

Development and Evaluation of a Real-Time PCR Multiplex Assay for the Detection of Allergenic Peanut Using Chloroplast DNA Markers

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Supporting Information

ABSTRACT: Peanut is one of the most commonly consumed allergy-causing foods in the United States. Prevention of accidental consumption by allergic individuals is assisted by methods that effectively identify the presence of peanut in food, even at trace levels. This study presents a multiplex real-time polymerase chain reaction (PCR) assay that uses chloroplast markers (*matK*, *rpl16*, and *trnH-psbA*) to specifically detect peanut in three types of foods: baked goods, chocolate, and tomato sauces. Food matrices were spiked with raw peanut at concentrations ranging from 0.1 to 10⁵ ppm. The assay was evaluated with respect to linear range and reaction efficiency. High reaction efficiencies were generally obtained across 6–7 orders of magnitude. Limits of detection were between 0.1 and 1 ppm, and reaction efficiencies were mostly within the preferred range of 100 ± 10%. Our results indicate that real-time PCR assays using chloroplast markers can be a valuable tool for peanut detection.

KEYWORDS: peanut, qPCR, food allergen detection, chloroplast markers

INTRODUCTION

It is estimated that about two percent of adults and five percent of the infant population in the United States suffer from food allergies. The Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) stipulates that eight major food groups, which account for 90% of food allergies, must be declared on the label. These are milk, eggs, fish, Crustacean shellfish, tree nuts, peanuts, wheat, and soybeans.¹

Peanut, *Arachis hypogaea* (Fabaceae), is one of the most consumed legumes in the world and is an important source of human nutrition. Nonetheless, peanut is also one of the most common allergy-causing foods in the United States and affects at least 3 million people. Peanut allergy is one of the most severe food allergies because of its persistence throughout the lifetime of individuals and its severity even at very low doses.^{2–4} Moreover, its prevalence in North America and Europe has steadily increased during the past 10 years, especially in children.^{5,6}

Numerous allergenic proteins have been identified in peanut.⁷ Traditionally, these allergens have been detected in food products using ELISA,^{8–11} and other protein-based methods such as Western blot,^{12,13} mass spectrometry,^{14–16} and biosensors.¹⁷ However, food composition and the manner in which food is processed (particularly thermal processing) can mask or modify proteins and impact the detection and quantitation of peanut using protein-based methods.¹⁸ In such cases, the detection of the allergenic food via DNA has some advantages even though it does not provide direct detection of the allergen itself.^{19,20}

Because DNA is more stable than proteins and other cell components during chemical and thermal processing,²⁰ the detection of the allergenic food DNA instead of direct allergenic protein detection can be a more effective method for processed food samples. DNA-based methods, polymerase chain reaction (PCR) in particular, provide a useful approach

for the detection and identification of multiple plant and animal products and have become a valuable tool in food safety.¹⁹ Real-time PCR (qPCR) assays for peanut have been developed using the allergen genes (Arah 1, 2, and 3) and the ribosomal nuclear internal transcribed spacer (ITS)^{10,21–24} and have been tested in matrices such as chocolate, baked products, cereals, legumes, and nuts (Table 1). Additional advantages of using PCR-based methods for allergen detection are their simplicity, sensitivity, robustness, and cost-effectiveness. These methods rely mainly on inexpensive reagents such as oligonucleotide primers and probes and do not have batch-to-batch sequence variation.²⁵ Nonetheless, PCR assays are suitable only when DNA is an indicative marker of the presence of the allergenic food, such as in peanuts.²⁵

The most optimal regions of the genome for specific, sensitive, and efficient real-time PCR assays are those with sufficient sequence variation to discriminate closely related species and high copy number. High sequence variation often results in higher specificity even when using small DNA fragments (<150 bp). This is particularly useful in the food industry, where degraded samples are typical given that common processing methods (e.g., baking, frying, and boiling) can deteriorate the DNA, making it challenging to detect.²⁰ High copy number provides sensitivity and robustness to the assay. Unlike the nuclear genome, which has been the target of previous real time PCR assays for peanut allergen detection,^{21–24} mitochondrial and chloroplast genomes are present in high copy numbers in the plant cell. Mitochondrial DNA, however, is not as variable as chloroplast DNA and does not have sufficiently high sequence variation for specific detection

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Table 1. DNA Regions Previously Used for qPCR Assays for Peanut Detection in Food Samples^a

target	amplicon size (bp)	sensitivity (limit of detection)	matrices spiked with peanut	foods tested for cross-reactivity	fluorescence type	reference
Arah 2	n/a	<10 ppm	chocolate, milk	cereals, tree nuts, legumes, cookies, yogurt, pudding, candy bar, chocolate	TaqMan	Stephan and Vieths, 2004
Arah 3	78, 105, 114	10 ppm	cookies	tree nuts, legumes, grains, chocolate, chicken, cattle meat, potato	TaqMan	Scaravelli et al. 2008
Arah 3	130	0.375 pg	no	legumes, tree nuts, grains, and cocoa	TaqMan	Bergerova et al. 2011
Arah 2	125	10 ppm	wheat flour, cocoa	tree nuts, legumes, grains, chocolate, biscuits, nut bars, nut beverages, rice cakes, potato, cattle, sheep, goat	TaqMan	Lopez-Calleja et al. 2013
ITS 1	90	0.1 ppm	wheat flour, cocoa	tree nuts, legumes, grains, chocolate, biscuits, nut bars, nut beverages, rice cakes, potato, cattle, sheep, goat	TaqMan	Lopez-Calleja et al. 2013
Arah 1	137	0.01%	wheat powder	cereals, legumes, fruits, biscuits, powders, drinks, candy, nuts, shrimp, crab, oyster, salmon, milk	SYBR Green	Zhang et al. 2015

^abp: base pairs.

