DEVELOPMENT OF METHODS FOR THE IDENTIFICATION OF DUCK, PHEASANT, VENISON, HORSE, DONKEY AND WILD BOAR IN MEAT PRODUCTS

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Development of methods for the identification of duck, pheasant, venison, horse, donkey and wild boar in meat products

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(TaqMan™) PCR

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ABBREVIATIONS

Ct	Threshold cycle
CTAB	Cetytrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphates
EDTA	Ethylenediaminetetraacetic acid
mCyt b	Mitochondrial cytochrome b gene
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
w/w	Weight for weight

EXECUTIVE SUMMARY

The objective of this study was to design and optimise real-time PCR assays for the specific detection of horse, donkey, duck, deer, pheasant and wild boar in commercial products. The assays were to be easy to use and applicable for use by public analysts and other laboratories. Although the assays were primarily for use with real-time PCR equipment, they were also to be available for use by laboratories which did not have real-time PCR capability, but which did have experience of gel electrophoresis. Finally, the outputs of the project were to include a comprehensive set of standard operating procedures to facilitate technology transfer to other laboratories.

The objectives of the study were broadly met, with assays specific for the detection of all target species successfully designed and optimised, with the exception of wild boar. After extensive sequence analysis, and utilising the results of population genetics work at CSL (a MAFF funded project, Goulding and Smith 1998), it was concluded that the identification of wild boar in commercial products was not achievable using this real-time PCR based approach and that a microsatellite based approach might be more applicable.

The real-time PCR based approach was found to be applicable to the detection of duck, deer, horse, donkey and pheasant. Sensitive and specific assays were designed and optimised for each species. These were shown to be capable of detecting their target species in both heat and pressure processed commercial products. The design of the assays made them fully portable between real-time PCR and gel electrophoresis equipment. Finally technology transfer has been facilitated by the early submission of two manuscripts to peer reviewed journals detailing the design and optimisation of the horse, donkey and duck assays, both of which have been accepted for publication. Additionally, a comprehensive set of standard operating procedures has been provided for use by Public Analysts and other laboratories.

The final part of this project sought to evaluate an emerging methodology for species identification which utilises chip technology. The Food Expert ID system, developed by bioMerieux, was loaned to CSL for a period of 6 months and its operation and applicability to the identification of species in food products evaluated. The range of species included on the chip allowed samples from 3 FSA funded projects to be assayed: white fish mixtures from the CCFRA fish project, meat mixtures from the

DNA quality project (Q01033 & Q01034) and exotic meats from the Exotic meat project (Q01083). Additionally the chip was assessed using tuna standards, originally from the FSA tuna survey, conducted in 1999. The system was able to detect both single species and species in mixtures, even after heat and pressure processing. Cod was detected in a mixture of cod and haddock, and pork, beef and chicken were also detected, all below the 5% level given as the sensitivity of the assay. A range of tuna species were correctly identified from raw muscle, however canned tuna could not be detected, even at 100%. The system was easy to use however unexplained variability was encountered when visualising the chip intensities and the system did not always give the expected results. When compared to the routine set up RT-PCR systems the current chip methodology as currently presented was found to be lengthy, expensive and inflexible. Therefore at present RT-PCR assays would still be favoured for the routine testing of samples for common species.

INTRODUCTION

Food labelling regulations require the identity of meat in meat products to be accurately labelled. This has resulted in a need for tests which will reliably identify the species of meat present in a food sample and which must also be sensitive and robust enough to be applied to complex food matrices. A range of analytical approaches have been taken to meet these demands, broadly based on detecting either protein or DNA. Of the protein based methods, immunoassay is the most widely used with several companies supplying kits for a range of species. However, proteins are denatured during heat and pressure processing, making the detection of species present in a processed sample more difficult. DNA has the advantage of being a relatively stable molecule, and is more able to withstand heat processing.

DNA methods have commonly been based around the use of species-specific primers in PCR followed by signal detection using gel electrophoresis (Meyer, Hofelein, Luthy, & Candrian, 1995; Bottero *et al*., 2003; Rodriguez *et al*., 2003). More recently published reports have focused on the use of specific primers in real-time PCR using TaqMan™ technology (Brodmann & Moor, 2003; Dooley, *et al*., 2004; Mendoza-Romero *et al*., 2004; Laube *et al*., 2003; Hird *et al.,* 2004). This technique utilises fluorescently labelled probes which allow signal generation to be measured in real time, thus eliminating the need for electrophoresis, end point determination and consequently the subjective analysis of the results.

The development of tests which will reliably identify the species of meat present in food has historically focused on species of high economic importance, including pork, beef and chicken. Many different tests now exist for each of these species (Dooley *et al*., 2004, Lahiff *et al*., 2001, Laube *et al*., 2003), whereas the range of assays for the detection for less commonly used meat species, for example horse and donkey, is relatively limited. This project focused on the development of assays for the identification of more exotic meat types including horse *(Equus caballus)*, donkey *(Equus asinus)*, wild boar *(Sus scrofa scrofa)*, pheasant *(Phasianus colchicus)*, deer (*Cervus elaphus, C. dama, C. nippon, Capreolus capreolus and Muntiacus muntjak*) and duck *(Anas platyrhynchos* and *Cairina moschate)*.

There are no assays currently available which can identify horse, donkey, wild boar or pheasant in commercial products and the assays available for duck and deer are focused upon particular varieties that could not identify all commercially important varieties of these species. For example Rodriguez *et al* (2003a & b) used conventional PCR for the detection of Mule duck, a cross between Mallard and Muscovy duck (*Anas platyrhynchos x Cairina moschate*), using separate primer sets for two genes: 12S ribosomal RNA and α-actin genes. Rodriguez *et al* (2004) went onto develop a real-time PCR assay for the detection of Mule duck. However this assay was based on Mallard DNA sequence and would not detect wild type Muscovy duck, a duck species now commonly used for human consumption in the UK.

This report outlines the development and optimisation of real-time PCR assays for the identification of horse, donkey, pheasant, duck and deer in commercial products. These assays have been designed to be fully portable between real-time PCR and gel electrophoresis equipment. Additionally we report on the sequence analysis performed on wild boar samples outlining the genetic relatedness of wild boar to pig. The report includes a comprehensive set of standard operating procedures for use by Public Analysts and other laboratories and technology transfer has been facilitated by the early submission of two manuscripts to peer reviewed journals detailing the design and optimisation of the horse, donkey and duck assays.

