

Chapter 11

Fast Real-Time PCR for the Detection of Crustacean Allergens in Foods

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Abstract

Crustaceans are one of the most common allergens causing severe food reaction. Hypersensitivity reactions associated with seafood intake are one of the most common food allergies in adults. Crustaceans including shrimps, prawns, crabs, lobster, and crayfish are a common cause of anaphylaxis or hypersensitivity, with shrimps and crabs being the most common causes of allergy. Symptoms occur most often when food or cooking water are ingested.

These food allergens are a health problem, and they have become very important; as evidenced by the existence of several regulations that establish that labeling must be present regarding these allergens to warn consumers.

The methodology herein exposed allows the detection of crustaceans in any type of product, including those where very aggressive treatments of temperature and pressure are used during the manufacturing process.

The main features of this method are its high sensitivity and specificity, and reduced analysis time of real-time PCR (40 min). This assay is a potential tool in issues related to the labeling of products and food security to protect the allergic consumer.

Key words Crustacean, Detection, Allergen, Fast real-time PCR, LNA probe

1 Introduction

An allergen is a substance that can induce a hypersensitivity or allergic reaction in susceptible people who have previously been in contact with this substance. Food allergens can act at very low doses (traces) and potentially cause fatal reactions depending on factors such as the individual's tolerance or health, because there is no curative therapy for these allergic diseases; and the most effective way to prevent allergic reactions is to remove the sensitive components that trigger adverse effect from the diet.

For this reason, consumers should have all the necessary information on food composition on the label, to make a choice that suits their needs. Changes in labeling legislation have allowed a significant improvement in the labeling of ingredients with allergenic capacity.

The traces are minimal amounts of a substance or ingredient, which are included in a food. The presence of trace amounts of allergenic substances may occur due to cross-contamination or because they were included in one of the raw materials used in the manufacture of a product. When there is a demonstrable risk for the presence of these substances in trace quantities they must be declared as a precaution, with a preventive label containing a warning message.

Allergens can also be present in some products as a result of common practice in the food industry and do not appear in the label. This is mainly due to cross-contamination between raw materials, production lines, or equipment, caused by insufficient cleaning of them. For this reason, it acquires special attention conducting surface controls to detect these traces of allergens in plants where food production is done.

Given all the issues previously highlighted, detection methods are needed for determining the presence or absence of allergenic substances in trace amounts, because small amounts are sufficient to produce allergic reactions in sensitized individuals.

In this chapter a protocol based on the real-time PCR TaqMan assay using specific set primers/probe is described, which allows the detection of crustaceans in food in trace amounts, and responds to the needs of the food industry, allowing to indicate the presence/absence of crustaceans in food.

2 Materials, Equipment, and Reagents

2.1 Materials

1. Sterile microtubes of 1.5 and 2 mL.
2. MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems).
3. Optical adhesive films.
4. Tubes of 50 mL.
5. Foil.
6. Sterile bags.

2.2 Equipments

1. Thermomixer (Eppendorf).
2. Vortemp.
3. Refrigerated centrifuges for microtubes.
4. Micropipettes of variable volume and their respective tips (0.1–2.5 µL; 0.5–10 µL; 10–100 µL; 100–1000 µL; 1–10 mL).
5. Laminar flow hoods or PCR cabinet.
6. Hot plate.
7. Water purification system (Milli-Q, Millipore).
8. Tweezers.

- 9. Spectrophotometer (NanoDrop).
- 10. Vortex mixer.
- 11. Tissue homogenizer.
- 12. Autoclave.
- 13. Balances.
- 14. Freezer.
- 15. Centrifuge for PCR plates.
- 16. Maxwell® 16 MDx Research Instrument.
- 17. ViiA 7 Real-time PCR System (Applied Biosystems).

