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Molecular Detection of the Major Peanut Allergen Ara h 1 in Food Products Using a Duplex Polymerase Chain Reaction Assay

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ABSTRACT

Peanut allergy is a major global health concern, and undeclared peanut residues in processed foods pose serious risks to sensitized individuals. Reliable analytical methods are essential for accurate labeling and consumer protection, particularly because high-heat processing can cause protein denaturation and DNA fragmentation.

This study assessed the applicability and analytical reliability of a short-amplicon duplex quantitative polymerase chain reaction (qPCR) system targeting the *Ara h 1* gene across diverse peanut genotypes, thermally processed samples, and commercial food matrices. Genomic DNA was extracted from raw peanuts representing 11 varieties, roasted and autoclaved samples, and commercial foods using a modified cetyltrimethylammonium bromide (CTAB)-based protocol. The duplex qPCR assay targeted the *Ara h 1* gene and incorporated *18S rRNA* as an internal amplification control on the Rotor-Gene Q platform.

The assay detected all peanut varieties consistently, with a limit of detection (LOD) of 100 pg of DNA. The short amplicon (<150 bp) enabled reliable detection even after severe thermal processing (autoclaving at 121°C for 15 min). Validation across commercial food products demonstrated that the *18S rRNA* internal control effectively prevented false-negative results in inhibitor-rich matrices, such as chocolate and cocoa-based foods.

These findings confirm that *Ara h 1* is a stable and robust molecular target for detecting trace peanut residues in both raw and processed food products, supporting regulatory compliance and food safety for allergic consumers.

Keywords: Allergens; Arachis; DNA; Food analysis; Food safety; Molecular diagnostic techniques; Plant proteins; Plant; Real-time polymerase chain reaction

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INTRODUCTION

Peanut allergy is a major global health concern due to its early onset, lifelong persistence, and risk of severe

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anaphylaxis.[1,2] Its prevalence has risen markedly in recent decades, especially in Western populations, now affecting about 1% to 3% of children and many adults.[1,3] Because even trace amounts of peanut can provoke serious reactions, precise detection and labeling of peanut-containing foods are essential for consumer safety and allergy management.[2,4]

Ara h 1, a vicilin-type 7S globulin storage protein, is one of the major peanut allergens with strong immunoglobulin E (IgE)-binding and high immunogenicity.[5] Most peanut-allergic individuals are sensitized to Ara h 1, which is closely linked to clinically significant reactions.[6] Owing to its abundance and immunodominance, Ara h 1 serves as a key molecular marker for peanut detection in food products.[7]

Detection of peanut allergens in foods is challenging because processing methods, including roasting, boiling, and autoclaving, modify protein structure through denaturation, aggregation, and Maillard reactions, affecting allergenicity and detectability.[8,9] Thermal treatment can alter IgE binding, with roasting increasing allergenicity compared with raw peanuts.[10] In addition, complex matrices may impair extraction and detection, leading to false-negative or underestimated results.[11] To overcome these issues, DNA-based approaches, such as quantitative polymerase chain reaction (qPCR), are applied, since fragmented DNA remains amplifiable when short targets are used. Designing short amplicons within stable regions of the *Ara h 1* gene enables sensitive detection of peanut traces even in highly processed foods.

Variability among peanut cultivars can affect allergen composition and expression, as genetic differences influence the levels of major allergens, such as Ara h 1, Ara h 2, and Ara h 3.[12] Understanding this variability is important for risk assessment and for standardizing detection methods in regions with multiple cultivars. Reliable analytical tools are therefore essential to ensure regulatory compliance, accurate labeling, and consumer protection.[11,13] However, processing-induced protein modifications may reduce antibody recognition in immunoassays, whereas DNA-based methods offer greater stability and specificity, particularly in highly processed foods.[11,14] Further evaluation of allergen detectability across cultivars, processing conditions, and commercial products remains necessary to improve food allergy risk management.

This study aimed to evaluate the detection of the major peanut allergen Ara h 1 in different peanut varieties, heat-processed samples, and selected commercial foods. By assessing the effects of thermal processing and sample type on detectability, it supports improved allergen monitoring and evidence-based food safety management. We hypothesized that the short-amplicon duplex qPCR system would maintain high sensitivity (limit of detection [LOD] = 100 pg) and diagnostic reliability across diverse genotypes and severe processing conditions, such as autoclaving, providing a more robust alternative to protein-based assays that may lose sensitivity after thermal denaturation.

MATERIALS AND METHODS

Peanut Germplasm Selection, Sampling Strategy, and Matrix Preparation

The study protocol was reviewed and approved by the Ethics Committee of Iran University of Medical Sciences (Approval Code: IR.IUMS.FMD.REC.1402.022).

A panel of 11 peanut cultivars, including 3 Iranian varieties (Guil, Gorgani, and Jonobi) and 8 international cultivars (NC-2, NC-7, Mersin, Improved 306, Improved 308, Shullomit, Filler, and Altyka), was selected to evaluate inclusivity. To ensure high specificity, 11 non-target species, including tree nuts, legumes, and cereals, were included.

For practical validation, a total of 8 commercial food products were purchased from chain supermarkets in Tehran, Iran, including 4 products with declared peanut content and 4 products without peanut declaration (Table 1). These products represented common processed categories with potential allergen cross-contact risk, such as cookies, chocolates, snacks, and desserts. To account for potential batch-to-batch variation, 2 different production lots were analyzed for each commercial product where available. To minimize operator bias and ensure the objectivity of the results, all laboratory-spiked and commercial samples were blind-coded by an independent researcher before DNA extraction and qPCR analysis.

