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ORIGINAL ARTICLE

Detection of Allergy-Related *TPSAB1* Copy Number Variation by MALDI-TOF Nucleic Acid Mass Spectrometry

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ABSTRACT

Background: **Allergic diseases have been linked to genetic variations in *TPSAB1* and *TPSB2*. This study aimed to develop a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) method for simultaneous copy number detection of *TPSAB1* and *TPSB2*.**
Methods: **We established a multiplex PCR-tandem MALDI-TOF MS assay for *TPSAB1* and *TPSB2* copy number analysis. To validate the method, 35 standard samples pre-validated by droplet digital PCR (ddPCR) were used to assess accuracy, sensitivity, repeatability, and stability. Additionally, we applied the method to 274 randomly selected population samples for clinical evaluation.**
Results: **The developed MALDI-TOF MS assay demonstrated high reliability, achieving 100% accuracy in standard samples. The detection limit for the standard template was 20 ng, with repeatability (CV: 8.41 - 13.60%) and stability (CV: 7.75 - 17.38%) within acceptable ranges. In the population cohort, the carrier rate for *TPSAB1* copy number gain (> 2 copies) was 12.41%.**
Conclusions: **The MALDI-TOF MS-based method is a robust, cost-effective, and high-throughput molecular diagnostic tool, particularly suited for differentiating highly homologous TPS genes. Its high accuracy and reliability make it a promising clinical assay for *TPSAB1* and *TPSB2* copy number analysis.**
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INTRODUCTION

Allergic diseases, systemic disorders arising from immune dysregulation, constitute a growing global public health challenge with steadily increasing incidence worldwide. Epidemiological studies reveal that the lifetime prevalence of systemic anaphylaxis ranges from 0.05% to 2% in the United States and reaches approximately 3% in European populations. These conditions are classified into four pathophysiological categories based on tissue damage mechanisms: type I (immediate hypersensitivity), type II (cytotoxic), type III (immune

complex-mediated), and type IV (delayed-type hypersensitivity). Among these, mast cell disorders (MCDs) represent a distinct clinical entity characterized by aberrant mast cell proliferation or activation, resulting in pathological degranulation.

Trypsin, accounting for about 25% of total mast cell granule protein content, serves as a key mediator in these processes. The trypsin-like protease gene cluster on chromosome 16p13.3, comprising *TPSG1*, *TPSB2*, *TPSAB1*, and *TPSD1*, exhibits particularly notable genomic organization: *TPSB2* and *TPSAB1* share 99% sequence homology and are arranged in reverse tandem orientation. Critically, a gene dosage effect has been established between *TPSAB1* copy number variation (CNV) and serum basal tryptase (BST) levels, with each additional *TPSAB1* copy elevating BST by 9.5 ng/mL. This robust genotype-phenotype correlation underscores the clinical imperative for precise *TPSAB1*/*TPSB2* CNV quantification in MCD diagnosis and management.

Current molecular diagnostic approaches, including multiple ligation probe amplification (MLPA), chromosomal microarray analysis (CMA), next-generation sequencing (NGS), and droplet digital PCR (ddPCR), face three fundamental limitations: Firstly, procedural complexity requiring specialized expertise; secondly, prohibitive infrastructure and reagent costs; and thirdly, computationally intensive bioinformatics pipelines. These constraints are particularly acute for differentiating between highly homologous sequences like *TPSAB1*/*TPSB2*, necessitating innovative solutions for clinical application.

To address these challenges, we developed a novel single-tube MALDI-TOF MS assay for parallel *TPSAB1*/*TPSB2* CNV quantification. Our methodology combines: Multiplex PCR amplification of target loci, single-base extension (SBE) probe strategy for sequence differentiation and peak height ratio-based quantification of copy numbers. The clinical validity of this platform was rigorously assessed using 274 population-derived samples, demonstrating its potential as a high-throughput, cost-effective diagnostic tool for MCDs.

MATERIALS AND METHODS

Samples

A series of plasmids were constructed by use of directly cloning or site-directed mutagenesis technology with PUC57 Vector (Sangon Biotech, Shanghai, China), including the target genes of *TPSAB1*, *TPSB2*, *TPSD1,* and *TPSG1*, as well as *ACTB*. The copies of target alleles in these plasmids were determined with ddPCR (QX200, BIO- RAD, USA). The standard/control samples with different ratios of target genes were prepared by mixing these plasmids.

A total of 274 peripheral blood samples were randomly recruited from Shenzhen Guangming District Maternal and Child Health Hospital. All participants provided informed consents before sampling. Blood gDNA was purified using the QIAamp DNA Blood Kit (Qiagen, Germany), according to the manufacturer's instructions.

Development of quantitative ddPCR system

Using *ACTB* as the reference, two ddPCR assays were developed to quantify the target gene copies of *TPSAB1* and *TPSB2*, respectively. The primers and probes of these ddPCR assays are listed in Table 1. The reference sequences were obtained from the NCBI RefSeq database under the following accession numbers:
NC_000016.10:1242225..1242374 and
NC_000016.10:1228 516..1228665.