In addition this report includes an assessment of a recently developed chip based system developed by bioMerieux. The bioMerieux FoodExpert-ID system is designed for the qualitative detection and identification of vertebrate species or groups of species present in human or animal food. The system utilises a reverse dot technique on a DNA microarray to allow the identification of over 30 species of fish birds and mammals. The system was assessed using a variety of samples from previously funded FSA projects: white fish mixtures from the CCFRA fish project,

meat mixtures from the DNA quality project (Q01033 & Q01034) and exotic meats from the Exotic meat project (Q01083). Additionally the chip was assessed using tuna standards, originally from the FSA tuna survey, conducted in 1999.

RESULTS AND DISCUSSION

The work involved in the design, optimisation and application of the primer and probe sets for horse, donkey and duck species has been written up into two manuscripts which have been accepted for publication in peer reviewed journals. These manuscripts have been included as Appendices 2 and 3. Project work which lead up to these manuscripts or which did not form part of these publications has been outlined below.

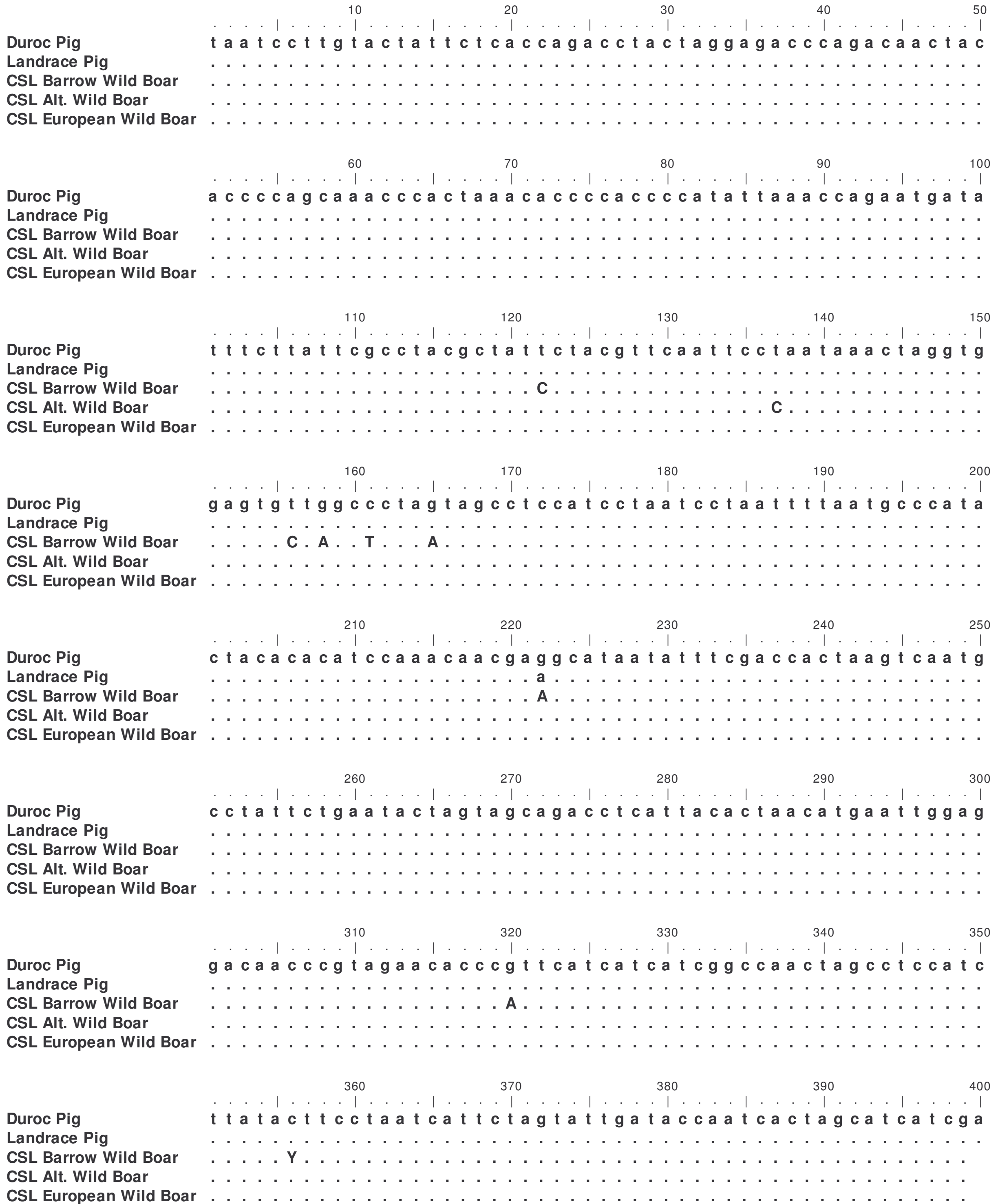
Sequence confirmation

The first phase of the project involved the verification of the sequence for commercially important species of pheasant, wild boar, horse, donkey, duck and deer. This project was to use the mitochondrial cytochrome b gene (mCyt b): a gene with significant heterogeneity between species allowing the design of species specific assays. In a previously funded FSA project (ANO695) it was noted that mCyt b gene sequence in the NCBI database was not always for varieties of animals commonly available in the UK (Hird *et al*., 2003). It was therefore important to sequence the mCyt b gene to obtain sequence relevant to the UK and Europe. Fragments of the mCyt b gene were amplified using universal primers and these fragments sequenced using both forward and reverse primers. The consensus sequence was then determined using the sequencing results in comparison to the sequence data available in the NCBI database.

The sequence obtained for pheasant, deer species, duck species, horse and donkey were found to be similar to the sequences available in the NCBI database, with only a few base changes. These sequence data were therefore suitable for use in designing real-time PCR primers and probe sets.

The sequencing data derived for the wild boar samples collected for this project, showed a high degree of homology between domestic pig and wild boar. An alignment of the sequence data for the wild boar samples and pig is shown in Figure 1, which highlights these sequence similarities. These data were not suitable for

Figure 1 Alignment of pig and wild boar sequence



designing species specific real-time primer and probe sets since there were no regions of heterogeneity between wild boar and pig which were conserved across all the wild boar samples. This is due to the very close genetic relationship between wild boar and pigs, making them indistinguishable using mitochondrial cytochrome b sequence data.

Microsatellite analysis of wild boar samples.

The Central Science Laboratory had a MAFF commissioned project (Goulding and Smith 1998) to perform genetic analysis on wild boar populations. This work identified and used microsatellite analysis to determine the relatedness of wild boar populations in Europe. The microsatellites were used to build a genetic fingerprint of the wild boar samples obtained for this project, which were then compared with the results from the MAFF project. It was found that none of the microsatellites were able to completely distinguish wild boar from pig, and that the fingerprints for the wild boar were not consistent over the range of wild boar studied. For example, for microsatellite 1ys, alleles of 119 and 123 were only found in wild boar, while alleles of 115, 121 and 127 were only found in pigs, however the alleles only found in wild boar were not found in all wild boar samples and similarly the alleles only found in pigs were not found in all pigs (Table 1).

