

Chapter 12

Fast Real-Time PCR Method for Detection of Soy in Foods

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Abstract

Soy is used as an additive in the manufacturing of diverse products, because of their ability of emulsification, water and fat absorption, contributing to the consistency of food products. Moreover, soy is recognized as a potential allergen, so its presence should be indicated in all the food products.

These issues highlight the need for techniques that allow the detection of soy in foods. This work describes a real-time PCR method for the detection of soy in a wide range of foodstuffs. The main features of this technique are its reliability and sensitivity, allowing the detection of trace amounts of soybean in processed products. TaqMan real-time PCR is one of the simplest and fastest molecular biology techniques, with a high potential for automation. Therefore, it is one of the techniques most used for screening a variety of substances.

The methodology herein described is of great value in issues regarding the presence of soy protein in processed products, especially in verifying labeling and security regulations to protect consumer's rights.

Key words Detection, Allergen, Emulsifier, Adulteration, Authenticity, Additives

1 Introduction

In the food industry, many of the additives used in the manufacture of diverse products are vegetable protein, being soybeans one of the most used and referenced [1, 2]. Among the properties of soy stand out the emulsification capabilities and the water and fat absorption, contributing to more consistent food products. In the case of meat products and seafood, the water absorption and retention properties of textured soy proteins can be used to bind moisture in fish or meat blocks and reduce fat and jelly deposits liberated during the manufacturing process, obtaining a firmer final product. Increased water retention results in end products with a higher weight; which means that soy protein replaces raw material, usually with a higher cost. This makes the food products more cost-effective; there is a considerable decrease in production costs to the benefit of the manufacturers. In addition to the fraudulent use of soy, the other negative side is its allergenicity, as it is recognized as a potential allergen by the *Codex Alimentarius* [3]. Therefore, the

detection of this food additive in food products focuses on ensuring food quality and safety, as the adulteration of food not only causes harm to consumers, but also unfair competition between industries in the food sector.

Due to all the issues outlined above, a methodology to detect soy protein in food products is necessary to ensure food safety, consumer protection and, at the same time, to guarantee fair competition between producers. This protocol is based on the real-time PCR TaqMan assay using specific set primers/probe developed by Espiñeira et al. [4].

2 Materials, Equipment, Reagents, and Preparations

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. Variable volume micropipettes of 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. ViiA 7 Real-time PCR System (Applied Biosystems).

2.3 Reagents

1. 1 M Tris (Tris hydroxymethyl aminomethane), pH 8.0. For 1 L of final volume, add the following amounts: 121.1 g of

Tris, 700 mL of Milli-Q H₂O. Dissolve the Tris and bring to 900 mL. Adjust the pH to 8.0 with concentrated HCl (will need ~50 mL). Bring to 1 L.

2. 5 M NaCl. For 1 L of final volume, add the following amounts: 292.2 g of NaCl, 700 mL of Milli-Q H₂O. Dissolve and bring to 1 L.
3. NaOH pellets.
4. 0.5 M EDTA (ethylenediaminetetraacetic Acid), pH 8.0. For 1 L of final volume, add the following amounts: 186.12 g of EDTA, 750 mL of Milli-Q H₂O. Add about 20 g of NaOH pellets. Slowly add more NaOH until pH is 8.0, EDTA will not dissolve until the pH is near 8.0. Bring to 1 L.
5. CTAB (cetyl trimethyl ammonium bromide). For 1 L of CTAB buffer: 100 mL of 1 M Tris, pH 8.0, 280 mL of 5 M NaCl, 40 mL of 0.5 M EDTA, 20 g of CTAB. Add Milli-Q H₂O to 1 L. To facilitate the dissolution of the components, preheat on a hot plate with stirring at 65 °C.
6. RNAAsA.
7. Polyvinylpyrrolidone mol. Weight 40,000 (PVP-40). β-mercaptoethanol.
8. Proteinase k.
9. Chloroform–isoamyl alcohol (24:1).
10. 7.5 M Ammonium Acetate. For a final volume of 250 mL, add the following amount: 144.5 g of ammonium acetate and bring to volume with Milli-Q H₂O.
11. Isopropanol (2-propanol).
12. 70 and 95% ethanol.
13. TaqMan® Universal PCR Master Mix (Applied Biosystems).
14. Oligonucleotide primers (Table 1).
15. FAMTM-TAMRATM-labeled oligonucleotide probe (Table 1).

