Application Note

Identifying meat species using RFLP-PCR and the QIAxcel® Advanced system

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Food producers are obliged to monitor the manufacturing process for compliance with regulations on the quality and originof food products. Technology for rapid and accurate meat species authentication is crucial to the meat industry. This paperlooks at a sensitive method using restriction fragment length polymorphism PCR and the QIAxcel Advanced system.

Introduction

The requirement to put labels on food products providing consumers with information about the quality and origin of ingredients first appeared in the European Union in 1992. Directives on the legal protection of geographical indications and designations of origin for agricultural products and foodstuffs followed a few years later (1).

To protect consumers' interests, it was necessary to develop effective methods to authenticate the species composition of various food products, including meat. The prices for high quality meat are higher, so fraud does occur. Species identification requirements are also connected with the prohibition of sale of meat from certain protected animal species. Furthermore, certain cultures have religious restrictions on the meat composition of food. Finally, allergy sufferers need to know that they are getting meat that is safe for their consumption.

Fortunately, it is now possible to identify individual food species using molecular biology techniques, some of which allow unequivocal species identification in both raw and processed food.

Protein-based methods are not sensitive enough and cannot be used with processed meat because soluble muscle proteins are destroyed by processing. Therefore, most methods use PCR-based DNA amplification. PCR is characterized by high specificity and a relatively short analysis time.

The most commonly used PCR methods for meat identification are:

1. **PCR with species-specific primers** that are designed based on cytochrome b mitochondrial DNA (2) has been used in multiplex PCR for the qualitative identification of 6 meat species: cattle, swine, chicken, sheep, goat, and horse (3). Multiplex PCR has also been used for the distinct and specific detection of chicken, turkey, duck, goose, pheasant, quail, and guinea fowl in raw meat and processed meat products (4). These experimental setups enabled authentication for correct food labeling and for compliance with ingredient composition covering the range of all common domestic poultry species available on the EU market. ▷

2. **RFLP-PCR** allows the identification of various mammal, bird, and fish species (5). Wolf et al. developed a method that allows recognition of 25 animal species. The DNA fragment obtained by amplifying a specific region of the mitochondrial genome (tRNAGlu/cytochrome b) is treated with 11 different restriction endonucleases.

3. **Real-time PCR** allows quantitative contamination assessment, including the identification of meats of different, even closely related, animal species. It is very efficient in detecting traces of specific animal DNA, even if the DNA has been degraded during a meat preparation process. As such, it serves an excellent screening tool for high-throughput routine testing where the target is known.

RFLP-PCR satisfies crucial aspects such as specificity, sensitivity, flexibility, and efficiency. Previous studies demonstrated that RFLP-PCR (5, 6, 7, 8) is successful in identifying meat species.

Sample analysis using slab gel electrophoresis is unsuitable for routine work because the method is time-consuming, requires more manual handling, and uses hazardous products such as ethidium bromide. The results are difficult to interpret and may require specific software.

Native capillary electrophoresis with the QIAxcel Advanced system overcomes these issues. The automated procedure is fast and inexpensive. The QIAxcel ScreenGel® Software analyzes the electrophoresis data and provides the sizes and concentrations, so no other software is needed.

The purpose of this study was to optimize a procedure using RFLP-PCR in conjunction with the QIAxcel Advanced system for use in rapid (results in less than 8 h) and accurate routine analyses. The method involves amplification of a 359-bp product that is common to all vertebrates, followed by one or more enzymatic digestions. The proportion of individual meats could range from 1 to 99%. By applying four enzymes, it was possible to distinguish meat from 15 different animals. In addition, 6 animals served as contamination markers.

Criteria for optimization

We defined four criteria for optimizing the meat authentication method: sensitivity, flexibility, speed, and simplicity.

The limit of detection (LOD) should range between < 0.5% and 1%. Several different pure animal samples were tested and a sensitivity of 0.01% was validated for beef, pork and chicken. The meat was tested either as a water dilution or in a maize mixture.

The method should be suitable for a broad range of sample material to give maximum flexibility of application. It should take less than one working day to receive results as longer delays lead to higher costs for food producers. Finally, since the method is intended for routine analyses, it should be easy to implement and perform.