The population genetics of wild boar are complex and negated the use of a single real-time PCR primer and probe set for the definitive identification of wild boar in a food product, also likely to contain pig. .

Table 1. Allele sizes for microsatellite markers 1ys and 1bs for wild boar and pig

	Allele 1ys		Allele 1bs	
	Allele sizes	Notes	Allele sizes	Notes
Wild Boar	113, 119, 123	123 in 2 of 6 boar	93, 103, 107, 109, 113	93 in 4 of 6 boar and 107 in 2 of 6 boar
Pig	113, 115, 121, 127	115 and 127 in 2 of 4 pigs and 121 in 1 of 4 pigs	97, 103, 109, 111	97 in 2 of 4 pigs and 111 in 2 of 4 pigs

Pheasant

Primer and probe design

Sequence analysis data was aligned with sequence data from other commercially popular species and areas of mismatch identified (Figure 2). Using Primer Express® software, a primer and probe set was designed to have mismatches to all other species at the 3' position of both sense and antisense primers (Hird *et al*., 2003).

Figure 2 Position of pheasant primer set showing 3' mismatch with other commercially important species

sense primer

```
Pheasant     t a t c g g a c g c g g c c t c t a t t a a
Partridge    c . . . . . . . . . . . . . . a . . c . . .
Grouse       c . . . . . . . . t . . a . . . . . c . . .
Quail        c . . . . . . . . a . . . . . g . . c . . .
Chicken      c . . . . . . . . a . . . . . a . . c . . .
Sheep        . g . a . . . . . a . . . . . a . . c . . .
Muscovy      c . . t . . t . . a . . . t . . . . c . . .
Donkey       c g . a . . g . . . . . . . . . . . c . . .
Horse        c g . a . . . . . . . . . . . . . . c . . .
Pig          c g . a . . c . . a . . . . . a . . c . . .
```

antisense primer

```
Pheasant     t c g t c c t a c t c c t c a c a c t c a t a
Grouse       . a a . . . . c t . . . . t g . . . . . . . .
Red grouse   . a a . t . . g . . . . . . . . . . . a . . .
Chicken      . a a . . t . g . . t . . . . . . . . . . . .
Quail        . a a . . . . g . . . . . . . . . . . a . . .
chicken      . a a . . . . c . . . . . . . . . . . . . . .
Sheep        . a a . . . . c . . a t . t g . g a c a . . .
Donkey       . t a . . . . . . . t t . . . . . g . a . . .
Cow          . a a . . . . t . . g . . . . . . g . a . . .
Mallard      . a . . . . . c . . a . . . g . c . . . . . g
Horse        . . a . . . . . . . t t . . . . . g . t . . .
Pig          . a . . . . . . . . a t . t . . c g . t . . .
```

Primer Truncation & Specificity

Following initial screening of the pheasant primer and probe set using the DNA from a range of species as template, it became apparent that the only non-target species that they cross amplified was donkey despite the 3' mismatches. This was unexpected since no cross amplification had occurred with species which had a higher degree of sequence homology to pheasant than the donkey.

We used the strategy outlined in Hird *et al.* (2004) to increase the specificity of the primers by truncation at the 5' position, a strategy which reduces the melting temperature of the primer, thereby reducing the temperature differential between primer melting temperature and the TaqMan™ cycling conditions. Following significant levels of truncation of sense and antisense primers, specific primer combinations were identified (Table 2).

Table 2 Effect of truncation of pheasant primers on assay specificity

Primer truncation Sense	Primer truncation Antisense	Pheasant DNA Ct	Donkey DNA Ct
0	0	15.65	36.40
-2	0	15.34	34.77
-3	0	15.33	36.17
-4	0	15.23	35.87
-2	-1	15.5	*36.39*
-2	-2	15.28	*37.22*
-2	-3	15.39	*36.22*
-2	-4	15.36	N

Negative figures indicate the number of bases removed form the '5 end of the original primer. 0 indicates full length primer, N indicates no amplification signal could be detected after 40 cycles. Figures are averages of triplicate well readings, those in italics include failures (assigned Ct 40) within the triplicate.

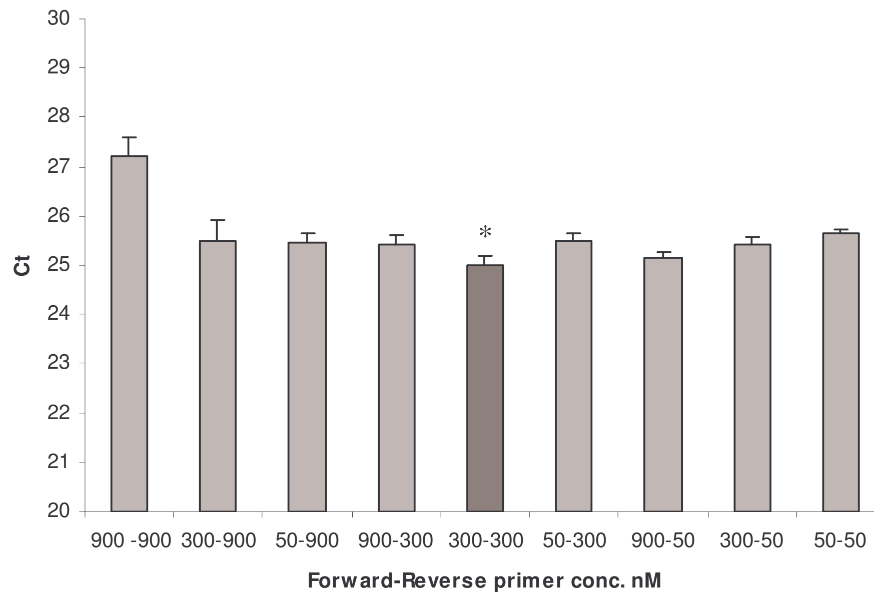
No cross-species amplification was observed using template DNA isolated from donkey, pig, deer, pheasant, duck, rabbit, guinea fowl, grouse, goose, pigeon, quail, cow, partridge, sheep, turkey, chicken, and horse, for the truncated pheasant primer and probe set (Table 3) thus demonstrating specificity. Following optimisation of primer concentrations, forward and reverse primers at 300nM (Figure 3) were used for subsequent assays.

