# On the origin of meat - DNA techniques for species identification in meat products

**Johannes A. Lenstra, Jacob B. Buntjer* and Frederik W. Janssen****

Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Yalelaan 1, 3584 CL Utrecht, The Netherlands
* Keygene, P.O. Box 216, 6700 AE Wageningen, The Netherlands.
** Inspectorate for Health Protection, P.O. Box 9012, 7200 GN Zutphen, The Netherlands

## Introduction

**The conventional methodology used for the determination of species origin in meat products has been predominantly based on the immunochemical and electrophoretic analysis of proteins. More modern techniques now allow the identification of species-specific DNA sequences, which has two major advantages over protein analysis: samples heated to as high as 120°C can still be analysed and discrimination between related species, such as sheep and goat or chicken and turkey, is possible.**

During the early development of DNA sequence analysis, genomic DNA was used as a species-specific probe and was hybridised to DNA extracted from meat samples [1, 7, 9, 24]. The subsequent development of probes derived from species-specific satellite repetitive DNA sequences has greatly improved the specificity of the assay, now making it possible to detect admixtures that contribute as little as 5 %, or less, to a product. Two satellite hybridisation procedures [4, 6, 10, 11, 13], which differ in technical implementation rather than in principle or performance, are described below in Sections 1 and 2.

An alternative DNA detection system is based on the polymerase chain reaction (PCR) amplification of a segment of the mitochondrial cytochrome *b* gene [15, 18, 24]. Subsequent cleavage by a restriction enzyme gives rise to a species-specific pattern on an agarose gel (Figure 1). This method does not require the development of species-specific probes and, because it is PCR-based, is most suitable for critical samples in which DNA is largely degraded. Section 3 describes the application of this method.

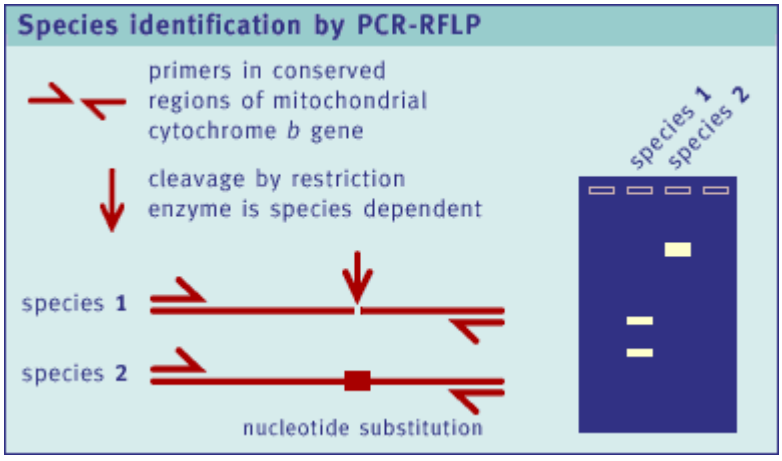


**Figure 1.** Species identification by PCR-RFLP of mitochondrial DNA.

Several other methods have been described for the determination of species origin in meat products, the most straightforward of which is sequencing of the mitochondrial PCR product [2, 19]. This method is suitable for the identification of unknown species, but cannot be used to test samples of dual species origin. An alternative is the MIR-specific PCR, which generates species-specific fingerprints by amplification of DNA segments positioned between MIR (mammalian interspersed repeat) elements [5]. A third method uses one generic primer and several species-specific primers to differentiate between the most common meat species [14]. Other PCR-based methods have been designed specifically for the detection of porcine [17], bovine [22], ostrich and emu [8] derived DNA. Table 1 summarises the methods available currently and highlights their main features.

**DNA-based methods for species-identification in meat products**

| Method | Features | Disadvantages |
|---|---|---|
| Hybridisation to oligonucleotide | * versatile screening of many samples with several different probes<br>* rapid procedure<br>* detection of admixture | * hybridisation dependent on exact experimental condition<br>* species-specific probes need to be developed |
| Hybridisation to PCR-generated | * convenient screening of many samples<br>* detection of admixtures | * species-specific probes need to be developed |
| PCR-RFLP of mitochondrial DNA | * most suitable for degraded samples<br>* detection of admixture | * sensitive to contaminations |
| PCR and sequencing of mitochondrial DNA | * most suitable for degraded samples<br>* one procedure for all species | * sensitive to contaminations<br>* not suitable for samples of mixed origin |
| MIR-PCR [See Ref.5] | * one fast procedure for all mammalian species<br>* detection of unknown species<br>* suitable for degraded samples | * requires special equipment and software<br>* not validated in practice<br>* not suitable for samples of mixed origin |

**Table 1.**

## Hybridisation to satellite-specific oligonucleotides

DNA-DNA hybridisation is based on the spontaneous association of two single strands of DNA that have complementary sequences, so forming the classical DNA duplex described by Watson and Crick [23] (Figure 2). The single strands may be derived from the same or similar DNA sequences. In a typical hybridisation experiment, double-stranded DNA is denatured and both constituent single strands are immobilised on a nylon membrane (the 'blot'). The other partner in the association process, the 'probe', is either a second denatured DNA molecule or a short synthetic oligonucleotide, which binds to only one strand of the DNA immobilised on the blot. By labelling the probe with an isotope ($^{32}P$), a fluorescent group (FITC), an antigen-hapten (digoxigenin), or an enzyme (alkaline phosphatase), its position on the blot after hybridisation is determined.