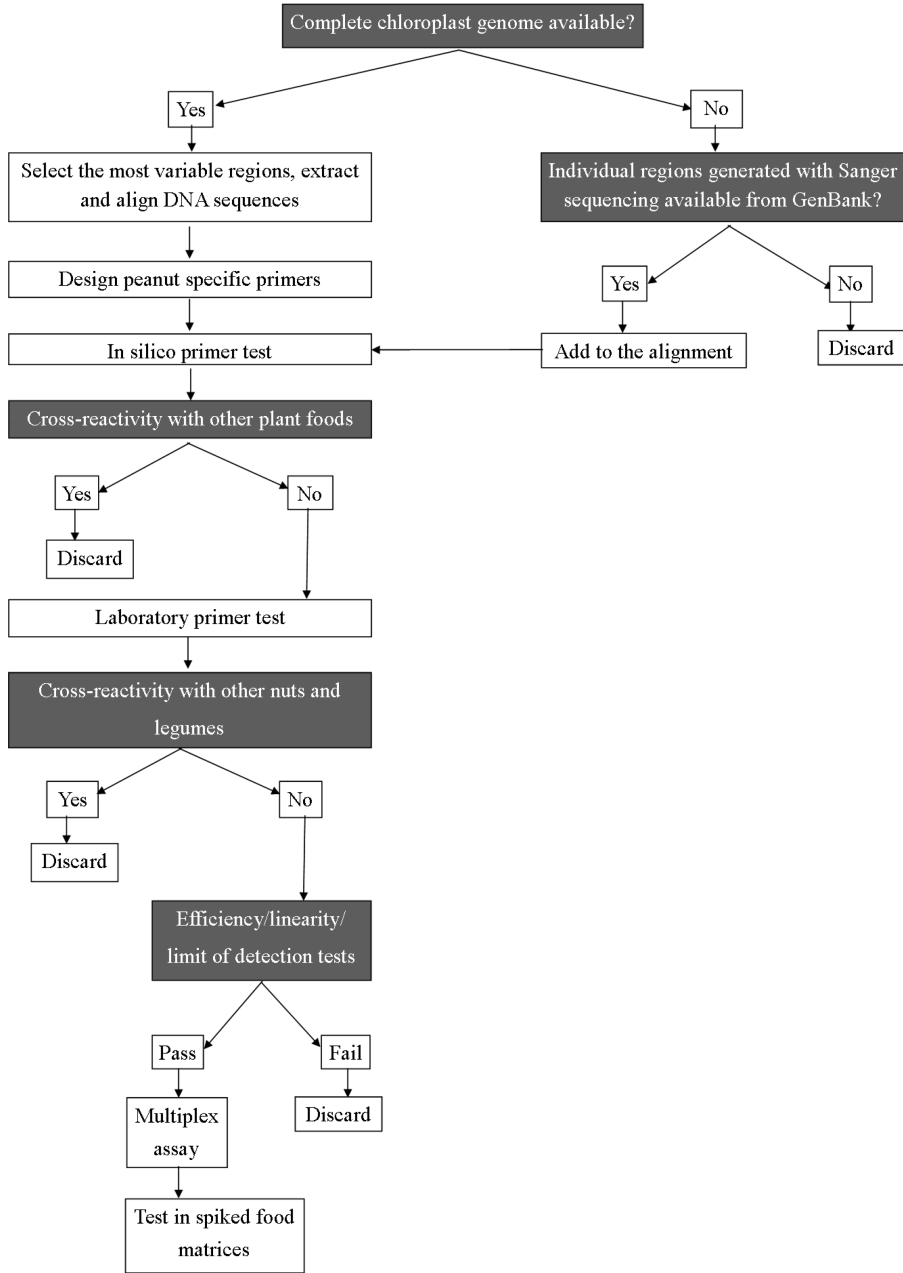


Figure 1. Workflow to identify and select suitable chloroplast markers for the development of a multiplex qPCR assay for peanut detection.

of plant material. Chloroplast genomes (also known as plastomes) are single, small (107–218 kb) molecules that harbor between 120 and 130 genes, depending on the plant group, and are generally conserved in structure and organization.²⁶ This structural consistency facilitates the sequence comparison and alignment across plant groups required for the design of highly specific primers and probes. Altogether, these characteristics make chloroplast regions excellent candidates for the development of real-time PCR assays for peanut detection in food matrices. Despite these advantages, few studies have used chloroplast regions for plant-based allergen detection.^{20,27,28}

The aim of this study was to develop and validate a qPCR-based detection method for peanut in complex food matrices. We did this by first carrying out a thorough and systematic evaluation of multiple plastid regions across numerous plant species that are commonly used in food products and selecting the most sensitive and specific chloroplast regions for a multiplex real-time PCR assay. This is the first qPCR assay for peanut allergen detection that (1) targets chloroplast markers, (2) simultaneously targets multiple regions in a single reaction, and (3) has been systematically tested in three different food matrices.

MATERIALS AND METHODS

Sample Preparation and DNA Extraction. An initial peanut homogenate was prepared by mixing 5 g of raw seeds with 15 mL of 2% SDS, then blended on high speed with the IKA Tube Mill control (IKA, Wilmington, NC, U.S.A.) for 3 min. Additional 2% SDS was added to reach a total volume of 20 mL and a concentration of 0.25 g of peanut/mL. Subsequently, serial dilutions were prepared with 9 mL of 1% SDS and 1 mL of the homogenate to produce lower concentrations of peanut (Table S1). Food matrices were then spiked at these concentrations by blending 18 mL of 1% SDS, 5 g of food matrix, and 2 mL of peanut homogenate to achieve the desired ppm level (Table S1). For all samples (peanut, tree nuts, legumes, and food matrices), DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, U.S.A.) following manufacturer instructions. DNA concentrations were quantified using a NanoDrop Lite Spectrophotometer (ThermoFisher Scientific, Wilmington, DE, U.S.A.) and normalized to 10 ng/ μ L prior to PCR.

Primer and Probe Design and Evaluation. A data set that included chloroplast genomes for plant species that are likely to be found in food products such as tree nuts, legumes, grains, fruits, and spices was built from GenomeTrakrCP²⁹ and GenBank (Table S2) using Geneious 10.0.7 (<http://www.geneious.com>).³⁰ In cases where the full plastome was not available, individual region sequences were mined from GenBank. Primers and probes were designed to target the most variable and peanut specific sections in the plastome³¹ and evaluated systematically (Figure 1). All primer sets were empirically tested and assessed with respect to (1) cross-reactivity and (2) reaction efficiency, statistical R^2 value, and linear range. Initial cross-reactivity tests included mostly nuts and legumes (1 ng/mL of DNA) because they are more likely to result in false positives. Limits of detection (LOD) were established by serially diluting peanut DNA in TE buffer at levels of 10 000–0.01 pg/ μ L (50 000–0.05 pg per reaction), equivalent to 10⁶–0.1 ppm in spiked food matrices.

Samples were run in triplicate with a CFX96 Touch real-time PCR system with a C1000 thermal cycler (Bio-Rad, Hercules, CA, U.S.A.). Each PCR reaction included an internal amplification control (IAC)³² as previously described by Eischeid et al.³³ and contained the following in a 12.5 μ L reaction volume: 1 $\times$ PCR buffer, 200 nM of each primer and probe for the internal amplification control (IAC), a constant copy number of 5 $\times$ 10⁵ of the internal control template, 0.625 units of Platinum Taq polymerase (Invitrogen/Life Technologies, Grand Island, NY, U.S.A.), and 5 μ L of diluted DNA. Single and multiplex assay validation in buffer was carried out using 5 mM

Mg and 1.2 mM dNTPs; validation in food matrices was adjusted to 7 mM Mg and 2 mM dNTPs. Primers and probes for peanut targets were optimized at 500 and 200 nM, respectively. The effects of different thermal profiles on PCR performance were initially determined with three annealing temperatures (60, 65, and 72 $^{\circ}$ C) in serially diluted peanut DNA in TE buffer. An annealing temperature of 65 $^{\circ}$ C was found to be the optimal for most primers and probes and was therefore used in all assays. DNA amplification was performed under the following conditions: 95 $^{\circ}$ C for 3 min followed by 45 cycles of denaturation at 95 $^{\circ}$ C for 15 s, annealing at 65 $^{\circ}$ C for 30 s, and extension at 65 $^{\circ}$ C for 2 min. Fluorescent signal was read at the end of the annealing step. All resulting data were analyzed using the CFX Manager v3.1 software (Bio-Rad, Hercules, CA, U.S.A.).