Table 1. Summary of sample types, varieties, sources, and scientific rationale for selection

Sample Category	Specific Samples / Varieties	Source	Rationale for Selection
Peanut Cultivars	Local Iranian landraces (Guil, Gorgani, and Jonobi) International: NC-2, NC-7, Mersin, Improved 306, Improved 308, Shullomit, Filler, and Altyka	Certified seed banks and agricultural suppliers	To validate assay inclusivity by ensuring reliable detection of the *Ara h 1* gene across diverse genetic backgrounds and geographic origins
Non-Target Species	Tree Nuts: Walnut, Almond, Hazelnut, and Cashew Legumes: Soybean, Chickpea, and Lentil Cereals: Wheat, Barley, and Maize	Local retail markets (Tehran, Iran)	To assess analytical specificity and rule out cross-reactivity with taxonomically related legumes or common food-grade cereals
Thermal-Treated Peanuts	Raw peanut, Roasted (180 °C for 20 min), and Autoclaved (121 °C for 15 min) samples	Laboratory-processed	To investigate the impact of industrial thermal processing (roasting and sterilization) on DNA fragmentation and the stability of the short-amplicon target
Spiked Food Matrices	Cookie, Chocolate, Extruded snack, and Multi-ingredient food (laboratory-spiked)	Prepared in-house	To determine the Limit of Detection (LOD) and evaluate the impact of matrix-associated inhibitors (e.g., lipids and polyphenols) on amplification efficiency[a]
Commercial Foods (Market Samples)	8 products total: Positive (n=4): Peanut cookie, Chocolate with peanut, Peanut snack, and Peanut-containing dessert Negative (n=4): Plain cookie, Dark chocolate, Corn snack, and Processed food without peanut	Chain supermarkets (Tehran, Iran)	To validate practical applicability for regulatory compliance and to screen for undeclared peanut contamination in real-world market samples

LOD: limit of detection; NC-2: North Carolina 2; NC-7: North Carolina 7.

Model matrices, such as wheat and corn flour, were spiked with homogenized peanut at 1%, 0.1%, 0.05%, 0.01%, 0.005%, and 0.001% (w/w) and thoroughly mixed for uniform distribution. No naturally contaminated samples with independently confirmed peanut levels were included; the commercial samples served to evaluate the assay's performance in real-world processed matrices and to screen for undeclared peanut residues.

Controls and reagents included purified peanut DNA (positive control), genomic DNA from hazelnut, walnut, cashew, and almond (specificity panel), a plant DNA extraction kit, TaqMan qPCR master mix (Pishgam Plant DNA Isolation Kit; Pishgam Biotechnology Co., Tehran, Iran), Ara h 1-specific primers/probe, and an 18S rRNA internal amplification control (IAC). All samples were milled to fine powder, sealed sterile, and stored at −20°C until DNA extraction and qPCR analysis.

No hazardous or biohazardous materials were used, and all procedures followed standard laboratory biosafety guidelines.

Thermal Processing Models to Assess Allergen Stability

To evaluate the effect of thermal intensity on the

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stability and detectability of the peanut allergen Ara h 1, 2 standardized processing models representing domestic and industrial conditions were applied to selected cultivars and spiked food matrices. In the dry-heat model, kernels were roasted at 180 °C for 20 minutes, promoting Maillard reactions and protein denaturation that may affect allergen extractability and qPCR detection. In the moist-heat model, samples were autoclaved at 121°C for 15 minutes (103.4 kPa) to simulate industrial sterilization, which can cause significant protein and DNA degradation. After treatment, samples were cooled, ground, homogenized, and stored at −20°C until DNA extraction. These models enabled assessment of processing severity on Ara h 1 stability and qPCR detection in raw, spiked, and commercial matrices.

Genomic DNA Extraction and Quality Assessment

Genomic DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol optimized for high-lipid peanut matrices. Briefly, ~100 mg of ground sample was lysed in CTAB buffer, including Tris-HCl, NaCl, ethylenediaminetetraacetic acid (EDTA), CTAB, and β-mercaptoethanol, and incubated at 65°C. Following centrifugation, the aqueous phase was recovered, and DNA was precipitated with cold isopropanol and sodium acetate, incubated at −20°C, washed with 70% ethanol, air-dried, and resuspended in TE buffer. DNA concentration and purity were measured by NanoDrop (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA), and extracts with A260/280 ratios of 1.8 to 2.0 were deemed suitable for real-time PCR. The protocol consistently produced amplifiable DNA from raw, processed, and commercial samples. Contamination was prevented by physical separation of pre- and post-PCR workflows, the use of aerosol-resistant tips, and dedicated equipment. Surfaces were routinely decontaminated, and no-template controls (NTCs) were included in all runs to ensure the integrity of the results.

Molecular Detection of Ara h 1 Allergen-Encoding Gene

The *Ara h 1* gene was detected using a duplex TaqMan qPCR assay targeting a conserved short region of the *Ara h 1* gene (GenBank L34402), enabling efficient amplification from fragmented DNA. Primer and probe sequences for *Ara h 1* (6-carboxyfluorescein [FAM]/black hole quencher-1 [BHQ-1]) and the 18S rRNA internal control (hexachloro-fluorescein [HEX]/black hole quencher-1 [BHQ-1]) are provided in Supplementary Table 1 and were validated using Primer3, OligoAnalyzer, and BLAST.