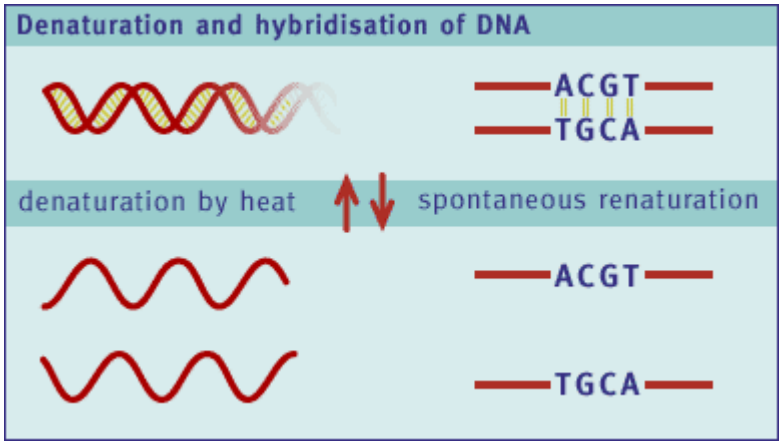


**Figure 2.** Hybridisation of DNA, or formation of a DNA double helix by mutual recognition of two single_stranded DNA strands that have complementary sequences to one another.

DNA can be extracted from meat samples by using one of three methods: rapid alkaline lysis (Protocol 1), a similar procedure to Protocol 1 but with extra purification steps (Protocol 2), the common proteinase K/sodium dodecyl sulphate (SDS) procedure (Protocol 4). The latter is the most elaborate of these methods, but has a better yield and produces double- rather than single-stranded DNA. Species-specific probes are derived from tandem repeated satellite DNA, which accounts for up to 20 % of the total genome and has a species-specific sequence. In Protocol 3, the oligonucleotide probe is coupled directly to alkaline phosphatase (AP). A positive reaction is determined by the detection of a chemiluminescent AP product (Figure 3). Protocol 5 describes the preparation and digoxigenin (DIG) labelling of 200-1000 bp probes by PCR amplification of satellite DNA segments in the presence of DIG-dUTP. After hybridisation, the probe is detected by the generation of a coloured AP product (Protocol 6; Figure 4).

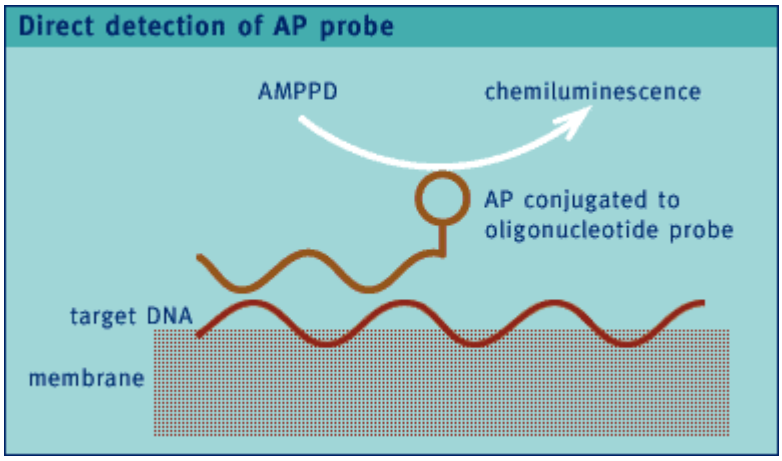


**Figure 3**. Direct detection of the hybridisation of oligonucleotides conjugated to alkaline phosphatase (AP). Hydrolysis of a special alkaline phosphate (AP) substrate (AMPPD) generates chemiluminescence, which is detected subsequently by autoradiography.
AMPPD = [3_(2'_spiroadamantane)_4_methoxy_4_(3'' phosphoryloxy)_phenyl_1,2_dioxetan]

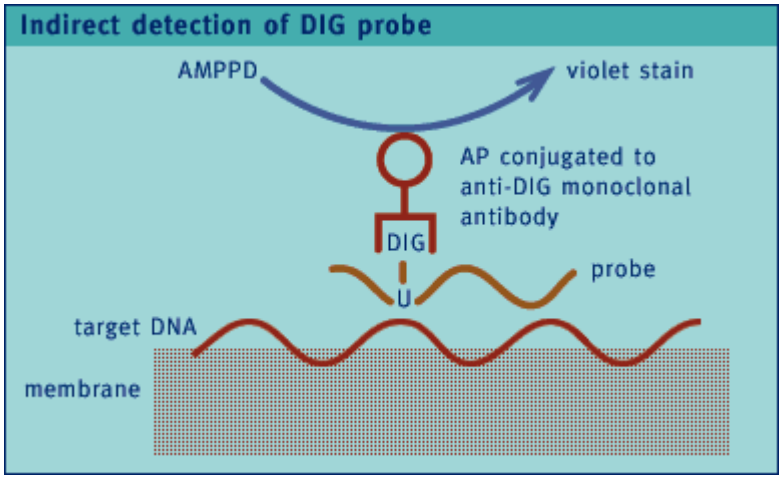


**Figure 4.** Indirect detection of the hybridisation of oligonucleotides conjugated to digoxygenin (DIG). DIG groups are recognised by an anti_DIG monoclonal antibody conjugated to alkaline phosphates (AP). Hydrolysis of a chromogenic substrate (AMPPD) generates a violet staining.
AMPPD = [3_(2'_spiroadamantane)_4_methoxy_4_(3'' phosphoryloxy)_phenyl_1,2_dioxetan]

Protocols 1 (or 2) and 3, and Protocols 4, 5 and 6, form two integrated procedures for species identification, both of which have been validated with test samples. The first procedure is carried out in a 96-well microtitre plate and is convenient for testing relatively few samples with several different probes. For the second, many samples are spotted onto one large filter, making it more efficient for screening many samples with few probes. Protocols from the two integrated procedures can be interchanged [see 10].