Design of assay scheme

A technical scheme was designed for this MALDI-TOF MS assay, in order to simultaneously detection the copy number of target genes. As shown in Figure 1, the templates of target genes (*TPSAB1*, *TPSB2*, *TPSD1,* and *TPSG1*) reference (*ACTB*) were amplified with a single-tube multiplex PCR. Subsequently, SBE was performed with the amplified products, and then detected on MALDI-TOF MS. Based on the mass spectrum result, target alleles were distinguished according to their specific molecular weight, while the allelic copy numbers were analyzed according to their mass spectrum peak height.

The multiplex PCR amplification strategy is schematically illustrated in Figure 1A. Specifically, three primer pairs were designed to co-amplify five target sequences: *TPSAB1*, *TPSB2*, *TPSD1*, *TPSG1*, and the reference gene *ACTB*, with corresponding primer sequences detailed in Table 2. The reference sequences were obtained from the NCBI RefSeq database under the following accession numbers:
NC_000016.10:1258419..12558598,
NC_000016.10:1242163..1242342,
NC_000016.10:1228548..1228727,
NC_000016.10:1223157..1223360, and
NC_000007.14:5528609..5528897. Each 20 μL reaction mixture contained the following components: 40 ng genomic DNA (gDNA), 0.1875 mM deoxyribonucleotide triphosphates (dNTPs), 0.4 M betaine (Sigma-Aldrich, St. Louis, MO, USA), 4.0% (v/v) dimethyl sulfoxide (DMSO), primers at concentrations ranging from 200 to 2,000 nM, and 1.5 U of high-affinity HotStart Taq polymerase in 1× HA buffer (Tiangen Biotech, Beijing, China).The thermal cycling program began with an initial denaturation step at 95°C for 7 minutes, followed by 40 cycles consisting of: denaturation at 95°C for 30 seconds, annealing at 67°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension was performed at 72°C for 10 minutes.

The second step used SBE technology to generate sequence-specific products (Figure 1A). Copy number determination was achieved through relative peak height (RPH) analysis, comparing target gene signals between test and standard samples. Standard samples with known diploid copies were first analyzed to establish baseline peak heights (P1) for each gene. Test sample

peak heights (P2) were then normalized against reference genes, enabling calculation of P2/P1 ratios for determining absolute copy numbers of *TPSAB1* and *TPSB2* and their intergenic copy number ratios. After the PCR cycles, 5 µL of the 20 µL amplification product was mixed with 2 µL of a solution containing 0.51 U shrimp alkaline phosphatase (SAP) and 0.17 µL of 10× SAP buffer (Guangzhou Darui Biotech, China) and dispensed into a 96-well plate. The mixture was incubated at 37°C for 40 minutes and then at 85°C for 5 minutes to remove residual dNTPs. Following this, 2 µL of expansion reagent was added, which contained an expansion probe mixture, 1.312 U Pro Enzyme, and a 1-fold expansion reaction mixture containing ddNTPs (Darui). The extension thermal cycling conditions began with an initial denaturation step at 95°C for 30 seconds, followed by 150 cycles of denaturation at 95°C for 5 seconds and a 5-second extension cycle step at 52°C. A final 3-minute extension step was performed at 72°C, followed by incubation at 4°C.

The final analytical phase involved MALDI-TOF MS detection (Figure 1A). Extended products were diluted with 41 µL ddH_2O and processed using the DR MassARRAY® system (Darui Biotech) following manufacturers´ protocols. Key steps included: Resin-based desalination (10 µL), chip spotting (MALDI chips; Darui Biotech), matrix-assisted laser desorption/ionization and time-of-flight separation. Mass spectra were acquired and analyzed by Typer 4.1 software (Agena), which determined copy numbers by quantifying peak molecular weights (MWs) corresponding to extended products (reference MWs provided in Table 3). This integrated SBE-MALDI platform enables precise *TPSAB1* copy number quantification, establishing a reliable high-throughput genotyping solution.

Methodological validation of the MALDI-TOF MS analytical system

To validate the proposed method, we first assessed its accuracy using a set of 14 standard samples with distinct genetic profiles. The sensitivity and detection limit were defined as the lowest DNA concentration at which all three genotypes could be accurately identified. Sensitivity was constructed with three *TPSAB1*:*TPSB2* ratios (2:2, 3:2, and 4:2). For each ratio, the total DNA content was serially diluted (40 ng, 20 ng, 10 ng, 5 ng, and 2.5 ng), yielding 15 unique standard samples. This constituted a preliminary, single-run experiment.

To evaluate repeatability and stability, two sets of standard samples, each comprising the three *TPSAB1*:*TPSB2* ratios (2:2, 3:2, and 4:2), were employed (six standards total). Repeatability was determined by analyzing one set of standards three times. Stability was assessed by analyzing the second set at three distinct time points. The reproducibility for both metrics was expressed using the coefficient of variation.

Finally, to evaluate the clinical applicability of the MALDI-TOF MS technique, we tested 274 randomly selected population samples.

