8)

the second PCR. This protocol thus can be used for both research and clinical purposes.

A limitation of this protocol is time-consuming in comparison with other methods such as real-time PCR which requires approximately two hours.²³ Nevertheless, the total time for the test including DNA extraction is four hours, enabling to return genotyping results much earlier than sequencing by an outsourcing unit. In addition, this protocol does not require specially trained workers or expensive reagents and equipment; thus, it can be used for screening patients at risk of allopurinol-induced SCARs in local hospitals in developing countries. The total cost for reagents in this method is less than \$2, while the costs for high-throughput methods are usually higher.²³

Another limitation of this PCR-based protocol is the probability of false-positive results due to cross-contamination during electrophoresis or preparation of DNA template. The validation on 100 samples showed one sample with false-positive result (Table 3). For a screening test, sensitivity is more important than specificity. However, a validation on a larger sample size is needed for a comprehensive evaluation of the protocol.

To date, there has been a report on the *HLA-C*03:02* frequency in 170 unrelated Vietnamese Kinh people in Hanoi (the North of Vietnam).⁹ The present study on 810 unrelated Vietnamese Kinh people evenly distributed in the North, the South and the Centre of Vietnam had a significantly bigger and more representative sample of Vietnamese population. The allele frequency (AF) of the *HLA-C*03:02* allele in this study was 7.5%, higher than the AF in the North of Vietnam (6.8%). The *HLA-C*03:02* AF of the Vietnamese Kinh people in our study was the same as that of the Korean people (7.42%)²⁴ and the Thai people (7.77%),¹⁶ more than that of the Chinese people (5.9%)²⁵ and much more than that of the Japanese people (0.6%),²⁶ the Italian people (0.6%),²⁷ the Swiss people (0.54–0.72%),²⁸ the African American people (0.975%)²⁹ or the European American people (0.358%).³⁰ The frequency of *HLA-C*03:02* carriers was notably high (14.2%), which was similar to that of the Thai people (14.68%).¹⁶ These comparisons indicate a diversity of the *HLA-C*03:02* allele distribution among various populations and explain a significant association of the *HLA-C*03:02* allele and the risk of allopurinol-induced SCARs in certain Asian populations with high *HLA-C*03:02* AF such as the Koreans.⁸ This AS-PCR protocol can be used for the *HLA-C*03:02* allele detection in not only Vietnamese people but some other Asian populations with similar genetic characteristics as well.

Abbreviations

AF, allele frequency; AS, allele specific; DRESS, drug reactions with eosinophilia and systemic symptoms; HLA, human leukocyte antigen; SBT, sequencing-based typing; SCAR, severe cutaneous adverse drug reactions; SJS, Steven-Johnson syndrome; SNP, Single nucleotide polymorphism; SSO, sequence specific oligonucleotide; TEN, toxic epidermal necrolysis.

Acknowledgments

The authors would like to thank Dr. Duong Tuan Linh (National Institute of Nutrition) for his valuable supports and comments on the research.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

This study was funded by Vietnam Ministry of Health, Grant# 4694/QD-BYT.

Disclosure

The authors report no conflicts of interest for this work.

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