Development and Validation of a Real-Time PCR Assay for *HLA-A*31:01* Genotyping in Pharmacogenetic Screening

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ABSTRACT Carbamazepine (CBZ) is a widely prescribed anticonvulsant for the treatment of epilepsy, trigeminal neuralgia, and bipolar disorder. Although therapeutically effective, CBZ can trigger severe adverse drug reactions (SADRs), including life-threatening severe cutaneous adverse reactions (SCARs). Robust pharmacogenomic evidence has linked CBZ-induced SADRs to specific human leukocyte antigen (HLA) alleles, particularly *HLA-B*15:02* (along with other alleles in the HLA-B75 serotype group) and *HLA-A*31:01*. While clinical testing for *HLA-B*15:02/B75* is widely accessible in Thailand, *HLA-A*31:01* genotyping remains unavailable in many settings, leaving a critical gap in comprehensive pharmacogenetic risk assessment. This gap is of particular concern because *HLA-A*31:01* is associated with a broader spectrum of CBZ hypersensitivity and is more prevalent in populations where *HLA-B*15:02* is uncommon. To close this limitation, we developed and validated a real-time PCR assay for *HLA-A*31:01* to complement existing genotyping services. Clinical validation was performed on 198 genomic DNA samples with known *HLA-A* genotypes, including 14 *HLA-A*31:01*-positive and 184 negative samples. The assay demonstrated high sensitivity with a reliable detection range of 0.78–200.00 ng DNA/reaction and revealed 100% concordance with the reference method. Its simplicity, speed, and compatibility with standard laboratory workflows highlight its clinical utility. This assay represents a significant step toward safer, personalized CBZ therapy by enabling comprehensive, preemptive screening for SADR-associated *HLA* alleles.

Keywords: *HLA-A*31:01*, Real-time PCR, Carbamazepine, Severe adverse drug reactions (SADRs), Pharmacogenetics

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Introduction

Carbamazepine (CBZ) is a commonly prescribed anticonvulsant used to manage epilepsy, trigeminal neuralgia, and bipolar disorder.[1] Its mechanism of action involves inhibiting voltage-gated sodium channels in the brain, thereby mitigating seizures and neuropathic pain.[2] Despite its clinical efficacy, CBZ is a known cause of severe drug hypersensitivity reactions, particularly severe cutaneous adverse reactions (SCARs) such as Stevens-Johnson Syndrome (SJS), Toxic Epidermal Necrolysis (TEN), and Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS), which can lead to significant morbidity and mortality.[2-4]

Pharmacogenomic studies have identified strong associations between CBZ-induced severe drug hypersensitivity reactions and specific *Human Leukocyte Antigen (HLA)* alleles, notably *HLA-B*15:02* and *HLA-A*31:01*.[1, 5-7] *HLA-B*15:02* is a key member of the B75 serotype, which also includes *HLA-B*15:08, HLA-B*15:11, HLA-B*15:21*, and *HLA-B*15:31*. *HLA-B*15:02* is strongly associated with CBZ-SCARs, predominantly among East and Southeast Asian populations. Its prevalence reaches 10–15% in Han Chinese, 8–12% in Thai populations, and approximately 12% in Malaysians, but remains rare among Europeans, Japanese, and Africans.[3,8] Other *B75 alleles*, including *HLA-B*15:08, HLA-B*15:11,* and *HLA-B*15:21,* though found at lower frequencies, have also been implicated in CBZ-induced SCARs.[7, 9-11]

Conversely, *HLA-A*31:01* is associated with a broader spectrum of CBZ hypersensitivity reactions, including maculopapular eruptions and DRESS. Its prevalence is approximately 8% in Japanese, 4–7% in Koreans, 2–5% in Europeans, and 1–2% in South Asians.[1,5] Based on these associations, the Clinical Pharmacogenetics Implementation Consortium (CPIC) and U.S. Food and Drug Administration (FDA) recommend genetic testing for both *HLA-B*15:02* and *HLA-A*31:01* before CBZ therapy initiation.[6,12]

HLA typing can be conducted using various technologies. Sanger sequencing and bead-based hybridization have been used for decades.[13] They are resource-intensive and require specialized personnel. Next-Generation Sequencing (NGS) offers high resolution but is limited in routine clinical use due to its high cost and complexity.[14] In contrast, real-time PCR and conventional PCR provide faster turnaround, cost-efficiency, and are suitable for most molecular laboratories.[15-17] Although Single-Nucleotide Polymorphism (SNP)-based real-time PCR assays using tag-SNPs, such as rs1061235 or rs17179220, have been investigated for *HLA-A*31:01* detection due to their cost-effectiveness.[18,19] These assays often show diminished specificity, especially in admixed populations, which restricts their practical application and underscores the superiority of allele-specific real-time PCR as a more dependable alternative.