2.3 Reagents

- 1. Maxwell® 16 FFS Nucleic Acid Extraction Kit X9431 (Promega).
- 2. CTAB (cetyl trimethyl ammonium bromide). For 1 L of Maxwell CTAB buffer: 20 g of CTAB, 81.9 g of NaCl, 100 mL of 1 M Tris hydrochloride solution, 40 mL of 0.5 M EDTA pH 8.0. Dissolve and bring to 1 L (to facilitate the dissolution of the components, preheat on a hot plate with stirring at 65 °C). Autoclave for sterilizing the solution.
- 3. NaCl.
- 4. 1 M Tris.
- 5. 0.5 M EDTA pH 8.
- 6. TaqMan® Universal PCR Master Mix (Applied Biosystems).
- 7. Oligonucleotide primers (Table 1).
- 8. FAM™-LNA labeled oligonucleotide probe (Table 1).

3 Methods

3.1 DNA Extraction

- 1. Prepare CTAB buffer as described in the Subheading 2.3.2, use within 3 months, store capped in the refrigerator at 4 °C. Preheat the CTAB solution at 65 °C before use to dissolve the precipitate particles.

Table 1
Primers and probe used in this protocol for the detection of crustaceans in food with the protocol herein described

Oligonucleotide designation	Sequence (5'–3')	Molecular marker	Amplicon size (bp)	Reference
Forward primer	TAA AGT CTG GCC TGC CCA	16S rRNA	205	[1]
Reverse primer	GCT TTA TAG GGT CTT ATC GT			
Probe	6-FAM-TGC TAC CTT IGC ACG GTC A-BHQ1(LNA)			[2]

2. Perform DNA extraction using a kit for automated isolation. The example given is for the Maxwell® 16 FFS Nucleic Acid Extraction Kit X9431. The procedure with sample varies depending on the type of sample, which can be classified into two groups:

3. For the DNA extraction from the sample, continue as described:

Sample Type 1: fish, meat, and processed meat products (raw and cooked, as for instance cured products, tinned food, convenience food, hamburgers, doner kebab). Proceed as described below:

- (a) Weigh 50 mg of sample material into a 1.5 mL tube.
- (b) Add 600 μ L CTAB buffer, 30 μ L of proteinase K (20 mg/mL), and 2 μ L of RNase A solution.
- (c) Vortex vigorously and incubate for at least 60 min at 60 °C in a thermo shaker (1400 rpm).
- (d) Centrifuge for 10 min at $\geq 14,000 \times g$.

Sample Type 2: food, feed, seed (e.g., rapeseed, soybean, corn, rice, chocolate bars, milk powder). Proceed as described below:

- (a) Grind sample material and weigh 100 mg (rapeseed, soybean) or 200 mg (corn) into a 2 mL tube.
- (b) Add 1 mL CTAB buffer, 40 μ L of proteinase K (20 mg/mL) and optionally 20 μ L of RNase A solution.
- (c) Vortex vigorously and incubate for 90 min at 65 °C.
- (d) Centrifuge for 10 min at $16,000 \times g$ (optional: centrifuge supernatant again).
- (a) Place the cartridge to be used into the Maxwell LEV cartridge rack and remove the seal.
- (b) Pipet 300 μ L of sample supernatant and 300 μ L of Lysis Buffer into well 1 of the Maxwell cartridge (Fig. 1).
- (c) Place one of the supplied elution tubes into the sample rack (position 2) and add 100 μ L of the supplied elution buffer (Fig. 1).
- (d) Place the plunger in the position 3 of the cartridge (Fig. 1).
- (e) Select LEV configuration of the Maxwell and select method: RUN $\rightarrow$ DNA $\rightarrow$ Blood $\rightarrow$ Start run.
- (f) After the extraction your sample is ready-to-use for downstream applications.
4. The DNA obtained from the samples must be quantified with a spectrophotometer using the absorbance at 260 and 280 nm, and the concentration must be normalized to use in the downstream steps (*see* **Note 1**).

