

Detection of Food Allergens by Taqman Real-Time PCR Methodology

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Abstract

Real-time PCR (polymerase chain reaction) has shown to be a very effective technology for the detection of food allergens. The protocol described herein consists on a real-time PCR assay targeting the plant ITS (Internal Transcribed Spacer) region, using species-specific primers and hydrolysis probes (Taqman) dual labeled with a reporter fluorophore at the 5' end (6-carboxyfluorescein, FAM) and a quencher fluorophore at the 3' end (Blackberry, BBQ). The species-specific real-time PCR systems (primers/probe) described in this work allowed the detection of different nuts (peanut, hazelnut, pistachio, almond, cashew, macadamia, walnut and pecan), common allergens present in commercial food products, with a detection limit of 0.1 mg/kg.

Key words Real-time polymerase chain reaction, Taqman probe, Nuts, Food allergy

1 Introduction

Within the food industry and official food control agencies, the currently most commonly used methods to assess the presence of allergens in food products are based in protein detection [1]. Particularly, the enzyme-linked immunosorbent assay (ELISA), based on the interaction of species-specific proteins with specific antibodies either raised in animals [2, 3] or produced by recombinant technologies [4, 5], is the most widespread and commonly used protein-based test for allergen detection. Immunochemical methods can be highly sensitive and specific; however, depending on the epitopes recognized by the antibodies, they may be biased by cross-reactivity with other species. Moreover, the detection of the target proteins can be compromised by the variety of food

processing methodologies, like heat treatment or roasting, which can strongly affect their integrity and quality [2]. An alternative to the direct detection of proteins is provided by DNA-based methods, where the target molecules are specific DNA sequences which are amplified by the polymerase chain reaction (PCR), either by qualitative end-point PCR (distinguished by their differential migration through agarose gel electrophoresis) or by real-time PCR using fluorescent-labeled probes or dyes. In real-time PCR, the crossing point value (C_p), which refers to the cycle number where the sample's fluorescence increases above the background level, is related to the amount of amplicon generated, so it provides quantitative information about the presence of the target species.

Real-time PCR has several advantages compared to conventional end-point PCR, for example: (1) the ability to detect very small size products, since it eliminates the need for gel visualization, (2) the reduced risks for crossover contamination, as reactions are kept confined during amplification and analysis, (3) the capability for large-scale processing and high automated throughput, and (4) the improvement in precision, sensitivity, dynamic range and resolution, since data are analyzed at the exponential phase of the PCR reaction.

Initially, intercalating dyes were used to measure real-time PCR products, but they have the disadvantage that both, specific and nonspecific PCR products, are detected. Besides, TaqMan[®] probes consisting on single-stranded oligonucleotides containing a fluorescent reporter (donor) dye on the 5' end and a quencher (acceptor) dye on the 3' end, are designed to hybridize within the amplification primers resulting in specific system detection. The proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) mechanism. However, the hydrolysis of the probe during the amplification due to the 5'-3' double-strand-specific exonuclease activity of the *Taq* polymerase separates the donor fluorophore from the quencher resulting in a fluorescence increase. Fluorescence intensity registered will be proportional to the amount of target DNA present in the sample [6].

Here, we describe the development of a high-resolution species-specific Taqman[®] real-time PCR protocol to detect peanut [7], hazelnut [8], pistachio [9], almond [10], cashew, macadamia [11], walnut and pecan [12]; nuts that are included within the 14 major food allergens that should be declared in food labeling according to EU Regulation 1169/2011 [13].

2 Materials

Prepare all solutions using ultrapure water (PCR water if indicated) and analytical grade reagents (*see Note 1*).