Currently, PCR-based pharmacogenetic testing for *HLA-B*15:02* and the broader *HLA-B75* group is available through the Department of Medical Sciences under Thailand's National Health Security Office scheme.[20] However, these tests do not include *HLA-A*31:01* genotyping, leaving a diagnostic gap in the preemptive screening of CBZ-induced severe drug hypersensitivity reactions.

In contrast, medical schools and high-end private hospitals typically use HLA typing, laboratory-developed tests, or commercial kits for *HLA-A*31:01*, which are not aligned with the methodology implemented in the Department of Medical Sciences (DMSc) network. Remarkably, to facilitate nationwide implementation, DMSc completed the technical transfer of *HLA-B75* real-time PCR testing to the private sector in 2024. Developing an additional real-time PCR assay for *HLA-A*31:01* can bridge this gap and support broader clinical adoption. This study addressed that need by developing and validating a real-time PCR assay specifically for *HLA-A*31:01*, aiming to expand the detection of CBZ-induced adverse reactions. Wider availability of this assay could improve drug safety across diverse Thai populations and strengthen national pharmacovigilance and precision medicine in Thailand.

Materials and Methods

DNA samples

This study utilized leftover genomic DNA (gDNA) from EDTA-anticoagulated blood samples originally collected during the Third Thailand National Health Examination Survey (NHES III). A total of 650 gDNA samples had previously undergone HLA-A allele typing using a bead-based hybridization assay (WAKFlow HLA Typing Kit and WAKFlow Typing Software, WAKUNAGA, Japan) as part of a prior study.[21] For the present study, a subset of 198 DNA samples was selected for clinical validation, comprising 31 *HLA-A31:01*-positive and *167 HLA-A31:01*-negative samples.

The gDNA used in this study was previously extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germany), following the manufacturer's protocol. Each extraction involved processing 200 µL of EDTA-anticoagulated blood to obtain 50 µL of genomic DNA. For the current study, the quality and concentration of the leftover gDNA were reassessed using NanoDrop™ 2000 spectrophotometry (Thermo Scientific, USA), ensuring an OD_{260}/OD_{280} ratio between 1.60 and 2.00 and a minimum DNA concentration of 20 ng/µL.

Ethical approval

Ethics approval for this research was granted by the Department of Medical Sciences, Ministry of Public Health, Thailand (Protocol No. 33/2566, December 6, 2023).

Plasmid controls

A 500-bp synthetic DNA fragment corresponding to the *HLA-A*31:01:02.01* allele (spanning exon 2 to exon 3, including intron 2) was cloned into the pMK-RQ (KanR) vector. Additionally, a 980-bp segment of the *Tissue Inhibitor of Metalloproteinase 1* (*TIMP1*) gene, located on the human X chromosome, was inserted into the pMK-RQ (AmpR) vector to serve as an internal control. Both plasmids were custom-designed and synthesized by Invitrogen (Invitrogen, USA).

The *TIMP1* plasmid was diluted to a final concentration of 50 copies/µL and used as a plasmid negative control (pNC). Equal concentrations of 100 copies/µL for both the *HLA-A*31:01* and *TIMP1* plasmids were combined to create a plasmid positive control (pPC). Quality control for each batch of genomic

DNA (gDNA) testing included the pNC, pPC, and nuclease-free water as a no-template control (NTC).

Primers and probes design

A comprehensive multiple sequence alignment of all known *HLA-A* alleles spanning exons 1 to 4 was conducted using *HLA-A* 31:01:02.01* as the reference sequence. The alignment was performed with the web-based Major Histocompatibility Complex database (dbMHC) Sequence Alignment Viewer (https://www.ncbi.nlm.nih.gov/Web/Newsltr/Summer03/dbMHC.html), which identified unique polymorphic regions for primer and probe design. Primers and probes were designed using Primer Express Software v3.0.1 (Applied Biosystems, USA) to optimize melting temperature (Tm), GC content, and reduce the potential for secondary structures and dimer formation.

