



Development and validation of a duplex real-time PCR assay with locked nucleic acid (LNA) probes for the specific detection of allergenic walnut in complex food matrices

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ABSTRACT

Walnut is one of the most widely consumed and highly allergenic tree nuts in North America. The two species most frequently consumed in the United States are English walnut (*Juglans regia*) and black walnut (*Juglans nigra*). Ensuring accurate labeling to protect allergic individuals from accidental consumption demands methods that accurately identify the presence of walnut in food. We developed a novel real-time PCR assay that targets two chloroplast regions (*rbcL-accD* and *psbM-trnD*) and implements LNA probes to specifically detect walnut in three types of foods: baked goods, salad greens, and creamy dips. This is the first assay for walnut detection that uses LNA oligos to increase specificity and prevent significant cross-reactivity with pecan and other tree nuts by blocking their amplification signal. Food matrices were spiked separately with English and black walnut at concentrations that ranged from 0.1 to 10⁵ mg/kg (parts per million, or ppm). Reaction efficiencies within the optimal range of 100 ± 10% were obtained across 6 to 7 orders of magnitude with a limit of detection of 0.1 ppm. Our results show that real-time PCR assays using chloroplast markers and LNA probes are an effective and accurate tool for allergenic walnut detection.

1. Introduction

Tree nut allergies have become an increasing concern in the United States as consumption of tree nuts, as well as the number of allergic individuals, continues to rise (Weinberger & Sicherer, 2018). With the aim of protecting allergic consumers from accidental consumption, the Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) requires all major allergenic food groups, including tree nuts such as walnuts, be declared on the label (U.S. FDA, 2006). Walnut is one of the most commonly consumed tree nuts, and it is also the most allergenic in North America. Thirty-four percent of individuals with a tree nut allergy react to walnut (Costa et al., 2014; Weinberger & Sicherer, 2018). Although all 24 species in the genus *Juglans* are edible, the two species most frequently consumed in the United States are black walnut (*J. nigra*) and English walnut (*J. regia*). Both walnut and pecan (*Carya illinoensis*) belong to the botanical family Juglandaceae. Their allergenic proteins are of the same type and family and can be difficult to discriminate (Costa et al., 2014). Allergies to these two nuts often

co-occur (Andorf et al., 2017); nonetheless, not all individuals have such co-occurring allergies, and FALCPA mandates that for tree nuts, type of tree nut must be specified on package labeling. Therefore, the development of an assay that specifically detects walnut and discriminates it from pecan would be valuable not only for detection in food products, but also to more accurately assess the co-occurrence of these two allergies and ensure reliable detection of potential allergenic contaminants in food production and processing facilities (Cho et al., 2018). Allergen management in such facilities is often challenged by the absence of testing and reference materials, as well as the lack of consensus regarding the most effective and reliable methods for allergen detection and quantification (Costa et al., 2014). Currently, the two most common approaches are protein- and DNA-based.

The most frequently used protein-based methods are immunoassays, such as ELISA, and more recently, mass spectrometry (MS). They both target the allergen proteins directly. Walnut harbors multiple allergenic proteins: Jug r 1, 2, 3, 4, and 5, and Jug n1 and 2. They are all of clinical importance and tend to cross-react (Cabanillas et al., 2014; Costa et al.,

Abbreviations: 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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2014). ELISA is widely used for walnut allergen detection because it allows for fast detection of the allergenic protein without advanced laboratory equipment and requires a low level of expertise. However, cross-contamination and cross-reactivity with pecan and other nuts and plant species are frequent. MS methods, on the other hand, provide higher sensitivity, specificity, and reproducibility but their application is restricted by the requirement of expensive equipment and specialized expertise to perform (Croote & Quake, 2016).

DNA-based methods, namely PCR, have emerged as a robust alternative for allergen detection. Although they do not target the allergenic protein directly, they can be implemented as a confirmatory tool for the identification of allergenic foods. PCR-based assays, and especially real-time PCR (qPCR), can be highly specific and sensitive tools when using markers that are sufficiently variable and high copy number (Linacero et al., 2019). A number of PCR-based assays have been developed for walnut detection (Brezna et al., 2006; Costa et al., 2013; Linacero et al., 2016, 2019; Lopez-Calleja et al., 2015; Yano et al., 2007) but most of them have been tested only for English walnut (*J. regia*), not Black walnut (*J. nigra*), and report cross-reactivity with pecan (Linacero et al., 2016). These assays have traditionally targeted the allergenic genes with the exception of Yano et al. (2007), who targeted the chloroplast region *matK*. Chloroplast DNA contains a large number of potentially suitable markers for the development of qPCR assays (Puente-Lelievre & Eischeid, 2018). Nonetheless, sequence divergence is lower in closely related species, and finding regions of the genome that are not only distinct but also suitable for primer and probe design can be limited to single nucleotide polymorphisms (SNPs). In such cases, amplification of the non-target species, which results in cross-reactivity, can occur when primers and probes bind even with one or two mismatches. SNP recognition is generally important in molecular biology methods, and in qPCR in particular, it can determine the specificity of the assay. In cases where DNA sequence variation is reduced to single mismatches, the experimental design can be modified by incorporating Locked Nucleic Acids (LNA). LNA probes improve stability and mismatch discrimination by forming a covalent bridge and creating a conformation that results in higher affinity for the complementary DNA sequence (You et al., 2006). This approach has successfully been used in qPCR assays that aim to differentiate closely related species and even populations within the same species (wild-type and mutant) that genetically differ by only a SNP (Ahn et al., 2016, 2017; Handy et al., 2013; Hansen et al., 2015).

We developed and validated a qPCR assay that specifically detects both English and black walnut in six different complex food matrices with no significant cross-reactivity with pecan. To achieve this, we carried out a thorough and systematic evaluation of multiple chloroplast regions across numerous plant species that are commonly used in food products and targeted the regions that would discriminate walnut from pecan. Multiple regions were targeted to provide greater confidence in the accuracy of results. Two suitable regions were found; the second required design of two different LNA oligonucleotides: one to increase affinity for walnut and one to block the amplification signal from pecan. This is the first qPCR assay for walnut detection that simultaneously targets two chloroplast regions, and uses LNA oligos to specifically prevent cross-reactivity from pecan.

2. Materials and methods

2.1. Sample preparation and DNA extraction

Preparation of allergen-spiked food matrices was carried out as previously described by Puente-Lelievre and Eischeid (2018). Walnut homogenates were prepared by mixing 5 g of raw seeds with 15 mL of 2% SDS and blending at high speed with the IKA Tube Mill control blender (IKA, Wilmington, NC, USA) for 3 min. Additional 2% SDS was added to reach a total volume of 20 mL and a concentration of 0.25 g of walnut/mL. Next, serial dilutions of this initial homogenate were used to spike food matrices at final concentrations of 0.1–10⁵ mg walnut per kg

Table 1

Number of primer sets tested for each region of the chloroplast genome.

Region	No. primer sets tested
<i>atpF-atpH</i>	3
<i>atpH-atpI</i>	2
<i>rpl14-rpl16</i>	1
<i>rpl12</i>	1
<i>psbM-trnD</i> (LNA)	3
<i>rbcl-accD</i>	2
<i>rpl16</i>	1
<i>rpO-trnC</i>	1
<i>ycf3</i>	1
<i>clpP</i>	1
<i>ndhC-trnV</i>	1
Total	17

food matrix (ppm) as per Puente-Lelievre and Eischeid (2018). For all samples (English and black walnut, pecan and other nuts and grains for cross-reactivity tests, and blank/spiked food matrices), DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) following manufacturer instructions. DNA concentrations were quantified using the QIAxpert System (Qiagen, Germantown, MD, USA), and equivalent amounts of DNA were used in all reactions.