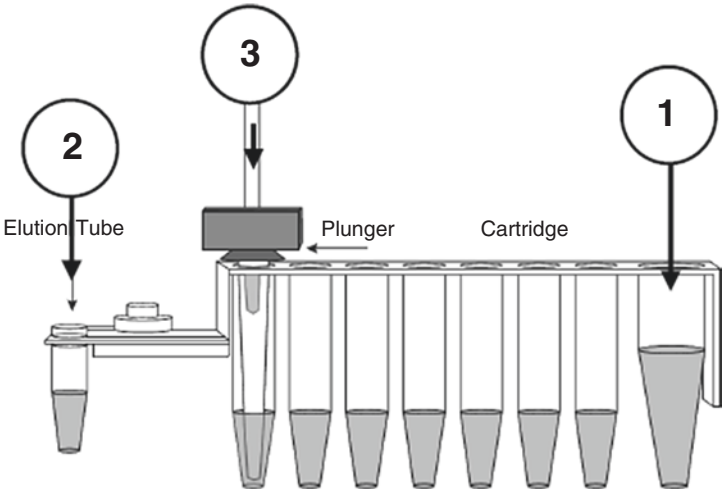


Fig. 1 Schematics of a Maxwell cartridge. *Circled numbers* refer to positions described in the text

Table 2
Composition of PCR reactions

Mix components	Mix 1	Mix N
TaqMan® Universal PCR Master Mix (2×)	10 µL	10 × N µL
Forward primer 10 µM	1.8 µL	1.8 × N µL
Reverse primer 10 µM	1.8 µL	1.8 × N µL
Probe 10 µM	0.5 µL	0.5 × N µL
Molecular biology grade water	to 20 µL	to 20 × N µL

3.2 Real-Time PCR

While the PCR reactions are being set up, precautions should be taken at all stages to avoid contamination of samples and reagents (*see Note 2*).

1. Mix well all reagents by inverting the tubes a number of times or for small volumes by flicking the tube and spin briefly (~5 s) in a centrifuge.
2. Prepare a PCR master mix in 1.5 mL microcentrifuge tubes, following the proportions indicated in the Table 2 to maximize uniformity and minimize labor when working with multiple tubes or plates. The final volume of each master mix depends on the number of reactions required for each real-time PCR. Master mixes can be made in excess of what is actually needed to account for pipetting variations, i.e., prepare 10% excess of master mix. The probe must be handled with extreme caution (*see Note 3*).

Table 3
PCR amplification profile of the real-time PCR system herein described

Step	Time (s)	Temperature (°C)
Hold	20	95
PCR (×40 cycles)		
Denaturation	1	95
Annealing	20	60

3. Mix the master mix thoroughly and dispense equal aliquots into each thin-walled PCR tube. Set up the real-time PCR in a 96-well plate, including nontemplate (NTC), negative controls, positive controls and the test samples, each at least in duplicate (*see* **Note 4**).
4. Add DNA template (125 ng of DNA per sample to each well) to each reaction tube as needed (be sure to include a negative control that does not receive any template). When pipetting is finished, seal the plate with optical adhesive film.
5. Centrifuge the reaction plate at low speed for 1 min and then load the plate onto a ViiA 7 real-time PCR System.

**3.3 Program
and Run Applied
Biosystems ViiA 7
System**

1. Operate the Applied Biosystems ViiA 7 System and SDS (Sequence Detection System) software according to the manufacturer’s recommendations. Set reaction volume to 20 µL, and the amplification program as described in Table 3 (unless other parameters are established in the internal methodological validation). Use FAM/LNA as detector/quencher.
2. Briefly, launch the SDS software and open a new plate template window to denote well locations on the 96-well plate for the controls, and testing samples. Save the template window with the recorded data as a SDS run file.
3. Select the “Connect” button, and then load the 96-well plate into the instrument. Select the “Start” button to begin the actual run.
4. Run the PCR reaction to determine Ct (cycle threshold) values for each sample.

**3.4 Data Collection
and Analysis**

After the run is completed, select baseline, and place the threshold line at the exponential phase of amplification. Remove the plate from the instrument and save the results of the run. When the analysis button is selected in the SDS software, the results will be analyzed automatically if the standards and testing sample information were recorded as noted in the previous section. The Ct values obtained should be thoroughly reviewed (*see* **Note 5**) and interpretation of

the results must be made in relation to data obtained in the internal validation performed in each laboratory for a given detection limit (*see* **Notes 6–8**), allowing to determine whether a sample contains or does not contain crustaceans in its composition.