The *HLA-A*31:01*-specific probe was labeled with Cy5.5 at the 5′ end and quenched with a minor groove binder (MGB). The *TIMP1* internal control probe was labeled with Cy5 and also quenched with MGB for spectral resolution in duplex PCR. The detailed characteristics of the primers and probes used in this study are presented in Table 1.

The designed primers and probes used in this assay have been submitted for petty patent registration with the Department of Intellectual Property, Thailand (Application No. 250300202).

Table 1 Sequences and properties of primers and probes for *HLA-A*31:01* and *TIMP1* detection

Primer/Probe name	Sequence (5' to 3')	Size (bp)	Tm (°C)	CG (%)	PCR product (bp)
A3101-F	GGC TCC CAC TCC ATG AGG TAT TTA AC	26	65.6	54	237
A3101-R	CCC CAG GTC CAC TCG GTC AA	20	64.0	65	
A3101-probe	Cy5.5- CTC CTG GTC CCA ATA CTC AGG CCT - MGB	24	78.0	58	
TIMP1-F	CAT GGA GAG TGT CTG CGG ATA CTT	24	60.5	50	74
TIMP1-R	TGC CTC ACC AGC AAT GAG AAA	21	60.3	48	
TIMP1-probe	CY5- ACA GGT CCC ACA ACC GCA GCG A -MGB	22	80.0	65	

Real-time PCR optimization for the detection of *HLA-A*31:01*

Real-time PCR optimization involved titration of primers and probes (0.1 to 2.0 µM). Reactions (12 µL) consisted of 2 µL DNA and 10 µL KAPA PROBE FAST qPCR Master Mix (Roche, USA) with appropriate primer and probe concentrations. Temperature optimization was performed via gradient PCR (57.0°C to 72.0°C) using the CFX Opus Real-Time PCR System (BioRad, USA), which automatically generated eight temperature points: 57.0°C, 58.0°C, 60.0°C, 62.9°C, 66.5°C, 69.5°C, 71.2°C, and 72.0°C. Amplification curves and Ct values were analyzed using CFX Maestro Software (BioRad, USA). An *HLA-A*31:01*-positive and an *HLA-A*31:01*-negative gDNA samples (20 ng/µL) were used to identify the optimal conditions for specific and efficient amplification.

Method validation

Analytical performance was assessed in duplicate with a two-fold serial dilution of DNA across 12 dilution points, from 100 ng/μL down to 0.049 ng/μL. Amplifications were run under optimized PCR conditions, and reactions yielding Ct values below 30 for both Cy5 (*TIMP1*) and Cy5.5 (*HLA-A*31:01*) were interpreted as positive.

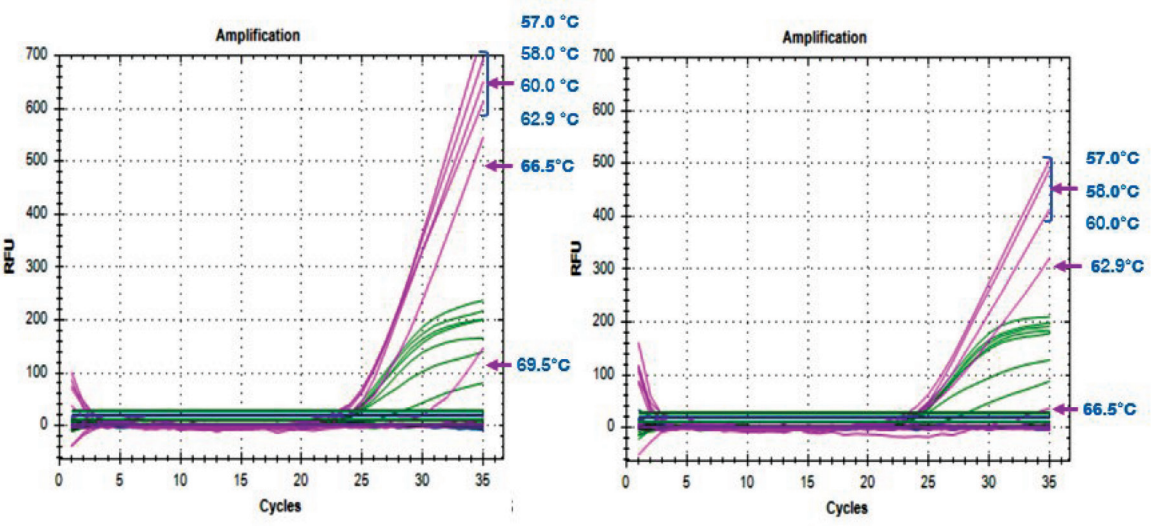
Validation of clinical sample testing was performed using 198 gDNA samples, comparing real-time PCR results with those obtained from the bead-based hybridization. Discordant samples were re-evaluated by Oxford Nanopore Technology (ONT) long-read sequencing at the Siriraj Long-Read Lab.[22]