## DNA extraction

Protocol 1 provides a rapid procedure for DNA extraction by using an alkaline extraction buffer. It yields single-stranded DNA (6-15 μg DNA/g meat), which can be spotted directly onto a positively charged nylon membrane for subsequent hybridisation. However, if the alkaline extracted DNA sample is used as a template in a PCR procedure, it should first be diluted or neutralised. DNA isolated in this manner cannot be digested by restriction enzymes. Protocol 2 is a larger scale operation, which reduces the amount of protein by using an organic extraction procedure.

## Protocol 1 - Rapid alkaline extraction of DNA from meat

### Materials

- Extraction buffer: 0.5 M NaOH, 10 mM EDTA

### Method

1. Weigh 100 mg tissue in a 2-ml Eppendorf tube.
2. Add 0.2 ml extraction buffer, vortex and incubate at 100°C for 7 min.
3. Centrifuge for 2 min at 12,000 rpm and transfer supernatant to a new tube, avoiding floating fat.
4. Repeat step 3.

## Protocol 2 - Alkaline and organic extraction of DNA from meat

### Materials

- Extraction buffer: 0.5 M NaOH, 10 mM EDTA
- Alkaline solution: 2 M NaOH
- Phenol chloroform isoamylalchohol (PCI) solution: 0.05 % (w/v) hydroxyquinoline, 2 % (v/v) isoamylalcohol, 49 % (v/v) phenol, water-saturated and neutralised with Tris/HCl [13], 49 % (v/v)CHCl3

### Method

1. Weigh 1 g ground tissue into a 10-ml tube.
2. Preheat extraction buffer to 100°C, add 2 ml to the tissue and incubate at 100°C for 7 min.
3. Centrifuge at 10,000-15,000 rpm for 2 min and transfer the supernatant to a fresh tube, avoiding the floating fat layer.
4. Centrifuge at 10,000-15,000 rpm for 2 min and transfer 100 ml supernatant to an Eppendorf vial.
5. Add 50 ml PCI solution and leave for 5 min, vortexing occasionally.
6. Centrifuge at 10,000-15,000 rpm for 3 min in a tabletop minifuge and transfer the (upper) aqueous phase to a new Eppendorf vial.
7. Add 25 μl 2M NaOH to the aqueous phase.

## Hybridisation to AP-labelled oligonucleotides

Hybridisation in the wells of a 96-well microtitre plate reduces the volume of hybridisation fluid required and allows the use of several different probes in one experiment.

## Protocol 3 - Hybridisation to AP-labelled oligonucleotide probes

### Materials

- Microtitre plate (96 wells)
- Whatman 3MM paper
- Hybond N+ hybridisation membrane (Pharmacia Biotech)
- Neutralisation buffer: 0.5 M Tris/HCl, pH 8.0, 1.5 M NaCl
- Pre-hybridisation buffer: Quantum Yield blocking buffer (Promega)
- Standard sodium citrate (SSC) 20×: 3 M NaCl, 0.3 M trisodium citrate, pH 7.0
- Hybridisation buffer: Quantum Yield high stringency hybridisation buffer
- Hybridisation wash buffer: 2×SSC/0.1 % (w/v) SDS or 0.5× SSC/0.1 % (w/v) SDS
- Equilibration buffer: 100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$
- Chemiluminescent substrate: 0.25 mM [3-(2'-spiroadamantane)-4-methoxy-4-(3'' phosphoryloxy)-phenyl-1,2-dioxetan] (AMPPD) or disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1$^{3,7}$]decan}-4-yl)phenyl phosphate (CSPD; Boehringer) in equilibration buffer
- Oligonucleotide probes: label oligonucleotides (Table 2) by using the Promega Quantum Yield AP Labelling System and dilute 1:500

| satellite oligonucleotide probes for species in meat products | | |
|---|---|---|
| **Species** | **Probe** | **Sequence (5'→3')** |
| Chicken | GMRS | gcgttttctcttgcaaatcc |
| Turkey | MMRS | gtatttvtvvagaaaaaggg |
| Goose | TGrev | ctggcaccactggggatgcag |
| Pig | SSAS | attgaatccactgcattcaatc |
| Cattle | 1.706 | aatcatgcagctcagcaggcaat |
| Male cattle | BovY | tcagccctgtgccctggyra |
| Sheep/goat | OASL | tcgcctttcctgtggagagc |
| Deer | CCSI | tcagggtccctctcacatac |
| Roe deer | CcsIII | ccctcgctctccaatgaaagc |
| Horse | HMSR | ctacttcagccagatcaggc |
| Human | Alpha | tcaactcacagagttgaacgatc |
| Microsatellite (positive control) | CACA | $(ca)_{25}$ |