Development and Evaluation of the Multiplex Real-Time PCR Assay. Once primer sets were selected based on their performance, they were pooled in a single reaction and evaluated following the United States Food and Drug Administration (FDA) guidelines for the validation of chemical methods³⁴ in three types of commercial food matrices representative of different commodity groups: (1) high in starch and/or protein content and low water and fat content (baked products—blueberry muffin and chocolate chip cookie), (2) “Difficult or unique commodities” (chocolate, e.g., cocoa powder and milk chocolate), and (3) high in acid and water content (tomato based sauces, e.g., pasta sauce and tomato salsa). Matrices were spiked as described in Sample Preparation and DNA Extraction. These food matrices were chosen because they are likely to show peanut cross-contamination in food processing facilities and have been the object of safety recalls by the FDA (<https://www.fda.gov/Safety/Recalls/ArchiveRecalls/default.htm>). Moreover, chocolate and acids have shown to be challenging food matrices for both DNA- and protein-based allergen detection.²⁵

RESULTS AND DISCUSSION

Primer and Probe Design and Evaluation. A total of 77 plastomes were included in the data set used for primer and probe design. Eight regions were extracted and aligned for primer design and evaluation: *ndhA*, *matK*, *rpl16*, *trnL-F*, *trnL*, *ndhC-trnV*, *trnH-psbA*, and *rbcL*. A total of 33 primer and probe sets were generated and tested in 24 samples comprising legumes and nuts (Table 2). Of these, the three targets that

Table 2. Chloroplast DNA Regions and Number of Primers Tested for Each Region

chloroplast region	no. accessions	no. primer sets
<i>ndhA</i>	53	4
<i>matK</i>	47	3
<i>rpl16</i>	60	5
<i>trnL-F</i>	77	3
<i>trnL</i>	79	4
<i>ndhC-trnV</i>	47	0
<i>trnH-psbA</i>	84	2
<i>rbcL</i>	91	12
Total	538	33

showed no significant cross-reactivity and the highest reaction efficiency and linearity were selected: *matK*, *trnH-psbA*, and *rpl16* (Table 3). Late amplification ($C_T > 32$) of one of the three targets selected was only observed in soybean, pecan, and pistachio (Table 4). This amplification was not considered significant because it was $\sim$ 100 000-fold weaker than peanut and appeared at least 15 cycles later.

PCR Performance and Assay Optimization. Single Marker. Primer and probe evaluation and PCR optimization were carried out for each target separately using serial dilutions

Table 3. Primer and Probe Sequences for the Three Targets Selected for the Multiplex Assay Based on Their Specificity, Sensitivity, and Reaction Efficiency and Linearity^a

target	<i>rpl16</i>	<i>matK</i>	<i>trnH-psbA</i>
forward primer	GCGATGGGAACGACGAAAAC	AAGCGAAATTTTGTATATATTAGG	AGGAGCAATAGAACTGCGT
reverse primer	TTAGTTCGTTCCGCCATCCC	CTGCATATCCGCAAATAC	TTTTTGTCTTAAGGGATACGAGT
probe	56-FAM/ACCTAAGAT/ZEN/ TCATTGACGGGA/3IABkFQ/	/5Cy5/CATCCCATT/TAO/ AGTAAGCCCGTTTG/3IABRQSp/	/STEX61S/ TGATATTGCTCCTTTACTTTCAAAA/ 3IABRQSp/
amplicon length (bp)	69	100	68

^abp: base pairs.**Table 4. Cross-Reactivity Data at 1 ng/μL^a**

common name	scientific name	family	average C _T value		
			<i>matK</i>	<i>trnH-psbA</i>	<i>rpl16</i>
adzuki beans	<i>Vigna angularis</i>	Fabaceae	n/a	n/a	n/a
almonds	<i>Prunus dulcis</i>	Rosaceae	n/a	n/a	n/a
black turtle beans	<i>Phaseolus vulgaris</i>	Fabaceae	n/a	n/a	n/a
Brazil nut	<i>Bertholletia excelsa</i>	Lecythidaceae	n/a	n/a	n/a
cannellini beans	<i>Phaseolus vulgaris</i>	Fabaceae	n/a	n/a	n/a
chestnut	<i>Castanea mollissima</i>	Fagaceae	n/a	n/a	n/a
chickpeas	<i>Cicer arietinum</i>	Fabaceae	n/a	n/a	n/a
coconut	<i>Cocos nucifera</i>	Arecaceae	n/a	n/a	n/a
English walnut	<i>Juglans regia</i>	Juglandaceae	n/a	n/a	n/a
fava beans	<i>Vicia faba</i>	Fabaceae	n/a	n/a	n/a
green lentils	<i>Lens culinaris</i>	Fabaceae	n/a	n/a	n/a
hazelnut	<i>Corylus avellana</i>	Betulaceae	n/a	n/a	n/a
jumbo raw peanut	<i>Arachis hypogaea</i>	Fabaceae	17.6	19.2	17.9
lupini beans	<i>Lupinus</i> sp.	Fabaceae	n/a	n/a	n/a
macadamia	<i>Macadamia integrifolia</i>	Proteaceae	n/a	n/a	n/a
mung beans	<i>Vigna radiata</i>	Fabaceae	n/a	n/a	n/a
navy beans	<i>Phaseolus vulgaris</i>	Fabaceae	n/a	n/a	n/a
pecan	<i>Carya illinoensis</i>	Juglandaceae	n/a	32.4	34.1
pinenut	<i>Pinus</i> sp.	Pinaceae	n/a	n/a	n/a
pinto beans	<i>Phaseolus vulgaris</i>	Fabaceae	n/a	n/a	n/a
red kidney beans	<i>Phaseolus vulgaris</i>	Fabaceae	n/a	n/a	n/a
red lentils	<i>Lens culinaris</i>	Fabaceae	n/a	n/a	n/a
soy bean	<i>Glycine soja</i>	Fabaceae	37.2	n/a	n/a
Turkish pistachio	<i>Pistacia vera</i>	Anacardiaceae	n/a	n/a	41.6
sesame seeds	<i>Sesamum indicum</i>	Pedaliaceae	n/a	n/a	n/a