Results

Real-time PCR optimization for the detection of *HLA-A*31:01*

The final real-time PCR assay was established based on conditions optimized during the development phase that yielded the best results. Each 12 μL reaction included 2 μL of DNA template and 10 μL of master mix. The mix comprised 2.0 μM each of *HLA-A* 31:01*-specific forward and reverse primers, 1.0 μM of the Cy5.5-labeled probe, and 100 nM and 40 nM of *TIMP1* primers and Cy5-labeled probe, respectively.

For thermal optimization using gradient PCR, strong and specific Cy5.5 fluorescence signals were consistently observed in an *HLA-A*31:01*-positive sample between 57.0°C and 69.5°C, although the signal was poor at 69.5°C, with a Ct value of 30.50. In contrast, weaker and non-specific signals (Ct < 30) were detected in an *HLA-A*31:01*-negative sample at temperatures between 57.0°C and 62.9°C. The *HLA-A*31:01*-negative sample also gave a very low signal at 66.5°C. From these findings, 67°C was selected as the optimal annealing/extension temperature, balancing specificity and workflow compatibility with our current B75 genotyping. The amplification plots for positive and negative samples at various temperatures are shown in Figure 1.



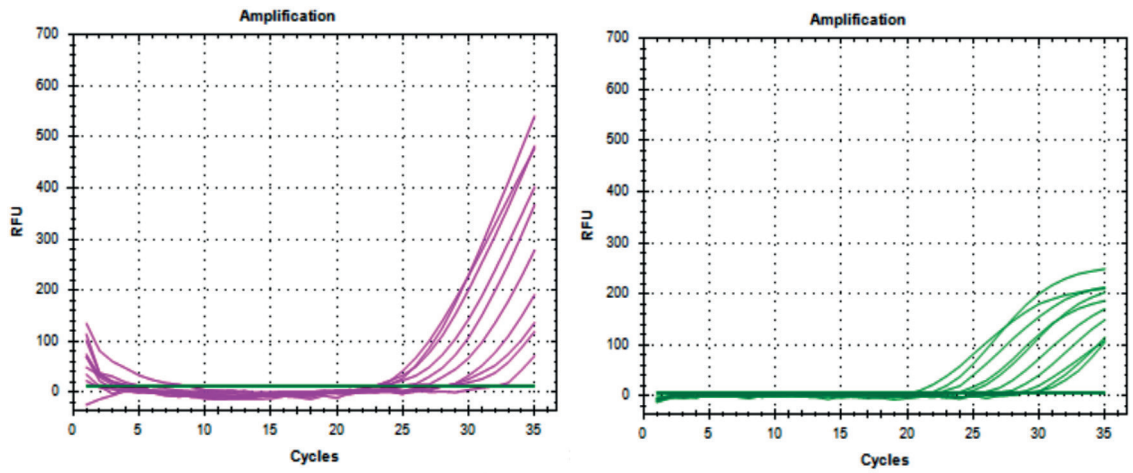
1A: *HLA-A*31:01* positive gDNA 1B: *HLA-A*31:01* negative gDNA

Figure 1 Amplification plot of an *HLA-A*31:01* positive gDNA (1A) and an *HLA-A*31:01* negative gDNA (1B) at eight temperature points; 57.0°C, 58.0°C, 60.0°C, 62.9°C, 66.5°C, 69.5°C, 71.2°C and 72.0°C. The pink curves represent Cy5.5 fluorescence from the *HLA-A*3101* probe; the green curves represent Cy5 fluorescence from the TIMP1 probe.