2.2. Primer and probe design and evaluation

A data set that included chloroplast genomes for plant species that are likely to be present in food products, such as tree nuts, legumes, grains, fruits, and spices, was built as in Puente-Lelievre and Eischeid (2018) from GenomeTrakrCP (Zhang et al., 2017) and GenBank using Geneious R11 (Kearse et al., 2012; www.geneious.com). Primers and probes were designed to target the most variable and walnut-specific sections of the plastome (Shaw et al., 2014) and evaluated as described by Puente-Lelievre and Eischeid (2018), with particular emphasis on regions that contained SNPs present in English and black walnut and absent in pecan (Table 1). All primer sets were empirically tested and assessed with respect to 1) cross-reactivity and 2) quantitative performance including reaction efficiency, statistical R² value, and linear range. Initial cross-reactivity tests were performed with 1 ng/μL of DNA and included pecan and other tree nuts because they are more likely to result in false positives. Legumes, grains and cereals were also included as they are extensively used in food products.

Two targets were selected for validation: *rbcl-accD*, detected with standard oligonucleotide primers and probes, and *psbM-trnD*, detected using an LNA detection probe combined with an LNA blocking oligo (Table 2 and see below). Limits of detection for the chosen targets (LOD) were established by serially diluting walnut DNA in TE buffer at levels of 10,000–0.01 pg/μL (50,000–0.05 pg per reaction), equivalent to 10⁶–0.1 ppm in spiked food matrices; primer and probe concentrations which gave the best reaction efficiency, range, and linearity were determined experimentally.

2.3. Development of the LNA probe

An LNA probe was developed to target a SNP present in the intergenic region *psbM-accD* in English walnut and black walnut (*J. regia* and *J. nigra*), and absent in pecan (*C. illionensis*). This walnut detection probe was 11 base pairs long with a 5' TEX615 dye and a 3' IABRQSp quencher on the 3' end. It contained 6 LNA bases represented by "+" as follows: AT + C C + C + C + TT + T + CT (Table 2). In order to block nonspecific fluorescent signal potentially generated from pecan, we also designed a pecan blocking LNA oligo. This is a non-fluorescent oligo, instead of a probe, that competes and specifically binds to pecan in the probe binding region without generating any fluorescence. Consequently, only binding by the walnut detection probe generates a fluorescent signal. The pecan blocking oligo contained 13 base pairs and 6 LNA bases: AG

Table 2

Primers and probes used in final version of duplex assay.

Target	Oligonucleotide	Sequence	Number of pairbases	Amplicon size
<i>psbM-trnD</i>	Forward primer 585F	GAG AGA TAG CCC TTT CTA A	19	88
	Reverse primer 672R	GCA TCC CAT AAT TAA TCC AA	20	
	LNA walnut detection probe	/5TEX615/AT + C C + C + C + TT + T + CT/3IAbRQSp/	11	
	LNA pecan blocking oligo	AG + G A + TC C + C + A + TT + T C	13	
<i>rbcl-accD</i>	Forward primer 268F	CCG TAG TTT CCG ATC A	16	108
	Reverse primer 375R	TCT CGT CTA CTA ATA AGT TTC	21	
	Probe 348P	/56-FAM/AAC ACA GAC/ZEN/CTA AAA GGA CAA/	21	
		3IABkFQ/		

C/A: Single Nucleotide Polymorphism

+ G A + TC C + C + A + TT + T C (Table 2). The LNA probe and primers were purchased from Integrated DNA Technologies, and the LNA oligo was acquired from QIAGEN.

2.4. Optimization of single target assays

Samples were run in triplicate in a CFX96 Touch real-time PCR system with a C1000 thermal cycler (Bio-Rad, Hercules, CA, USA). Each PCR reaction contained the following in a 12.5 µl reaction volume: 1 × PCR buffer, 200 nM of each primer and probe for the internal amplification control (IAC; Deer et al., 2010) as described by Eischeid et al. (2013), a constant copy number of 5×10^5 of the IAC template, 0.125 units of Platinum Taq polymerase (Invitrogen/Life Technologies, Grand Island, NY, USA), and 5 µl of DNA, 3 mM Mg and 1.2 mM dNTPs. For *rbcl-accD*, 1000 nM of primers and 500 nM of the probe were added. For *psbM-trnD*, 500 nM of primers and 200 nM of the LNA probe and oligo were used. These concentrations were the same for validation of the duplex assay in both buffer and food matrices. DNA amplification was performed under the following conditions: 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 65 °C for 15 s, and extension at 60 °C for 45 s. Fluorescent signal was read at the end of the annealing step. All resulting data (Figs. S1 and S2) were analyzed using the CFX Manager v3.1 software (Bio-Rad, Hercules, CA, USA).

2.5. Optimization of duplex assay

Two parameters were adjusted for optimal reaction efficiency: annealing temperature (Figs. S3 and S4) and magnesium (Mg^{2+}) concentration (Fig. S5). The effect of the annealing temperature (60, 65, and 68 °C) and the Mg concentration (5 and 8 mM) was evaluated separately in serially diluted English and black walnut DNA in TE buffer. A Mg^{2+} concentration of 5 mM and an annealing temperature of 68 °C were found to be the optimal balance between cross-reactivity, reaction efficiency, and linearity. Optimized amplification conditions were as follows: 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 68 °C for 20 s, and extension at 60 °C for 45 s.

2.6. Testing in spiked food matrices

Following the United States Food and Drug Administration's Guidelines (U.S. FDA, 2019a; 2019b), the assay was tested in three types of commercial food matrices representative of different commodity groups: 1) High water content (leafy vegetables: Caesar salad kit and spinach and kale salad mix), 2) high oil and very low water content (dips: hummus and artichoke dip), and 3) high starch/protein and low water content (baked products: granola bars and chocolate chip cookies). These food matrices were selected because they are likely to cross-contaminate with walnuts in food processing facilities, and have previously been subject to several safety recalls in the United States (<https://www.fda.gov/Safety/Recalls/Archive> Recalls/default.htm). Furthermore, we wanted to evaluate the performance of the assay in challenging food matrices for both DNA- and protein-based allergen

Table 3

Results of assay validation in buffer.

Assay	PCR parameters	English walnut		Black walnut	
		<i>rbcl-accD</i>	<i>psbM-trnD</i>	<i>rbcl-accD</i>	<i>psbM-trnD</i>
Single target	Reaction efficiency (%)	93.9	93.7	97.8	102
	Average C_T value highest DNA concentration (10000 pg/µl)	11.9	10.3	11.9	10
	Average C_T value lowest DNA concentration (0.01 pg/µl)	32.4	31.3	31.3	28.8
Duplex	Reaction efficiency (%)	91.8	92.7	97.8	91.4
	Average C_T value highest DNA concentration (10000 pg/µl)	11.9	11.7	13.9	11.8
	Average C_T value lowest DNA concentration (0.01 pg/µl)	32.8	32.6	33.6	30.8

detection, such as those with high oil and very low water content. Matrices were spiked as described in Sample preparation and DNA extraction (Section 2.1). Assay performance was evaluated at two annealing temperatures (65 and 68 °C) to confirm the robustness of the optimized amplification parameters. The remaining variables were the same as previously described (section 2.5).