^an/a: C_T value not available because there was not amplification. C_T values provided are the means of the values obtained from two separate runs, each one done in triplicates.**Table 5. PCR Data for Individual and Multiplex Assays in Buffer^a**

target	Single target					Multiplex				
	efficiency	R ²	slope	C _T value	IAC C _T + SD	efficiency	R ²	slope	C _T value	IAC C _T + SD
<i>matK</i>	88	0.98	−3.8	17.6	23.32 ± 0.09	81	0.99	−3.88	13.76	21.06 ± 0.09
<i>trnH-psbA</i>	100	0.99	−3.32	16.9	20.94 ± 0.09	98	0.99	−3.36	12.58	21.06 ± 0.09
<i>rpl16</i>	104	0.99	−3.23	16.5	20.87 ± 0.11	92	0.99	−3.53	13.06	21.06 ± 0.09

^aOptimal range for reaction efficiency: 90–110%; R² > 0.98; and slope ~−3.32.

of 10 000–0.01 pg/ul target peanut DNA in TE buffer. C_T values were determined from amplification plots (Figure S1) and used to generate linear standard curves (Figure S2). The standard curve data showed that for all three markers the PCR was linear over 7 orders of magnitude with R² values > 0.98 and reaction efficiencies of 88 (*matK*), 100 (*trnH-psbA*), and 104% (*rpl16*). In each assay replicate, C_T values were similar across the three targeted regions: 17.6, 16.9, and 16.5 respectively (Table 5). The internal control was added to all PCR reactions at a constant copy number. As expected, it

amplified consistently across all samples, regardless the amount of peanut DNA (Table 5).

Multiplex Assay. The performance of the three primer sets selected, *matK* (Cy5), *rpl16* (FAM), and *trnH-psbA* (TEX), was initially evaluated in buffer (Figure 2, Table 5). Because each set of primers and probes amplified different regions of the chloroplast, cross amplification or interference between them within the multiplex assay was not observed. Although C_T values lowered and the efficiency for all three markers changed when combined in a single reaction, they remained

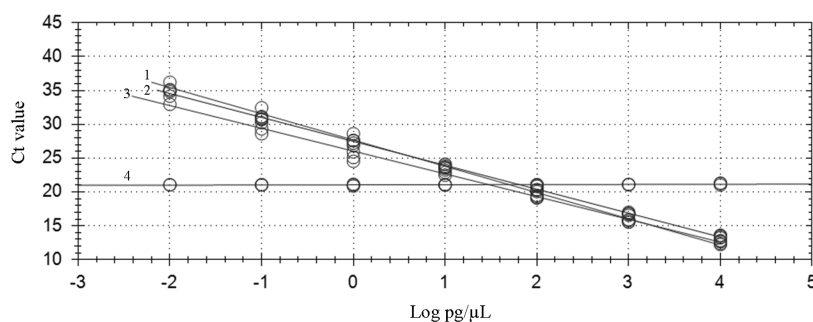


Figure 2. Standard curves generated for peanut DNA in TE buffer multiplexing *matK*, *rpl16*, and *trnH-psbA*. C_T values were calculated from the point at which amplification curves cross threshold and plotted versus (log) peanut DNA concentration ($\text{pg}/\mu\text{L}$). For all three targets the lowest limit of detection is $0.01 \text{ pg}/\mu\text{L}$, $C_T = 32\text{--}38$. 1, *rpl16* (FAM); 2, *matK* (CyS); 3, *trnH-psbA* (TEX); 4, internal amplification control (HEX).

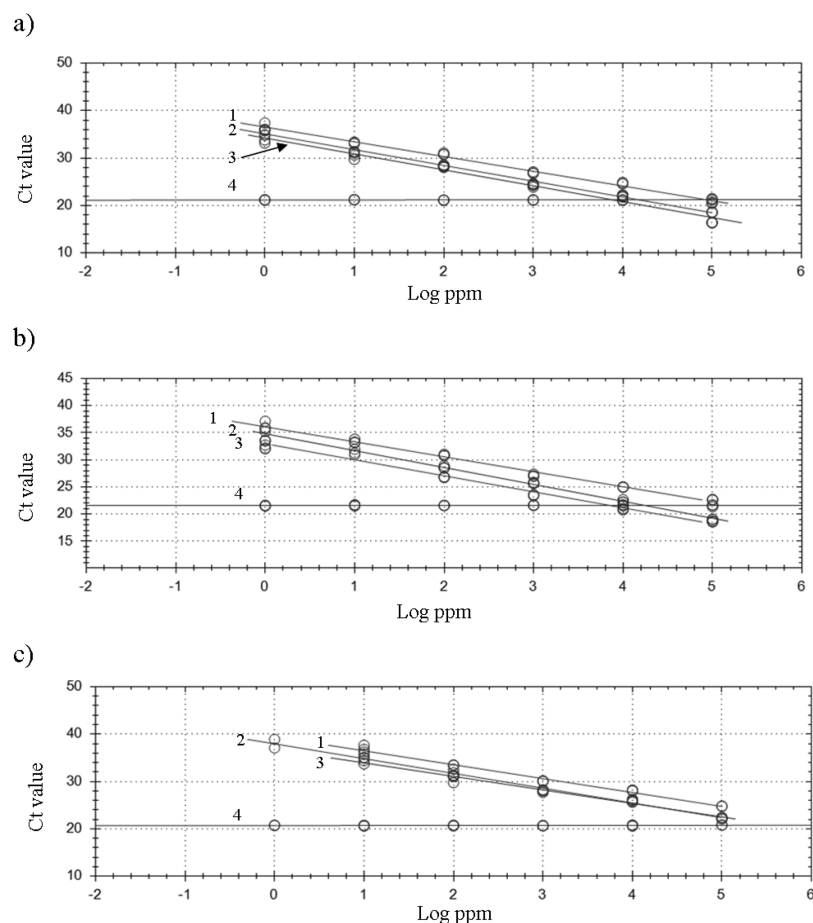


Figure 3. Examples of standard curves generated from the multiplex assay in spiked food matrices showing C_T values at different peanut concentrations. For all three matrices the lowest limit of detection was 1 ppm, $C_T = 32\text{--}38$. (a) Chocolate chip cookie, (b) tomato sauces, and (c) milk chocolate. 1, *rpl16* (FAM); 2, *matK* (CyS); 3, *trnH-psbA* (TEX); 4, internal amplification control (HEX).

within the expected range (90–110%) except for *matK* (81%). The internal control was also added to the multiplex assay at the same constant copy number as the single marker assays. Its amplification was equally consistent (Table 5).