Based on the results from gradient temperature optimization, the finalized thermal profile included an initial denaturation at 95°C for 3 minutes, followed by 35 cycles at 95°C for 20 seconds and 67°C for 1 minute. This configuration ensured reliable and reproducible amplification of the *HLA-A*31:01* allele in both clinical and research samples.

Validation of the detection range

The analytical performance was assessed by performing two-fold serial dilutions of genomic DNA ranging from 100 ng/μL to 0.049 ng/μL in duplicate. Robust and consistent amplification signals (Ct < 30) were observed in 9 out of 12 dilution points, corresponding to DNA amount between 200.00 and 0.78 ng per reaction, for both the *HLA-A*31:01* target and the *TIMP1* internal control. Even the dilution point at 0.39 ng/reaction showed an amplification curve and detectable signal; its Ct value was 32.5, which exceeded the threshold of 30 and was thus interpreted as negative. Amplification curves for all 12 DNA dilutions are shown in Figure 2.



2A 2B

Figure 2 Amplification plot of an *HLA-A31:01*-positive gDNA sample across a series of concentrations. A two-fold serial dilution of 100 ng/μL from an *HLA-A31:01*-positive gDNA sample was performed, resulting in 12 concentrations ranging from 100 ng/μL to 0.049 ng/μL. Positive signals were observed in 9 out of the 12 tested concentrations (equivalent to 200.00 to 0.78 ng per reaction). Panel 2A displays the Cy5.5 signal (pink) generated by the A3101 probe, while Panel 2B illustrates the Cy5 signal (green) from the TIMP1 probe. Panels 2A and 2B represent data from the same real-time PCR experiment.

Validation of clinical sample testing

Among the 198 clinical DNA samples tested, the real-time PCR assay accurately identified 14 samples as *HLA-A*31:01*-positive. These 14 positive samples showed Ct values below 30 for both Cy5.5 (*HLA-A*31:01*) and Cy5 (*TIMP1*) signals, with mean ± SD of 24.77±2.17 and 20.36±0.77, respectively. The 167 negative samples yielded only Cy5 (*TIMP1*) signal with a mean ± SD of 20.53±0.92.

In comparison, the bead-based hybridization method previously identified 31 positive samples.[21] To resolve this discrepancy, the 17 discordant samples (reported positive by

hybridization but negative by PCR), along with 3 concordant samples (positive by both assays), were retested using Oxford Nanopore Technology (ONT) long-read sequencing at the Siriraj Long-Read Lab. ONT confirmed that the 3 concordant samples were true carriers of *HLA-A*31:01*. However, all 17 discordant samples were negative for *HLA-A*31:01*, fully aligning with the PCR assay's results and indicating that the hybridization method had overinterpreted positives due to misclassification. This analysis established 100% concordance between the developed PCR assay and ONT-confirmed genotypes, validating its accuracy and clinical reliability. Figure 3 illustrates the amplification performance of 37 gDNA samples, as a representative subset from the 198 samples analyzed during such validation. Among these, two *HLA-A*31:01*-positive samples were detected. The plot demonstrates a clear differentiation of the CY5.5 signal between positive reactions (gDNA and the plasmid positive control (pPC) and negative ones (negative samples (pNC) and no-template control (NTC)).