**Table 2.** For satellite oligonucleotide probes listed above see [4 and 13]. For other oligonucleotides refer to [13].

## Method

1. Cut 4×4 mm pieces of hybridisation membrane and place the membranes on Whatman paper moistened with extraction buffer.
2. Spot 5-7 µl DNA extract (Protocols 1 or 2) on each membrane and leave for 10 min. Include the following controls: water; 1, 10 and 100 ng purified DNA of the species to be detected; 100 ng DNA of other species; appropriate mixtures of purified DNA samples for the analysis of admixtures.
3. Transfer each membrane to a well in a 96-well microtitre plate and add 200 ml neutralisation buffer to each well.
4. Incubate 1 min and remove the buffer.
5. Repeat steps 3 and 4.
6. Put the microtitre plate on a water bath at 50°C
7. Add 200 ml preheated pre-hybridisation buffer to each well, cover the plate with parafilm and incubate for at least 30 min.
8. Remove the pre-hybridisation buffer and add 200 ml probe (diluted 1:500). Avoid drying of the blots. Do not remove the plate from the incubator in order to prevent non-specific binding at lower temperatures.
9. Incubate for 30 min.
10. Remove the probe, add 200 ml hybridisation wash buffer and incubate for 10 min. Again avoid drying of the blots.
11. Remove wash buffer, add 200 ml hybridisation wash buffer and incubate for 10 min.
12. Remove the plate from the bath and discard the wash buffer.
13. Add 200 µl equilibration buffer and leave for 1 min.
14. Take the blots out of the wells, drain excess buffer on clean Whatman paper and place on a plastic sheet.
15. Drop 5-10 ml chemiluminescent substrate on each blot and incubate 5-10 min.
16. Transfer to a new plastic sheet and cover with a second plastic sheet.
17. Expose X-ray film (Kodak) to the blots for 60 min at room temperature and compare the intensities of the spots with the controls ie. the blank and purified DNA samples.

Notes

1. With AP-probes, hybridisation should not be carried out at temperatures higher than 55°C to avoid enzyme denaturation. If necessary, increase the stringency of the reaction by lowering the salt concentration in the wash buffer.
2. This protocol can be combined with the DNA isolation of Protocol 4.
3. The procedure can also be combined with the less sensitive colorimetric detection (see Protocol 5).
4. Alternatively, the oligonucleotides may be labelled with DIG and hybridised using the protocols provided by Boehringer [10].

## Hybridisation to satellite probes generated by PCR

This procedure [11] is used routinely by the Dutch Inspection for Health Protection and a typical test result is shown in Figure 5. The procedure is slightly more time consuming than the preceding one, but does not require specialised chemicals or equipment for the AP-labelling and subsequent chemiluminescent detection steps. Samples are spotted onto a single membrane which facilitates the testing of many samples with one probe. By using longer probes, the hybridisation conditions are less critical. Probes have been validated for the most common meat species (Table 3). Specific satellite or short interspersed nuclear element (SINE) probes for other species [11] have not yet been tested in meat samples.

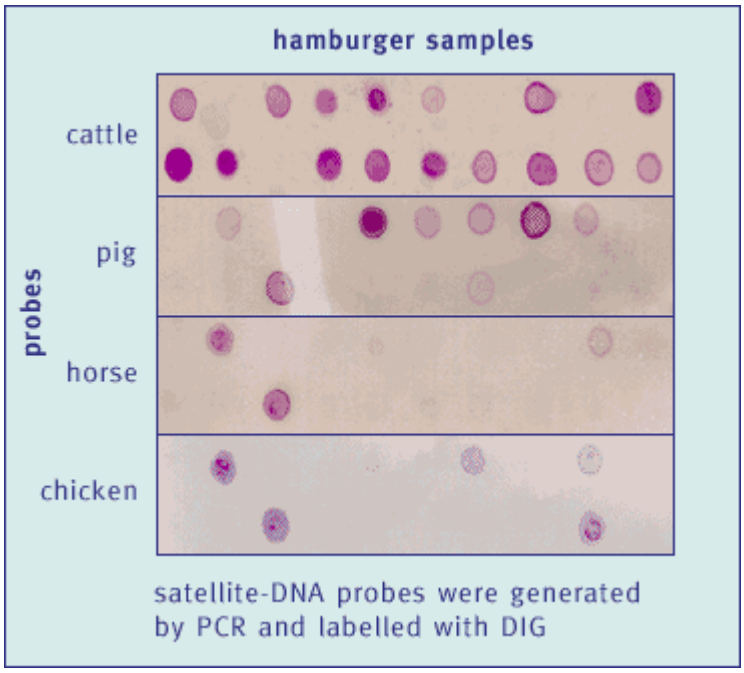


**Figure 5.** The species origin of components in 20 hamburger samples determined using probes specific for beef, horse meat, pork and chicken meat. Samples are spotted onto a positively charged nylon membrane. After hybridisation species-specific probes are detected via the indirect procedure (Figure 4).

| Primers for the generation of species-specific satellite oligonucleotide probes | | | | |
|---|---|---|---|---|
| **Species-specific repeat** | **Genbank reference** | **Primers (5' → 3')** | **Annealing temperature** | **Length of PCR product** |
| Chicken CNM | X51431 | gcgttttctcttcgcaaatcc<br>acgcgtgattttcgcttaaatg | 55°C | 50-bp multimers |
| TurkeyTM | X66696 | gtatttgtgggagaaaaaggg<br>cacaaatacctgtttttacacg | 55°C | 50-bp multimers |
| Pig Ac2 satellite | X51561-51565 | ggagcgtggcccaatgca<br>attgaatccactgcattcaatc | 55°C | ⩽100 bp |
| Cattle satellite IV | X00979 | aagcttgtgacagatagaacgat<br>caagctgtctagaattcaggga | 55°C | 603 bp |
| Sheep satellite I | X01839 | gttaggtgtaattagcctcgcgagaa<br>aagcatgacattgctgctaagttc | 60°C | 374 bp |
| Deer CcsatI | [See Ref. 12] | tgcagagcaattccttgttgc<br>tcagggtccctctcacatac | 55°C | roe deer: 500 bp<br>red deer : 300 bp |
| Horse major satellite | X70916 | ttctgctctgggtgtgctactt<br>ctacttcagccagatcaggc | 55°C | 221-bp multimers |