The duplex qPCR reactions were performed in a total volume of 20 µL. Each reaction contained 10 µL of 2X Real-time PCR Master Mix (Pishgam Biotechnology Co., Tehran, Iran), 300 nM of each forward and reverse primer, 200 nM of each TaqMan probe, and 1.5 µL of template DNA. The specific sequences for the primers and probes used in this study are detailed in Supplementary Table 1. Amplification and detection were carried out using the Rotor-Gene Q platform (Qiagen, Hilden, Germany). The thermal cycling conditions consisted of an initial activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds and a combined annealing/extension step at 60°C for 45 seconds. Fluorescence signals were recorded during the extension phase. To ensure reliability, positive controls and no-template controls (NTCs) were included in each run, and all samples were analyzed at least in duplicate. For performance evaluation experiments, such as repeatability and reproducibility, reactions were run in triplicate. Results were accepted only when the internal control (18S rRNA) produced Ct values within the expected range.

Assessment of PCR Inhibition and Internal Amplification Control

PCR inhibition was assessed by continuously monitoring the internal amplification control (IAC). The 18S rRNA target, detected with a HEX-labeled probe (Supplementary Table 1), was co-amplified in each duplex reaction to assess DNA quality and matrix-derived inhibitors. Sample IAC Ct values were compared with non-inhibitory reference reactions containing purified peanut DNA. Samples were considered non-inhibitory when 18S Ct remained within ±1 to 1.5 cycles of the reference mean. A larger Ct shift or lack of IAC amplification indicated partial or complete inhibition, prompting re-extraction or dilution (1:5 or 1:10) before re-testing. This IAC strategy ensured that *Ara h 1* signals reflected true detection rather than inhibition-related false negatives.

Analytical Performance Evaluation

The analytical performance of the duplex qPCR assay was evaluated according to EN ISO 24276 and

Codex Alimentarius (CAC/GL 74-2010)[15].

The assay was further described in line with key Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) reporting principles.[27] A 10-fold serial dilution of peanut DNA (1000 to 0.1 pg per reaction) was used to construct standard curves. Amplification efficiency (E) was calculated as: $E = (10^{-1/\text{slope}}-1)\times 100$. The limit of detection (LOD) was defined as the lowest peanut DNA concentration that produced consistent amplification in at least 95% of replicate reactions and was determined to be 100 pg per reaction. The limit of quantification (LOQ) was considered the lowest concentration at which amplification remained sufficiently reproducible for reliable quantification; concentrations of 10 pg and below showed variable amplification and were therefore considered below the reliable quantification range.

Analytical specificity was assessed using genomic DNA from *Arachis hypogaea* and various non-target plant species. The absence of *Ara h 1* amplification in non-targets and consistent 18S rRNA amplification confirmed assay specificity, DNA integrity, and lack of PCR inhibition.

Repeatability and inter-run reproducibility were tested with peanut DNA at 1000 pg and 100 pg per reaction across 3 independent runs, each analyzed in triplicate. Ct values for Ara h 1 and 18S rRNA were used to calculate intra- and inter-assay standard deviation (SD) and coefficient of variation (CV%), and run-to-run variation was compared.

To assess the overall workflow reproducibility, including extraction-to-extraction variability, biological replicates were analyzed. Specifically, 3 independent DNA extractions were performed from separate aliquots of the same homogenized food matrices, such as spiked cookie and spiked chocolate. Each extract was then analyzed by duplex qPCR in triplicate, and extraction-to-extraction variability was expressed as the coefficient of variation (CV%) of Ct values for Ara h 1 and the 18S rRNA internal control.

Assay robustness was verified using a 10-fold serial dilution of peanut DNA, where stable 18S rRNA amplification confirmed consistent performance across a broad template range.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Data distribution was assessed for normality using the Shapiro-Wilk test. As the data showed normal distribution, parametric tests were applied. Differences among treatment groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey post hoc test, while comparisons between 2 groups were performed using the unpaired Student t-test .Ct values are presented as mean±standard deviation (SD). Repeatability and inter-assay reproducibility were assessed by calculating the SD and coefficient of variation (CV%). In addition to SD and CV%, analytical sensitivity was evaluated by determining the LOD, defined as the lowest peanut DNA concentration consistently detected in at least 95% of replicate reactions. A $p<0.05$ was considered statistically significant.

RESULTS

Assay Performance and Internal Control Verification

The duplex assay showed clean background signals, with the no-template control remaining undetermined for both targets (Table 2). The positive control (10 ng peanut DNA) yielded strong amplification, with Ara h 1 at 22.1±0.3 and the internal control at 15.4±0.2. Raw and processed peanut samples were clearly positive, with Ct values increasing from 23.8±0.5 in raw kernels to 27.6±0.4 after roasting and 30.9±0.6 following autoclaving. In spiked food matrices, Ara h 1 was consistently detected (Ct 26.4 to 28.7), and the internal control remained within 16.0 to 17.5, indicating no significant inhibition. Non-spiked wheat flour and chocolate showed undetermined Ara h 1 but valid internal control Cts (16.0±0.3 and 17.2±0.3), confirming them as true negatives.

Detection of Ara h 1 across peanut varieties

The Ara h 1 target was successfully detected in all eleven peanut varieties, including the three Iranian varieties Guil, Gorgani, and Jonobi, as well as the eight international cultivars North Carolina 2 (NC-2), North Carolina 7 (NC-7), Mersin, improved 306, Improved 308, Shullomit, Filler, and Altyka. As shown in Table 3, the Ct values for Ara h 1 exhibited a narrow range across all varieties (17.5–18.2), with no statistically significant differences observed ($p>0.05$). This high level of consistency demonstrates that genetic diversity among cultivars does not influence assay performance. The internal control (18S rRNA) also showed stable

amplification across samples, confirming comparable DNA quality and the absence of inhibitors. Overall, these results indicate that the developed duplex qPCR assay provides reliable and cultivar-independent detection of Ara h 1.