2.1 Components and Reagents for DNA Extraction

1. DNA extraction buffer: 10 mM Trizma® base, 150 mM NaCl, 2 mM EDTA, 1% SDS, pH 8.0. Add about 200 mL water to a beaker provided with a magnetic stirring bar. Weight and transfer to the beaker: 0.6 g Trizma® base, 4.38 g NaCl, 0.37 g EDTA and 5 g SDS. Mix until complete dissolution of solids and adjust pH to 8.0 with 1 N HCl. Make up in a graduated flask to 500 mL with water and transfer the solution to a 1 L glass bottle. Autoclave for 20 min at 121 °C. Make 15–20 mL aliquots and store at room temperature for a period no longer than 3 months.
2. Guanidine hydrochloride (5 M): add about 80 mL water to a beaker provided with a magnetic stirring bar. Weight 47.76 g of guanidine hydrochloride and transfer into the beaker. Mix until complete dissolution and make up in a graduated flask to 100 mL with water. Autoclave for 20 min at 121 °C and make 500 µL aliquots in 1.5 mL microcentrifuge tubes. Store at room temperature for a period no longer than 3 months (*see Note 2*).
3. Proteinase K (20 mg/mL): weight in precision balance 20 mg of proteinase K and transfer to a microcentrifuge tube with 1 mL of PCR water. Vortex until complete dissolution. Make 200 µL aliquots into 500 µL microcentrifuge tubes and store at 4 °C.
4. Isopropanol (80%): prepare 50 mL by mixing 40 mL of isopropanol with 10 mL of PCR water in a conical tube. Make 2 mL aliquots in 2 mL microcentrifuge tubes and store at room temperature.
5. Chloroform. The volume required will be aliquoted in a conical tube and discarded after use.
6. DNA elution solution: water (DNase, RNase-safe certified, 0.1 µm filtered) or Tris-EDTA buffer solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Make 1 mL disposable aliquots in 1.5 mL microcentrifuge tubes and store at –20 °C.
7. Kit Wizard® DNA Clean-Up System (Promega, Madison, WI, USA): each Kit contains sufficient reagents for 100 samples (100 mL Wizard® DNA Clean-Up Resin, 100 Wizard® minicolumns and 100 syringe barrels). Aliquot the whole content of the resin into 1.5 mL microcentrifuge tubes at a volume of 1 mL. Store at room temperature and protected from direct sunlight exposure (*see Note 3*).

8. Vac-Man[®] Laboratory Vacuum Manifold (Promega): vacuum manifold capable of processing 20 samples simultaneously and provided with 20 one-way luer locks stopcock, so each port is controlled individually.
9. Vacuum pump “Fisher Scientific” mod. EYELA A-3S.
10. Incubator at 55 °C provided with a shaker.
11. Thermoblock at 65 °C.

2.2 Components and Reagents for Real-Time PCR

1. Water (DNase, RNase-safe certified, 0.1 µm filtered).
2. LightCycler[®] TaqMan[®] Master Mix (Roche Diagnostics GmbH, Mannheim, Germany): briefly spin one vial of enzyme (1a) and one vial of “reaction mix” (1b), pipet 10 µL from vial 1a into vial 1b and mix gently by pipetting up and down (do not vortex enzyme solutions). Re-label this master mix vial with number 1 (*see Note 4*). Vial number 2 contains water, PCR grade (*see Note 5*). Kit components must be stored at −20 °C.
3. Primers (forward and reverse) (*see Note 6*): resuspend the lyophilized oligonucleotides in 1 mL water to get the stock solution. Vortex and spin the stock solution before making two 200 µL aliquots into 1.5 mL microcentrifuge tubes to obtain two work solutions (label these solutions as A and B). Always vortex and gently spin the primer work solution before preparing the dilutions for real-time PCR assay. Into 500 µL microcentrifuge tubes, add the necessary volume of work solution A and PCR water to obtain the final primer concentration used in the PCR reaction (forward primers at 300 nM/µL and reverse primers at 900 nM/µL). Make 50 µL disposable aliquots and store at −20 °C.
4. *Taqman*[®] probes: resuspend the lyophilized oligonucleotides in the necessary volume (see manufacturer indications) of water to obtain a final concentration of 20 pmol/µL (stock solution). Once it is completely resuspended by pipetting up and down (do not vortex labeled oligonucleotides), prepare two 100 µL aliquots into 1.5 mL microcentrifuge tubes to obtain two work solutions (label solutions as A and B). Using 200 µL microcentrifuge tubes, prepare disposable 1/10 dilutions (2 pmol/µL) by mixing 3 µL of the work solution and 27 µL of PCR water. Store these aliquots at −20 °C.

The primers and probes used for real-time PCR detection of each food allergen are listed and described in Table 1.

5. LightCycler[®] 2.0 (Roche Applied Science, Germany) equipped with LightCycler[®] 2.0 Sample Carousel.
6. LightCycler[®] Capillaries (*see Note 7*).
7. LightCycler[®] Capping Tools.