**Table 3.**

## Protocol 4 - Isolation of double-stranded DNA from meat

### Materials

- Homogenisation buffer: 0.4 M NaCl, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0
- SDS 10×
- Proteinase K solution: 1 mg/ml proteinase K freshly dissolved in 1 % (w/v) SDS, 2 mM EDTA
- Saturated NaCl
- Ethanol (-20°C)
- TE: 10 mM Tris/HCl, 1 mM EDTA, pH 7.5, autoclaved

### Method

1. Homogenise 5 g homogeneous meat sample in 20 ml homogenisation buffer for 1 min in a Waring Blendor beaker.
2. Transfer 750 ml to an Eppendorf tube, add 50 µl 10 (w/v) SDS and 125 µl proteinase K solution and incubate overnight at 65°C.
3. Add 250 µl saturated NaCl and vortex 15 s.
4. Let fat solidify by cooling for 30 min in a refrigerator, centrifuge for 15 min at 10,000 rpm, 10°C and transfer 500 µl (without fat) to a new tube.
5. Add 1 ml (-20°C) ethanol and centrifuge again.
6. Remove the ethanol and dry the pellet at 50°C.
7. Add 50 µl TE and let dissolve for 2 h at 37°C. Vortex occasionally.
8. Store at -20°C.

**Notes**

1. For the analysis of heated meat samples, process 3 to 5 Eppendorf tubes per sample, combine the DNA and purify on a Wizard Clean Up column (Promega) or a comparable resin system following the instructions of the appropriate manufacturer.
2. If the sample after spotting (Protocol 6) leaves a white stain, contaminants interfere with the binding of DNA to the blot. Purify these samples on a Wizard Clean Up column. (Promega).
3. Yield and quality of the DNA can be checked by agarose gel electrophoresis [13]. Typically, most of the DNA has a length of ≤ 100 bp, but Promega's Wizard purification method yields DNA with a length of several hundred of bp.
4. Reference DNA can be purified from tissue or blood samples by common procedures [13]. Check the purity and concentration by measuring the absorbance at 260 and 280 nm.

## Protocol 5 - Amplification of satellite probes

**Materials**

- PCR reaction buffer 10×: 100 mM Tris/HCl, 500 mM KCl, 15 mM $MgCl_2$, 0.1 % gelatine
- Primers: ordered from specialist oligonucleotide synthesisers (customer service) and diluted to 50 ng/µl (7.5 µM for a 20-mer)
- DIG dNTP mix: 2 mM dATP, 2 mM dCTP, 2 mM dGTP, 1.9 mM dTTP and 0.1 mM DIG-dUTP (Boehringer).
- Amplitaq DNA polymerase, 5 U/µl (PE Applied Biosystems)
- Purified genomic DNA of the appropriate species, 0.5 ng/10 µl.
- Mineral oil for thermocyclers without a heated lid

**Method**

The pre-PCR pipetting should preferably be carried out in one room, using special filter tips to prevent contamination of the reagents and the automatic pipette. In a different room the DNA templates should be prepared and added to the PCR mix. After completing the cycling amplification step, products should be handled in a third room. Do not take any pipettes, racks, or other equipment, from one room to another.

1. Pipette in the sequence indicated: for n-1 samples n × 53.5 µl $H_2O$, n × 10 µl PCR reaction buffer (10×), n × 10 µl DIG dNTP mix, 10 × 0.5 µl Amplitaq DNA polymerase, n × 8 µl of one primer, n × 8 µl of the other primer (Table 3).
2. Mix by vortexing and dispense 90 µl in n-1 PCR tubes.
3. For PCR machines without heated lid, add 50 µl mineral oil. Keep cool.
4. In another room: add 10 µl (5 ng) genomic DNA
5. Mix, pellet and carry out one of the following cycling programmes: 30-35 cycles of: 1 min 93°C, 2 min at the annealing temperature indicated in Table 3, 2 min at 72°C.
6. In the third room analyse 5 µl PCR reaction material by agarose gel electrophoresis [21].

### Notes

1. With the exception of the annealing temperature, most PCR parameters are not critical. If necessary, consult the textbooks about adjusting these parameters or designing other primers.
2. If the PCR product is not pure enough, run the samples on a low melting point (LMP) agarose gel and cut out the fragment of the indicated length. Just before use in Protocol 6 melt/denaturate at 100°C and use directly as probe.
3. If species from different mammalian orders have to be discriminated (e.g. horse or kangaroo vs. cattle), alternative probes may be derived from genomic DNA. Label 1 μg DNA by random priming using the Boehringer DIG-DNA labelling and detection kit.

## Protocol 6 - Hybridisation and detection of probe

### Materials

- Reference samples: purified DNA of each of the appropriate species at 1, 10, 100 and 1000 ng/μl, respectively
- Positively charged nylon membrane
- SSC 20×: 3 M NaCl, 0.3 M trisodium citrate, pH 7.0
- 100× Denhardt's: 2 % (w/v) polyvinylpyrollidone, 2 % (w/v) BSA, 2 % (w/v) Ficoll 400
- Herring sperm DNA, 10 mg/ml in $H_2O$, boiled just before use
- Pre-hybridisation mix: 50 (v/v) formamide, 5× SSC, 50 mM sodium phosphate, pH 7.0, 5 mM EDTA, 5× Denhardt's, 0.25 mg/ml herring sperm DNA
- Anti-digoxygenin(DIG)-AP, Fab-fragments (Boehringer)
- Washing buffer I: 2× SSC, 0.1 % (w/v) SDS
- Washing buffer II: 0.1× SSC, 0.1 % (w/v) SDS
- Washing buffer III: 100 mM Tris/HCl-oplossing, pH 7.5, 150 mM NaCl
- Blocking solution: 0.5 % (w/v) sodium caseinate in washing buffer III
- Staining buffer: 50 mM $MgCl_2$, 100 mM Tris/HCl, pH 7.5, 100 mM NaCl
- Naphthol AS-E phosphate stock (Serva): 1 % (w/v) in DMSO
- Substrate solution: prepare just before use 0.05 % (w/v) Fast Violet B (6-Benzamido-4-methoxymethanilamide-diazonium salt) in staining buffer, add 5 % (v/v) naphthol AS-E phosphate stock and mix.