3. Results and discussion**3.1. Primer and probe design and evaluation**

A total of 78 full chloroplast genomes were included in the data set used for primer and probe design (Puente-Lelievre & Eischeid, 2018). Based on their level of variation, eleven regions were extracted and aligned separately for primer design (Table 1). Seventeen primer and probe sets were generated and evaluated in 24 samples that included grains, fruits, legumes and nuts as per Puente-Lelievre and Eischeid (2018). Of these, only *rbcl-accD* showed high reaction efficiency and linearity with no significant cross-reactivity with any other nut, including pecan. Initially, *psbM-trnD* showed cross-reactivity with pecan ($C_T = 30$), which resulted from a SNP in the region where the probe binds. The pecan-blocking LNA oligo (Table 2), was designed by locking the nucleotides surrounding the SNP to increase the melting temperature and reduce non-specific binding; this effectively prevented significant cross-reactivity with pecan, and amplification signals were obtained only for English and black walnut.

3.2. PCR performance and assay optimization**3.2.1. Single marker**

Each target was evaluated and optimized separately in both English and black walnut using serial dilutions of DNA in TE buffer that ranged from 10,000–0.01 pg/ul. C_T values were determined from amplification plots (Fig. S1) and used to generate linear standard curves (Fig. S2). Standard curve data indicated that for both markers the PCR was linear

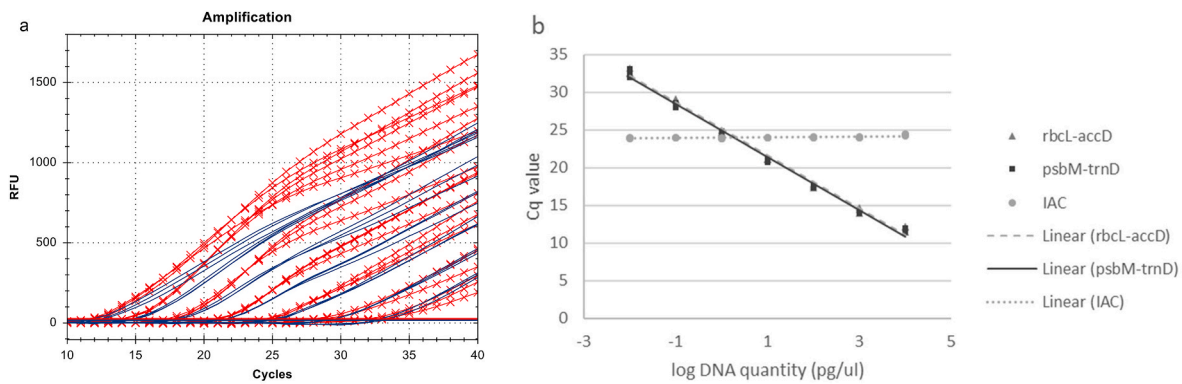


Fig. 1. a) Amplification plots* for duplex assay generated from serial dilutions of English walnut DNA in TE buffer at 68 °C; b) Standard curve generated from amplification curves. IAC: Internal Amplification Control. *note: 45 cycles were used for all PCR runs; in cases with no amplification in later cycles, the software performed an automatic cutoff.

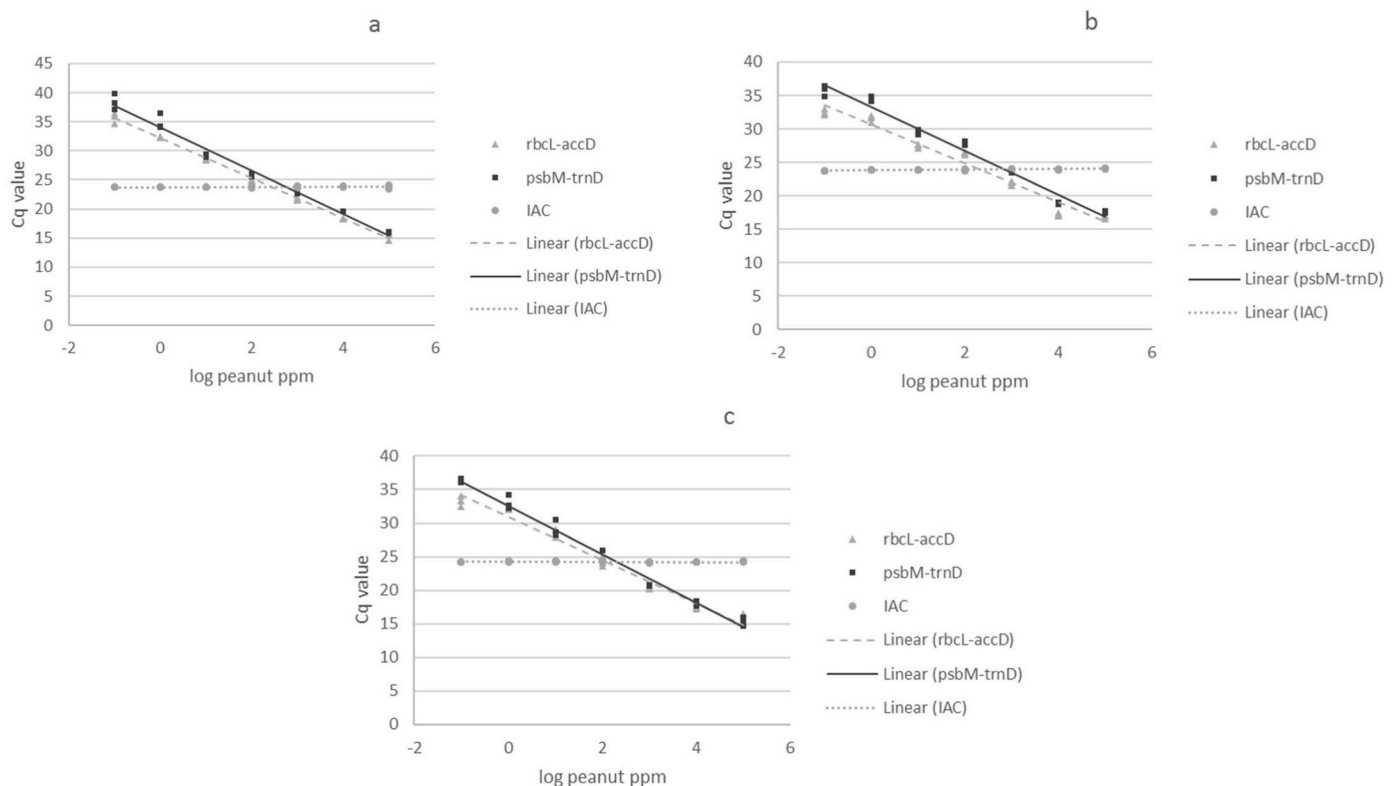


Fig. 2. Standard curves for duplex assay in food matrices spiked with black walnut. a) Granola bar b) Kale spinach salad mix c) Artichoke dip.

over 7 orders of magnitude, with R^2 values > 0.99. Reaction efficiencies for English and black walnut were within the optimal range of 90–110% for both *rbcL-accD* and *psbM-trnD*. Average C_T values were 10–12 at the highest target DNA concentration (10,000 pg/ul) and 28–32.5 at the lowest concentration (0.01 pg/ul) (Table 3). These low C_T values suggest that the copy number of the target is high, and it can be detected and quantified even at the lowest concentration, which results in a very sensitive assay. The internal control was added to all PCR reactions at a constant copy number with the purpose of detecting potential PCR inhibitors. It amplified consistently across all samples except with *rbcL-accD* in buffer. This was most likely caused by an excess of walnut target interfering with amplification of the internal control at high walnut concentrations.