Table 1
DNA sequences and description of the primers and probes listed alphabetically

Primers	Length (bp)	Sequence (5'/3')	Description	Target gene	Fragment length (bp)	Reference
AlmondITSfw	19	CTAGCCGAACGACCCGAGA	Almond-specific forward primer	ITS	76	[10]
AlmondITSrev	19	CCGAGATAAAGGGGACGAG	Almond-specific reverse primer			
CashewITSfw	19	GAACGAACCCGATGATCC	Cashew-specific forward primer	ITS	69	[11]
CashewITSrev	19	CCATCGAGGGTCAAGGAG	Cashew-specific reverse primer			
HazelnutITSfw	20	GAGACACTCGTGCCCTTCTTG	Hazelnut-specific forward primer	ITS	70	[8]
HazelnutITSrev	17	GGAGCACTCTTTAGTTGAAGTTCC	Hazelnut-specific reverse primer			
MacITSfw	19	AGTCAAGGGCTCCTCGTGT	Macadamia-specific forward primer	ITS	72	[11]
MacITSrev	20	ATGTCCGAACAATGGCAAAG	Macadamia-specific reverse primer			
PeanutITSfw	20	AAGCCAAACGTTTCTGCTCT	Peanut-specific forward primer	ITS	90	[7]
PeanutITSrev	17	CGTTGCCGAGAGTCGTT	Peanut-specific reverse primer			
PecITSfw	18	ATGAAAGCTGCCACCCGC	Pecan-specific forward primer	ITS	69	[12]
PecITSrev	18	CATTGTTCCGCCGGGAAG	Pecan-specific reverse primer			
PistITSfw	20	GGCGTTCGAAACAGAACCTA	Pistachio-specific forward primer	ITS	72	[9]
PistITSrev	20	CGAGGGTCAAAAGAGTCCGTA	Pistachio-specific reverse primer			
WalITSfw	20	GACAATCGGTGTTTGAGAAA	Walnut-specific forward primer	ITS	70	[12]
WalITSrev	20	GTCGAGGAGCACCTTCACAG	Walnut-specific reverse primer			
18Sfw	16	TGGTGCCAGCAGCCGC	Positive control forward primer	18S rRNA	77	[7-12]
18Srev	25	TCCAACCTACGAGCTTTTAACTGCA	Positive control reverse primer			
AlmondITSP	22	6FAM-ACTAGTTTCAAAGCGGGG-BBQ	Almond probe	ITS		[10]
CashewITSP	22	6FAM-ACGCGCTCTCTGTGCC-BBQ	Cashew probe	ITS		[11]
HazelnutITSP	18	6FAM-GCGCCGGGGTTCGTTGTT-BBQ	Hazelnut probe	ITS		[8]
MacITSP	19	6FAM-TCACGATGAGTGGGGTC-BBQ	Mac probe	ITS		[11]
PeanutITSP	24	6FAM-TAACTCTTGTGGTCACTCGTCGCC-BBQ	Peanut probe	ITS		[7]
PecITSP	19	6FAM-CGGGTCAGTCTCCTCGTTTC-BBQ	Pecan probe	ITS		[12]
PistITSP	22	6FAM-TCGTGGGTTGGGTTCTACTGT-BBQ	Pistachio probe	ITS		[9]
WalITSP	18	6FAM-TGACCCGTCGTGTGTGCCCC-BBQ	Walnut probe	ITS		[12]
18SP	22	6FAM-CGCTATTGGAGCTGGAATTACC-BBQ	Universal probe	18S rRNA		[7-12]

8. LightCycler® Centrifuge Adapters in a dedicated aluminum cooling block. Store at 4 °C (*see Note 8*).
9. Centrifuge provided with a microcentrifuge tubes rotor.

3 Methods

3.1 Sample Digestion

1. Prepare working areas (*see Note 9*).
2. Grind sample in order to facilitate the DNA extraction. A pestle and mortar or a kitchen robot can either be used.
3. With a disposable spatula weigh 200 mg of the sample (in case of liquid sample, pipette 200 µL) into a 2 mL microcentrifuge tube and add 860 µL of extraction buffer, 100 µL of guanidine hydrochloride (5 M) and 40 µL of proteinase K (20 mg/mL) (*see Note 10*).
4. Homogenize samples with a vortex mixer and incubate overnight at 55 °C with shaking at 60 rpm.