Table 2. Performance of the duplex assay in controls and representative samples

Sample Type	*Ara h 1* Ct (Mean±SD)[a]	Internal Control Ct (Mean±SD)	Interpretation
No-template control (NTC)	Undetermined	Undetermined	No amplification
Positive control (peanut DNA 10 ng)	15.2±0.4	15.4±0.2	Valid positive
Raw peanut sample	23.8±0.5	16.2±0.3	Positive
Roasted peanut (180 °C, 20 min)	27.6±0.4	16.8±0.3	Positive (expected Ct shift)
Autoclaved peanut (121 °C, 15 min)	29.7±0.7	17.5±0.4	Positive (reduced signal)
Wheat flour spiked (1%)	26.4±0.4	16.1±0.2	Positive
Chocolate matrix spiked (1%)	28.7±0.5	17.3±0.4	Positive (matrix effect mild)
Non-spiked wheat flour	Undetermined	16.0±0.3	True negative
Non-spiked chocolate	Undetermined	17.2±0.3	True negative

Ct value represents the mean±SD obtained from independent experimental runs.
Ct: cycle threshold; NTC: no-template control; SD: standard deviation.

Table 3. Detection of the peanut allergen Ara h 1 across different peanut cultivars using duplex qPCR

No.	Peanut Variety / Cultivar	Origin	Ct (Ara h 1) (Mean±SD)[a]	Ct (18S rRNA) (Mean±SD)	Interpretation
1	Guil	Iranian	17.5±0.21	16.8±0.3	Positive
2	Gorgani	Iranian	17.6±0.3	16.9±0.3	Positive
3	Jonobi	Iranian	17.7±0.5	17.0±0.2	Positive
4	North Carolina 2 (NC-2)	International	17.8±0.53	16.7±0.4	Positive
5	North Carolina 7 (NC-7)	International	17.9±0.11	16.8±0.41	Positive
6	Mersin	International	18.0±0.7	16.9±0.6	Positive
7	Improved 306	International	17.6±0.5	17.1±0.33	Positive
8	Improved 308	International	17.8±0.66	16.8±0.5	Positive
9	Shullomit	International	17.9±0.35	16.9±0.7	Positive
10	Filler	International	18.1±0.4	17.0±0.1	Positive
11	Altyka	International	18.2±0.2	17.1±0.4	Positive

Ct: cycle threshold; rRNA: ribosomal RNA; SD: standard deviation.

Detection of Ara h 1 in Peanut Samples Under Different Processing Conditions

As shown in Figure 1, raw peanuts had the lowest Ct (23.8±0.5), indicating high DNA integrity. These values are from processed ground material, whereas the lower Ct values in Table 3 (17.5 to 18.2) correspond to purified genomic DNA from different cultivars. Roasting at 180 °C for 20 minutes increased the Ct to 26.8±0.5, and autoclaving at 121 °C for 15 minutes yielded the highest Ct (29.7±0.7), consistent with progressive DNA degradation.

Despite these increases, Ara h 1 remained detectable after autoclaving, underscoring the advantage of the short-amplicon design (<150 bp) for sensitive detection in degraded DNA. Internal control Ct values were stable across treatments, confirming consistent DNA quality and the absence of PCR inhibition. Thus, although heating elevated Ct values, the duplex qPCR assay reliably detected Ara h 1 under all tested conditions.

Detection of Ara h 1 in Spiked and Commercial Food Matrices

The duplex qPCR assay reliably detected Ara h 1 in all spiked processed food matrices (Table 4). Furthermore, the assay was applied to 8 commercial market samples (4 declared positive and 4 without peanut declaration) to verify its practical applicability. Cookies showed the lowest Ct (24.7±0.5) due to efficient DNA recovery, while chocolate produced higher values (27.8±0.6), likely due to cocoa-associated inhibition. Extruded snacks showed intermediate Ct (26.3±0.4), and composite matrices the highest (29.6±0.7). Internal control Ct values remained stable (16.5±0.3 to 17.9±0.5), confirming the absence of PCR inhibition.

Commercial products with declared peanut produced clear Ara h 1 amplification (Ct = 20.7±0.25 to 23.5±0.34), whereas peanut-free samples were negative with consistent internal control signals (Ct ≈ 14). Overall, the assay showed robust *Ara h 1* detection across diverse food matrices.

Sensitivity and Precision of the Duplex qPCR Assay

As shown in Table 5, the duplex qPCR assay demonstrated high analytical sensitivity and precision across a 10-fold dilution series (1000 pg to 0.1 pg). Ara h 1 was reliably detected at 1000 pg and 100 pg, with mean Ct values of 17.9±0.28 and 21.3±0.33, respectively, showing low inter-assay SD (0.15 to 0.20) and CV% < 1%, confirming good repeatability and reproducibility at and above the LOD (100 pg).

