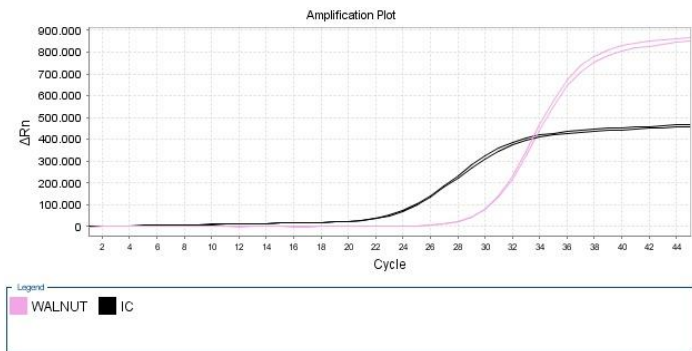
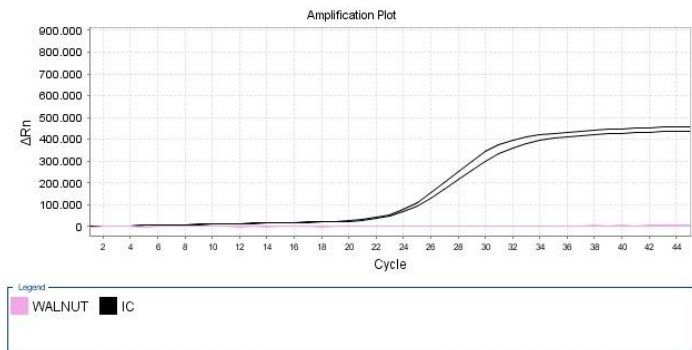


Positive Control –



Negative Control –



12. SPECIFICITY/INCLUSIVITY

a) 94 % Exclusivity, determined using 6 meat species, 19 sea animal species, and 26 plant species, including all the plant allergens from Directive 2007/68/EC.

3 non-target species showed a positive result: Goji berries (*Lycium barbarum*), Zucchini courgette (*Curcubita pepo L.*) and Pistachio (*Pistacia vera*).

b) 100 % Inclusivity, determined in 5 food samples obtained from different commercial sources.

13. SENSITIVITY

The limit of detection (LoD) for REAL TIME DETECTION KIT WALNUT is 10 fg of target DNA.

The method's detection limit can detect 0.00001% of walnut DNA in 100 ng of total DNA.

No false negatives were obtained, either in the defined PCR conditions or in the deviations used for robustness, increasing the stringency.

REAL TIME DETECTION KIT WALNUT

Ref: BIOPFS-0062

1. PRODUCT DESCRIPTION

Allergen detection kits provide a simple, reliable, and rapid procedure for detecting the presence of an animal species. The assay is based on 5'nuclease real time PCR reactions to amplify a unique genomic sequence in the target organism.

2. TECHNOLOGY DESCRIPTION

PCR is a method used to amplify a specific DNA sequence in a reaction containing among other components, a thermostable DNA polymerase, nucleotides, and primers complementary to the target sequence. The DNA molecule denatures when this solution is heated, separating into two strands. As the solution cools, the primers anneal to the target sequences in the separated DNA strands and the DNA polymerase synthesizes a new strand by extending the primers with nucleotides, creating a copy of the DNA sequence (amplicons). When repeated, this cycle of denaturing, annealing, and extending exponentially increases the number of target amplicons. In Real Time PCR, the signal is measured in each cycle, using in most cases specific fluorescent probes. Fluorescence is measured by a detector and the associated software plots the fluorescence intensity versus the number of cycles, allowing the determination of the presence or absence of the target organism.

3. SCOPE

The severity of the reactions triggered by food allergens varies from mild urticarial to potentially lethal anaphylactic shocks, even after the ingestion of minimal doses of the allergenic food. The prevalence of clinically confirmed food allergy indicates that up to 2.5% of the adult population and 6–8% of children less than 3 years of age are affected by food allergies. However, population surveys have estimated the rate of prevalence of perceived food allergy in adults to be between 12 and 20%. This confirms that food allergy represents a major threat to human health. Within the European Union, the presence of the most important food allergens needs to be declared on the label of food products whenever they are used as ingredients as required by Directive 2006/142/EC and is maintained in Directive 2007/68/EC. Walnut is one of the allergens listed in this directive. Several methods for allergen detection are based on the identification of proteins, especially by means of immunological methods. However, these methods are not reliable for application in highly processed and heated products due to protein deterioration. DNA is more stable than proteins during processing and although it can be fragmented by several processes, it continues to be detectable by PCR. Real time PCR techniques are especially suitable for these products because small fragments of DNA can still be amplified and identified with high sensitivity and specificity.

4. INTERNAL CONTROL (IC)

Allergen detection kits include an Internal Control (IC) in the master mix. This control is amplified at the same time as the target DNA sequence, but using a different set of primers and a probe labeled with a different fluorophore. The inclusion of the IC in each reaction allows the evaluation of PCR inhibitors in negative results.

5. APPLICATION

REAL TIME DETECTION KIT WALNUT is intended for the rapid detection of walnut (*Juglans regia L.*) DNA in food products, after DNA extraction. It can also be used with animal feedstuff and other samples in which walnut species detection is needed. The kit was validated in the instruments ABI PRISM® 7500 Fast and ThermoScientific® PikoReal. The kit is compatible with all thermocyclers working in **FAM** and **ROX** channels. The detection kit must not be used for diagnostic procedures. For Food use only.