3.2.2. Duplex assay

The performance of the combined primer sets selected – *rbcL-accD* (FAM) and *psbM-trnD* (TEX) – was first evaluated in buffer (Fig. 1, Table 3) at different annealing temperatures (60, 65 and 68 °C) to confirm the optimal conditions to achieve high reaction efficiency and linearity without compromising specificity (Fig. S3). Reaction efficiencies at 65 and 68 °C were equivalent for *rbcL-accD* (92.6 and 91.8%, respectively) and showed small variation for *psbM-trnD* (100.8 and 92.7%, respectively) (Fig. S4). No significant cross-reactivity with pecan was detected at 68 °C (Fig. 1) while a small amplification for pecan was observed at 65 °C 16 cycles after walnut amplification (C_T = 27–28.5) (Fig. S3). Consequently, we selected 68 °C as the most satisfactory annealing temperature. No cross-amplification or interference between the two targets was detected. This was expected since the primers and probes targeted different regions of the chloroplast. C_T values were

Table 4

Results of assay validation in food matrices.

Matrix	rbcL-accD			psbM-trnD		
	Efficiency (%)	R2	Slope	Efficiency (%)	R2	Slope
Granola bar	95.6	1.00	−3.43	91.5	0.99	−3.55
Choc chip cookie	101.4	0.99	−3.29	100.2	0.99	−3.32
Caesar salad mix	115.7	0.96	−3.00	103.2	0.98	−3.25
Kale and spinach salad mix	93.3	0.99	−3.49	92.6	0.98	−3.51
Hummus*	110.6	0.98	−3.09	98.2	0.99	−3.37
Artichoke dip*	96.9	0.99	−3.40	90.4	0.98	−3.57

* Six orders of magnitude, LOQ = 1 ppm.

consistent between the single marker and duplex assays (Table 3). The internal control was also used in the duplex assay at the same constant copy number as the single marker assays. Its amplification was equally consistent (Fig. 1b). The effect of magnesium concentration was assessed at 5 and 8 mM. Reaction efficiencies obtained were within the 90–110% range at both concentrations, but cross-reactivity was significantly higher at 8 mM (Fig. S5). Thus, 5 mM was selected as the preferred parameter to proceed with the validation of the assay in food matrices.

3.3. Validation of duplex assay in food matrices

The robustness and performance of the assay was examined in six food matrices representative of three commodity groups: baked goods (chocolate chip cookies and granola bars), salad greens (Caesar and spinach and kale salads), and creamy dips (hummus and artichoke dip). 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 (Fig. 2). *psbM-trnD* performed within the preferred reaction efficiency range in all food matrices whereas reaction efficiencies with *rbcL-accD* were slightly higher than 110% in the Caesar salad mix and hummus (Table 4). A more consistent assay performance with *psbM-trnD* may be related to the use of a LNA probe and a blocking oligo since they provide more stability to the reaction (You et al., 2006). Even though potential amplification inhibitors were expected in low water/high fat content food matrices (ie. hummus and artichoke dip), none were observed in any of the food matrices. Reaction efficiencies for this type of food matrix stayed within the expected range for 6 orders of magnitude with a limit of quantitation of 1 ppm instead of 0.1 as in the other food matrices (Table 4).

3.4. Conclusions

The assay developed in this study is important for both the enforcement of FALCPA and optimal consumer protection because it enables the differentiation of walnut from other tree nuts and can help allergic consumers make more appropriate food choices. This is a novel contribution to the allergen detection field because it uses a LNA probe to increase specificity for walnut and prevent cross-reactivity with other closely related nuts, and a LNA oligo to specifically block potential cross-reactivity with pecan. LNA probes have successfully been used in assays that aim for higher discrimination power (Sanchiz et al., 2018). This assay not only distinguishes walnut from pecan, it also actively prevents amplification signal from pecan. Moreover, real-time PCR approaches for walnut detection traditionally target the genes for the allergenic proteins that are single copy nuclear regions. The assay presented here targets two high-copy number chloroplast regions in a single reaction, which increases the assay sensitivity as well as its robustness and specificity by having two independent sources of detection. Our results show LODs of 0.01 pg/μL in buffer and most food matrices, and 1 ppm in creamy dips. Finally, this newly developed assay 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 walnut. These included foods not previously tested that can be difficult and unique commodities for allergen detection (e.g. creamy dips and green salads) and products prone to cross-contact with tree nuts in food processing facilities (e.g. baked goods).

CRedit authorship contribution statement

Caroline Puente-Lelievre: Investigation, Formal analysis, Visualization, Writing - original draft. **Anne C. Eischeid:** Conceptualization, Supervision, Methodology, Visualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2020.107644>.

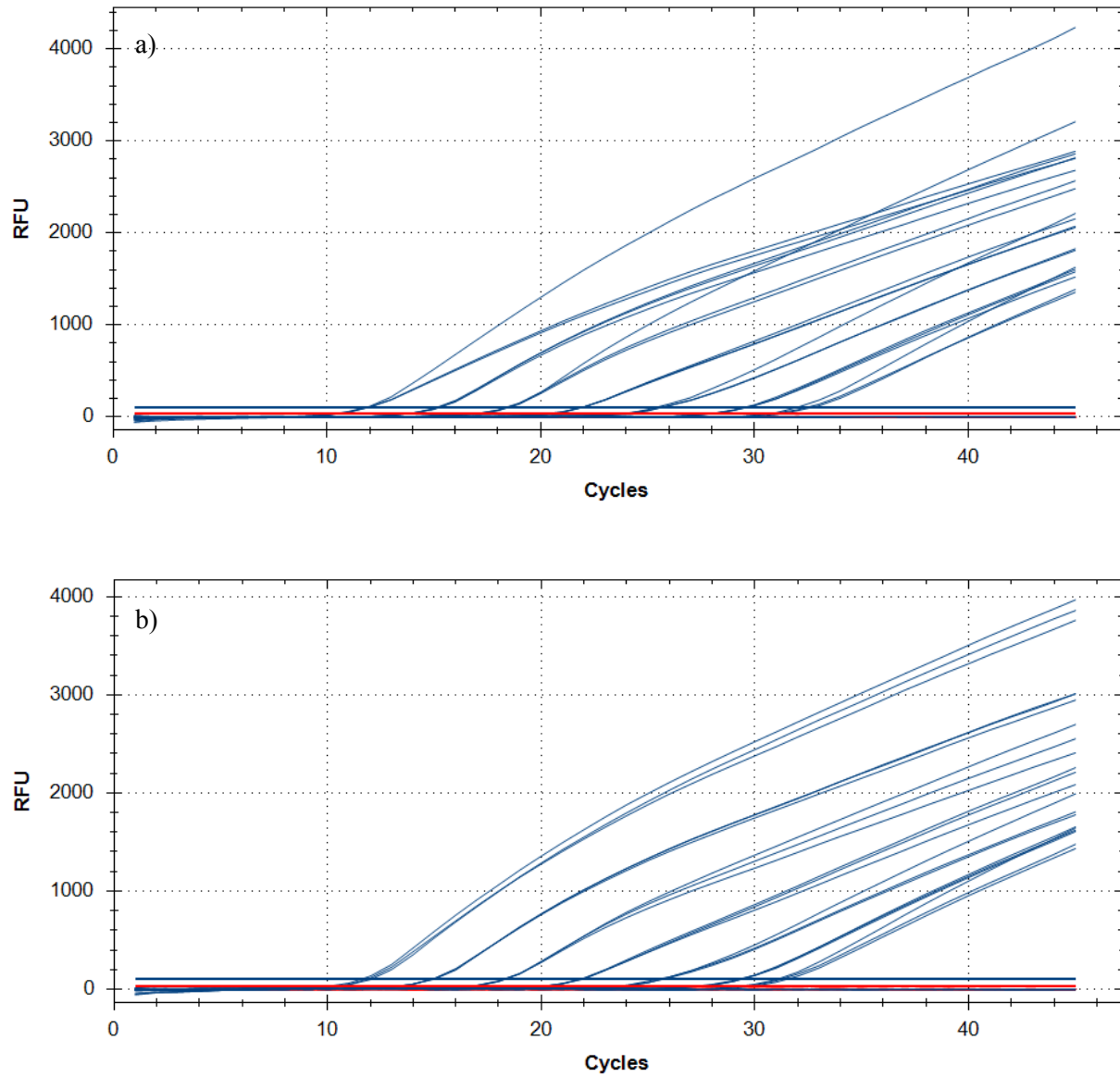
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Supporting information

Figure S1. Amplification plots generated for single markers from serial dilutions of walnut DNA in TE buffer. a) *rbcL-accD* in English walnut, b) *rbcL-accD* in black walnut, c) *psbM-trnD* in English walnut, d) *psbM-trnD* in black walnut.