Statistical analysis

Statistical analysis and data description were performed using IBM SPSS Statistics 19.0.1 and GraphPad Prism 8.0.1 software. All ratios are presented as the mean ± standard deviation (SD).

RESULTS

MALDI-TOF MS genotyping analysis of allergy-associated genes

Figure 2 presents representative MALDI-TOF MS results. As depicted in Figure 2A, samples exhibiting an identical copy number of *TPSAB1* and *TPSB2* (*TPAB1*:*TPSB2* = 2:2) demonstrate a CNR approximately equal to 1. Conversely, samples with an additional *TPSAB1* copy (*TPAB1*:*TPSB2* = 3:2) display a CNR approximately equal to 1.5. These samples exemplify the disparity between theoretical and observed ratios with increasing *TPSAB1* copy numbers. In this investigation, *ACTB* serves as the reference gene for analyzing the copy numbers of *TPSB2*, *TPSAB1*, *TPSD1*, and *TPSG1* (Figure 2C, D, E, F, and G). As depicted in Figure 2C, samples with identical copy numbers of *TPSB2* and *ACTB* (*TPSB2*:*ACTB* = 2:2) exhibit a CNR approximately equal to 1, whereas samples lacking a *TPSB2* copy (*TPSB2*:*ACTB* = 1:2) showcase a CNR approximately equal to 0.5. These samples elucidate the discrepancy between theoretical and observed ratios as the copy number of the target gene varies.

Methodological validation of the MALDI-TOF MS analytical system

The analytical performance of our MALDI-TOF MS-based genotyping platform was rigorously evaluated using 14 certified reference standards encompassing various TPSAB1:*TPSB2* allelic configurations, TPSAB1-2:*TPSB2* haplotypes, and precisely quantified *TPSB2* copy number variants. Quantitative analysis demonstrated exceptional method accuracy, with all standard samples achieving 100% concordance in genotyping results. As shown in Figure 3, the normalized signal intensity ratios for both TPSAB1 and TPSAB1-2 probes relative to *TPSB2* established well-defined diagnostic thresholds: a ratio range of 0.49 - 0.71 corresponds to a single-copy state, 0.71 - 1.28 indicates diploid configuration, and 1.28 - 1.69 reflects copy-number gain. The graphical representation clearly depicts these analytical parameters, with black trend lines showing mean ± standard deviation values that demonstrate measurement precision. We applied the red-line method to assign copy number in the subsequent validation phase, which included both methodological tests and analysis of 274 clinical samples.

The sensitivity of the proposed MALDI-TOF MS-based assay was rigorously evaluated using 15 standardized reference samples with predetermined copy number variations. As detailed in Table 4, the method's detection capability was systematically examined across a range

Table 1. The primers and probes of ddPCR assays.

Primer/Probe name	Sequence (5'-3')	Length of product	Gene
TPS-F	ccccaaaaagccgtgagtc	150 bp	TPSAB1/TPSB2
TPS-R	ttaggacaggaaaggggcactc		
TPSB2-P	AcactggagagccagcccctcA	/	TPSB2
TPSAB1-P	AcactggaggaccaacccctgA		TPSAB1

The uppercase indicates base mismatch in template.

Table 2. The primers sequence and length of multiplex PCR product.

Primer name	Sequence (5'-3')	Length of product	Amplicon
TPS-F	CTATAcccaaccggcctggcatctac	190 bp	TPSAB1/TPSB2/TPSD1
TPS-R	CTAGTgggaaggtgtgggggggcagtc		
TPSG-F	CTAtcaggaggcagattgcaagctcag	210 bp	TPSG1
TPSG-R	CTAgcagaggcctccacttggtcttga		
Actb-F	CTATAcccgtcaccggagtccatcac	296 bp	ACTB
Actb-R	TAggtgtaaagcggccttggagtgtg		

The uppercase indicates base mismatch in template.

Table 3. Expected molecular mass of probes and products of multiplex single-base extension.

Probe	Sequence (5'-3')	Probe Mass (Da)	Product Mass (Da)	Target gene
TPSB2D	cctggggtgtccacc	4,544.91	4,792.11 (+ddC)	TPSD1
			4,872.01 (+ddT)	TPSB2
TPSG1	gaaggtgggcaaccag	4,980.25	5,227.45 (+ddC)	TPSG1
TPSAB1	Aggaggaccaacccctg	5,189.37	5,436.57 (+ddC)	TPSAB1
TPSAB1-2	agtggtgttttggacagc	5,585.60	5,856.80 (+ddA)	
ACTB	agcctggatagcaacgtac	5,821.78	6,092.98 (+ddA)	ACTB

The uppercase indicates base mismatch in template.

Table 4. Statistics of the sensitivity evaluation.

Total amount of standard samples (ng)	*TPSAB1*:*TPSB2* standard samples		
	2:2	3:2	4:2
40	+	+	+
20	+	+	+
10	+	-	-
5	+	-	-
2.5	+	-	-

+: indicates that the copy number matches the ddPCR result, -: indicates that the copy number does not match the ddPCR result.