Table 1

Primers and probe used in this protocol for the soy detection with the protocol herein described

Oligonucleotide designation		Sequence (5'-3')	Molecular marker	Amplicon size (bp)	References
Forward primer	LEC IF	CTT CTT TCT CGC ACC AAT	Lectin gene	100	[5]
Reverse primer	LEC IR	CTC AAC AGC GAC GAC TTG			
Probe	LE-Probe	6-FAM-CAC ATG CAG GTT ATC TTG GTC-TAMRA			[1]

3 Methods

3.1 DNA Extraction

1. Prepare CTAB solution as described in the materials section (Subheading 2.3, item 5), use within 3 months, store capped in the refrigerator at 4 °C. Prior to use, add to 100 mL of CTAB, 4 g polyvinylpyrrolidone MW 40,000 (PVP-40) and 500 μ L β -mercaptoethanol and stir to dissolve before starting extractions. Preheat the CTAB solution at 65 °C before use to dissolve the precipitate particles.
2. Weigh out 10 g of triturated and homogenized sample that will be analyzed.
3. Add 10 mL of preheated CTAB buffer and 100 μ L proteinase K (10 mg/mL).
4. Transfer the solution to a 50-mL tube. *Optional*: Add 10 μ L of RNase A (2 mg/mL) and mix by inverting.
5. Incubate samples in a thermomixer (or similar equipment) at 55 °C for 1 h. Then centrifuge for 5 min at maximum speed and transfer 1 mL of aqueous phase to a 2 mL microtube (in duplicate).
6. Add 500 μ L of 24:1 chloroform–isoamyl alcohol and mix well the tubes with hands (by inversion), until a homogeneous emulsion is formed.
7. Centrifuge for 8–10 min at maximum speed (12,000–13,000 rpm or 9600–11300 relative centrifugal force). Following centrifugation, you should have three layers. Top: aqueous phase; middle: debris and proteins; and bottom: chloroform with cellular debris. Proceed to the next step quickly, so the phases do not remix.
8. Pipette off the aqueous phase (top) taking care not to suck up any of the middle or chloroform phases.
9. Place the aqueous phase into a new labeled 1.5 mL tube.
10. Add 30 μ L of cold 7.5 M ammonium acetate.
11. Add 200 μ L of cold isopropanol (–20 °C, to the combined volume of aqueous phase and added ammonium acetate).
12. Mix well by inversion.
13. Let sit in freezer for 45 min to an hour.
14. Centrifuge for 3 min at maximum speed. Orient tubes in an equal fashion to facilitate subsequent removal of supernatant without disturbing resultant DNA pellet.
15. Pipette off the liquid, being careful not to lose the pellet with your DNA. The DNA pellet at this stage is very loose and difficult to see.

16. Add 700 μL of 70% ethanol at $-20\text{ }^{\circ}\text{C}$ and mix by gentle inversion.
17. Centrifuge for 1 min at maximum speed.
18. Pipette off the liquid, being careful not to lose the pellet with your DNA.
19. Add 700 μL of 95% ethanol at $-20\text{ }^{\circ}\text{C}$ and invert to mix.
20. Centrifuge for 1 min at maximum speed.
21. Pipette off the liquid, being careful not to lose the pellet with your DNA.
22. Dry the pellet in a vacuum centrifuge or on a hot plate at $55\text{ }^{\circ}\text{C}$.
23. Resuspend the DNA of the samples with 100 μL of water of molecular biology grade. Store the DNA at $-20\text{ }^{\circ}\text{C}$ until required. The DNA can be stored at $4\text{ }^{\circ}\text{C}$ if used in the next few days, or in the freezer at $-20\text{ }^{\circ}\text{C}$ for long-term storage.
24. The DNA obtained from the samples must be quantified with a spectrophotometer or similar equipment (for instance NanoDrop), and the concentration must be normalized at 100 $\text{ng}/\mu\text{L}$ or multiple, to use in the following steps. The purity of the DNA solution must be higher than 1.8 measured as the ratio of absorbance at 260 and 280 nm (*see Note 1*).