Multiplex Assay Validation in Food Matrices. Assay performance and robustness were evaluated by spiking known amounts of peanut (Table S1) in six food matrices representative of three commodity groups: blueberry muffin, chocolate chip cookies, cocoa powder, milk chocolate, tomato pasta sauce, and tomato salsa. Linear standard curves were generated by plotting the C_T values as a function of parts per million spiking level and analyzed as previously described

(Figure 3). After further optimization by increasing the amount of magnesium, probes, and dNTPs in the master mix (see Materials and Methods), all three targets performed well in all foods tested (Table 6). *matK* was most efficient in chocolate chip cookie (95%), milk chocolate (101%), and tomato sauces (107 and 109%). *trnH-psbA* performed the best in baked goods (100 and 101%) and cocoa powder (101%). *rpl16* showed the best efficiency in baked goods (100–102%). Because there was performance variability among the targets, having more than one marker increases the assay robustness and specificity by having three separate sources of detection. Amplification of the peanut target was only inhibited in

Table 6. PCR Data for the Multiplex Assay in Food Matrices^a

matrix	<i>matK</i>			<i>trnH-psbA</i>			<i>rpl16</i>			IAC
	efficiency	R ²	slope	efficiency	R ²	slope	efficiency	R ²	slope	C _T ± SD
muffin	87	0.95	−3.70	100	0.97	−3.37	100	0.97	−3.31	20.49 ± 0.08
cookie	95	0.98	−3.44	101	0.99	−3.38	102	0.99	−3.36	21.09 ± 0.07
pasta sauce	109	0.98	−3.12	108	0.99	−3.15	124	0.99	−2.91	21.24 ± 0.09
tomato salsa	107	0.98	−3.17	112	0.97	−3.07	131	0.99	−2.75	21.16 ± 0.08
cocoa powder ^b	131	0.97	−2.75	101	0.95	−3.06	113	0.99	−3.08	20.74 ± 0.08
milk chocolate	101	0.98	−3.32	124	0.97	−2.87	132	0.98	−2.74	21.14 ± 0.09

^aIAC: internal amplification control. Optimal range for reaction efficiency: 90–110%; R² > 0.98; and slope ~−3.32. ^bResults after diluting DNA to 1 ng/μL.

samples containing excessive amounts of cocoa powder DNA (Figure S3). This inhibition was confirmed by the IAC and overcome by diluting the DNA 10-fold (to 1 ng/μL).

The assay developed in this study offers a novel and systematic approach because it targets three separate, high-copy number chloroplast regions in a single reaction. Furthermore, it has been strategically evaluated in three different commodity groups that have been the object of FDA recalls in recent years due to the presence of undeclared peanut. These included foods not previously tested such as tomato-based sauces, which can be cross-contaminated through the spices they contain; chocolate, considered a difficult and unique commodity for allergen detection; and baked goods, which are prone to peanut cross-contact in food processing facilities. Our results show LODs of 0.01 pg/μL in buffer and 1 ppm in food matrices along with high robustness and specificity. In contrast, previously published studies have targeted a single, nuclear, low-copy number region. LODs achieved using one of the allergen genes (*Arah*) were 10–50 ppm in food samples.^{10,21,22} López-Calleja et al. also implemented ITS and reached an LOD of 1 ppm.²² However, our recent work with ITS 1 and 2 has shown significant cross-reactivity between peanuts and tree nuts,³⁵ making this marker a less desirable alternative for this purpose.

This paper has described the development and validation of a highly sensitive multiplex real-time PCR assay using chloroplast DNA markers to specifically detect plant-based allergens, such as peanuts, in a variety of complex and potentially challenging food matrices. Future research will aim to develop similar assays for other allergenic nuts and evaluate and compare their performance with commercial ELISA and PCR kits.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02053.

Table S1: Preparation of peanut homogenate, spiking levels, and ppm calculations. Table S2: GenBank accession numbers for chloroplast genomes and single sequences used for primer and probe design. Figure S1: Amplification plots generated by serial dilution of peanut DNA in TE buffer (a) *matK*, (b) *rpl16*, and (c) *trnH-psbA*. Figure S2: Standard curves generated from amplification plots in Figure S1. C_T values were calculated from the point at which amplification curves cross threshold and plotted versus (log) peanut DNA spiking concentration (ppm). IAC: Internal Amplification Control. Figure S3: Example of standard curve for

multiplex assay in cocoa powder prior to DNA dilutions (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DNA, Deoxyribonucleic acid
FDA, United States Food and Drug Administration
IAC, Internal Amplification Control
ITS, Internal transcribed spacer
qPCR, Real-time PCR

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SUPPLEMENTARY MATERIAL

Table S1: Preparation of peanut homogenate, spiking levels, and ppm calculations

homogenate #	mg/mL peanut	mg peanut in 2 mL homogenate	mg food matrix (5 grams)	peanut parts per million (ppm)*
1	250	500	5000	100000
2	25	50	5000	10000
3	2.5	5	5000	1000
4	0.25	0.5	5000	100
5	0.025	0.05	5000	10
6	0.0025	0.005	5000	1
7	0.00025	0.0005	5000	0.1
8	0.000025	0.00005	5000	0.01

*parts per million = $10^6 \times (\text{mg peanut} / \text{mg food matrix})$

Table S2. GenBank accession numbers for the chloroplast genomes and sequences used for primer and probe design.

Scientific name	Common Name	Chloroplast	GenBank accession					
			<i>rbcL</i>	<i>matK</i>	<i>trnL</i>	<i>trnL-trnF</i>	<i>trnH-psbA</i>	<i>rpl16</i>
<i>Allium cepa</i>	Onion	NC024813	*	*	*	*	*	*
<i>Anacardium occidentale</i>	Cashew	*	AY462008	AY594459	DQ131556, JQ769096	AY594497, KR081693	KR075990	KR055826, KF664263
<i>Arachis hypogaea</i>	Peanut	KX257487	*	*	*	*	*	*
<i>Arachis hypogaea</i>	Peanut	KJ468094	*	*	*	*	*	*
<i>Avena sativa</i>	Oat	KM974733	*	*	EU276606	KP711124	*	*
<i>Bertholletia excelsa</i>	Brazil nut	*	Z80178	*	AF077635, JQ769095	DQ417933, JN221844	AY172783, JN221778	*