Table 5. Statistics of repeatability and stability evaluation results of the detection system.

Standard samples	Repeatability		Stability	
	Mean ± SD	CV %	Mean ± SD	CV %
2:2	0.74 ± 0.10	13.60	0.86 ± 0.15	17.38
3:2	1.39 ± 0.16	11.17	1.52 ± 0.12	7.75
4:2	1.29 ± 0.11	8.41	1.45 ± 0.15	10.27

To ensure robust data collection, each of the six standard samples was subjected to three independent measurements. Number of repetitions (n = 3).

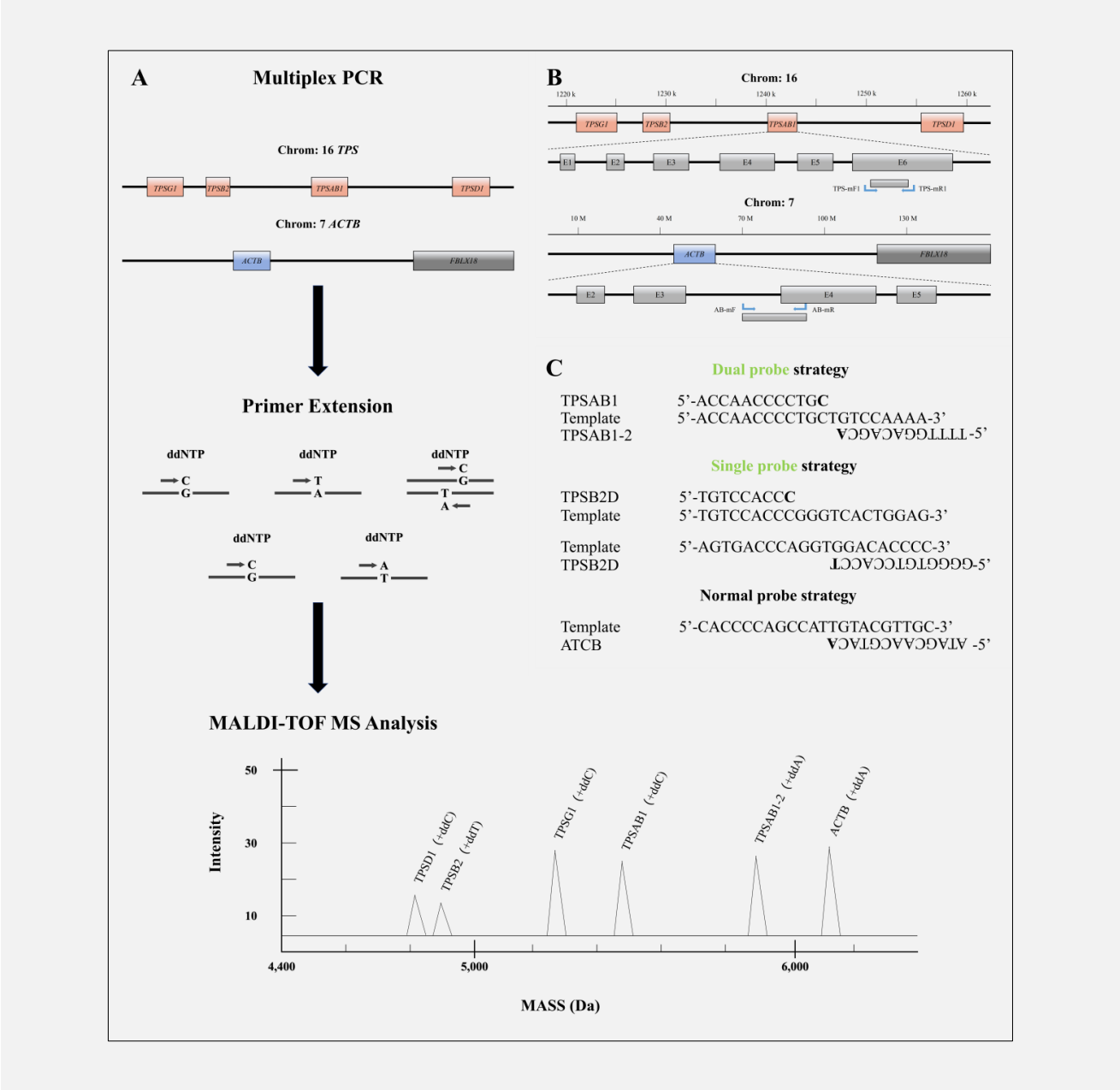
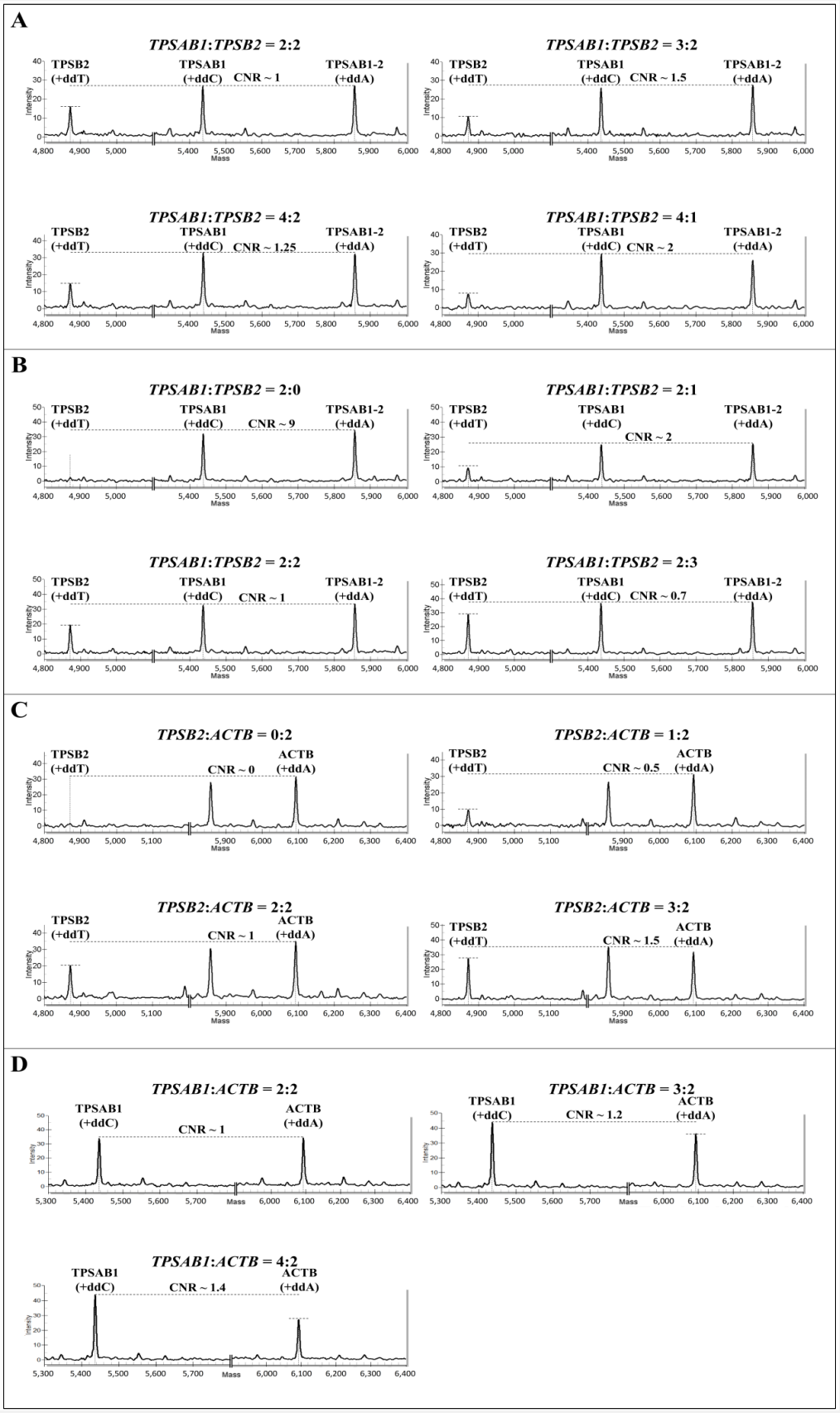