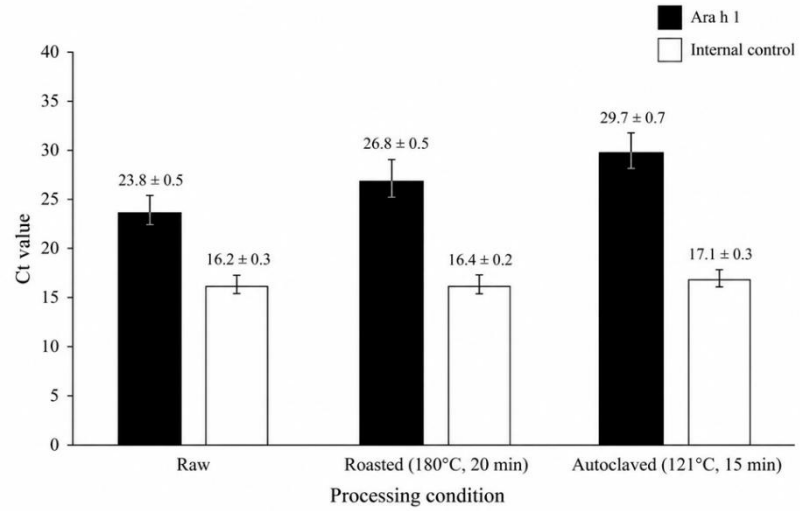

Figure 1. Comparison of Ct values for *Ara h 1* and 18S rRNA internal control in peanut samples under different thermal treatments. Raw peanuts showed strong Ara h 1 amplification; roasting at 180°C for 20 min led to a moderate increase in Ct, while autoclaving at 121°C for 15 min resulted in the highest Ct values, indicating progressive DNA degradation. The stability of the 18S rRNA internal control across all treatments confirms consistent DNA integrity and assay performance. Data represent the mean±SD from independent duplicate runs. Abbreviations: Ct: cycle threshold; rRNA: ribosomal RNA; SD: standard deviation.

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Table 4. Detection of Ara h 1 in spiked and commercial food matrices using the duplex qPCR assay

Sample Category	Sample Type / Product	Ara h 1 Ct (Mean±SD)	Internal Control Ct (Mean±SD)	Interpretation / Result
Spiked processed matrices	Cookie matrix (spiked)	24.7±0.5	16.5±0.3	Positive
	Chocolate matrix (spiked)	27.8±0.6	17.2±0.4	Positive (matrix effect)
	Extruded snack matrix (spiked)	26.3±0.4	16.8±0.3	Positive
	Multi-ingredient food matrix (spiked)	29.6±0.7	17.9±0.5	Positive (complex matrix)
Commercial food products (market samples)[a]	Peanut cookie	21.3±0.28	14.1±0.22	Detected
	Chocolate with peanut	22.0±0.31	14.3±0.24	Detected
	Peanut snack	20.7±0.25	13.9±0.20	Detected
	Peanut-containing dessert	23.5±0.34	14.4±0.27	Detected
	Plain cookie (no peanut declared)	ND	14.2±0.23	ND
	Dark chocolate (no peanut declared)	ND	14.0±0.21	ND
	Corn snack (no peanut declared)	ND	14.3±0.26	ND
	Processed food without peanut	ND	14.1±0.24	ND

[a]Commercial products without peanut declaration were screened for potential hidden/undeclared peanut contamination.
[b]Ct: cycle threshold; ND: not detected (no amplification before cycle 40); qPCR: quantitative polymerase chain reaction; SD: standard deviation.

Table 5. Sensitivity and precision of the duplex qPCR assay for Ara h 1 detection across serial DNA dilutions

DNA Concentration, pg/reaction	*Ara h 1* Ct (Mean±SD)[a]	18S rRNA Ct (Mean±SD)	SD (Inter-assay)	CV% (Intra-assay)	CV% (Inter-assay)	CV% (Between Extractions)	Detection Outcome
1000	17.9±0.28	14.1±0.22	0.15	1.42	0.85	1.56	Detected
100 (LOD)	21.3±0.33	14.2±0.24	0.20	1.45	0.93	1.82	Detected (LOD)
10	25.0±0.41	14.3±0.26	NA	NA	NA	NA	Detected (variable)
1	28.7±0.52	14.4±0.28	NA	NA	NA	NA	Weak / inconsistent
0.1	33.5±0.63	14.6±0.30	NA	NA	NA	NA	Sporadic / unreliable

[a]Values represent mean Ct±SD from three independent experiments. Amplification at ≤10 pg/reaction was inconsistent and considered below the limit of quantification (LOQ).
[b]Ct: cycle threshold; CV: coefficient of variation; LOD: limit of detection; LOQ: limit of quantification; NA: not applicable; qPCR: quantitative polymerase chain reaction; rRNA: ribosomal RNA; SD: standard deviation.

Table 5 also shows that the assay maintained high reproducibility across independent DNA extractions. In addition to technical precision, the reproducibility of the assay across independent DNA extractions was also evaluated using representative spiked food matrices. For spiked cookie and chocolate samples, mean Ct values for *Ara h 1* remained consistent across 3 independent extraction procedures. The extraction-to-extraction variability, representing biological replicates, showed a coefficient of variation (CV%) of less than 3.5%, while intra-assay and inter-assay CV% remained below 2.1%. These findings indicate that the developed method is robust and that DNA extraction efficiency is sufficiently consistent even in complex food matrices, supporting reliable detection of peanut traces.

Amplification Profiles and Standard Curves

For *Ara h 1*, amplification curves progressively shifted right as DNA concentration decreased from 1000 pg to 0.1 pg per reaction, indicating increasing Ct values. The standard curve showed strong linearity (R^2=0.99086), a slope of −3.346, and ~100% amplification efficiency, confirming reliable quantification across the assay's dynamic range.