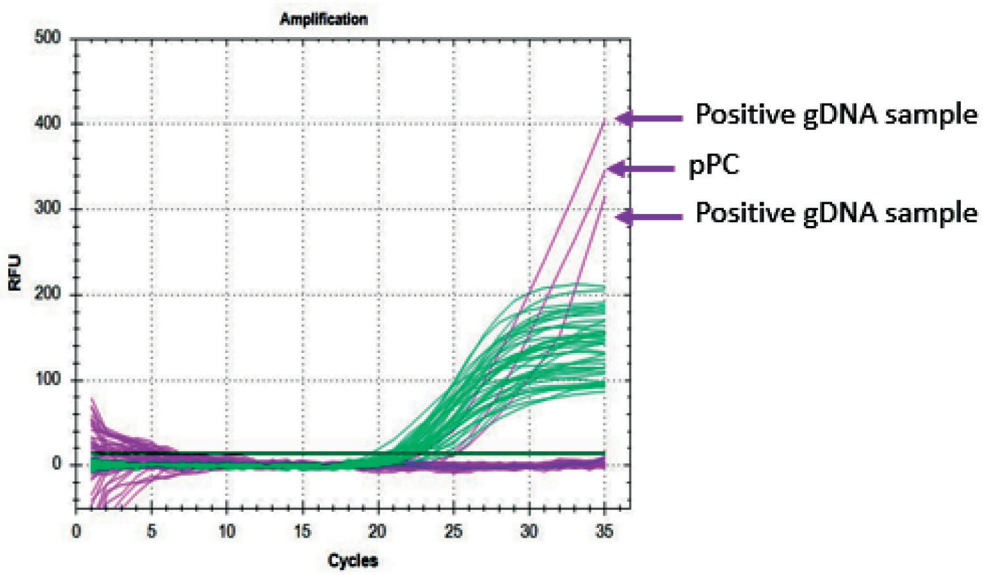


Figure 3 Amplification plot of 40 reactions, including two *HLA-A*31:01*-positive gDNA samples, 35 negative gDNA samples, and 3 controls: plasmid positive control (pPC), plasmid negative control (pNC), and no-template control (NTC). The Cy5.5 signal (pink) represents amplification from the A3101 probe, while the Cy5 signal (green) indicates amplification from the TIMP1 probe.

Discussion

This study presented a validated real-time PCR assay for *HLA-A31:01* detection, demonstrating high analytical and clinical performance with complete concordance to Oxford Nanopore Technology (ONT)-based Long-Read Sequencing. Compared to the bead-based hybridization, which overestimated *HLA-A*31:01* positivity due to potential allele misclassification, this allele-specific real-time PCR assay offers superior specificity. Seventeen discordant samples initially identified as

HLA-A31:01:07 by hybridization were accurately genotyped as *HLA-A33:03:01* using ONT, highlighting the precision of the designed primer-probe set.

Several real-time PCR assays for *HLA-A*31:01* genotyping have been developed and validated in specific populations. For instance, Zhang et al. designed a TaqMan-based multiplex assay validated in Han Chinese and Tibetan populations, achieving 100% sensitivity and 98.9% specificity.[15] However, their probe design targeting an 82-bp amplicon demonstrated cross-reactivity with *HLA-A*33:01*. Nguyen et al. developed a triplex assay for detecting *HLA-A31:01* and *HLA-B15:02*, incorporating an internal *β-actin* control.[23] This assay achieved high sensitivity, detecting DNA as low as 0.037 ng/μL.

In contrast, the real-time PCR assay developed in this study was uniquely designed to target three defining polymorphisms in a cis configuration across exons 2 of *HLA-A*31:01*. This molecular configuration enhanced allele specificity and eliminated cross-reactivity with *HLA-A*33:01*, a known limitation in other designs. While our detection limit (0.78-200.00 ng/reaction) was less sensitive than Nguyen's, it adequately encompassed the DNA concentrations typically used in clinical pharmacogenetic settings where sample quality and quantity might vary.

Furthermore, this assay was specifically designed to be compatible with the existing real-time PCR platform used for *HLA-B75* testing. By sharing a consistent thermal profile and reagent compatibility, it facilitated rapid adoption in laboratory settings. Incorporation of spectrally distinct fluorophores, Cy5.5 for *HLA-A*31:01* and Cy5 for TIMP1, further enhanced its utility by enabling potential multiplexing with FAM and HEX probes currently employed in HLA-B75 assays. With further validation, this approach could evolve into a single-tube multiplex format, streamline pharmacogenetic testing, reducing reagent costs, and increasing throughput.

While high-resolution technologies such as NGS and ONT deliver comprehensive pharmacogenomic insights, their high cost and technical complexity limit their practicality for routine clinical use. In contrast, real-time PCR offers a cost-effective and scalable solution, making it particularly suitable for resource-limited healthcare systems. However, this study had some limitations, including the relatively small number of *HLA-A*31:01*-positive samples, reflecting the allele's low frequency in Thailand, and the use of pre-characterized samples, which might inflate the perceived performance of the assay. Expanding validation efforts to include larger and more diverse multi-ethnic cohorts will enhance the assay's generalizability, confirming its diagnostic reliability and broad applicability across different populations.