Table 3 Pheasant assay specificity

Species	Ct
Pheasant	15.95
Chicken	N
Cow	N
Deer group	N
Donkey	N
Goose	N
Grouse	N
Guinea fowl	N
Horse	N
Mallard duck	N
Muscovy duck	N
Partridge	N
Pig	N
Pigeon	N
Quail	N
Rabbit	N
Sheep	N

N indicates no amplification signal could be detected after 40 cycles.

Figure 3. Effect of varying pheasant sense and antisense primer concentrations on Ct value using pheasant DNA as template



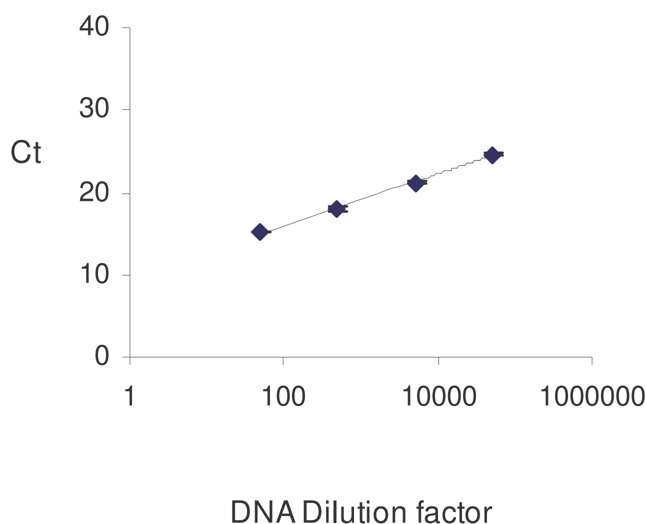
* denotes concentration of primers used in pheasant assay

Limit of detection of pheasant assay.

The limit of detection for the pheasant primer and probe set was determined using ten fold dilutions of pheasant DNA diluted in water. The limit of detection for the pheasant primer and probe set extended to a 1:50,000 dilution (average Ct 24.60)

(Figure 4). The dilutions relate to the detection of 0.002% (w/w) pheasant meat in a sample. The assay was therefore highly sensitive. Although these limits of detection were calculated from dilutions of DNA in water, detection at these orders of magnitude means that the assays could be used as a basis for enforcing accurate product labelling and provide reassurance to consumers concerned about even the smallest levels of contamination of pheasant meat in products.

Figure 4. Limit of detection of pheasant assay



Application of pheasant assay to model and commercial samples

The optimised pheasant assay was used to test DNA extracted from test meat samples containing high levels (48% w/w) of either fat (beef lard), carbohydrate (soya flour), or protein (pork) spiked with 4, 2 and 1% (w/w) of pheasant muscle and prepared in a meat muscle background. The primer set successfully detected the spikes at all three concentrations in all three matrices, returning comparatively low Ct values (<23) as shown in Figure 5. The higher Ct values returned from the high carbohydrate samples are probably caused by enzyme activity within the soya flour matrix causing degradation of the target DNA. Finally, DNA was extracted from commercial products and assayed using the pheasant specific primer and probe set, which successfully detected the presence of pheasant DNA in all samples (Table 4). No gross matrix effects were found in the successful testing of the matrix samples and commercial products, indicating the applicability of the assays to detection in complex food matrices.

Figure 5. Effect of carbohydrate, fat and protein on detection of pheasant in model samples by pheasant assay

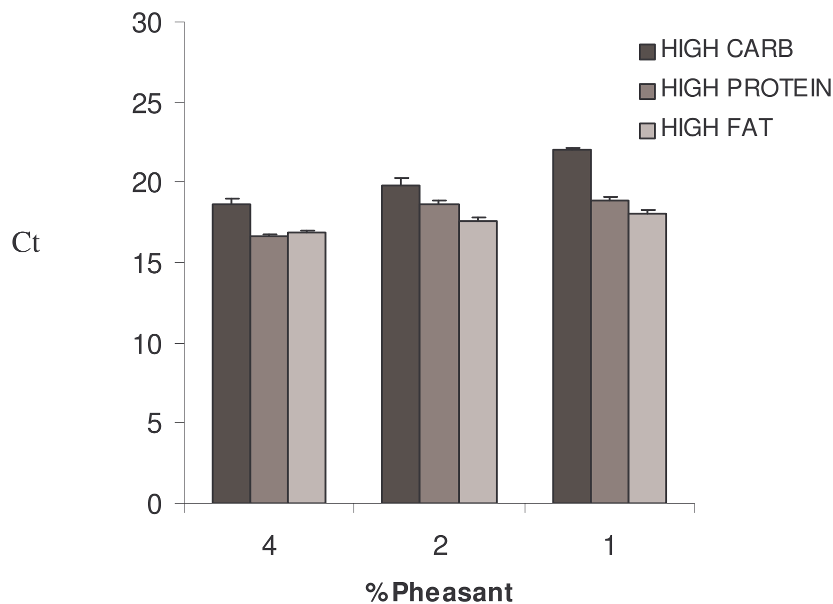


Table 4 Real-time PCR analysis of commercial products using the pheasant assay.

Product	Declared meat species or animal content	Average Ct value ± SD
Pheasant pate	24% pork, 24% pork liver, 19%pheasant, 8% turkey liver, 6% dried apricots, 3% pork fat, 3% port	17.40 ± 0.16
Game casserole	Raw pheasant, partridge, Mallard duck, venison, pigeon and rabbit	11.22 ± 0.13
Friskies game, turkey and vegetable cat food (pouch)	Min 4% game, 4% turkey	Not detected
Whiskers game cat food (pouch)	Meat and animal derivatives including min 4% game	Not detected
Whiskers poultry cat food (pouch)	Meat and animal derivatives including min 4% poultry	Not detected

Deer

Sequence analysis.

There are five commercially important species of deer in the UK: red, roe, fallow, muntjac and sika. Although these deer all belong to the same family they are relatively distantly related and analysis of the sequence data from each species illustrated the divergence of these species (Figure 6).