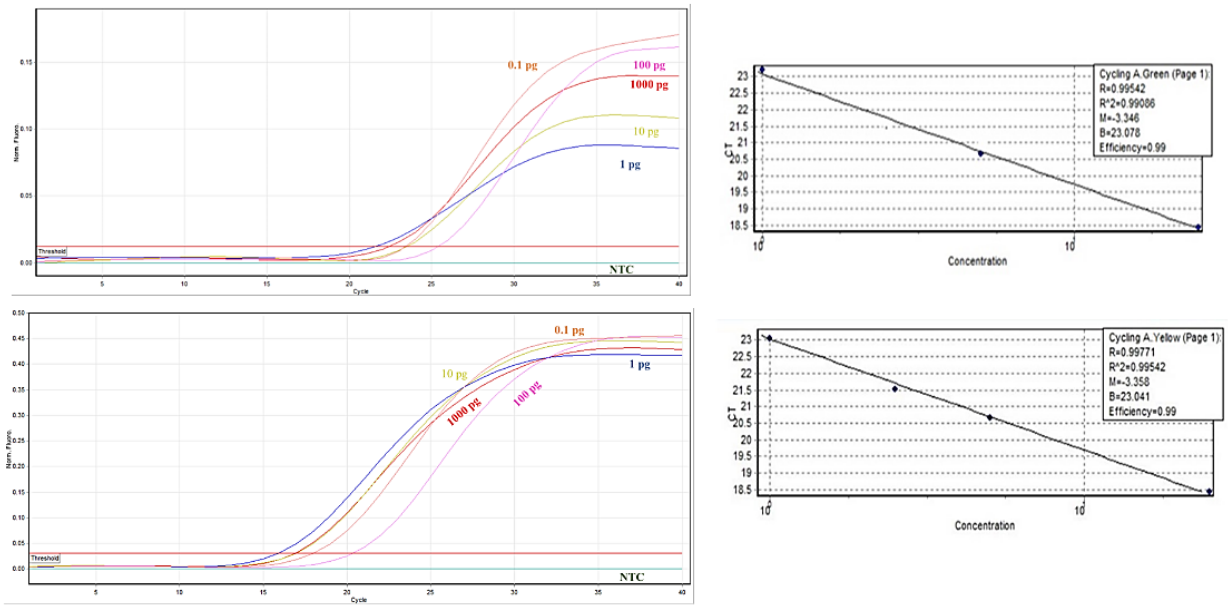

Figure 2. Amplification plots and standard curves for *Ara h 1* and 18S rRNA generated from 10-fold serial dilutions of peanut genomic DNA (1000 to 0.1 pg). A. *Ara h 1* curves show a consistent shift toward higher Ct values with decreasing DNA concentrations, demonstrating high linearity (R^2=0.99086) and 99% efficiency. B. The 18S rRNA internal control shows stable amplification across dilutions with high linearity (R^2=0.99542) and 99% efficiency, confirming assay reliability. Abbreviations: Ct: cycle threshold; pg: picogram; rRNA: ribosomal RNA.

Non-Target Amplification Assessment

The specificity of the duplex qPCR assay was assessed using DNA from non-target plant species commonly found in processed foods, including tree nuts, legumes, and cereal grains. As anticipated, none of the non-peanut samples showed any detectable Ara h 1 amplification, while Ct values for the internal control (18S rRNA) remained consistent, confirming DNA quality and the absence of inhibition. Strong Ara h 1 signals were observed exclusively in peanut samples, verifying high analytical specificity and no cross-reactivity with closely related or co-processed species. Detailed results are summarized in Table 6, which shows true-positive amplification for *Arachis hypogaea* and true-negative results for all other tested species.

Assay Consistency Across Runs

The stability of the duplex qPCR assay was confirmed through 3 independent runs at 1000 pg and 100 pg DNA inputs. At 1000 pg, *Ara h 1* showed tightly clustered Ct values (17.5±0.25 to 17.8±0.27), while 18S rRNA remained similarly stable (13.9±0.18 to

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14.1±0.21). At the LOD level (100 pg), Ara h 1 exhibited only minor run-to-run variation (21.2±0.29 to 21.6±0.34), with consistent 18S rRNA amplification (14.1±0.22 to 14.3±0.25). These narrow Ct ranges and low variability demonstrate good inter-assay precision, confirming the robustness and reproducibility of the duplex assay across independent experiments. Minor Ct fluctuations in raw peanut samples also remained within acceptable inter-assay limits (CV%<2.5%), further supporting platform stability. Together with the low extraction-to-extraction variability observed for representative spiked matrices, these findings further confirm the overall reproducibility of the assay workflow.

Table 6. Assessment of non-target amplification using duplex qPCR

Common Name	Scientific Name	*Ara h 1* Ct (Mean±SD)	18S rRNA Ct (Mean±SD)	Interpretation
Peanut (Positive control)	*Arachis hypogaea*	16.8±0.27	12.9±0.21	True positive
Walnut	*Juglans regia*	ND	14.7±0.23	True negative
Almond	*Prunus dulcis*	ND	15.1±0.26	True negative
Hazelnut	*Corylus avellana*	ND	15.4±0.28	True negative
Cashew	*Anacardium occidentale*	ND	15.8±0.32	True negative
Soybean	*Glycine max*	ND	16.2±0.35	True negative
Chickpea	*Cicer arietinum*	ND	16.4±0.31	True negative
Lentil	*Lens culinaris*	ND	16.9±0.38	True negative
Wheat	*Triticum aestivum*	ND	17.1±0.40	True negative
Barley	*Hordeum vulgare*	ND	17.3±0.42	True negative
Maize	*Zea mays*	ND	17.6±0.45	True negative

[a]Ct: cycle threshold; ND: not detected; rRNA: ribosomal RNA; SD: standard deviation.