Figure 1. Design of assay scheme.

A: Elucidation of the fundamental technical procedures involved in MALDI-TOF MS analysis. B: Map of multiple PCR amplicons. C: Design diagram of probes using different strategies.


A
TPSAB1:TPSB2 = 2:2
TPSB2 (+ddT)
TPSAB1 (+ddC)
CNR ~ 1
TPSAB1-2 (+ddA)
TPSAB1:TPSB2 = 3:2
CNR ~ 1.5
TPSAB1:TPSB2 = 4:2
CNR ~ 1.25
TPSAB1:TPSB2 = 4:1
CNR ~ 2
B
TPSAB1:TPSB2 = 2:0
CNR ~ 9
TPSAB1:TPSB2 = 2:1
CNR ~ 2
TPSAB1:TPSB2 = 2:2
CNR ~ 1
TPSAB1:TPSB2 = 2:3
CNR ~ 0.7
C
TPSB2:ACTB = 0:2
ACTB (+ddA)
CNR ~ 0
TPSB2:ACTB = 1:2
CNR ~ 0.5
TPSB2:ACTB = 2:2
CNR ~ 1
TPSB2:ACTB = 3:2
CNR ~ 1.5
D
TPSAB1:ACTB = 2:2
TPSAB1 (+ddC)
CNR ~ 1
ACTB (+ddA)
TPSAB1:ACTB = 3:2
CNR ~ 1.2
TPSAB1:ACTB = 4:2
CNR ~ 1.4
Intensity
Mass