The procedure includes the following main steps:



Sample
Processing

DNA
extraction

PCR
set-up and run

Results and
Interpretation



BPMP – Production and Development, Lda. Amadora INOVA
Rua Henrique de Paiva Couceiro, 10
2700 – 453 Falagueira – Venda Nova Amadora, PORTUGAL
Tel: +351 211 451 410
E-mail: sales.support@biopremier.com
Website: <http://www.biopremier.com>

BPMP is certified ISO 9001:2015

6. CONTENTS AND STORAGE

The kit contains reagents for 100 assays

CONTENTS	UNITS	COMPOSITION
Master Mix (blue cap)	● 2 tubes (2 x 840 µl)	Buffer, dNTPs, DNA polymerase
Assay Mix (brown cap)	● 1 tube (1 x 210 µl)	Primers and Probes
Negative Control (clear cap)	○ 1 tube (1 x 70 µl)	Nuclease-free water
Positive Control (red cap)	● 1 tube (1 x 70 µl)	Target DNA

Store all contents at -20°C and protect them from light as excessive exposure to light may affect the fluorescent probes. Minimize freeze-thaw cycles. Reagents stored as recommended may be used until the expiration date indicated in the tube.

7. MATERIAL REQUIRED AND NOT SUPPLIED

- Microcentrifuge
- Laminar Air Flow Cabinets/PCR Cabinets
- Disposable powder-free gloves
- Micropipettes and nuclease-free filter tips
- Real-time PCR instrument
- Tubes/Strips/Multiwell plates and accessories specific for each Instrument
- DNA extraction kit (example: BIOPEXT-0609)

8. PRECAUTIONS AND RECOMMENDATIONS

Molecular Biology procedures, such as DNA extractions and PCR amplification, require qualified staff to prevent the risk of erroneous results, especially due to sample contamination or degradation of the nucleic acids contained in the samples. It is strongly recommended to have dedicated areas, materials and equipments for the DNA extraction, preparation of the PCR and post-PCR procedures. Workflow in the laboratory must proceed in a unidirectional manner, beginning in the Extraction Area and moving to the Amplification and Detection Area. The user should always pay attention to the following:

- Read all the instructions provided before running the assay.
- Do not mix reagents from different batches.
- Wear proper PPE, including disposable gloves and laboratory coats.
- Store and extract positive material separately from all other reagents.

9. QUALITY CONTROL

In accordance with BPMR's ISO 9001, each lot of the kit is tested against predetermined specifications to ensure consistent product quality.

10. PROCEDURE

10.1 Sample preparation

Depending on the specific characteristics of the sample, procedures like homogenization and grinding may be necessary before DNA extraction.

10.2 DNA extraction

Use a kit or protocol suitable for DNA extraction from food products, such as BIOPREMIER DNA Extraction Kit from Food (ref: BIOPEXT-0609) or similar. Follow the manufacturer's or authors instructions.

10.3 PCR preparation

A – PCR mix

Always wear gloves for all PCR procedures.

1. Thaw the kit solutions. Mix thoroughly and centrifuge briefly
2. Prepare the reactions, as described below:

CONTENTS	N° OF SAMPLES	
	1	10 (10 + 1)
Master Mix ●	16 µl	176 µl
Assay Mix ●	2 µl	22 µl
Total Volume	18 µl	198 µl

Note: Prepare the PCR reaction for each sample, or in the alternative, prepare a Master Mix for the total number of reactions plus 10%, to cover pipetting losses (e.g. for 10 samples, prepare a volume for 11). In this case, prepare the Mix in a 1,5mL sterile, nuclease-free tube. Include 2 PCR reactions for the Positive and Negative controls.

3. Mix the prepared Mix by inverting the tube and centrifuge briefly
4. Dispense 18 µL aliquots of prepared Mix into the plate wells or PCR tube
5. For the negative control, pipette 2 µL of Negative Control tube (Clear Cap); Pipette 2 µL of DNA sample per well; and for the positive control, pipette 2 µL of Positive Control tube (Red Cap). Each PCR tube / well should have a final PCR volume of 20 µL
6. Centrifuge briefly the plate wells or PCR tubes
7. Place the reactions into the Real Time PCR instrument.

B – Program set up

Prepare the Real-Time PCR instrument according to the following temperature/time program:

PHASE	STEP	TEMPERATURE	TIME	ACQUISITION
Holding Stage	Step 1	50°C	2 min.	No
	Step 2	95°C	5 min.	No
Amplification - 45 cycles	Step 1	95°C	30 sec.	No
	Step 2	60°C	30 sec.	Yes
	Step 3	72°C	30 sec.	No

TARGET	CHANNELS
Detection of Walnut	FAM
Detection of Internal Control (IC)	ROX

11. DATA ANALYSIS

For analysis of PCR results, select fluorescence display options. Samples with positive Ct-values are considered positive.

Important: Please, also check amplification curves, not only Ct values. Samples should be inspected both in logarithmic and linear scale view and compared with the negative control. Adjust the Threshold, if necessary. Assessment of sample results should be performed after the positive and negative controls have been examined and determined to be valid. If the results of controls are not valid, the sample results cannot be interpreted. Interpretation of PCR data:

a) Controls

To validate the assay, the controls must have the following results:

	Walnut DETECTION FAM	IC DETECTION ROX
Negative Control	Negative	Positive
Positive Control	Positive	Positive

Note that if the controls do not match these results, the experiment must be repeated.

b) Samples

Interpretation of sample results is summarized in the following table:

Walnut DETECTION FAM	IC DETECTION ROX	INTERPRETATION
Positive	Positive/Negative**	DNA detected, sample Positive for walnut
Negative	Positive	No DNA detected, sample Negative for walnut
Negative	Negative	Invalid Result*

*When both walnut and IC detection are Negative, this means the presence of PCR inhibitors in the sample. Dilute the DNA sample (10-fold dilution) or perform another DNA extraction and repeat qPCR.

** High DNA concentration of the target in the sample can lead to a reduced or absent fluorescence signal of the IC.