<i>Brassica juncea</i>	Mustard greens	NC028272	*	*	*	*	*	*
<i>Brassica nigra</i>	Black mustard	NC030450	*	*	*	*	*	*
<i>Brassica oleracea</i> var. <i>capitata</i>	Cabbage	KR233156	*	*	*	*	*	*
<i>Carya illinoensis</i>	Pecan	*	U00436	KF419017	JQ769101	AF303798	KF201449	AB514142
<i>Castanea mollissima</i>	Chinese chestnut	NC014674	*	*	*	*	*	*
<i>Cicer arietinum</i>	Chickpea	EU835853	*	*	*	AB117648	*	*
<i>Cinnamomum verum</i>	Cinammon	*	JX414039	AJ247157	*	AB054234	AF268784, KF978092	*
<i>Cocos nucifera</i>	Coconut palm	KF285453	*	*	*	*	*	*
<i>Coffea arabica</i>	Coffee	NC008535	*	*	*	*	*	*
<i>Coffea canephora</i>	Coffee	NC030053	*	*	*	*	*	*
<i>Coriandrum sativum</i>	Coriander	NC029850	*	*	*	*	*	*
<i>Corylus avellana</i>	Hazelnut	NC031855	*	*	JQ769098, JX985260	FJ012026	*	*
<i>Cucurbita pepo</i> subsp. <i>pepo</i>	Pumpkin	KT898819	*	HQ438611	*	DQ536808	*	*
<i>Cuminum cyminum</i>	Cumin	*	JQ933290	KU499895	*	KR150210, KR150213	KX108702	*
<i>Fragaria chiloensis</i>	Strawberry	NC019601	*	*	*	*	*	*
<i>Fragaria vesca</i> subsp. <i>vesca</i>	Strawberry	NC015206	*	*	*	*	*	*
<i>Fragaria virginiana</i>	Strawberry	NC019602	*	*	*	*	*	*
<i>Fragaria x ananassa</i>	Strawberry	*	U06805	*	*	*	GQ476780, GQ476782	*
<i>Glycine max</i>	Soybean	DQ317523	*	*	*	*	*	*
<i>Glycine soja</i>	Soybean	KF611800	*	*	*	*	*	*
<i>Hordeum vulgare</i> subsp. <i>vulgare</i>	Barley	EF115541	*	*	*	*	*	*
<i>Juglans nigra</i>	Black walnut	*	U00437	AF118036, U92851	AY968575	AF303783	AY293339, HQ596736	*

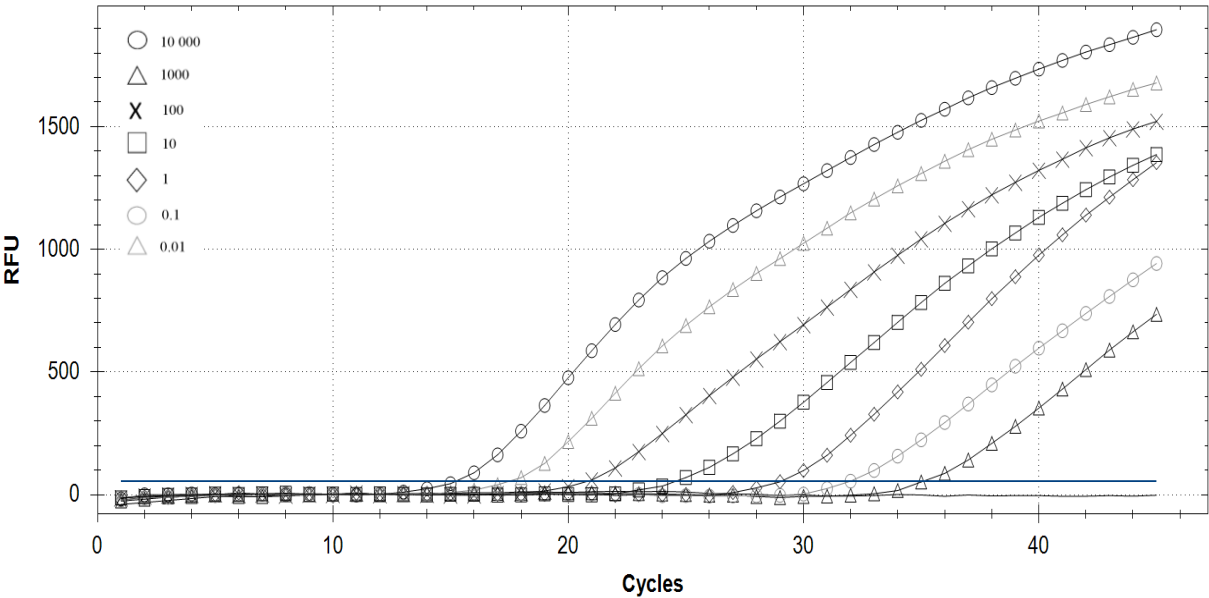
<i>Juglans regia</i>	English walnut	KT963008	*	*	*	*	*	*
<i>Juglans regia</i>	English walnut	KT870116	*	*	*	*	*	*
<i>Juglans sigillata</i>	Chinese walnut	NC031373	*	*	*	*	*	*
<i>Lactuca sativa</i>	Lettuce	NC007578	*	*	*	*	*	*
<i>Lens culinaris</i>	Lentil	NC027152	*	*	*	*	*	*
<i>Lupinus luteus</i>	Yellow lupine	NC023090	*	*	*	*	*	*
<i>Macadamia integrifolia</i>	Macadamia nut	KF862711	*	*	*	*	*	*
<i>Malus prunifolia</i>	Apple	NC031163	*	*	*	*	*	*
<i>Mentha longifolia</i>	Horsemint	NC032054	*	*	*	*	*	*
<i>Oryza glaberrima</i>	African rice	NC024175	*	*	AY792516, DQ143160	*	*	*
<i>Oryza sativa Indica Group</i>	Long-grained rice	KM103382	*	*	*	*	*	*
<i>Oryza sativa Indica Group</i>	Long-grained rice	AY522329	*	*	*	*	*	*
<i>Oryza sativa Japonica Group</i>	Japanese rice	X15901	*	*	GU595122	*	*	*
<i>Petroselinum crispum</i>	Parsley	HM596073	*	*	*	*	*	*
<i>Phaseolus acutifolius</i>	Terapy bean	*	*	DQ445951, DQ445952	*	*	*	*
<i>Phaseolus coccineus</i>	Butter bean	*	LT576851	DQ445965, DQ445966	GQ411558, GQ411559	GQ411774, GQ411776	FJ951184	*
<i>Phaseolus lunatus</i>	Lima bean	*	LT576852	DQ445985, DQ450861	EU909110	EU909056, EU909111	FJ951178	*
<i>Phaseolus vulgaris</i>	Common bean	NC009259	*	*	AY077944	AY077960	*	*
<i>Pinus armandii</i>	Pinenut	KP412541	*	*	*	*	*	*
<i>Pinus gerardiana</i>	Pinenut	EU998741	*	*	*	*	*	*
<i>Pinus koraiensis</i>	Pinenut	AY228468	*	*	*	*	*	*
<i>Pinus monophylla</i>	Pinenut	EU998745	*	*	*	*	*	*
<i>Pistacia vera</i>	Pistacio	matK	AJ235786	KF664257, KF664258	JQ769103, KJ173803	EF193139, KP055540	*	*
<i>Pisum sativum</i>	Pea	NC014057	*	*	*	*	*	*