### Method

1. Heat the test and reference samples for 5 min at 95°C and cool immediately in ice
2. Spot 2 μl of each sample on a nylon membrane. Include the following controls: water; 1, 10, 100 and 1000 ng purified DNA of the species to be detected; 1000 ng DNA of other species; appropriate mixtures of purified DNA samples for the analysis of admixtures. Handle the membrane with gloves.
3. Leave to dry for 10 min and fixate the DNA by exposure of the membrane to UV irradiation (312 nm or 254 nm) for 5 min. The membrane can be stored at room temperature.
4. Place the membrane in 25 ml pre-hybridisation mix per 100 $cm^2$ membrane and incubate 30 min at 42 °C. Use either a sealable plastic bag or a flat-bottomed plastic box with a tight lid. Ensure the membrane is completely covered in the fluid.
5. Prepare hybridisation mix: heat 200 μl probe/100 $cm^2$ membrane for 5 min at 100°C and add immediately to the pre-hybridisation mix (10 ml/100 $cm^2$ membrane).
6. Place the membrane in hybridisation mix (10 ml/100 $cm^2$ membrane) and incubate overnight at 42°C.
7. Pour off the hybridisation mix into a plastic tube and store at -20°C. Just before reuse, regenerate the probe by boiling for 15 min.

8. Place the membrane in washing buffer I (50 ml/100 $cm^2$ membrane), shake gently for 5 min at room temperature and pour off the washing buffer.
9. Repeat step 8.
10. Place the membrane in membrane washing buffer I, 50 ml/100 $cm^2$ preheated to 42°C, and shake gently for 15 min at 42°C.
11. Repeat step 10.
12. Wash the blot for 1 min in washing buffer III.
13. Dilute the anti-digoxygenin-AP 1:5000 in blocking solution (20 ml/100 $cm^2$ membrane).
14. Place the membrane in this antibody dilution and incubate for 30 min at room temperature.
15. Pour off and wash the blot 4 × 1 min in washing buffer III (50 ml/100 $cm^2$ membrane) at room temperature.
16. Wash once for 2 min in staining buffer (50 ml/100 $cm^2$ membrane).
17. Incubate in freshly prepared substrate solution (20 ml/100 $cm^2$ membrane) until the spot of 1 ng purified DNA is just detectable above the background signal of the blank.
18. Stop the staining by rinsing with $H_2O$ and store the membrane in the dark.
19. Compare the staining intensities of the samples with the reference signals. Check the negative controls (ie. the blank and the DNA samples of other species).

**Notes**

1. If the probe binds to DNA of other species, increase the stringency of the washing conditions (steps 10 and 11) by raising the temperature (e.g. to 65°C) or by lowering the salt concentration (to 0.5×, 0.2× or 0.1× SSC). When using genomic DNA probes, use washing buffer II (0.1×SSC).
2. The sheep probe will not discriminate between mutton and goat meat, and the cattle and sheep probes show a weak cross-reaction.

## PCR- RFLP analysis of mitochondrial DNA

Mitochondrial DNA accumulates about 10 times as many mutations per unit of time as nuclear DNA, and has thousands of copies per cell. Thus, amplification of a mitochondrial DNA segment is a relatively sensitive procedure, and identification of the species can be based on mutations in the amplification product. A simple and convenient way of testing for a mutation is RFLP (Restriction Fragment Length Polymorphism) analysis, which uses an enzyme with a recognition sequence created or abolished by the mutation. Species identification using PCR-RFLP of a mitochondrial cytochrome *b* segment has been well documented [15,16, 20] and the technique is equally applicable to the identification of species origin in cheese products (Figure 6) [3] as it is in meat products. Restriction enzymes that generate species-specific fragments are indicated in Table 4.