3.2 DNA Purification

1. Remove the samples from the incubator and leave them to cool at room temperature for 10 min before adding 500 µL of chloroform to the lysate (*see Note 11*).
2. Homogenize with a vortex mixer and centrifuge at 20,000 × *g* for 10 min (*see Note 12*).
3. Carefully pipet 500 µL of the lysate (the clear aqueous supernatant containing the DNA) into 1 mL aliquot of the resin and gently mix by pipetting up and down.
4. Pipet the resin/supernatant mix into the syringe, open the stopcocks and apply vacuum to draw the whole solution through the minicolumn. Close the stopcocks and break vacuum.
5. Add 2 mL aliquot of 80% isopropanol to the syringe to wash the column and re-apply vacuum following the same procedure. Apply vacuum for 30 s after the solution has been pulled through the column in order to encourage column drying.
6. Remove the syringe and transfer the minicolumn to a clean 1.5 mL microcentrifuge tube with the cap previously cut away.
7. Centrifuge the minicolumn at 20,000 × *g* for 2 min to remove any residual isopropanol.
8. Transfer the minicolumn to a new microtube with the cap previously cut away. To elute the DNA, add 50–100 µL of prewarmed (65 °C) water or TE buffer to the minicolumn and wait for 1 min.
9. Centrifuge the minicolumn for 1 min at 20,000 × *g* to elute the bound DNA.

10. Discard the minicolumn and distribute the eluted DNA (referred to as “DNA extract”) into two aliquots of 25–50 μL in 500 μL microcentrifuge tubes. DNA extracts may be stored at $-20\text{ }^{\circ}\text{C}$.
11. Make a disposable aliquot of 3 μL in a 200 μL microcentrifuge tube to measure the DNA concentration with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Delaware, United States) (*see* **Note 13**).
12. After each extraction, wash with 70% ethanol the stopcock one-way luer locks connected to the vacuum manifold and change vacuum pump water.

3.3 Real-Time Polymerase Chain Reaction Protocol

1. Prepare working areas (*see* **Note 14**).
2. Program the LightCycler[®] instrument before preparing the reaction mixes (*see* **Note 15**). In this work, program shown in (Table 2) was suitable for all the real-time PCR primers/probe systems.

The hydrolysis probes used in this assay are FAM-labeled at the 5' end so the fluorescence default channel employed was 530 λ (emission maximum of the fluorescence reporter dye) with a gain value “1”.

3. Prepare LightCycler[®] TaqMan[®] Master Mix and primers and probe solutions as previously indicated (forward primers at 300 nM/ μL , reverse primers at 900 nM/ μL , and probes at 2 pmol/ μL).
4. Dilute DNA extracts to obtain the required concentration for PCR (2 μL of sample will be added to each reaction) (*see* **Note 16**).

Table 2
PCR program used in all real-time systems

Analysis mode	Cycles	Segment	Target temperature ($^{\circ}\text{C}$)	Hold time	Acquisition mode
Pre-incubation None	1		95	10 min	None
Amplification Quantification	55	Denaturation	95	5 s	None
		Annealing	60	30 s	None
		Extension	72	1 s	Single
Cooling None	1		40	30 s	None
Temperature transition rate 20 $^{\circ}\text{C}/\text{s}$					

Table 3
PCR primer mixtures

Component	Concentration (pmol)	Volume per sample (μL)
Forward primer	3	1
Reverse primer	9	1
Probe	2	1
<i>LightCycler® TaqMan® Master</i> (vial 1)		2
PCR water (vial 2)		3
<i>Final volume PCR Mix</i>		8

5. In the reagents cabinet, prepare a rack with empty labeled 200 μL microcentrifuge tubes, one per test sample, including the negative controls: (a) PCR negative control (NC) and (b) the DNA negative extraction control (NEC) (*see Note 17*).
6. Prepare PCR Mix as described in (Table 3) for a volume of 10 μL per reaction (*see Note 18*).
7. Add 8 μL of PCR Mix to each tube.
8. In the tubes corresponding to the negative controls, NC and NEC, add 2 μL of PCR water (vial 2) and 2 μL of the negative extraction control, respectively.
9. Transfer the rack to the DNA cabinet and in the tubes corresponding to the test DNA samples, add 2 μL of DNA template at the required concentration.
10. Mix and briefly spin all the microcentrifuge tubes.
11. Bring the cooling block with the LightCycler® Centrifuge Adapters out of the fridge and clean it with 70% ethanol.
12. Fill the capillaries as follows: with clean forceps, carefully place a capillary into the adapter, pipet the complete PCR reaction volume (10 μL) into the capillary head and seal it with a stopper with the help of the LightCycler® Capping Tool (*see Note 19*).
13. Centrifuge the adapters at $500 \times g$ for 1 min (if a higher speed is used, the risk of capillaries breakage increases).
14. Transfer the capillaries to the LightCycler® 2.0 Sample Carousel.
15. Introduce Carousel in the instrument and run the real-time PCR program.
16. Obtain and interpret results: the crossing point value (C_p) is calculated automatically by the LightCycler® software as the first maximum of the second derivative of the curve (*see Note 20*).