<i>Prunus dulcis</i>	Almond	KY085904	*	*	HQ243717, JQ769094	AF318707, HQ243955	AY500625, HQ188713	*
<i>Salvia rosmarinus</i>	Rosemary	NC027259	*	*	*	*	*	*
<i>Sesamum indicum</i>	Sesame	NC016433	*	*	DQ221163, JQ769104	*	*	*
<i>Solanum lycopersicum</i>	Tomato	NC007898	*	*	*	*	*	*
<i>Solanum tuberosum</i>	Potato	NC008096	*	*	*	*	*	*
<i>Sorghum bicolor</i>	Sorghum	EF115542	*	*	*	*	*	*
<i>Sorghum timorense</i>	Sorghum	KF998272	*	*	*	*	*	*
<i>Spinacia oleracea</i>	Spinach	NC002202	*	*	*	*	*	*
<i>Theobroma cacao</i>	Cacao	HQ244500	*	*	*	*	*	*
<i>Thymus vulgaris</i>	Thyme	*	Z37472	*	*	*	HE819468	*
<i>Triticum aestivum</i>	Bread wheat	AB042240	*	*	*	*	*	*
<i>Triticum macha</i>	Wheat	LC005978	*	*	AB732943	AB732943	*	*
<i>Triticum monococcum</i>	Wheat	KC912690	*	*	*	*	GU575288	*
<i>Triticum timopheevii</i>	Wheat	KJ614410	*	*	*	*	*	*
<i>Triticum turgidum</i>	Wheat	KJ614397	*	*	*	*	*	*
<i>Triticum urartu</i>	Wheat	KC912693	*	*	*	*	KJ146067	*
<i>Vaccinium macrocarpon</i>	Cranberry	NC019616	*	*	*	*	*	*

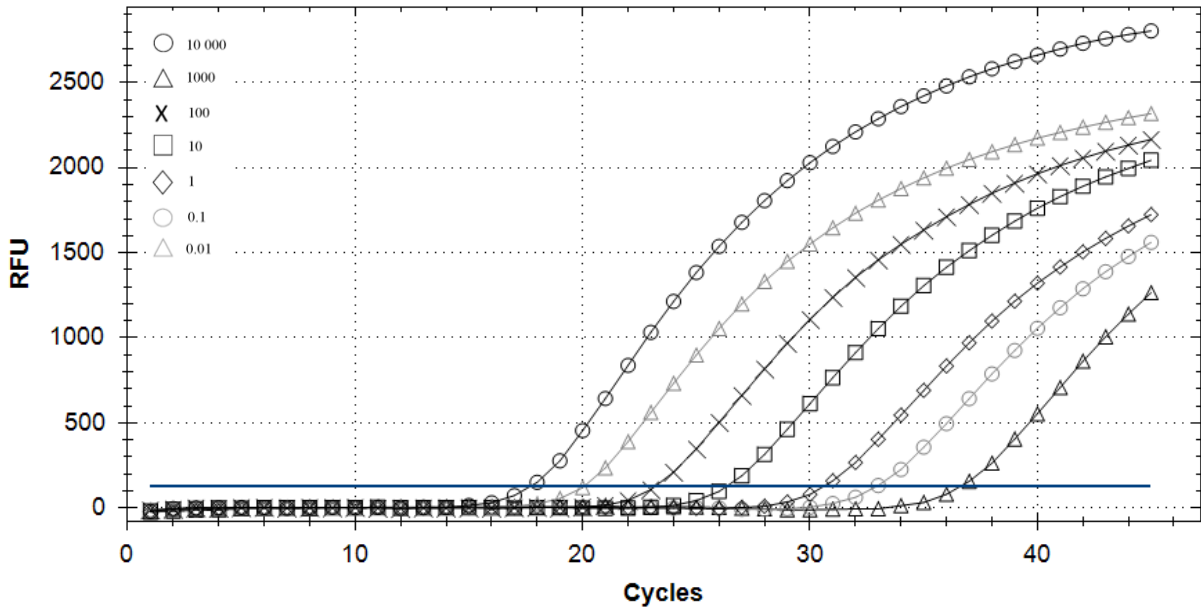
<i>Vicia faba</i>	Fava bean	KF042344	*	*	*	*	*	*
<i>Vigna aconitifolia</i>	Moth bean	*	KX087386	JN008214, JN008216	AB304069, JX233509	AB304069, JX233509	KX087541, KX087542	*
<i>Vigna angularis</i>	Adzuki bean	NC021091	*	*	*	*	*	*
<i>Vigna radiata</i>	mung bean	NC013843	*	*	*	*	*	*
<i>Vigna unguiculata</i>	Cowpea	NC018051	*	*	*	*	*	*
<i>Zea mays</i>	Corn	X86563	*	*	*	*	*	*

Figure S1. Amplification plots generated by serial dilution of peanut DNA in TE buffer. a) *matK*, b) *rpl16*, and c) *trnH-psbA*.

a)



b)



c)