3.2 Real-Time PCR

While PCR reactions are being set up, precautions should be taken at all stages to avoid contamination of samples and reagents. It is advisable to work in laboratories with unidirectional workflow, and at least the following rooms: DNA extraction room; master mix preparation room (in this room only reagents are handled, and no DNA enters, in order to avoid contamination of reagents); PCR room (in this room, the DNA is dispensed, and the master mix previously prepared is added); PCR room, where the thermocycler and PCR reactions are performed (*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 ($\sim 5\text{ s}$) in a centrifuge. The probe must be prepared as described in the **Note 3**.
2. Prepare the PCR master mix in 1.5 mL microcentrifuge tubes, as shown in Table 2 to maximize uniformity and minimize labor when working with multiple tubes. 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.
3. Mix the master mix thoroughly and dispense equal aliquots into each thin-walled PCR tube. Set up the real-time PCR in a

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	0.2 µL	0.2 × N µL
Molecular biology grade water	to 25 µL	to 25 × N µL

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

Step	Time (s)	Temperature (°C)
Hold	30	95
PCR (× 45 cycles)		
Denaturation	5	95
Annealing	30	58

96-well plate, including nontemplate (NTC) (*see Note 4*), negative controls, positive controls (*see Note 5*) and the test sample (*see Note 6*).

4. Add DNA template (200 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 2 min and then load the plate onto a Viia7 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 25 µL, and the amplification program as described in Table 3 (unless other parameters are established in the methodological validation). Use FAM/TAMRA 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 an 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 has been recorded as noted above. The Ct values obtained should be reviewed (*see* **Notes 7** and **8**) 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 9–11**), allowing to determine whether a sample contains or does not contain soy.

4 Notes

1. The quantity and purity of DNA extracted must be appropriate and sufficient to assess the presence of soy in a sample. If the amount is not enough (less than 200 ng) or the purity is not adequate, the DNA must be extracted again.
2. Precautions should be taken while PCR reactions are being set up to avoid contamination of samples and reagents. 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. Master mix preparation and addition of template should be performed in separate places with dedicated laboratory equipment. 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.
3. TaqMan probe and probe dilution: probes are usually received as a 100 pmol/μL 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. This may be due to amplicon contamination in the master mix 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 prep). If the problem disappears, one can proceed. If the problem persists, a new DNA extraction is necessary.
5. The positive control is a tube of DNA extracted from a specific food matrix contaminated with soybean reference material at the detection limit of the technique. The reference material used may be Soya Bean Powder from Sigma (reference ERMBF410BK), diluted in other certified materials as for instance corn, or alternatively food matrices that do not contain soybean.
6. Test samples should be analyzed in duplicate or triplicate according to individual laboratory statistical requirements.
7. Excessive variability between 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 has been prepared properly and well mixed, and that the micropipette is calibrated and is dispensing the proper amount of liquid reproducibly.
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).
9. 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 cutoff or C_t , which separates a positive sample of a negative will vary depending on the specific detection limit of each particular laboratory.
10. To consider a curve as positive, this must have an exponential appearance, and the threshold cycle must be less than the C_t determined in the methodological validation.
11. 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 com-

mercial dealers may vary in amplification efficiency, and consequently may affect the optimum temperature and the specificity of the technique, generating variations in observed Ct and amplification profile.

Substances included in the validation: In the phase of methodological design and development of the detection systems a great number of substances must be 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 matrix used in their production chain, since these could interfere with the results, giving rise to for instance false positives.

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