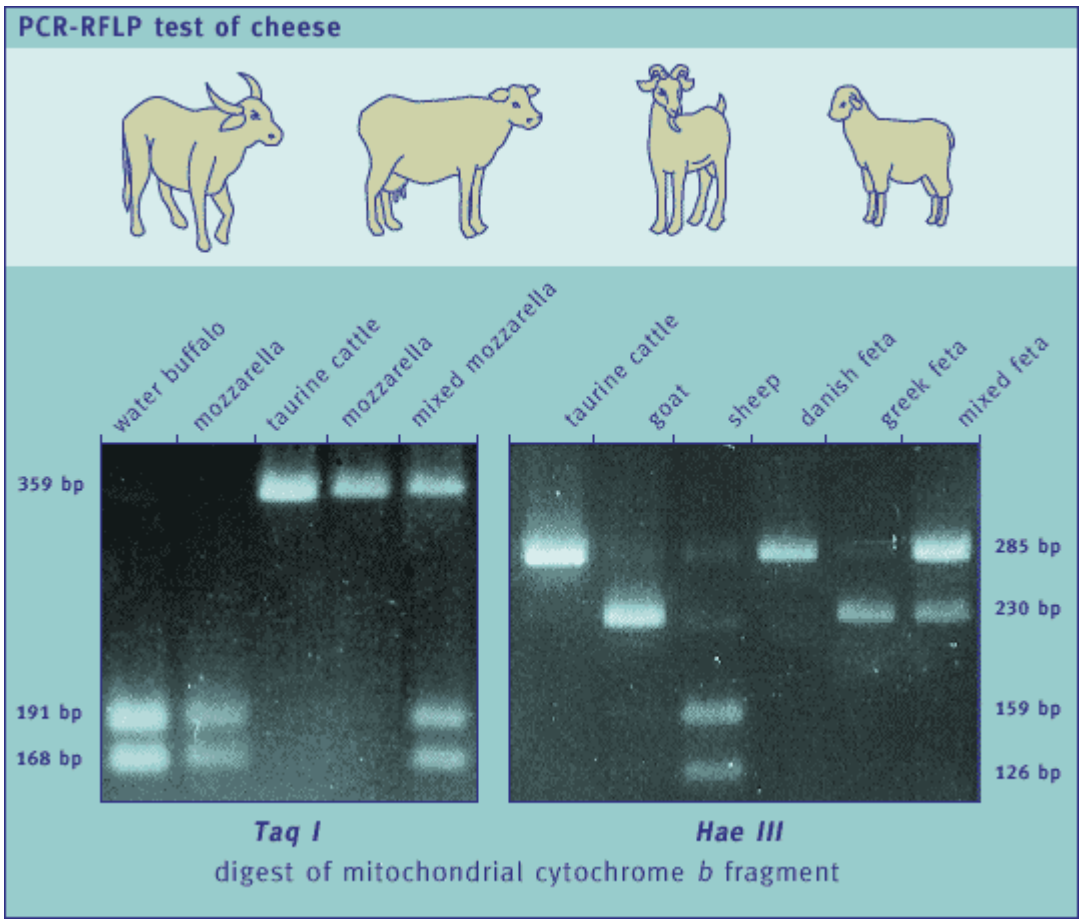


**Figure 6.** Mitochondrial PCR-RFLP analysis of cheese samples [3]. Mitochondrial cytochrome b fragments are amplified with DNA from purified lymphocyte DNA (taurine cattle, water buffalo, goat and sheep) or with DNA isolated from mozzarella and feta samples, as indicated. 'Mixed mozzarella' is a 1:1 mixture of water buffalo and taurine cattle mozzarella samples. 'Mixed feta' is a 1:1 mixture of cattle and sheep feta samples. Additional faint bands in the sheep and goat samples have been noted before [15] and probably result from co-amplification of nuclear copies of mitochondrial gene segments.

**Species-specific restriction enzyme fragments from the 359-bp cytochrome *c* PCR fragment generated by the primers indicated**

| Species | Enzyme | Fragment length (bp) |
|---|---|---|
| Chicken | RsaI | 210, 149 |
| Turkey | RsaI | 149, 109, 101 |
| Pig | AluI | 244, 115 |
| Cattle, water buffalo | AluI | 190, 169 |
| Cattle | HinfI | 198, 117, 44 |
| Water buffalo | TaqI | 191, 169 |
| Sheep, chicken, (pig) | HaeIII | 159, 126, 74 (153, 32, 74) |
| Sheep, water buffalo, pig | MboI | 244, 115 |
| Goat | HaeIII | 239, 74, 55 |
| Goat | MboI | 213, 115, 31 |
| Horse | AluI | 274, 169, 105, 85 |
| Man | MboI | 192, 115, 52 |

**Table 4.**

## Materials

- PCR buffer 10×: 500 mM KCl, 100 mM Tris/HCl, pH 8.3, 15 mM $MgCl_2$, 0.1 % gelatine, 1 % Triton X-100
- dNTP mix: 2 mM dATP, 2 mM dCTP, 2 mM dGTP and 2 mM dTTP
- cyt *b* primers: 5`-ccatccaacatctcagcatgatgaaa-3` and 5`-gcccctcagaatgatatttgtcctca-3`, 50 ng/ml
- Mineral oil for thermocyclers without a heated lid
- Taq polymerase (Amplitaq; Boehringer, ), 5 U/ml
- Restriction enzyme buffer
- Restriction enzymes
- DNA is isolated by following Protocol 4 or other isolation procedures [21], and finally dissolved in 10 ml $H_2O$/60mg meat.

## Method

As described in Protocol 5, the pre-PCR pipetting should be carried out in one room, using special filter tips to prevent contamination of the reagents and the automatic pipette, while the DNA templates should be prepared and added to the PCR mix in a different room. After the cycle steps, amplification products should be handled in a third room. Do not take any pipettes, racks, or other equipment from one room to another.

1. Pipette in the sequence indicated: for n-1 samples, n × 59.5 µl $H_2O$, n × 10 µl PCR reaction buffer 10x, n × 10 µl dNTP mix, 10 × 0.5 µl Amplitaq DNA polymerase, n × 5 µl of one primer, n × 5 µl of the other primer.
2. Mix by vortexing and dispense 90 µl in n-1 PCR tubes.
3. For PCR machines without a heated lid, add 50 µl mineral oil. Keep cool.
4. In another room: add 10 µl DNA samples. Include the appropriate positive (purified DNA) and negative ($H_2O$) controls.
5. Mix, pellet and carry out the following programme: 2 min at 94°C; then 35 cycles of 94°C for 15 sec, 50°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 2 min.
6. To 10-15 ml PCR amplification product, add $H_2O$ to a final volume of 20 µl, 2 µl of the appropriate 10 × enzyme reaction buffer and 5 U of the restriction enzyme that generates a specific pattern for the species under consideration (Table 4).
7. Incubate at 37°C (or at 65°C when using *Taq*I).
8. In the third room, analyse by electrophoresis on a 2 % agarose gel [21].
9. Identify species-specific patterns by comparing with reference DNA and the data from Table 4 [see 15, 16, 20].