3.4 Selection of Genetic Marker and Design of Primers and Probes

The next steps are followed to develop and assess the applicability of the real-time PCR assay to detect allergenic nut species in food products. In this work, the plant ITS-DNA region has been used as suitable genetic marker for the design of species-specific primers and Taqman[®] probes. NCBI (National Center of Biotechnology Information) database was used to search for the ITS sequences available for various plant species. EMMA program (EMBOSS software) allowed the alignment and computer analysis of the sequences. Primer Express 2.0 software helped in primer design. Taqman[®] probes were located within the gene fragment delimited by each species-specific primer pair and were designed and synthesized by TibMolBiol (Berlin, Germany). A positive amplification control (PAC) system (primers/probe) was designed in a conserved region of the nuclear 18S rRNA of eukaryotes (Table 1).

3.5 Assay Specificity and Efficiency (See Note 21)

1. Perform the DNA extraction from the target nuts: peanut, hazelnut, pistachio, almond, cashew, macadamia, walnut, and pecan (stored at room temperature in the dark). Whenever possible, try to test different available commercial varieties of each target species.
2. Perform the DNA extraction of different plant and animal species typically used in the manufacture of commercial food products (test at least 50 species for representative results).
3. Analyze all the samples with each species-specific PCR system, as well as with the PAC system.
4. When the species-specific system is employed, a *C_p* value should be obtained for the corresponding target DNA, whereas no amplification signal should be obtained for heterologous species DNA. For all the eukaryotic species, a *C_p* value should be obtained with the 18S rRNA system (PAC).
5. Prepare two binary mixtures of every allergenic target species (raw and heat-treated) in wheat flour at different concentrations ranging between 100,000 mg/kg and 0.1 mg/kg by (1) roasting 100 g of every sample at 160 °C for 13 min, (2) finely grinding the different varieties of nuts (both raw and roasted) with the aid of a pestle and mortar or a kitchen robot, (3) in order to obtain the first target concentration (100,000 mg/kg), mix thoroughly 50 g of ground sample with 450 g of wheat flour at maximum speed for 5 min in a kitchen robot (Thermomix, Vorwerk).
6. Repeat the process described in **item 5** with tenfold dilutions (50 g of previous mixture and 450 g of wheat flour) to obtain the rest of mixture concentrations: 10,000 mg/kg, 1,000 mg/kg, 100 mg/kg, 10 mg/kg, 1 mg/kg, and 0.1 mg/kg.
7. Perform DNA extraction for each binary mixture in triplicate.

8. Analyze the three replicates of both series of mixtures (raw and toasted) with the corresponding species-specific PCR system.
9. Construct the standard curve using a simple regression model by plotting the mean C_p values versus the log input DNA concentration ([]). The correlation between both variables is semilogarithmic where b is the slope and a is the intercept of the standard curve.
10. Calculate PCR efficiency (E) from the slope of the standard curve, according to the following formula:

$$E = \left(10^{(-1/\text{slope})} - 1\right) \times 100$$

Ideally the efficiency should be 100% (for each cycle the amount of product doubles the initial concentration), corresponding to a slope value of -3.32 . Optimally, a real-time PCR reaction should have efficiency between 90% and 110%, which corresponds to a slope ranging between -3.58 and -3.10 values. Other parameters such as Cochran’s test, regression analysis, or variance analysis with lack of fit can be evaluated following described validation protocols [14].

An example of species-specific Taqman real-time PCR assay developed for walnut detection [12] is shown in Fig. 1. The detection limit achieved for all the allergenic nut species described in this protocol is 0.1 mg/kg (*see* **Note 22**).