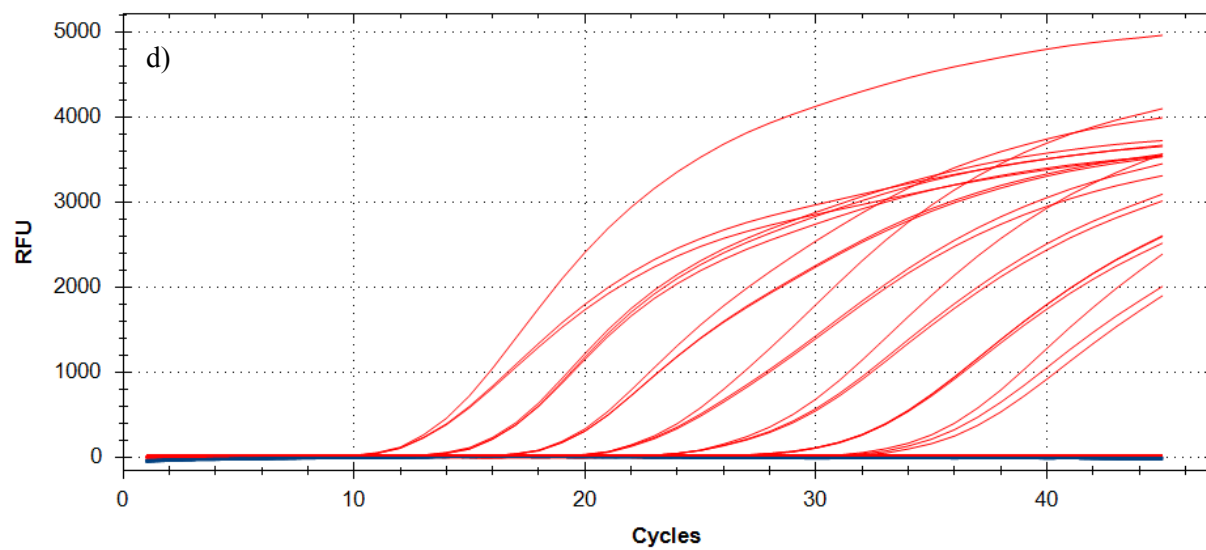
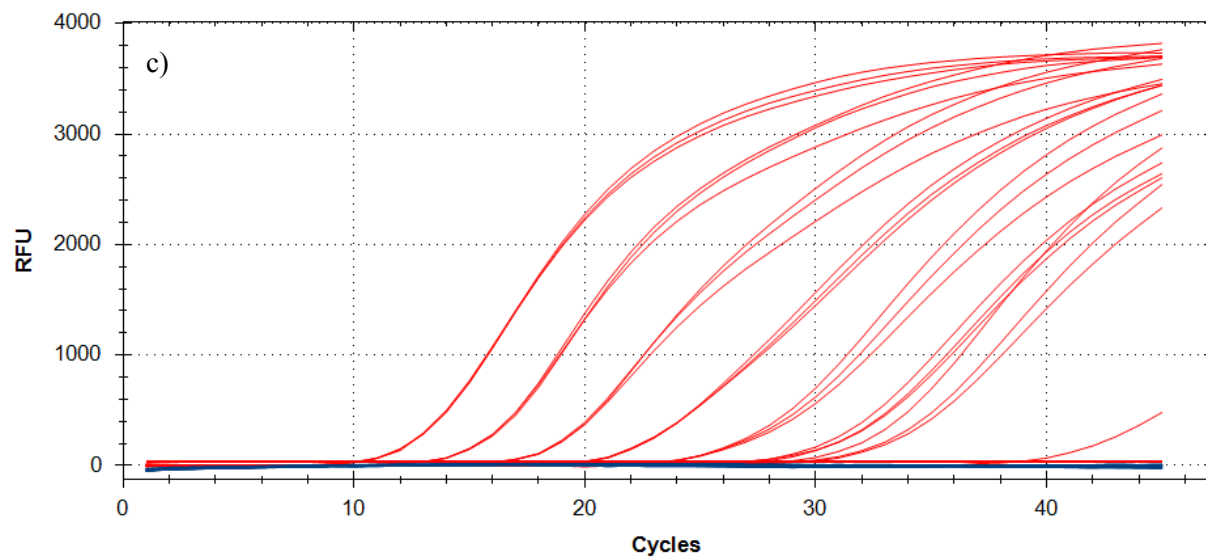
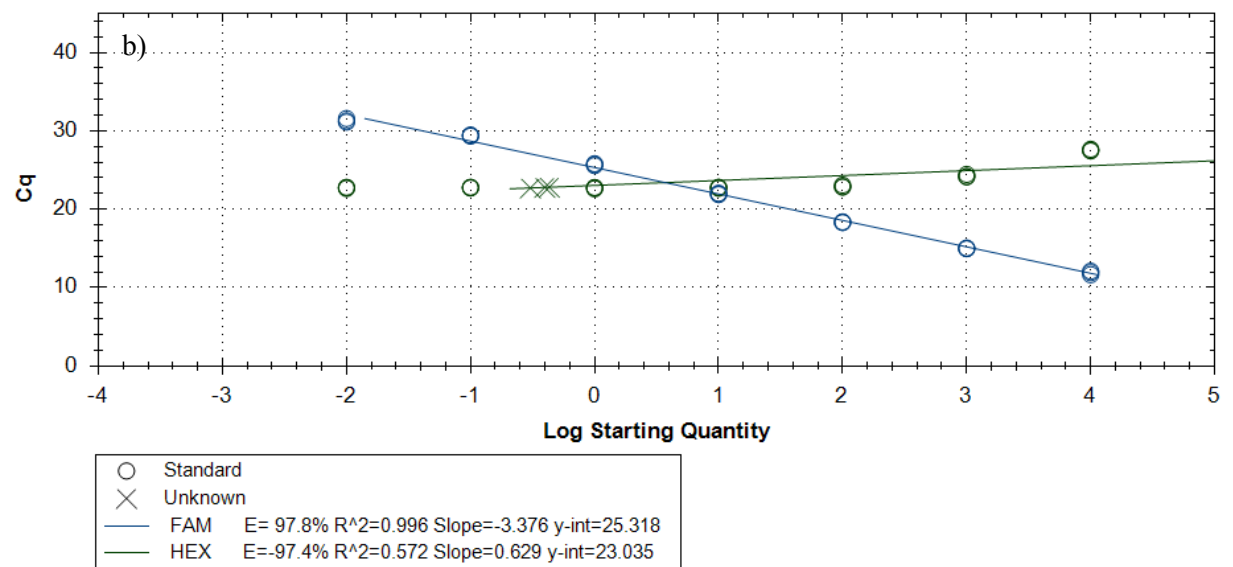
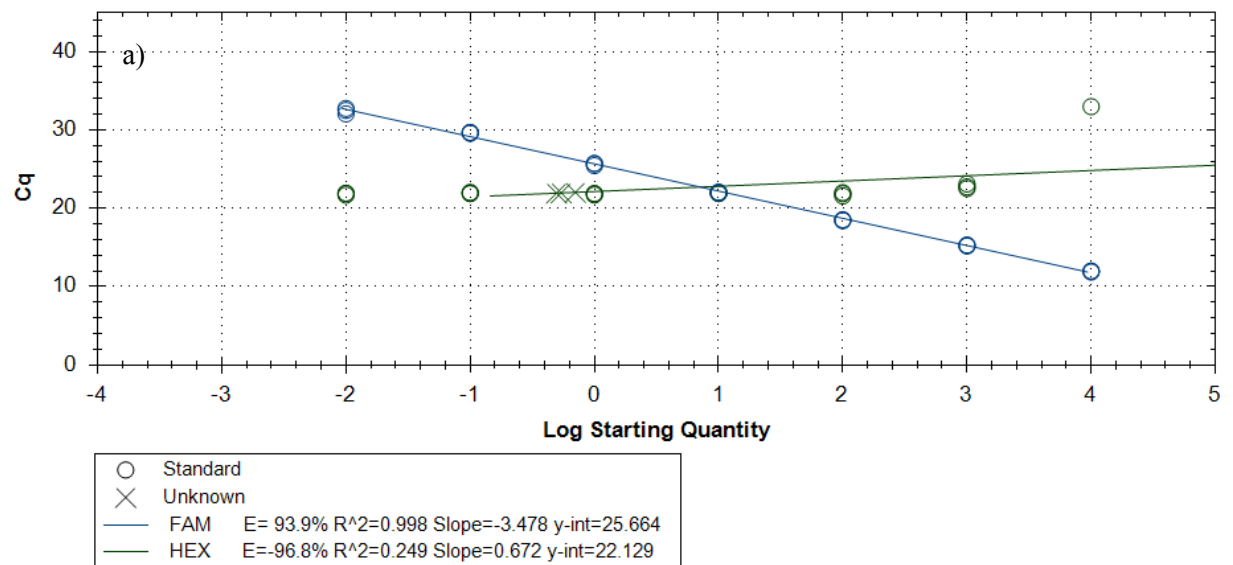