Fig 6 Mitochondrial cytochrome b gene sequence alignment for sika, muntjac, roe, red and fallow deer.

```
                10        20        30        40        50
Fallow  gtagacaaagcaaccttaactcgattcttcgctttccactttattctacc
Red     .....t.........c....c.....t....................c..
Sika    .....t.........c....c.....t.....c..............t..
Roe     ...............c.g........t.................c..c..
Muntjac .....t.....g...c.g..c...........c.....t........c..

                60        70        80        90       100
Fallow  attcatcattgcggcacttgctatagtacatttactctttcttcacgaga
Red     ...t.....c..a.....c...........c........c..........
Sika    ...t.....ca.a.....c...........c........c..........
Roe     ...t........a..............c........t..c..c.....a.
Muntjac ...t..t..c..a.....c........c..cc.g........c.....a.

               110       120       130       140       150
Fallow  caggatccaataacccaacaggaatcccatcagatgtagataaaattccc
Red     .........................t........c.c...c....c...
Sika    ..........c...................g..c.c...c....c...
Roe     .......a..c.....g.t.............a.c.cg...c.......a
Muntjac ..........c.........................c......g......

               160       170       180       190       200
Fallow  tttcatccctactacaccattaaagatattttaggcatcctattcctatt
Red     ..c.....t.....t..............c.........t..c.t...g.
Sika    ..c.....t.....t..............cc........t..c.t...g.
Roe     .....c........t..............cc....ag.t.........a.
Muntjac ..c..c..............c.........c....tg.....c.t...a.

               210       220       230       240       250
Fallow  tctcttcttaataacactagtactatttgcaccagacttgcttggagacc
Red     a......c.....tt.......t.....c.........c............
Sika    a......c.g...tt.......t.....c.........c..........t.
Roe     ...t.c.c.....tt......c......c.........c............
Muntjac .......c.....t........t.....ct.......c............

               260       270       280       290       300
Fallow  cagacaaatacactccagcaaatccactcaacacacctcctcatattaaa
Red     .......c..t..c.......................c.....c......
Sika    .......c.....c...........g...........c.....c..c...
Roe     ....t..c.....a..............t........c.....c......
Muntjac .c.....c.....c........c........t........c........g

               310       320       330       340       350
Fallow  cccgaatgatacttcctatttgcatacgcaatcctacgatcaattcccaa
Red     ..t........t.............................g........
Sika    ..t........t......................................
Roe     ..a............t.........................t.....t..
Muntjac ...........t....................t..............t..

               360       370       380       390       400
Fallow  taaattaggaggggtgttagccctagtctcatccatcctgatcctaattc
Red     c...c.......a..c.................t.....a...t.g....
Sika    c...c.......a..c.................t.....a.....g..c.
Roe     c...c.......a..ac........a.......a.....a...t.g..c.
Muntjac ....c.......a..cc........a.t...........a........c.
```

Primer and probe design

Sequence data derived from the sequencing analysis of this project was aligned with sequence data from other commercially popular species and areas of mismatch identified. Using Primer Express® software, a generic deer primer and probe set was designed to have mismatches to all other species at the 3' position of both sense and antisense primers (Hird *et al*., 2003). When DNA extracted from the five types of deer was used as template for this generic deer primer and probe set it was found that the rates of amplification efficiency were high for fallow and roe deer template DNA indicated by low Ct values, but low for red, muntjac and sika deer template DNA, indicated by high Ct values (Table 5).

Table 5 Effect on Ct of deer primer combinations

Primers Sense	Primers Antisense	Fallow DNA Ct	Roe DNA Ct	Sika DNA Ct	Red DNA Ct	Muntjac DNA Ct
Generic deer	Generic deer	14.18	15.14	24.02	25.94	28.52
Sika / Red	Sika / Red			15.01	32.12	
Red	Red				17.1	
Muntjac	Muntjac					20.51

The reaction efficiency reflected the degree of homology between the generic deer primers and the species sequence and therefore the primers for sika, red and muntjac assays were redesigned. The sika and red deer forward primer was substituted with a primer which was located at the same position as the generic deer primers and had a single base pair mismatch for the sika and red deer sequence, similarly muntjac deer forward and reverse primers were substituted with primers which matched the muntjac deer sequence at the same location as the original generic deer primers. When DNA from sika, muntjac and red deer were used as template for these alternative primer and probe sets it was found that the amplification efficiencies of the sika and muntjac primers had improved significantly, however the red deer reaction efficiencies remained low. It was therefore decided to design alternative sets of primers for red deer, at a new locations, but which would still utilise the same probe. In all 3 new primer sets were designed for the red deer and when used in forward and reverse combinations, were found to have improved amplification efficiency. Some

of combinations were shown to be red deer specific (Table 6). The improved specificity was most probably due to the increased number of 3' mismatches which had been incorporated into the primer design. The primer combination which gave the most efficient amplification, and which was red deer specific, was used for the remainder of the study.

Table 6 Effect of truncation of deer primers on assay specificity

Deer Species	Primers Sense	Antisense	Deer DNA Ct	Cow DNA Ct	Sheep DNA Ct
Fallow	Generic deer	Generic deer	14.18	33.27	30.26
	Generic deer -4	Generic deer	16.61	36.23	32.68
	Generic deer -5	Generic deer	17.67	N	N
Sika	Sika/Red	Generic deer	15.01	N	N
Red	Red 1	Red 1	17.1	N	37.63
	Red 2	Red 1	17.32	N	N
	Red 2	Red 2	17.30	N	N
	Red 2	Red 3	19.06	N	N
	Red 3	Red 1	20.97	N	N
	Red 3	Red 2	21.38	N	N
	Red 3	Red 3	22.46	N	N
Muntjac	Muntjac	Muntjac	20.51	30.73	37.04
	Muntjac –1	Muntjac	20.69	32.98	N
	Muntjac –2	Muntjac	20.47	35.04	N
	Muntjac –3	Muntjac	20.07	37.61	N
	Muntjac –4	Muntjac	24.7	N	N

Negative figures indicate the number of bases removed form the '5 end of the original primer. 0 indicates full length primer, N indicates no amplification signal could be detected after 40 cycles. Figures are averages of triplicate well readings, those in red include failures (assigned Ct 40) within the triplicate.

Primer Truncation & Specificity

Following initial screening of the generic deer, sika, and muntjac primer combinations using the DNA from a range of species as template, it became apparent that cow and sheep DNA was cross amplified despite the 3' mismatches.

We used the strategy outlined in Hird *et al.* (2004) to increase the specificity of the primers by truncation at the 5' position, a strategy which reduces the melting temperature of the primer, thereby reducing the temperature differential between

primer melting temperature and the TaqMan^{TM} cycling conditions. Following significant levels of truncation of sense primers, specific primer combinations were identified (Table 6). No cross-species amplification was observed using template DNA isolated from donkey, pig, pheasant, duck, rabbit, guinea fowl, grouse, goose, pigeon, quail, cow, partridge, sheep, turkey, chicken, and horse (Table 7) thus demonstrating specificity.

Table 7 Amplification of DNA isolated from commercially important species by deer group assay

Species	DNA Ct
Red deer	18.8
Roe	16.0
Muntjac	17.9
Fallow	15.6
Sika	16.4
Chicken	N
Cow	N
Donkey	N
Horse	N
Mallard duck	N
Muscovy duck	N
Pheasant	N
Pig	N
Rabbit	N
Sheep	N
Turkey	N

N indicates no amplification signal could be detected after 40 cycles.