If RFLP-PCR is intended for species identification, the reference samples must be analyzed using the same procedure and QIAxcel DNA kit as the unknown samples. The disadvantage of this technique is the possibility of incomplete digestion. In such cases, other methods, such as real-time PCR and/or sequencing, should be used for confirmation.

Materials and methods

Meat homogenization was followed by lysis with chemical (QIAGEN ATL lysis buffer), thermal (up to 65°C), and mechanical (1400 rpm agitation) treatment for up to one hour (internal method). DNA purification was performed on a QIAsymphony® SP using the QIAsymphony DSP DNA mini kit. PCR was performed as previously described (*7*, 8) using TopTaq Master Mix (QIAGEN) and the primersCYT b1 and CYT b2. This yielded a product of 359 bp that is common to all vertebrates. The PCR product was digested with different enzymes as previously described (*7*), and the digests were analyzed with the QIAxcel system, which provided information about the size and concentration of the products. The results were further interpreted using a programmed Excel sheet.

The sample analysis was performed according to method OM500 for the QIAxcel Advanced system, using the QIAxcel High Resolution Kit, QX Alignment Marker 15 bp/600 bp and QX DNA Size Marker 25 – 500 bp.

Results and discussion

We used the PCR-RFLP approach where the amplified mitochondrial DNA region encoding cytochrome b (*7*) undergoes enzyme digestion for species identification. This was achieved using four restriction enzymes: AluI, Hae III, Hinf I, and RsaI. The meat was tested raw and after processing methods, such as cooking, freezing, reheating, smoking, dehydration, and sterilization. The results proved that the PCR-RFLP method can successfully identify and authenticate the species of meat used in commercial products subjected to various processing.

The method was first tested on a range of reference samples containing either a single meat or mixed meat species. Initially, the samples were analyzed with real-time PCR and the results were compared to data obtained using RFLP-PCR to validate the procedure and establish whether detection using the QIAxcel system was comparable to detection using real-time PCR. A number of pure and mixed animal products were used as references (Figure 1). The number of enzymes needed for the analyses was species-specific. For example, AluI is sufficient to detect cattle DNA but not that of the other species. In our study, a maximum of four enzymes was needed to identify all of the animals tested.

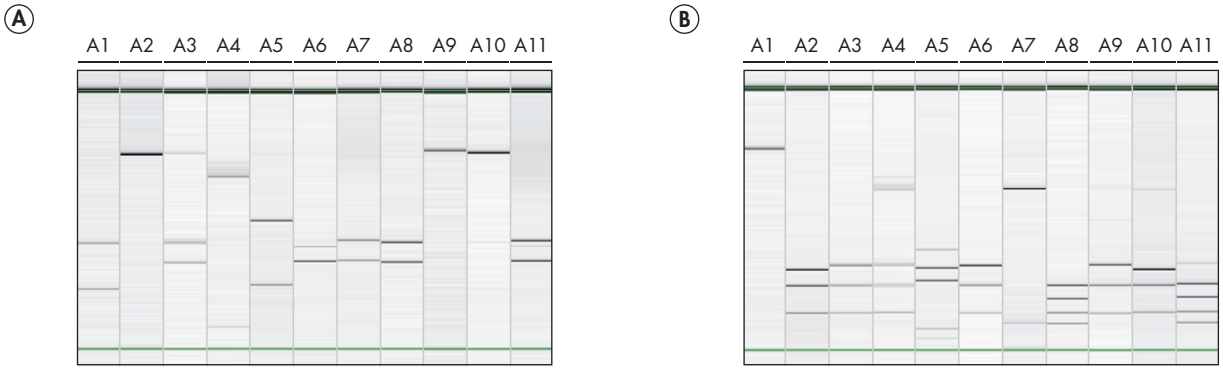

Figure 1. Successful identification of meat species using the QIAxcel Advanced System. The analysis of the 359-bp PCR fragment digested with **A.** HinfI enzyme and **B.** HaeIII enzyme. The 359-bp fragment was amplified from nine reference samples. The starting material was fresh meat. **Lane 1**: Cattle; **Lane 2**: Pig; **Lane 3**: Sheep; **Lane 4**: Deer; **Lane 5**: Rabbit; **Lane 6**: Chicken; **Lane 7**: Duck; **Lane 8**: Turkey; **Lane 9**: Goose. Only two mixed samples were prepared in the laboratory: **Lane 10**: Beef and pork and **Lane 11**: Chicken and turkey.