DISCUSSION

This study demonstrates the reliability of a short-amplicon duplex qPCR assay for detecting the major peanut allergen Ara h 1 across diverse matrices and processing conditions. The system consistently identified *Arachis hypogaea* DNA in multiple cultivars, heat-treated samples, and complex commercial products, supporting its robustness as a molecular tool for allergen monitoring.[16]

Real-time PCR has been widely used for peanut detection in processed foods because of its sensitivity and suitability for complex matrices. Stephan et al developed a peanut-specific real-time PCR assay targeting the *Ara h 2* coding region and reported a detection limit below 10 ppm in processed foods, with no cross-reactivity with 22 common food ingredients.[17] Puente-Lelievre and Eischeid later evaluated a multiplex real-time PCR assay using chloroplast DNA markers (*matK*, *rpl16*, and *trnH-psbA*) and achieved limits of detection ranging from 0.1 ppm to 1 ppm in several food matrices, including baked goods, chocolate, and tomato sauces.[18] In addition, Costa et al described and validated quantitative real-time PCR approaches for detecting allergenic species in foods, highlighting the importance of molecular methods for allergen monitoring and control in processed products.[19] In comparison with these studies, the duplex qPCR assay developed in the present study targets *Ara h 1* together with 18S rRNA as an internal amplification control. The inclusion of this endogenous control may improve the reliability of detection, particularly in complex or inhibitor-rich food matrices.

Consistent detection of *Ara h 1* across 11 peanut cultivars indicates that the primers and probe target a highly conserved genomic region, enabling reliable identification of local and imported varieties without false negatives from allelic variation. This agrees with genomic evidence showing strong conservation of major storage proteins, including the vicilin-type Ara h 1,[20] supporting its suitability for global allergen monitoring and food labeling compliance.

Thermal processing complicates allergen detection due to protein denaturation and DNA degradation. The selected roasting and autoclaving treatments were intended to model commercially relevant thermal

processing intensities. Roasting reflects dry-heat conditions commonly used in peanut-based snacks and confectionery products, whereas autoclaving represents a more severe thermal stress condition relevant to sterilized or highly processed composite foods. In this study, increasing Ct values from raw to roasted and autoclaved samples reflected heat-induced depurination and strand breakage.[21,22] However, Ara h 1 remained detectable after autoclaving at 121°C, highlighting the advantage of the <150-bp short-amplicon design, which is less susceptible to processing damage and enables reliable detection of fragmented DNA, consistent with recent studies on allergen detection in processed foods.[21,22]

The inclusion of 18S rRNA as an internal amplification control (IAC) ensured diagnostic reliability. Because complex foods, such as chocolate and biscuits, may contain PCR inhibitors, including polyphenols, polysaccharides, and lipids, stable 18S Ct values confirmed that the modified CTAB extraction effectively removed inhibitory compounds.[21,22] This interpretation was further supported by spike-recovery results, which showed no significant matrix-related loss of amplification efficiency in inhibitor-rich samples. Thus, the absence of Ara h 1 amplification in peanut-free foods represents true negatives rather than assay failure. Incorporating an internal control is also required by European genetically modified organism (GMO) laboratory guidelines and the Codex Alimentarius. 18S rRNA was selected as the IAC due to its high copy number and broad conservation across plant species. While we acknowledge that endogenous plant DNA levels may vary in highly processed or multi-ingredient foods, our validation demonstrated consistent 18S rRNA amplification across all tested matrices, including cookies, chocolates, and snacks. In this assay, the IAC functions primarily as a qualitative marker confirming DNA amplifiability and the absence of PCR inhibition. Accordingly, the 18S signal is interpreted as a reliable indicator of extraction success rather than a quantitative normalization factor for *Ara h 1* detection.

The assay showed high analytical sensitivity and precision, supporting routine use. It achieved an LOD of 100 pg peanut DNA per reaction, near-100% efficiency, and good linearity, enabling robust quantification across a wide dynamic range and meeting international validation criteria.[23] Repeatability and inter-run reproducibility showed negligible operator- or condition-related variation, supporting inter-laboratory harmonization.[23] Minor variations in Ct values observed across different experimental assessments represent normal inter-assay variability and remain within the acceptable precision range (CV%<2.5%), further supporting platform stability. Together with the low extraction-to-extraction variability observed for representative spiked matrices, these findings further confirm the overall reproducibility of the assay workflow.

The clinical relevance of the assay was also evaluated. The achieved LOD of 100 pg peanut DNA per reaction suggests that the method can detect trace contamination at levels below those generally considered clinically relevant for peanut-allergic consumers. Thus, the duplex qPCR assay provides sufficient analytical sensitivity for real-world allergen monitoring and food safety control.

PCR detection of the *Ara h 1* gene indicates the presence of peanut-derived material but does not directly measure intact allergenic proteins; therefore, molecular positivity should be interpreted as evidence of peanut contamination rather than a direct estimate of clinical risk.

Specificity evaluation against a panel of potentially cross-reactive species, including other nuts and legumes, revealed that amplification occurred exclusively for *Arachis hypogaea*. The absence of cross-reactivity validates the high discriminatory power of the designed primers and probes, aligning with recent molecular allergen studies that emphasize species-specific probe design to prevent misidentification in multi-ingredient foods.[24]

Although enzyme-linked immunosorbent assay (ELISA) is the standard method for food allergen detection, its reliance on intact protein epitopes may produce false negatives in processed foods where proteins are denatured or glycated by heat or Maillard reactions. These modifications can mask Ara h 1 epitopes and reduce ELISA sensitivity. In contrast, the duplex qPCR assay targets stable DNA fragments, enabling reliable detection even in heat-treated or chemically modified samples as long as amplifiable DNA persists. This method therefore provides a robust and sensitive approach for detecting hidden allergens in complex food matrices, with a detection limit of 100 pg that meets Codex Alimentarius performance criteria.[25,26]