Figure S2. Standard curves generated from amplification plots in figure S1. a) *rbcL-accD* in English walnut, b) *rbcL-accD* in black walnut, c) *psbM-trnD* in English walnut, d) *psbM-trnD* in black walnut. C_T values were calculated from the point at which amplification curves cross threshold and plotted versus (log) walnut DNA spiking concentration (ppm). IAC: Internal Amplification Control.



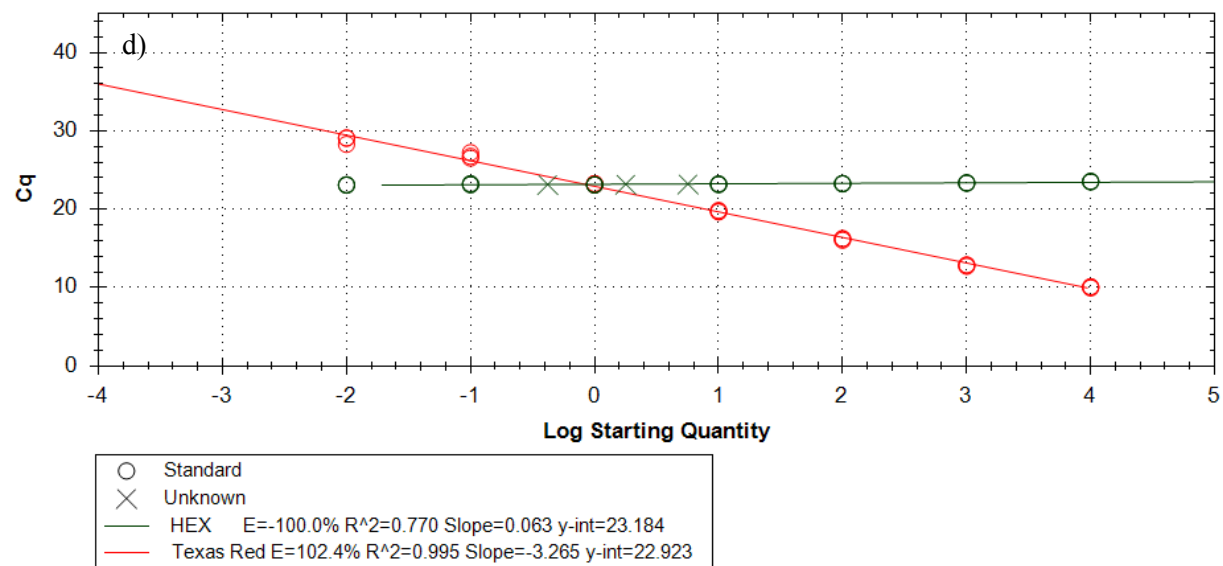
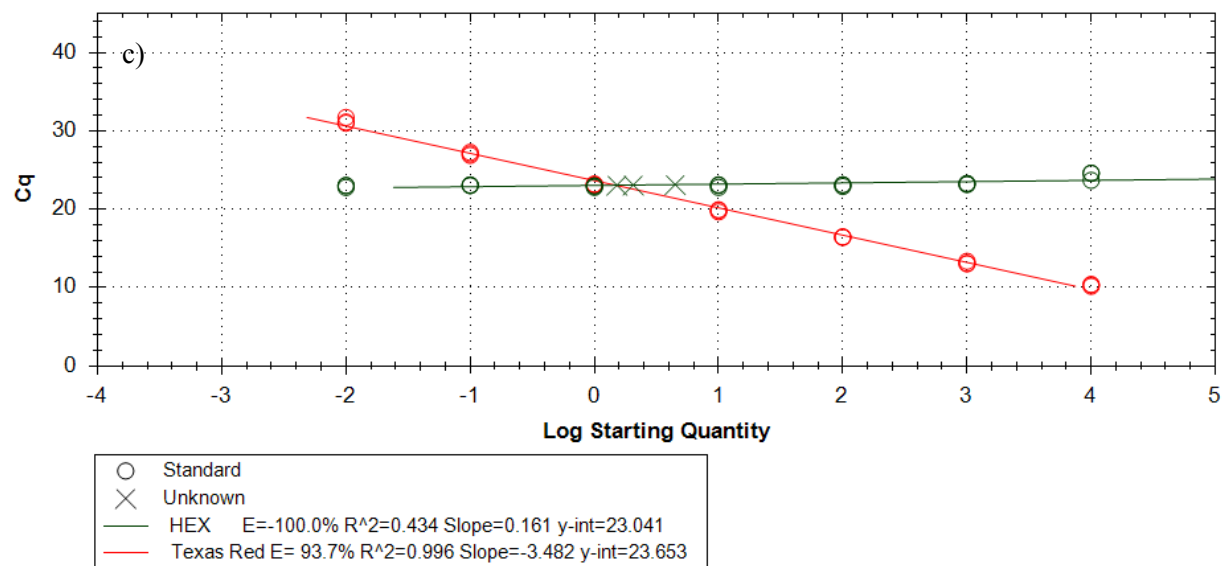
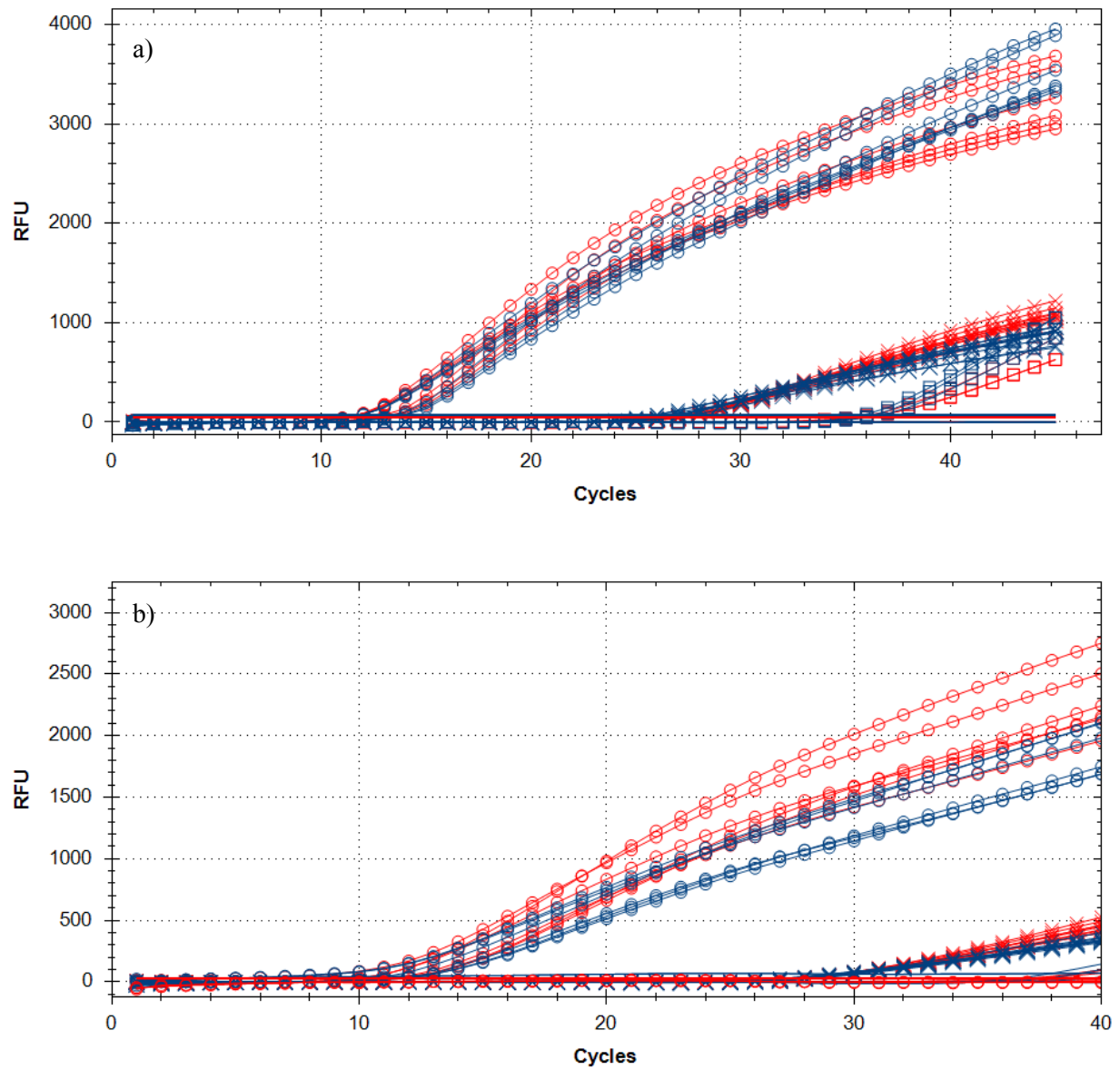