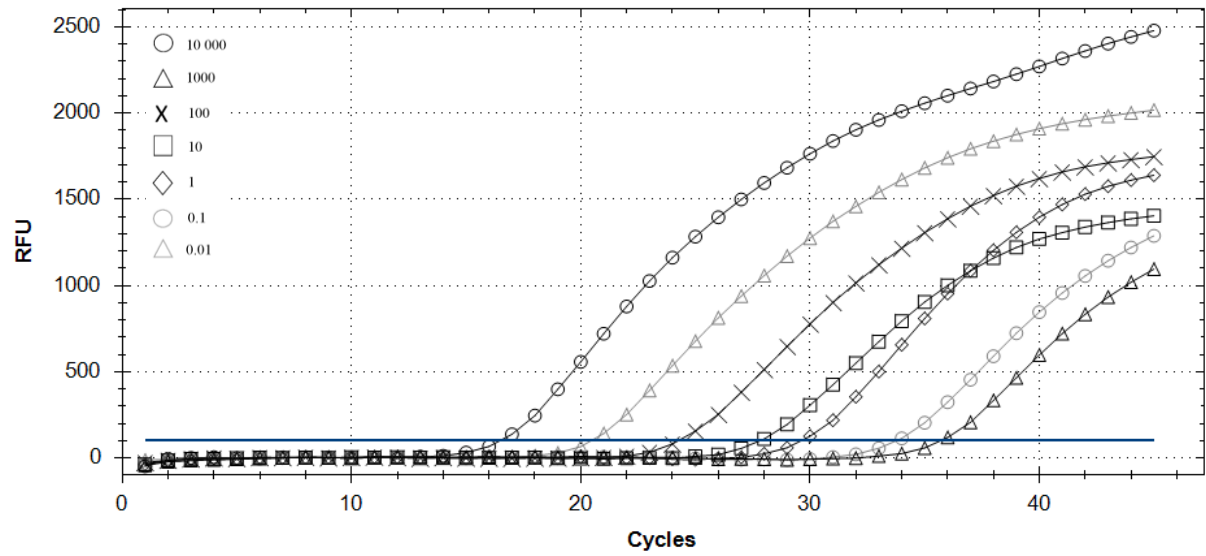
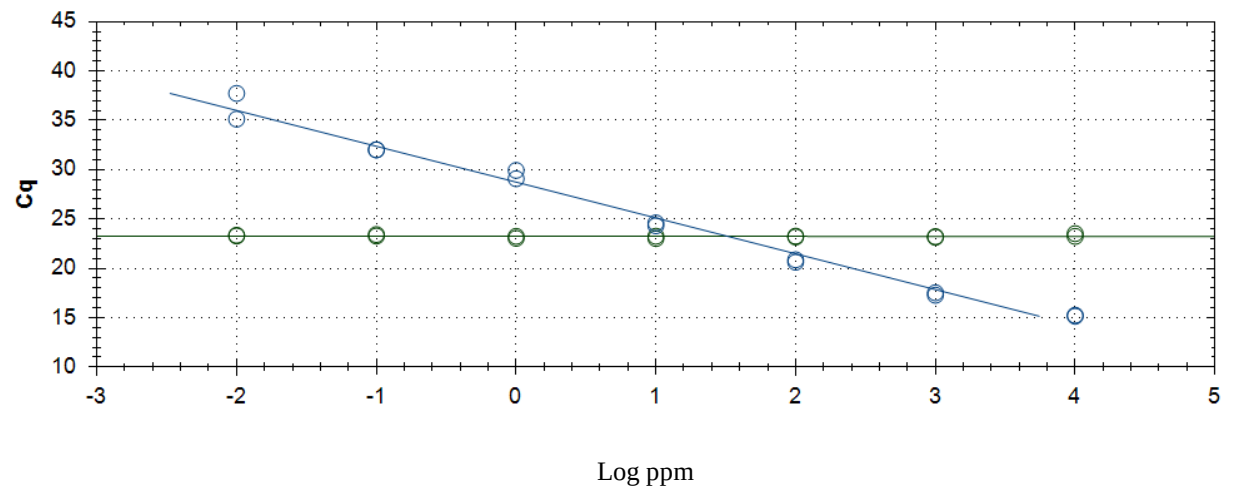
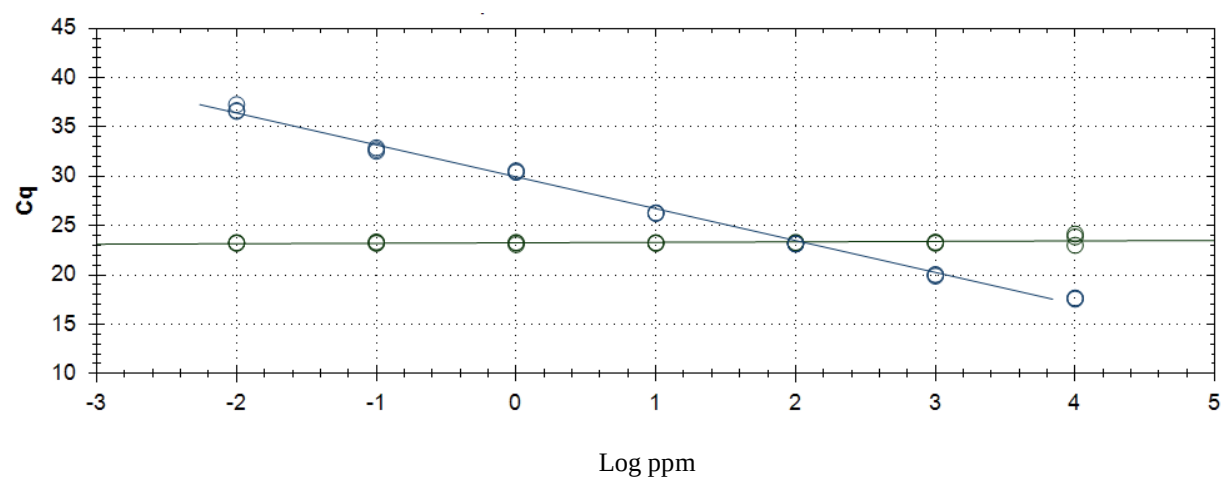


Figure S2. Standard curves generated from amplification plots. C_T values were calculated from the point at which amplification curves cross threshold and plotted versus (log) peanut DNA spiking concentration (ppm). a) *matK*, b) *rpl16*, and c) *trnH-psbA*. Internal Amplification Control (IAC).

a)



b)



c)

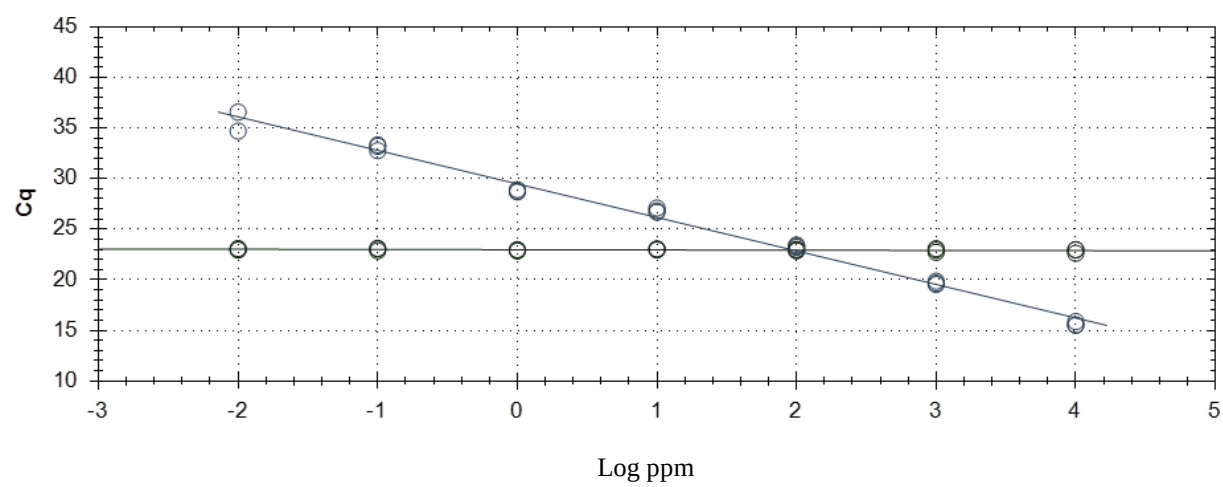


Figure S3. Example of standard curve for cocoa powder.