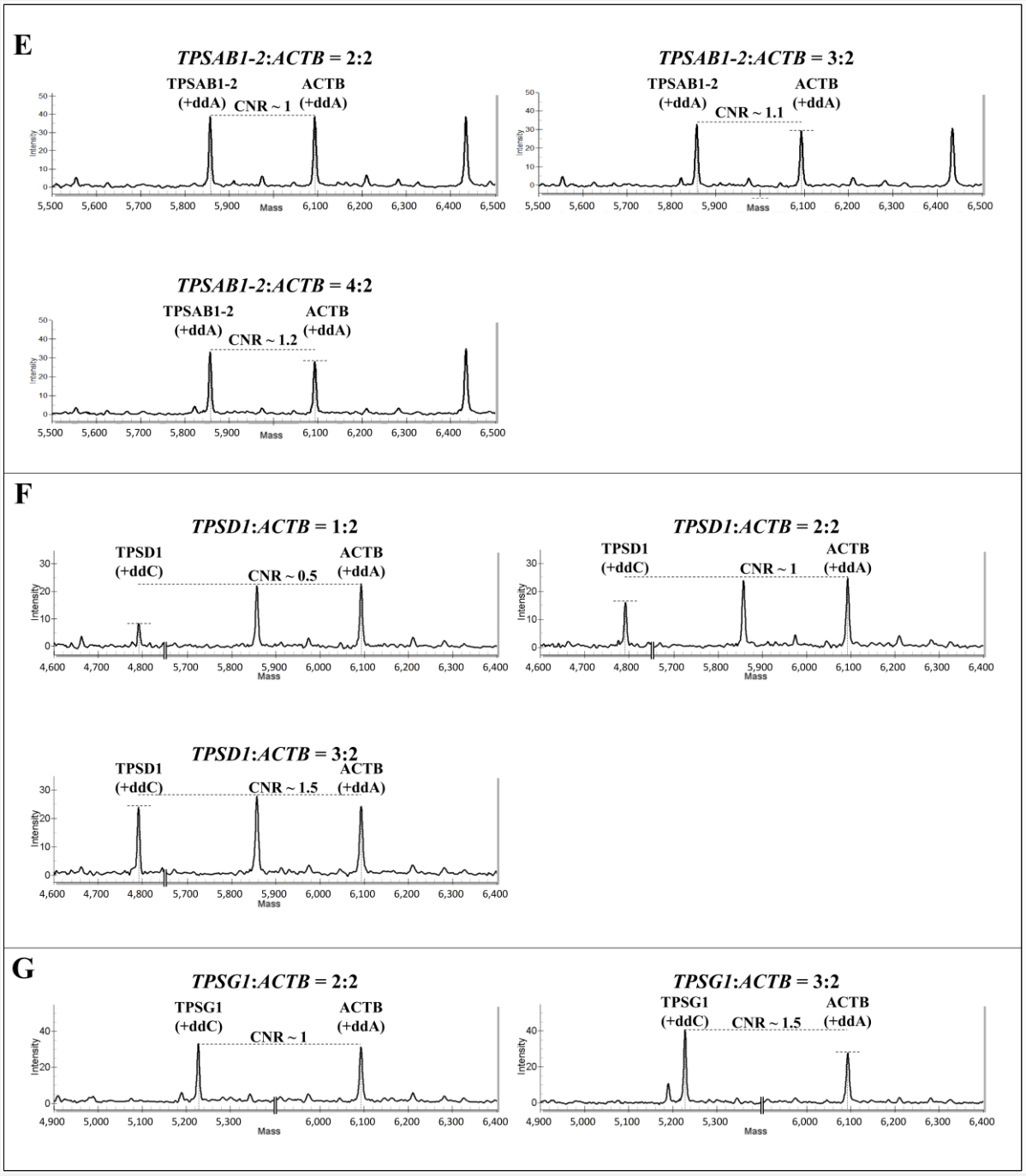


Figure 2. Mass spectrometry results of typical samples and standards.

Each mass spectrogram contains the peaks of target genes within the displayed MWs range.
CNR: The copy number ratio between target gene and reference gene. CNR = (Peak heighttarget(test)/Peak heighttarget(mean))/(Peak heightrefer(test)/Peak heightrefer(mean)).
A: MS results of different proportions of *TPSAB1*:*TPSB2* samples. B: MS results of different proportions of *TPSAB1*:*TPSB2* samples. C: MS results of different proportions of *TPSB2*:*ACTB* samples. D: MS results of different proportions of *TPSAB1*:*ACTB* samples. E: MS results of different proportions of *TPSAB1-2*:*ACTB* samples. F: MS results of different proportions of *TPSD1*:*ACTB* samples. G: MS results of different ::proportions of *TPSG1*:*ACTB* samples.