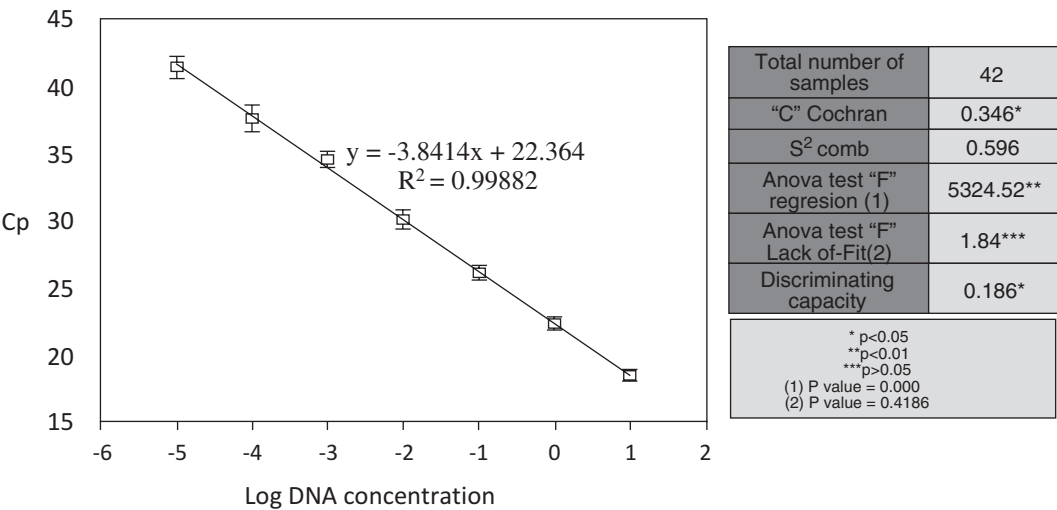


Fig. 1 Linearity test, regression line, and sensitivity parameters of the walnut Taqman real-time PCR system. Three different DNA extractions at seven concentrations (100,000–0.1 mg/kg) for both binary mixtures of raw and heat roasted (160 °C/13') walnut are used

4 Notes

1. Before and after use, all the flow laminar cabinets should be cleaned with Micro-90 (concentrated cleaning solution for critical cleaning applications, Cole-Parmer®, Illinois, United States), 70% ethanol and termi-DNA-Tor® (BioTools®, Madrid, Spain). To minimize the risk of contamination, we suggest to prepare all reagents in a cabinet (referred to as “reagents cabinet”) different to the one employed for DNA manipulation (referred to as “DNA cabinet”). Also, different micropipettes for reagents and DNA samples, as well as different color tube racks (i.e. white for reagents and red for DNA solutions) are used. Use sterile filter pipette tips and microcentrifuge tubes RNase-, DNase-, and pyrogen-safe certified. Store tubes in closed sterile containers inside the reagents cabinet and use forceps for their manipulation to avoid hand contamination. Wear clean protective gloves during the entire procedure.
2. To avoid contaminations, these aliquots may be discarded among different extractions.
3. Thoroughly mix the Wizard® DNA Clean-Up Resin before removing an aliquot. If crystals or aggregates are present, dissolve by warming the resin to 37 °C for 10 min. Cool to 25–30 °C before using the 1 mL aliquot for the extraction.
4. The volume of the resulting Master Mix (5×) is sufficient for 64 reactions with a final volume of 10 µL per reaction.
5. This water is for exclusive use in PCR Mix preparation. Make 100 µL disposable aliquots into 200 µL microcentrifuge tubes.
6. Do not freeze lyophilized oligonucleotides (primers and probes). They must be stored at 4 °C until resuspended. Employ work solution A for the experiment unless PCR contamination is detected and this result is confirmed with the aliquots from this work solution. In this case, discard solution A and use B work solution from there on. The optimum concentrations of primers and probes for PCR are experimentally determined for each PCR system as those yielding the highest endpoint fluorescence and the lowest C_p .
7. Do not touch the surface of the capillaries. Always wear gloves when handling the capillaries.
8. The LightCycler® Centrifuge Adapters are designed to hold and protect the LightCycler® Capillaries during pipetting and centrifugation, ensuring that the entire volume of reaction mix is concentrated into the tip of the capillary. The cooling block maintains a low temperature environment during real-time PCR setup, significantly reducing the risk of primer-dimer formation.