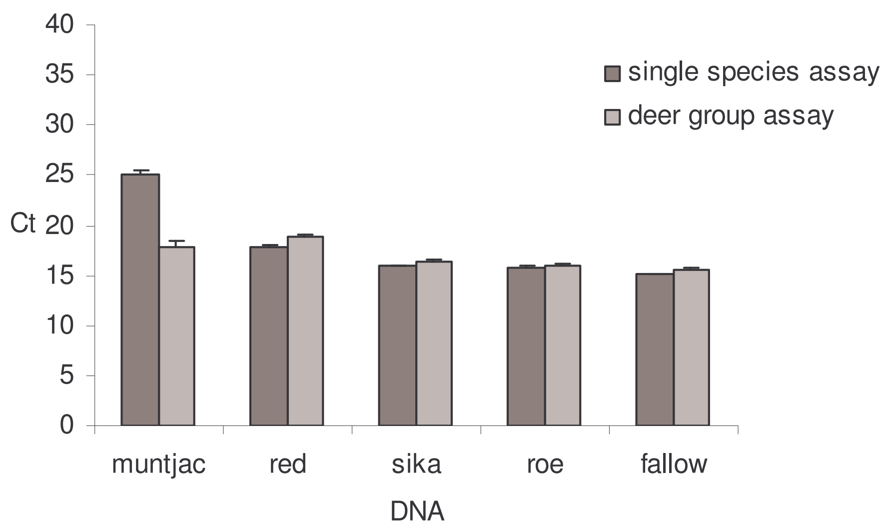
Following optimisation of primer concentrations, forward and reverse primers were used at 300nM for sika, red, roe and fallow and at 900nM for muntjac (Figure 7). A deer group assay was developed by mixing the forward and reverse primers for a deer species with the common probe.

This proved to be specific when checked for cross amplification using DNA extracted from donkey, horse, rabbit, duck, pheasant, cow, pig, sheep, chicken and turkey template DNA. Comparison of the Ct values returned by the single assays with the deer group assay on the 5 deer types also indicates cross amplification. The Ct values returned by the deer group assay are lower than those returned by the muntjac assay, indicating that amplification of the deer group assay is more efficient assay (Figure 8).

Figure 7. Effect of varying sense and antisense primer concentrations on Ct value using deer DNA as template

* denotes concentration of primers used in deer group assay (nM)

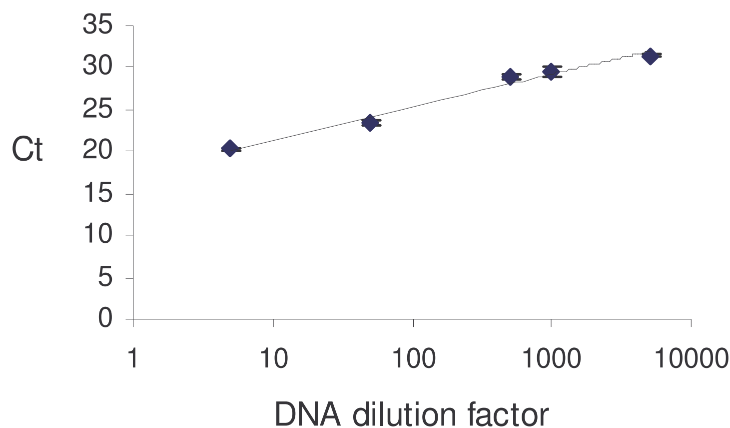
Figure 8. Effect of multiplexing on sensitivity of deer group real-time PCR assay.



Dynamic range and limit of detection of deer group assay.

Dynamic range of the deer group assay was determined using ten fold dilutions of red deer DNA diluted in water. The red deer primer and probe set was the least sensitive primer and probe set in the deer group assay and therefore the dynamic range determined for this primer and probe set would represent the smallest dynamic range for the combined primer and probe sets. The dynamic range extended to a 5000 fold dilution, at a Ct value of 31.4 (Figure 9) which relates to the detection of 0.02% (w/w) red deer meat in a sample. The cross amplification of the deer group assay with sheep DNA returned Ct values of 35.7. Work underway in another FSA funded project (Q01084) has produced guidelines for the application of real-time PCR based species specific assays. These guidelines include the positive and negative controls to be used for each assay and have been incorporated into the SOP outputs for this project (Appendix 4.1). These guidelines give a method for determining the limit of detection for each assay. When these guidelines are applied to the deer group assay the limit of detection falls at Ct values of 30.2, equating to the detection of 0.1% red deer in a sample and the Ct values returned by sheep template DNA fall below the limit of detection.

Figure 9. Dynamic range of deer group assay (Red deer shown)



Application of deer group assay to model and commercial samples

The optimised deer group assay was used to test DNA extracted from test meat samples containing high levels (48% w/w) of either fat (beef lard), carbohydrate (soya flour), or protein (pork) spiked with 4, 2 and 1% (w/w) of deer muscle from each species and prepared in a meat muscle background.

The deer group assay successfully detected the spikes all three matrices, as shown in Figure 10 (1% shown only). Unlike for the pheasant assay however, the carbohydrate matrix appeared to have no effect on the target DNA. Finally, DNA was extracted from commercial products and assayed using the deer group assay, which successfully detected the presence of deer DNA in all samples (Table 8). No gross matrix effects were found in the successful testing of the matrix samples and commercial products, indicating the applicability of the assays to detection in complex food matrices.

Figure 10. Effect of carbohydrate, fat and protein on detection of deer in model samples by deer group assay (1% of each deer type)

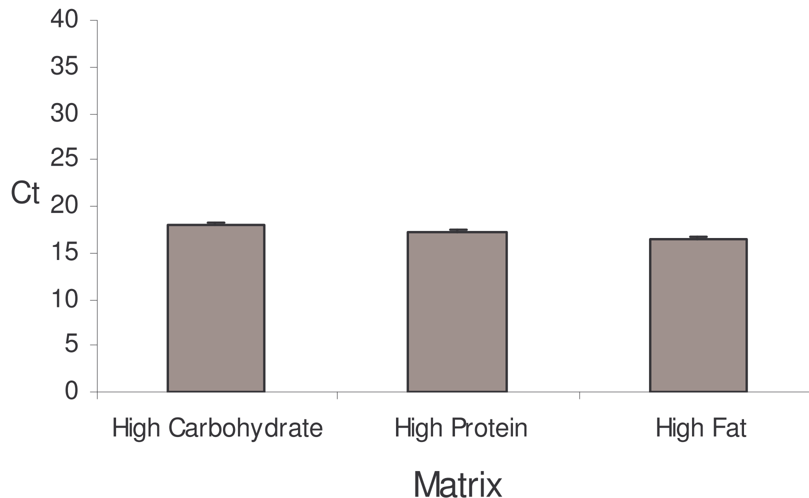


Table 8 Real-time PCR analysis of commercial products using the deer group assay.