Figure S3. Amplification plot for duplex assay at different annealing temperatures for English walnut. a) 60°C, b) 65°C, and c) 68°C. Later amplification (after 25 cycles) on these plots results from nontarget nuts and represents cross reactivity.



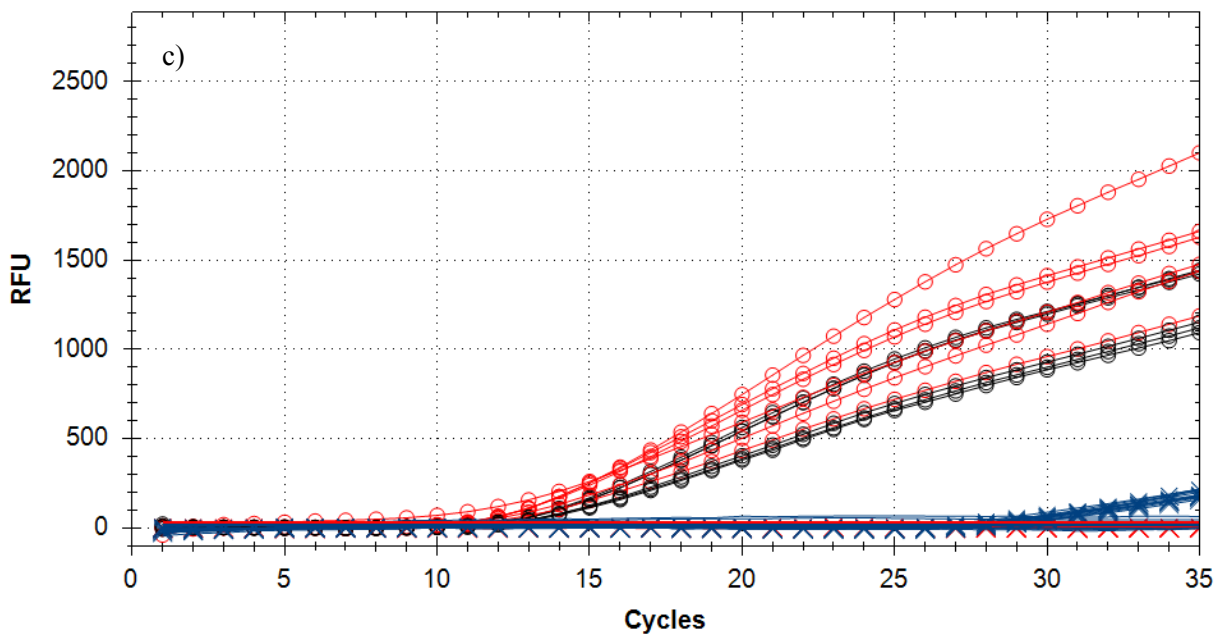
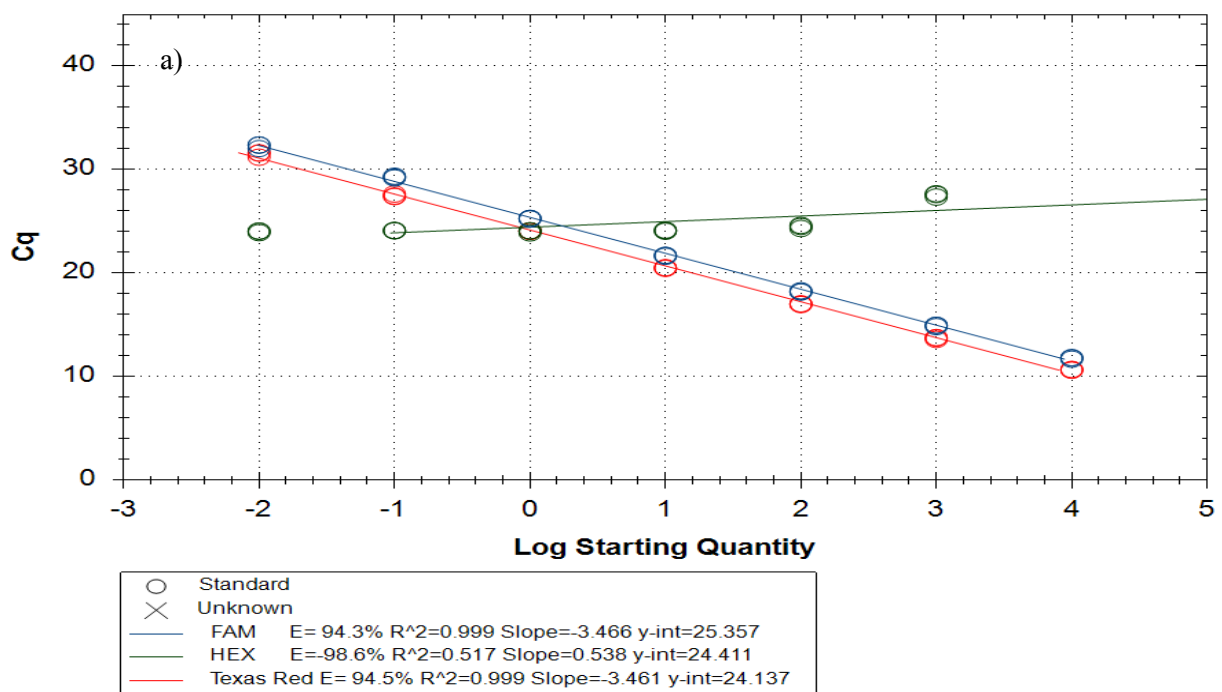