## Notes

1. Consult the textbooks about adjusting PCR parameters or designing other primers.
2. Inhibition of the PCR by components of the sample can be checked by analysing the amplification of a mixture of the test sample and purified DNA of the species to be detected.
3. The primers of the cytochrome *b* fragment have been designed on the basis of the human sequence and have mismatches with homologous sequences. As a result, amplification with one species may be more efficient than with another and a composite profile from a sample of mixed origin may not reflect quantitatively its species composition. For example, contaminating human DNA will be amplified relatively efficiently.
4. Diagnostic enzymes for other species may be identified by retrieving the sequence from the nucleotide sequence database and searching for restriction sites by a DNA sequence analysis programme package.

## References


1. Bauer, V.C., Teifel-Greding, J. and Liebhardt, E. (1987) Species identification of heat-denaturized meat samples by DNA analysis. Archiv für Lebensmittelhygiene 38, 149-176.

2. Bartlett SE and Davidson WS (1992) FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. BioTechniques 12, 408-411.

3. Branciari, R., Nijman, I.J., Plas, M.E., Di Antonio, E. and Lenstra, J.A. (2000) Species origin of milk in Italian mozzarella cheese and Greek feta. J. Food Prot. 63, 408-11.

4. Buntjer, J.B., Lenstra, J.A. and Haagsma, N. (1995) Rapid species identification by using satellite DNA probes. Z. Lebensm Unters. Forsch. 201, 577-582.

5. Buntjer, J.A. and Lenstra, J.A. (1998) Mammalian species identification by interspersed repeat PCR fingerprinting. Journal of Industrial Microbiology and Biotechnology 21, 121-127.

6. Buntjer, J.B., Lamine, A., Haagsma, N.and Lenstra, J.A. (1999) Species identification by oligonucleotide hybridisation: the influence of processing of meat products. J Sci. Food Agric. 79, 53-57

7. Chikuni, K., Ozutsumi, K., Koishikawa, T. and Kato, S. (1990) Species identification of cooked meats by DNA hybridization assay. Meat Science 27, 119-128.

8. Colombo, F., Viacava, R. and Giaretti, M. (2000) Differentiation of the species ostrich (Struthio camelus) and emu (Dromaius novaehollandiae) by polymerase chain reaction using an ostrich-specific primer pair. Meat Science 56, 15-17.

9. Ebbehøj, K.F. and Thomsen, P.D. (1991) Species differentiation of heated meat products by DNA hybridization. Meat Science 30, 221-234.

10. Hunt, D., Parkes, H.C. and Davies, I.D. (1997) Identification of the species of origin of raw and cooked meat products using oligonucleotide probes. J. Food Chemistry 60, 437-442.

11. Janssen, F.W., Buntjer, J.B. and Lenstra, J.A. (1998) Species identification in meat by using PCR-generated satellite-DNA probes. J. indust. Microbiol. Biotech. 21, 115-120.

12. Lee, C., Court, D.R., Cho, C., Haslett, J.L. and Lin, C.C. (1997) Higher-order organization of subrepeats and the evolution of cervid satellite I DNA. J. mol. Evolution 44, 327-335.

13. Lenstra, J.A. and Buntjer, J.B. (1999) On the origin of meat. Food Chemistry 64, 1.

14. Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamamda, J. and Shinmura, Y. (1999) A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Science 51, 143-148.

15. Meyer, R., Hofelein, C., Lüthy, J. and Candrian, U. (1995) Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food. J. Assoc. Off. Anal. Chem. Int. 78, 1542-1551.

16. Meyer, R. and Candrian, U. (1996) PCR based analysis for the identification and characterization of food components. Lebensmittel-Wissenschaft und -Technologie 29, 1-9.

17. Montiel-Sosa, J.F., Ruiz-Pesini, E., Montoya, J., Roncales, P., Lopez-Perez, M.J. and Perez-Martos, A. (2000) Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitochondrial DNA. J. Agric. Food Chem. 48, 2829-2832.

18. Murray, B.W., McClymonts, R.A. and Strobeck, C. (1995) Forensic identification of ungulate species using restriction digestion of PCR-amplified DNA. J. For. Sci. 40, 943-951.

19. Palumbi, S.R. and Cipriano, F. (1998) Species-identification using genetic tools: the value of nuclear and mitochondrial gene sequences in whale conservation. J. Hered. 89, 459-464.

20. Partis, L., Croan, D., Guo, Z., Clark, R., Coldham, T. and Murby, J. (2000) Evaluation of a DNA fingerprinting method for determining the species origin of meats. Meat Science 54, 369-376.

21. Sambrook J, Fritsch EF and Maniatis, T (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor. ISBN 0-87969-309-6

22. Tartaglia, M., Saulle, E., Pestalozza, S., Morelli, L., Antonucci, G. and Battaglia, P.A. (1998) Detection of bovine mitochondrial DNA in ruminant feeds: a molecular approach to test for the presence of bovine-derived material. J. Food Protect. 61, 513-518.

23. Unseld, M., Beyerman, B., Brandt, P. and Hiesel, R. (1995) Identification of the species origin in highly processed meat products by mitochondrial DNA sequences. PCR Methods and Applications 5, 241-243.

24. Watson, J.D. and Crick, F.H.C. (1953) Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. Nature 171, 737.

25. Winterø, A.K., Thomsen, P.D. and Davies, W. (1990) A comparison of DNA-hybridization, immunodiffusion, countercurrent immunoelectrophoresis and isoelectric focusing for detecting the admixture of pork to beef. Meat Science 27, 75-85.