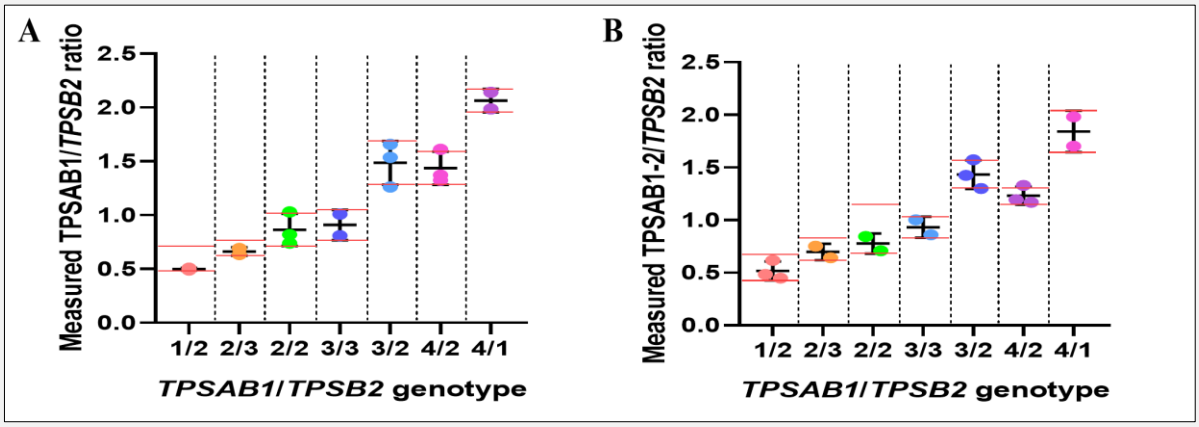


Figure 3. Accuracy evaluation results of standard products.

3A: and B: show the results of probe TPSAB1 to *TPSB2* ratio and probe TPSAB1-2 to *TPSB2* ratio, respectively.

of *TPSAB1*:*TPSB2* copy number ratios. Analytical sensitivity was determined using a stringent detection criterion requiring concurrent positive signals from both the TPSAB1 and TPSAB1-2 probe sets. The results establish a well-defined limit of detection (LOD) of 20 ng for total template DNA. This sensitivity threshold was consistently achieved across all tested copy number variants, confirming the assay's reliability for quantitative genotyping applications.

To evaluate the repeatability and stability of the proposed method, 6 standard samples were systematically analyzed. The repeatability of the method was determined through triplicate measurement of three standard samples within one analysis run, thereby limiting sources of extraneous variation. Method stability was established by performing three repeated analyses of three different standard samples over time under standardized conditions. As quantitatively demonstrated in Table 5, the method exhibited excellent precision performance, with coefficients of variation (CV) of 8.41 - 13.60% for repeatability measurements and 7.75 - 17.38% for stability assessments. These precision metrics confirm the method's robustness and reliability for clinical applications.

MALDI-TOF MS analysis of 274 random samples

A comprehensive MALDI-TOF MS analysis of 274 randomly selected samples was performed. By comparing the mass spectral peak heights to established standards, the prevalence of individuals carrying two or more copies of the *TPSAB1* gene was determined to be 12.41%. Following the removal of all personally identifiable information, the demographic data for these 274 samples - including age, gender, health status, and genotyping results - have been compiled in Supplementary Table S1.

DISCUSSION

Allergic diseases represent a significant global health burden characterized by systemic immune dysregulation [1]. The escalating prevalence of these disorders in recent decades has resulted in substantial healthcare expenditures and marked reductions in quality of life [2]. Their pathogenesis involves complex interactions between genetic predisposition (particularly cytokine-related and immune recognition genes), environmental factors, and epigenetic modifications [3,4]. Genomic studies have elucidated critical pathological mechanisms including Th2-mediated immunity, T-cell differentiation, TGF-β signaling, and regulatory T-cell dysfunction [5]. Among these conditions, mast cell disorders (MCDs) present unique diagnostic challenges due to characteristic mast cell proliferation and aberrant activation leading to pathological degranulation. Notably, trypsin constitutes approximately 25% of total mast cell protein content [6], with a demonstrated gene dosage effect between *TPSAB1* copy number variation and serum basal tryptase levels [7]. This relationship has been incorporated into current diagnostic criteria, where *TPSAB1* copy number analysis significantly improves diagnostic accuracy.

MALDI-TOF MS technology offers distinct advantages for genetic analysis, including high-throughput capability, tolerance to common contaminants, and versatile assay design [8]. While initial instrumentation costs may be higher than alternative methods, this is offset by su-

perior throughput and automation, making it cost-effective for CNV analysis [9-13]. Although primarily applied to single nucleotide variant (SNV) detection, its potential for CNV analysis remains underexplored [14, 15]. Technical challenges are particularly pronounced when analyzing highly homologous gene sequences, requiring specialized experimental approaches [16].

Current CNV detection methods each present limitations: MLPA offers precision but has limited throughput [17,18]. SMRT sequencing reveals structural variations but remains cost-prohibitive [19], while Sanger sequencing and modified PCR methods are vulnerable to amplification biases. Although ddPCR provides absolute quantification, its clinical implementation is constrained by cost [17,20,21]. Compared to established techniques (qPCR, ddPCR, HRM, PCR/CE), MALDI-TOF MS provides superior multiplexing capacity, automated analysis, and cost-efficiency [14,22].