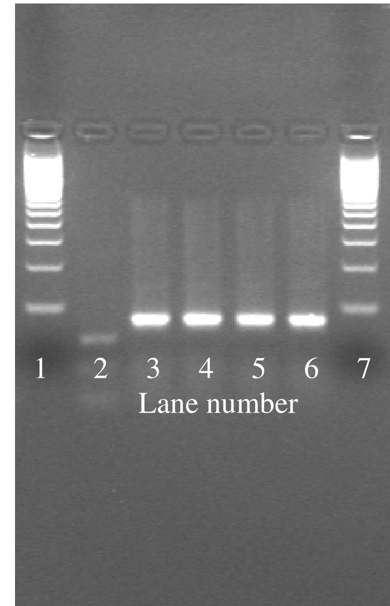
Product	Declared meat species or animal content	Average Ct value ± SD
Smoked Venison slices	97% venison	23.26 ± 0.28
Venison pate	32% pork, 24% pork liver, 24% venison, pork fat	16.28 ± 0.13
Game soup	Not stated	30.27 ± 0.45
Game casserole	Raw pheasant, partridge, Mallard duck, venison, pigeon and rabbit	23.81 ± 0.28
Whiskers game cat food (pouch)	Meat and animal derivatives including min 4% game	37.93 ± 1.48

Gel electrophoresis

The assays were designed for use with both real-time PCR and gel electrophoresis equipment. Real-time PCR involves the use of very small amplicons, which facilitate the production of efficient PCR reactions, however very small amplicons can be difficult to visualise using gel electrophoresis. The amplicon sizes for the species specific assays designed in this assay were 83, 84, 141, 144 and 198 base pairs for horse, pheasant, duck, donkey and deer respectively. Amplicons above 120 base pairs are easily visualised using a 2% (w/v) agarose gel, however amplicons below 120 base pairs are not routinely visualised in this manner. We amplified pheasant DNA using the pheasant primer set with the real-time PCR cycling conditions and then visualised the resulting amplicons using a 2% (w/v) agarose gel (Plate 1). The amplicons were visible as distinct bands and were easily distinguishable from the primer flare. We therefore concluded that these assays would be applicable for use by laboratories which did not have real-time PCR capability.

Plate 1 Amplicons from pheasant specific PCR using pheasant DNA as template specific assay

Lane number :
1 100bp marker
2 water control
3 pheasant DNA
4 pheasant DNA
5 pheasant DNA
6 pheasant DNA
7 100bp marker



ASSESSMENT OF BIOMERIEUX FOOD EXPERT ID SYSTEM

The bioMerieux FoodExpert ID is a detection system that involves 5 main steps, and the reagents for most of these steps are provided in kit form. Firstly DNA is extracted and purified from samples and then a portion of the mitochondrial cytochrome b gene is amplified using universal PCR primers supplied in the kit. The PCR products are labelled by the incorporation of a fluorescent nucleotide during transcription using an Ambion transcription kit, then the transcripts are cleaved and hybridised on DNA chip micro-arrays using the fluidics station. The microarray is scanned and analysed by the FoodExpert ID software to give a qualitative result of species and class presence or absence.

The system was assessed using a variety of samples from previously funded FSA projects: white fish mixtures from the CCFRA fish project, meat mixtures from the DNA quality project (Q01033 & Q01034) and exotic meat from the Exotic meat project (Q01083). Additionally the chip was assessed using tuna standards, originally from the FSA tuna survey, conducted in 1999.

Fish mixtures

To test levels of detection and allow comparison with the lab-on-a-chip RFLP system (Dooley et al., 2005), freeze dried fish mixtures of cod (10% 5% and 2%) in haddock and 100% cod were analysed. Haddock was a species which was not included on the chip and therefore the detection was of cod only. Initially cod was only detected in the 2% sample, but following repeat analysis cod was identified in the 100%, 10% and 5% samples (Table 9). The reasons for the failure of identification for the 100, 10 and 5% cod samples could not be identified, since the system had been shown to work in that batch of analysis for the 2% sample. To test the system further, a small number of frozen commercial products containing cod (fish pies) and tuna pate spiked with frozen cod (1:1) were analysed and resulted in the detection of cod. There was no detection of tuna species in the tuna paste sample, most probably due to the severe degree of processing used to make fish pastes. The effect of processing on the detection of species using the Food Expert-Id system will be discussed further in the following sections of this report.

Table 9 Detection of Cod in mixed matrices using the Food Expert Id detection system

Sample	Internal Control	Identified Species	Identified Class	Identification
CCFRA 100% Cod	-	-	-	×
CCFRA 10% Cod	-	-	-	×
CCFRA 5% Cod	+	-	Fish	×
CCFRA 2% Cod	-	Cod	-	✓
CCFRA 5% Cod Repeat	+	Cod	-	✓
CCFRA 10% Cod Repeat	-	Cod	Fish	✓
CCFRA 100% Cod Repeat	-	Cod	-	✓
PCR blank	+	-	-	✓
50% Tuna Paste & 50% cod	+	Cod	Fish	✓
Fisherman's Pie 31% cod	+	Cod	Fish	✓
Ocean Pie 6% cod	+	Cod	Fish	✓
PCR Blank	+	-	Mammal	✓

Meat mixtures

To examine the applicability of the chip system to meat mixtures, sausage samples from FSA project Q01084 were analysed using the kit exactly according to manufacturers instructions. The kit should be able to detect at least 2 species in a mixture of 2, 3, or 4 species when present at a level of at least 5% and therefore the samples from project Q01084 should have represented a challenge for the sensitivity of the chip. The samples were composed of chicken at 0.2%, 1%, 5% and 10% in 40-50% pork, and pork at 0.2%, 1%, 5%, and 10% in 40-50% beef.

In initial analysis of the samples both meat species were identified in the 1% and 5% mixtures, however only the major species component was identified in the 0.2% and 10% samples, and in the 10% chicken 40% pork sample no species were identified (Table 10). Since both species had already been identified at the 1% level it suggested that an error had occurred during analysis, similar to that had occurred in the cod samples.