Table 1. Fragment sizes after digestion of 359-bp mitochondrial cytb fragment with restriction enzymes: Data are presented for nine animal species. Fragment sizes are given in base pairs.

	Restriction enzyme			
Animal species	**HaeIII**	**AluI**	**HinfI**	**RsaI**
Cattle	74, 285	190, 169	44, 198, 117	359
Pig	74, 132, 153	115, 244	359	359
Sheep	74, 126, 159	359	161, 198	359
Deer	74, 285, 126, 159	359	44, 315	359
Rabbit	44, 132, 30	359	233, 126, 153	359
Chicken	74, 21, 159	359	105, 161, 188, 10	149, 210
Duck	55, 286, 18	359	161, 198	359
Turkey	74, 285	359	161, 198	149, 109, 101
Goose	74, 126, 159	130, 229	359	154, 205

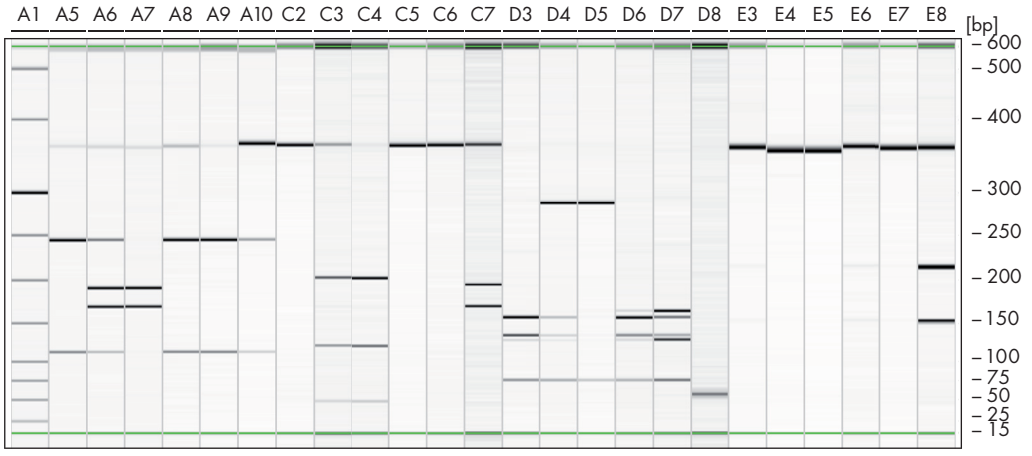

Figure 2. QIAxcel analysis of six commercial food samples. The samples were as follows — **FF1**: pork pâté (100% pig); **FF2**: hamburger meat (100% beef); **FF3**: Parmentier beef hash (100% beef); **FF4**: pork terrine (100% pig); **FF5**: pork liver mousse (100% pig); **FF6**: poultry liver terrine of (75% pig and 25% poultry). These samples were digested by the four studied enzymes, as follows — **Lanes A5–A10**: AluI; **Lanes C2–C7**: HinfI; **Lanes D3–D8**: HaeIII; **Lanes E3–E8**: RsaI. **Lane A1**: DNA Size Marker 25 – 500 bp.

Table 2. Summary of the analysis of the six samples, based on the data from Figure 2.

Sample	**Expected result**	**Observed result**	**Compliance**
FF1 (Pork pâté)	Pig	Pig	Yes
FF2 (Hamburger meat)	Beef (cattle)	Beef + Pig	No
FF3 (Parmentier beef hash)	Beef	Beef + Pig (traces)	No
FF4 (Pork terrine)	Pig	Pig	Yes
FF5 (Pork liver mousse)	Pig	Pig	Yes
FF6 (Poultry liver terrine)	Pig + Poultry	Pig + Chicken	Yes