9. If possible, sample digestion should be performed in a room different to the one used for the preparation of PCR reactions. Clean bench surface and scale plate with Micro-90, 70% ethanol, and termi-DNA-Tor. Work close to the sterile area around the Bunsen burner so that possible contaminants in the air are wafted away from your work area. After manipulation of each sample species, clean the bench again and move to a different one to avoid aerosol cross-contaminations within the different working species. Change your protective gloves between the different sample species.
10. Unless contrary indication, each DNA extraction is performed in duplicate. A negative DNA extraction control (NEC) is included for each DNA extraction. So, this control is handled in parallel, following the same procedure than the DNA samples: in the first step, add (in an empty vial) the extraction buffer, the guanidine hydrochloride, and the proteinase K. Then, follow the complete extraction procedure described.
11. During this 10 min interval, place a tube with the elution solvent (PCR water or TE buffer) in the thermoblock at 65 °C. For extraction of DNA >20 kb employ water or TE buffer pre-warmed to 80 °C. Also warm the Wizard® DNA Clean-Up Resin at 37 °C if crystals or aggregates are present.
12. Use this 10 min interval to prepare the vacuum manifold: attach the provided syringe barrel to the luer-lok extension of each minicolumn (one per sample, do not re-use). Insert the tip of the minicolumn/syringe assembly into the one-way luer lock stop-cocks and connect the vacuum manifold to the vacuum pump.
13. The Nanodrop® spectrophotometer measures sample absorbance at three different wavelengths: 230, 260, and 280 nm. Absorbance at 260 nm provides dsDNA quantification following the conversion factor that one absorbance unit (1 A.U.) corresponds to 50 µg/mL of dsDNA, according to the Lambert–Beer law. Ratios of absorbance 260/280 and 260/230 are used to assess the purity of the DNA extract. A ratio of 260/280 ~ 1.8 is generally accepted as pure for DNA. If this ratio is appreciably lower, it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at 280 nm. Expected 260/230 values are commonly in the range of 2.0–2.2. If the ratio is appreciably lower, it may indicate the presence of contaminants which absorb at 230 nm (EDTA, TRIzol, HCl, etc.).
14. Three different flow laminar cabinets will be used to perform real-time PCR assay: (1) “reagents cabinet” to prepare the master mix and other DNA-free solutions/reagents, (2) “DNA cabinet” for handling/pipetting DNA samples, and (3) a third cabinet to fill the capillaries. Before and after each use, switch

on the fan and UV for 10 min and clean all the cabinets with Micro-90, 70% ethanol, and termi-DNA-Tor.

15. Do not wear gloves or use new ones when handling computer and thermocycler equipment to prevent cross-contaminations.
16. We suggest the following steps to adjust the DNA concentration of the extracts in order to minimize the risk of contamination: (a) in the reagents cabinet and using forceps, place empty microcentrifuge tubes in the rack labeled as "PCR". Add the volume of water calculated for the dilution of each DNA extract employing micropipettes exclusive for reagents. (b) Thaw the DNA extracts, vortex and briefly spin the tubes. (c) In the DNA cabinet and employing micropipettes exclusive for handling DNA, add the necessary DNA volume for the calculated PCR concentration. Handle samples carefully and change gloves and clean surface and micropipettes when necessary.
17. All real-time PCR reactions are carried out in duplicate. The negative control of the PCR reaction (NC) and the negative control corresponding to each DNA extraction (NEC) must always be run in the same assay than the test DNA samples.
18. Although the required volume of PCR Mix will be prepared according to the number of reactions to be performed, it is advisable to calculate a little excess of PCR Mix volume to ensure enough amounts for all the tubes. Set the required number of PCR Mixes considering that each sample will be analyzed employing the species-specific PCR system for the allergens of interest and the universal positive amplification control (PAC) system.
19. We have observed that filling the capillaries with a certain speed minimizes the possibility of contamination.
20. If a C_p value is obtained for PCR negative control (NC), the result will not be valid, and the assay will be repeated. Similarly, if a C_p value is obtained for the negative extraction control (NEC), the assay can be firstly repeated. If the result is confirmed, repeat the sample DNA extraction before running again the PCR.
21. The close phylogenetic relationship among nuts, and the high number of plant and animal components that can be present in a commercial food, make it necessary to check the cross-reactivity of the PCR systems developed against a wide range of species.
22. As a further step, the applicability of the real-time PCR developed to detect the target species in foods is assessed by analyzing a range of commercial food products with different ingredients and label declarations.

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