Figure S4. Standard curves for duplex assay at different annealing temperatures for English walnut. a) 60°C, b) 65°C, and c) 68°C.



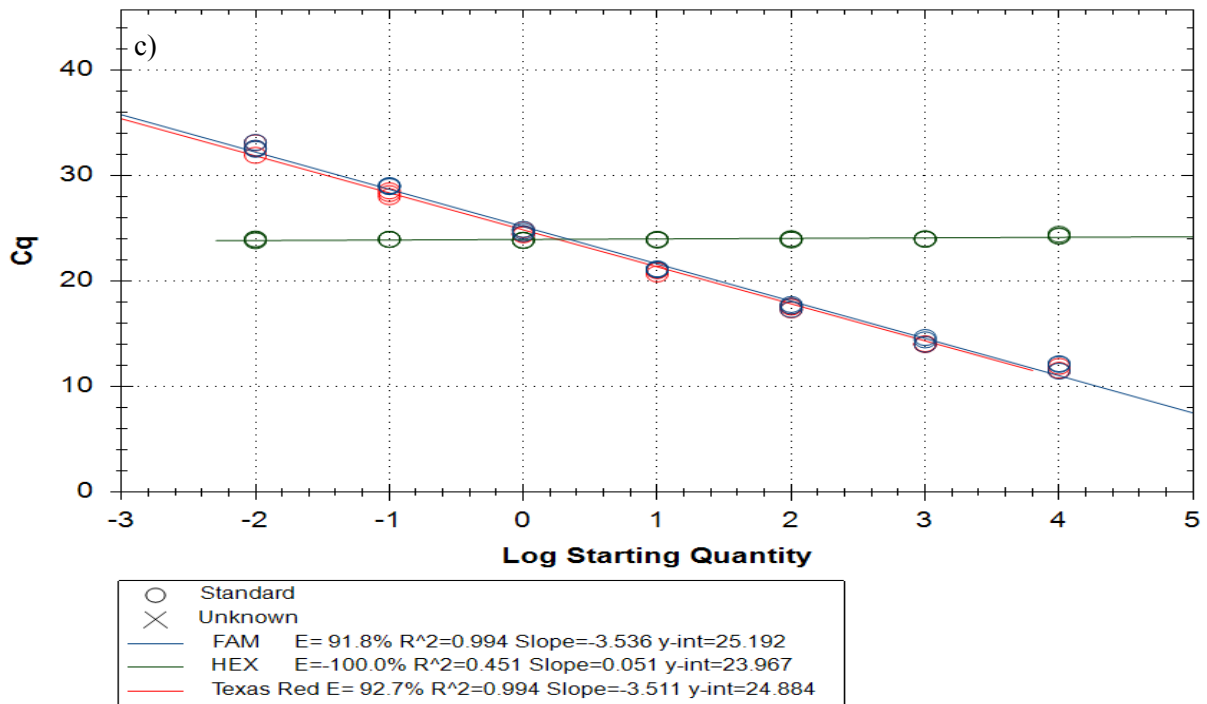
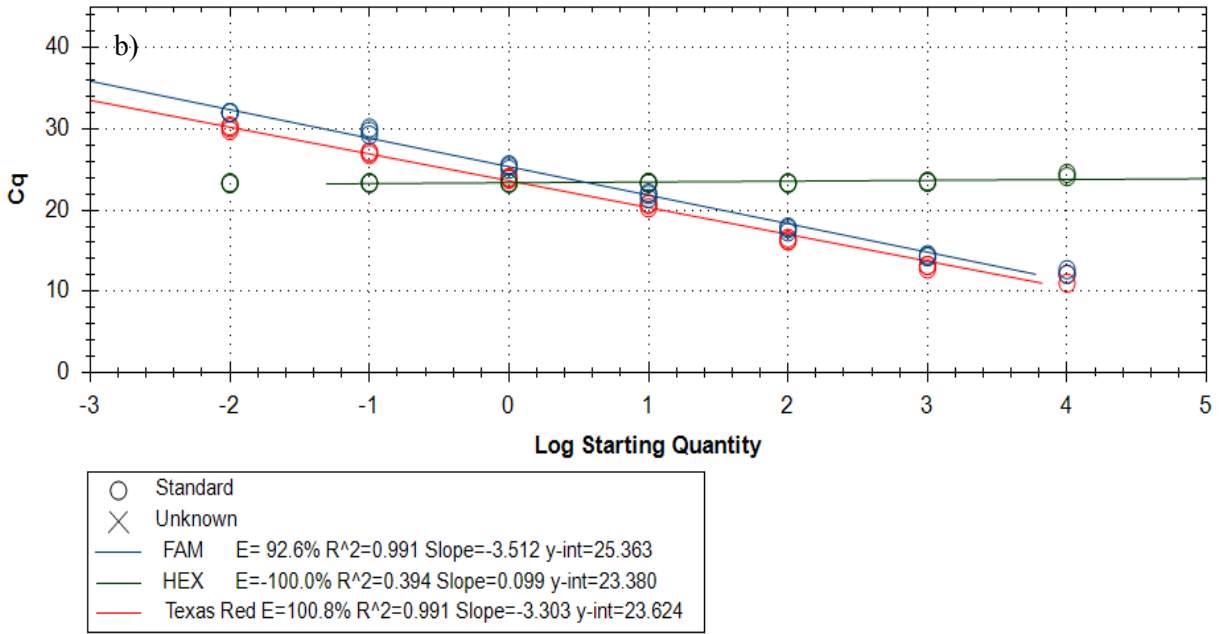


Figure S5. Standard curves for duplex assay at two different magnesium concentrations. a) 5 mM Mg with English walnut, b) 5mM Mg with black walnut, c) 8 mM Mg with English walnut, and d) 8 mM with black walnut.