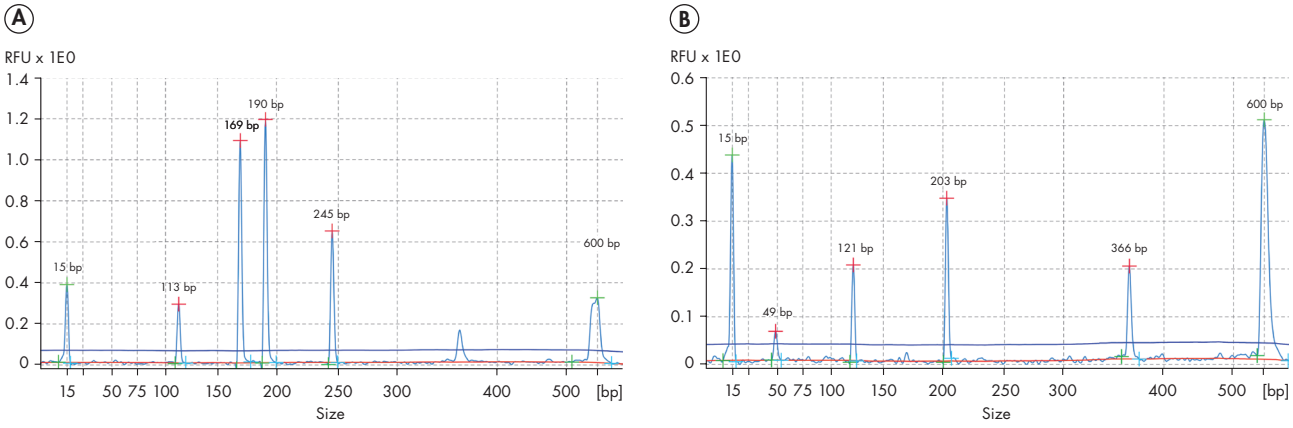

Figure 3. An electropherogram view of the data for the analyzed samples. A. Sample FF2 digested with AluI (corresponding to Lane A6 in Figure 2). **B.** Sample FF2 digested with HinfI (corresponding to Lane C3 in Figure 2).

In this study, four out of six samples were found to be correctly labeled with the RFLP-PCR yielding the expected results. However, we found that samples FF2 and FF3 were not properly labeled: they contained various amounts of pork despite being labeled as pure beef.

All of the commercial samples analyzed had undergone different production processes. The results indicated that the enzyme digestions were incomplete in some samples (lanes A5 to A9, Figure 2). Therefore, the samples were additionally analyzed with real-time PCR (results not shown), which confirmed that the digestion with AluI was incomplete for those samples.

Conclusion

- The QIAxcel Advanced System facilitates analysis of PCR-RFLP samples for meat species identification, especially for large-scale analyses.
- Using this native capillary electrophoresis system significantly reduces analysis time for the authentication of meat samples and minimizes the potential for procedural errors that would influence the accuracy of analysis.
- The method also eliminates exposure to hazardous chemicals and is easy to handle, making it an excellent routine method for the control of meat and meat products.

References

1. Council Regulation (EC) No 510/2006 of March 20, 2006
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5. Wolf, C. et al. (1999) PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. J. Agric. Food Chem. **47**, 1350.
6. Meyer, R. et al. (1995) Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis: A simple method for species identification in food. J. of AOAC Int. **78(6)**, 1542.
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Ordering Information

Product	Contents	Cat. no.
QIAxcel Advanced System	Capillary electrophoresis device: includes computer, QIAxcel ScreenGel software, and 1-year warranty on parts and labor	9001941
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High Resolution Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929002
Buffer ATL (4 x 50 ml)	4 x 50 ml lysis buffer for use in purification of nucleic acids using the QIAsymphony DSP DNA Mini Kit	939016
QIAsymphony SP	QIAsymphony sample prep module	9001297
QIAsymphony DSP DNA Mini Kit (192)	For 192 preps of 200 ul each: Includes 2 reagent cartridges and enzyme racks and accessories.	937236
TopTaq Master Mix Kit	For 200 x 50 ul reactions: 2x TopTaq Master Mix containing 250 units of TopTaq Polymerase in total, 10x CoralLoad Concentrate, and RNase-Free Water	200403
QX Alignment Marker 15 bp/600 bp (1.5 ml)	Alignment marker with 15 bp and 600 bp fragments	929530
QX DNA Size Marker 25–500 bp (50 ul) v2.0	DNA size marker with fragments of 25, 50, 75, 100, 150, 200, 250, 300, 400, and 500 bp; concentration 100 ng/ul	929560

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