In this study, we successfully adapted MALDI-TOF MS with SBE chemistry for TPS gene analysis. Our relative peak height (RPH) method enables precise quantification by comparing target gene signals against standardized references. Accurate CNV determination requires stringent optimization of multiplex PCR amplification, SAP treatment, and probe design to ensure specific allele detection. This approach overcomes the inherent challenges of analyzing highly homologous sequences while maintaining analytical precision. Our innovative single-tube assay combines: Dual-probe targeting of *TPSAB1* variants, single-probe detection of *TPSB2*/*TPSD1*, ddPCR-validated reference standards, and comprehensive copy number determination. This approach maintains the economic and efficiency advantages of MALDI-TOF MS while overcoming previous limitations in homologous sequence analysis. The methodology's robustness, combined with its throughput and cost-effectiveness, positions it as a valuable tool for both research and clinical applications in allergic disease diagnostics.

The establishment of the MALDI-TOF MS system in this study incorporated multiple methodological refinements to address specific technical challenges. Primer probe design was strategically optimized to overcome the high sequence homology characteristic of trypsin-like genes. This involved selecting targeted regions and deliberately incorporating mismatched bases at the 5' terminus to improve primer-template binding specificity, thereby significantly reducing nonspecific amplification artifacts. Furthermore, molecular weight modifications of primer probes through intentional base mismatches effectively minimized spectral interference from amplification primers, ensuring clear resolution of target peaks in mass spectrometric analysis. Critical optimization of primer and probe concentrations was systematically performed to balance amplification efficiency with product specificity. Suboptimal concentrations risked incomplete product formation and attenuated mass spectral signals, while excessive concentrations promoted primer-dimer artifacts that compromised both PCR efficiency and quantitative accuracy. These parameters were dynamically adjusted throughout experimental iterations to achieve optimal amplification yields and mass spectral peak intensities. For the amplification of GC-rich trypsin-like gene sequences (exceeding 60% GC content), the system incorporated betaine and DMSO as denaturation enhancers, with their concentrations carefully titrated to maximize amplification efficiency while maintaining specificity. Comprehensive reaction condition optimization encompassed precise calibration of thermal cycling parameters, including temperature profiles, incubation durations, and cycle numbers. During SBE system refinement, we implemented a combined annealing/extension protocol with reduced cycling to enhance throughput without compromising analytical sensitivity. These systematic optimizations collectively established a robust MALDI-TOF MS platform capable of highly reproducible detection and quantification of trypsin-like genes, significantly advancing mass spectrometric applications in molecular genetic analysis.

The clinical applicability of this methodology was demonstrated through analysis of 274 population-derived samples, enabling precise quantification of *TPSAB1*, *TPSB2*, *TPSD1*, and *TPSG1* gene copy numbers and determination of *TPSAB1* multi-copy prevalence in the Chinese population. MALDI-TOF MS analysis of 274 Chinese samples revealed a *TPSAB1* copy number gain carrier rate of 12.41%. This prevalence is significantly higher than the 4% - 8% reported in European populartions [7]. This notable disparity may be attributed to positive selection pressure exerted by local parasitic infections [23,24]. Elevated *TPSAB1* copy number, leading to increased levels of trypsin-like enzymes, potentially enhances type 2 immune responses against helminths [25]. Conversely, in European populations, reduced pathogen exposure - consistent with the hygiene hypothesis - likely diminishes the selective advantage associated with this genetic variant [26].

The genes in question, located on chromosome 16, exhibit remarkable sequence homology - particularly between *TPSAB1* and *TPSB2*, which share 99% sequence identity in their reverse-tandem arrangement. While traditional approaches relied on EcoRV restriction digestion for *TPSAB1*/*TPSB2* differentiation, our methodology achieves superior differentiation through comprehensive sequence analysis coupled with optimized primer-probe systems. The study specifically addresses the critical challenge of accurate *TPSAB1*/*TPSB2* copy number quantification. Conventional single-molecule sequencing approaches, while effective, require extensive positive controls and incur substantial costs. Our innovative solution combines T-vector cloning for reference plasmid generation with absolute quantification using ddPCR, establishing precise calibration standards for gene copy number ratios. This foundation enables subsequent high-throughput analysis of clinical samples through integrated amplicon-specific design, ddPCR, and nucleic acid mass spectrometry.

In summary, this study developed an enhanced MALDI-TOF MS platform integrating SBE principles for simultaneous *TPSAB1* and *TPSB2* copy number quantification. The methodology successfully addresses the technical challenges posed by the high homology and reverse orientation of *TPSAB1*/*TPSB2* while overcoming common obstacles including high GC content, primer-dimer formation, and secondary structure interference. These molecular diagnostic advances hold significant implications for allergic disease classification and staging [27,28], providing critical tools for mast cell infiltration assessment, allergic risk stratification, and targeted therapy selection [20,29,30]. Furthermore, the establishment of population-based *TPSAB1* copy number references facilitates more accurate determination of physiological α-tryptase ranges [29], with important implications for clinical decision-making and healthcare resource allocation.

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Ethical Approval Statement:
This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Shenzhen Guangming District Maternal and Child Health Hospital. Informed consent was obtained from all individual participants included in the study.

Declaration of Interest:
The authors have no relevant financial or non-financial interests to disclose.

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