Conclusion

This study successfully developed and validated a real-time PCR assay for *HLA-A*31:01* detection, demonstrating high specificity and complete concordance with long-read sequencing ONT results. The assay surpassed the performance of the bead-based hybridization by accurately distinguishing *HLA-A*31:01* from closely related alleles. Its robust amplification across a broad range of DNA concentrations

highlighted its practicality and efficiency for pharmacogenetic screening. Integrating this assay into clinical services, alongside *HLA-B*15:02/B75* testing, could significantly enhance preemptive risk assessment for carbamazepine-induced hypersensitivity. The widespread adoption of this cost-effective and rapid genotyping method could boost Thailand's pharmacovigilance infrastructure and advance personalized medicine, ultimately improving patient safety and public health outcomes.

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การพัฒนาและการประเมินประสิทธิภาพวิธี Real-Time PCR สำหรับการตรวจหายีน *HLA-A*31:01* ในการคัดกรองทางเภสัชพันธุศาสตร์

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[2]สถาบันชีววิทยาศาสตร์ทางการแพทย์ กรมวิทยาศาสตร์การแพทย์ นนทบุรี 11000

[3]กองแผนงานและวิชาการ กรมการแพทย์แผนไทยและการแพทย์ทางเลือก นนทบุรี 11000

บทคัดย่อ คาร์บามาซีพีน (Carbamazepine: CBZ) เป็นยากันชักที่ใช้กันอย่างแพร่หลายทางการแพทย์ในการรักษาโรคลมชัก โรคปวดเส้นประสาทใบหน้า และโรคอารมณ์สองขั้ว แม้ว่ายานี้จะมีประสิทธิภาพทางคลินิกสูง แต่สามารถก่อให้เกิดอาการไม่พึงประสงค์จากยารุนแรง (Severe Adverse Drug Reactions: SADRs) โดยเฉพาะกลุ่มอาการผื่นแพ้ยารุนแรงที่อาจเป็นอันตรายถึงชีวิต (Severe Cutaneous Adverse Reactions: SCARs) หลักฐานทางเภสัชพันธุศาสตร์ได้ยืนยันว่า *HLA-B*15:02* รวมถึง *HLA-B* alleles ในกลุ่ม *HLA-B75* serotype และ *HLA-A*31:01* เป็นปัจจัยเสี่ยงทางพันธุกรรมที่เกี่ยวข้องกับการเกิด SADRs จากการใช้ยา CBZ ปัจจุบันในประเทศไทยมีการให้บริการตรวจ *HLA-B*15:02/HLA-B75* อย่างแพร่หลายภายใต้ระบบประกันสุขภาพแห่งชาติ ในขณะที่การตรวจ *HLA-A*31:01* ยังมีจำกัด ส่งผลให้การประเมินความเสี่ยงทางเภสัชพันธุกรรมยังไม่ครอบคลุม โดยเฉพาะอย่างยิ่งในประชากรที่พบ *HLA-B*15:02* ในอัตราต่ำ แต่มีความชุกของ *HLA-A*31:01* สูง ซึ่งสัมพันธ์กับกลุ่มอาการแพ้ยาที่หลากหลายและซับซ้อนกว่า การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาและประเมินประสิทธิภาพของวิธีตรวจ *HLA-A*31:01* ด้วยเทคนิค Real-time PCR ผลการทดสอบในตัวอย่างดีเอ็นเอ 198 ตัวอย่าง ประกอบด้วย 14 ตัวอย่าง ที่มี *HLA-A*31:01* และ 184 ตัวอย่าง ที่ไม่มี *HLA-A*31:01* พบว่าวิธีนี้ให้ผลสอดคล้องกับวิธีตรวจอ้างอิงร้อยละ 100 และสามารถตรวจดีเอ็นเอได้ในช่วง 0.78–200.00 ng/ปฏิกิริยา แสดงถึงความแม่นยำสูง ใช้งานง่าย รวดเร็ว และเหมาะสมในการประยุกต์ใช้ในห้องปฏิบัติการทางคลินิก วิธีการตรวจนี้ช่วยเพิ่มความครอบคลุมในการคัดกรองความเสี่ยงทางพันธุกรรม และส่งเสริมความปลอดภัยของการใช้ยา CBZ ตามแนวทางการแพทย์แม่นยำ

คำสำคัญ: เอชแอลเอ-เอ 31:01, เรียลไทม์พีซีอาร์, คาร์บามาซีพีน, อาการไม่พึงประสงค์จากยารุนแรง, เภสัชพันธุศาสตร์