Recent advancements in allergen detection include multiplex qPCR, digital PCR (dPCR), and commercial kits. While multiplex platforms increase throughput by detecting multiple targets simultaneously, they often face challenges such as primer competition and complex optimization. In contrast, our duplex assay prioritizes

robustness and sensitivity for targeted *Ara h 1* detection. Although dPCR provides absolute quantification and higher tolerance to inhibitors, its high instrumentation costs and lower throughput currently limit its routine industrial deployment compared with qPCR.[27]

Compared with protein-based methods like ELISA, which are susceptible to epitope masking during thermal processing, our DNA-based approach targets a stable 77-base pair (bp) fragment, ensuring reliability in highly processed matrices.[27] While commercial kits offer standardized protocols, our assay provides full methodological transparency according to MIQE guidelines, offering a cost-effective and scalable solution for industrial quality control.[13,27] Consequently, this method serves as an efficient screening tool that can complement dPCR or ELISA for comprehensive allergen management. A comparison of commonly used allergen detection methods is summarized in Table 7.

Table 7. Comparison of commonly used allergen detection methods

Method	Sensitivity	Cost / Sample	Throughput	Strengths	Limitations
Singleplex qPCR (this study)	High	Low–moderate	High	Fast, scalable, and QC-friendly	DNA ≠ protein; needs extraction
Multiplex qPCR	High (sometimes slightly reduced)	Lower per target	Very high	Multi-allergen in one run	Complex optimization
ddPCR/dPCR	Very high	High	Moderate	Absolute quantification; robust	Expensive; slower
ELISA	Moderate–high	Moderate	Moderate	Protein-based relevance	Affected by processing/matrix
LFD	Moderate	Moderate	Very high	On-site rapid	Mostly qualitative

[a]ddPCR: droplet digital polymerase chain reaction; dPCR: digital polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; LFD: lateral flow device; QC: quality control; qPCR: quantitative polymerase chain reaction.

Overall, the analytical performance achieved in this study indicates that the developed duplex qPCR assay meets the main requirements expected for molecular allergen detection, including high inclusivity, specificity, sensitivity, precision, and tolerance to matrix-related effects.[16,23] However, the importance of these findings extends beyond analytical performance alone. For peanut-allergic consumers, even small amounts of undeclared or unintentionally introduced peanut material may represent a serious health concern. Therefore, a reliable molecular assay can contribute to public health protection by helping manufacturers and regulatory laboratories detect hidden peanut-derived material, reduce labeling errors, improve traceability, and strengthen confidence in food safety monitoring systems. In this context, implementation of a validated duplex qPCR system in industrial quality-control workflows may support regulatory compliance and enhance transparency in international food trade.[28]

Although Ara h 2 is clinically regarded as one of the most predictive allergens for severe peanut reactions, Ara h 1 was selected in the present assay for analytical reasons related to food-safety screening. Ara h 1 is an abundant and genetically conserved vicilin-type storage protein, making it a suitable molecular marker for detecting peanut-derived biomass across different cultivars and processing conditions. In addition, the use of a single peanut-specific target reduced the risk of primer-probe competition and helped maintain the high sensitivity of the duplex format, with an LOD of 100 pg. Nevertheless, this choice also has limitations. Because the assay relies on a single peanut genomic target, severe processing or extensive DNA fragmentation could potentially reduce amplification of the *Ara h 1* locus and lead to underestimation or false-negative results in some highly degraded samples. Therefore, future multiplex assays combining *Ara h 1, Ara h 2*, and *Ara h 3* would provide broader allergen coverage and may improve the reliability of peanut detection in more diverse food matrices.

While this study provides a strong analytical basis for peanut allergen detection, its scope should be interpreted carefully. The validation was performed in a

single-center laboratory setting, which allowed strict control of experimental conditions but does not fully represent the variability encountered in routine industrial testing. Real-world food products may differ in ingredient composition, processing intensity, DNA quality, and inhibitor content. Although the assay performed well across the tested high-risk commercial matrices, further evaluation using a wider range of naturally contaminated industrial samples is needed to better assess its diagnostic sensitivity under practical conditions. In addition, this study focused on molecular validation of the duplex qPCR system; therefore, direct comparative studies with protein-based methods, such as ELISA, and with commercial allergen detection kits would provide a more complete benchmark for its application in food-safety monitoring programs.

Future work should therefore focus on expanding the present duplex assay into a multiplex qPCR platform for simultaneous detection of multiple peanut allergens or other food allergen targets. Incorporating complementary technologies, such as digital PCR or next-generation sequencing, may also improve quantification, traceability, and confirmation of allergen contamination in highly processed foods. Finally, integration of this method into standardized monitoring programs, inter-laboratory validation studies, and routine quality-control workflows would support global harmonization, regulatory acceptance, and more effective protection of allergic consumers.

In conclusion, this study demonstrates the practical efficacy of a duplex qPCR system for the surveillance of peanut allergens in the modern food chain. The assay's ability to overcome genetic diversity among varieties and remain functional after severe thermal processing makes it a suitable candidate for integration into food safety quality control protocols. This approach not only enhances the accuracy of food labeling but also contributes to the safety of highly sensitive allergic consumers by providing a reliable tool for identifying even trace amounts of degraded peanut DNA.

STATEMENT OF ETHICS

The study protocol was approved by the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran (Approval Code: IR.IUMS.FMD.REC.1402.022). All procedures were performed in accordance with the ethical standards of the institutional and/or national research committee.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

The authors declare that no artificial intelligence (AI) or AI-assisted technologies were used in the conception, study design, data collection, analysis, or preparation of this manuscript.

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