4 Notes

1. The isolation and purification of DNA from the sample constitutes one of the most important steps of any real-time PCR protocol. The quality and integrity of both are essential to carry out an experiment successfully. One way to measure these characteristics of the DNA is using a spectrophotometer, measuring the absorbance at A260 and A280 nanometers. The ratio A260/A280 should be higher than 1.8. When lower values are obtained, this may indicate the presence of impurities or contaminants in the DNA solution as proteins or phenol, and which may affect the assay. Higher values than 2 may be due to the presence of RNA in the DNA solution. If the DNA extracted is not enough (less than 200 ng) or the purity is not adequate, DNA extraction should be repeated.
2. The following precautions should be taken to avoid contamination. It is paramount to work in at least three separate areas. One area should be designated just for DNA extraction; other for the preparation of the master mix; and other area designated just for adding the template. This is crucial to avoid contamination of the real-time PCR reagents with aerosols containing the template. Pipette sets must be dedicated for use in these areas and must not be moved between areas. Use only dedicated pipettes for preparing master mixes and another set for adding template DNA. Use only filter tips and sterile plasticware. Wear gloves and change them frequently.
3. TaqMan probe and probe dilution: probes are usually received as a 100 pmol/ μ L stock solution. This stock solution can be diluted with molecular biology grade water to achieve a working solution concentration. Probe working solutions should be aliquoted in volumes that are sufficient for one time use. These aliquots should be stored at -20°C and protected from light. In addition, repeated freeze–thaw cycles of probe solutions should be avoided.
4. Negative reaction control (NTC) produces amplification. The DNA may be contaminated. This may be due to amplicon contamination in the RT reagents or, in some cases, contamination in the DNA preparation. It is often best to simply repeat the real-time PCR reaction using new reagents (except DNA extraction). If the problem disappears, one can proceed. If the problem persists, it is necessary to make a new DNA extraction.

The positive control is a tube of DNA extracted from food matrix reference material contaminated with crustaceans at the detection limit of the technique. To determine this, serial dilutions of crustacean DNA and DNA from different species of seafood can be prepared to a final amount of 125 ng [2].

5. Test samples should be analyzed in duplicate or triplicate according to individual laboratory statistical requirements. Excessive variability between Ct of duplicate or triplicate samples: if the samples display large variability between duplicates and triplicates there may be a problem in the preparation of the master mix or a pipetting error. Check that the master mix was prepared properly and well mixed, and that the micropipette is calibrated and is dispensing the proper amount of liquid reproducibly.
6. Before applying these techniques in a particular laboratory, internal validations should be made taking into account the following points:

Thermocyclers: As previously mentioned, each thermocycler has its own characteristics that may require an adjustment in the working parameters (temperature or reaction times, as well as temperature ramp rate).

DNA polymerase enzyme: It is also necessary to make a validation if the polymerase employed is different from the herein described, since polymerases from different commercial dealers may vary in amplification efficiency, and consequently may affect the optimum temperature and the specificity of the technique, generating variations in observed cycle threshold and amplification profile.

Substances included in the validation: In the phase of methodological design and development of the detection system a great number of substances is included for evaluating the specificity of the technique, and so validate the correct working. However, each laboratory should perform an internal validation including all the matrixes used in their production chain, since these could interfere with the results, giving for instance false positives.

7. The detection limit should be calculated on the basis of the validation performed on each laboratory, as it will depend on the equipment and technical personnel. Similarly, the cut off or Ct, which separates a positive sample of a negative will vary depending on the specific detection limit of each particular laboratory.
8. To consider a test as valid, it must have a correct reading of the controls (the positive control in the detection limit must be positive, and the NTC and negative controls should not have amplification). Moreover, the amplification curve should have an exponential appearance, and the threshold cycle must be less than the Ct determined in the methodological validation.

References

1. Brzezinski JL (2005) Detection of crustacean DNA and species identification using a PCR–restriction fragment length polymorphism method. *J Food Prot* 68(9):1866–1873
2. Herrero B, Vieites JM, Espiñeira M (2012) Fast real-time PCR for the detection of crustacean allergen in foods. *J Agric Food Chem* 60(8):1893–1897