The 0.2% and 10% samples had been run as a batch of 4 on the fluidics station to hybridise, followed by the 1% and 5% samples as a second batch. When each batch of chips was scanned, the resulting image intensities of the two batches appeared to differ greatly. The image intensities of the first batch (0.2% and 10%), where only the major component meat, or no species were identified, were low, not differing greatly from the background. In contrast, the image intensities of the second batch (1% and 5%), where both meat components were identified, were visibly higher. The reason for this could not be immediately established. The 0.2% samples were re-run on new chips and the resultant scans were of high intensity and the identification of both meat components was achieved, indicating a better level of detection than anticipated given the manufacturers claim of consistent detection at the 5% level. Unlike the meat, the intensity of the scans in the cod samples was relatively similar between the first scan and the repeat.

Within the kit specifications it states that some canned pre-cooked meats subjected to 2 sterilisation cycles are rendered undetectable due to degradation of the DNA. We sought to examine the effect of processing on meat mixtures by analysing samples from FSA project Q01033 & Q01034 (DNA Quality). The samples contained 5% chicken in pork, 5% beef in pork, or 5% pork in beef, which had been baked or canned. When analysed both meat species were identified in each sample in all cases (Table 11), irrespective of whether the sample had been baked or canned.

Table 10 Detection of species in mixed meat matrices from project Q01084, using the Food Expert Id detection system

Sample	Internal Control	Identified Species	Identified Class	Correct ID
Sausage 10% pork 40% beef	-	Beef	Mammal	×
Sausage 0.2% pork 50% beef	-	Beef	Mammal	×
Sausage 10% chicken 40% pork	-	-	Mammal	×
Sausage 0.2% chicken 50% pork	-	Pig/Boar	Mammal	×
Sausage 5% pork 45% beef	+	Pig/Boar & Beef	Mammal	✓
Sausage 1% pork 50% beef	+	Pig/Boar & Beef	Mammal	✓
Sausage 5% chicken 45% pork	+	Chicken & Pork/Boar	Mammal & Bird	✓
Sausage 1% chicken 50% pork	+	Chicken & Pork/Boar	Mammal	✓
Sausage 0.2% pork 50% beef Repeat	+	Pig/Boar & Beef	Mammal	✓
Sausage 0.2% chicken 50% pork Repeat	+	Chicken & Pork/Boar	Mammal	✓
Extraction blank	+	-	-	
PCR blank	+	-	-	
Goose	-	Goose	Mammal	✓

Table 11 Detection of species in baked and canned mixed meat matrices from project Q01033 and Q01034, using the Food Expert Id detection system

Sample	Internal Control	Identified Species	Identified Class	Correct ID
5% pork 95% beef Baked	+	Pig/Boar & Beef	Mammal	✓
5% beef 95% pork Baked	+	Pig/Boar & Beef	Mammal	✓
5% chicken 95 % pork Baked	+	Chicken & Pork/Boar	Mammal & Bird	✓
5% pork 95% beef Canned	+	Pig/Boar & Beef	Mammal	✓
5% beef 95% pork Canned	+	Pig/Boar & Beef	Mammal	✓
5% chicken 95 % pork Canned	+	Chicken & Pork/Boar	Mammal & Bird	✓
PCR Blank	+	-	-	

Tuna

The FoodExpert ID system is designed to identify several tuna and bonito species. To test the veracity of these claims samples of authentic frozen tuna fish, sourced from Vigo (Spain), were analysed and included *Thunnus albacares* (yellowfin), *T. thynnus* (bluefin), *T. alalunga* (albacore), *T. obesus* (bigeye), *Euthynnus pelamis* (skipjack), *E. lineatus* (black skipjack) and *Sarda sarda* (bonito)

The data are presented in Table 12 and five of the seven tuna (yellowfin, skipjack, bluefin, bonito and albacore) were correctly identified. One of the five (bonito) also included detection of an unexpected species, the spotted tunny (*E. alleteratus*), that may have been detected due to cross contamination. Black Skip tuna was not one of the species that the chip was capable of detecting. Analysis of this sample resulted in its misidentification as spotted tunny. The bigeye sample was correctly identified at the Taxa level but not at the species level. The extraction blank tested positive for species of the Fish class. As the extraction blank was effectively also the PCR blank for this analysis we were unable to conclude at which stage contamination had occurred. This highlights one of the problems with analysing small numbers of samples with the chip system. Due to the high cost of chips the number of controls that are run have to be kept to a minimum and so not allow a full set of positive and negative and extraction blank controls that would be utilised in a Taqman PCR assay.

Analysis of a fresh tuna steak sample (species unknown) obtained from a supermarket resulted in the identification of Fish class but not of the fish species. The tuna species may not have been a species that the kit is able to identify, however it was expected that tuna would have been identified. The kit specifications state that in some canned fish samples the DNA will be rendered undetectable due to degradation. Following the correct identification of pure frozen Yellowfin and Skipjack tuna, samples were prepared to investigate the sensitivity of the system for samples which had been canned. Samples of pure canned Yellowfin and Skipjack tuna and samples containing low levels of canned Skipjack (10%, 1%, 0.1%) in canned Yellowfin tuna were prepared and analysed (Table 12). Two extraction methods were used on the canned samples, the fish extraction method (Hird *et al*., 2005) was used on the pure samples, and the NucleoSpin extraction method was used on the mixture samples. No species or class was identified in any of the canned samples, supporting the observation that high levels of processing can cause degradation of DNA rendering it undetectable using this system.

Table 12 Detection of tuna using the Food Expert Id detection system

Sample	Internal Control	Identified Species	Identified Class	Correct ID
Yellowfin Tuna	+	Tuna Gender	Fish	✓
Skipjack Tuna	+	Skipjack tuna	Fish	✓
Bluefin	+	Tuna gender	Fish	✓
Albacore	+	Tuna gender	Fish	✓
Bonito	+	Atlantic bonito,	Fish	✓
		Spotted tunny		×
Black skipjack tuna	+	Spotted tunny	Fish	×
Bigeye	+	-	Fish & Mammal	×
Extraction blank	+	-	Fish	
100% Yellowfin Canned	+	-	Mammal	×
100% Skipjack Canned	+	-	-	×
Extraction Blank	+	-	-	
90%Yellowfin	+	-	-	×
10% Skipjack Canned				
99%Yellowfin	+	-	-	×
1% Skipjack Canned				
99.9%Yellowfin	+	-	-	×
0.1% Skipjack Canned				
Extraction Blank	+	-	-	
PCR Blank	+	-	-	
Tuna steak	-	-	Fish	×
Tuna Fishcake	+	Beef	Mammal	×
PCR Blank	+	-	Mammal	

General comments on the FoodExpert ID system

The range of samples included on the chip is relatively limited and not particularly applicable to species of interest in the UK (Table 13). The species list did not contain any of the mammalian or avian species from the exotic meat project (horse, donkey, duck, pheasant, and commercially important deer species in the UK), and could not differentiation between pig and wild boar. The species included on the chip should

be widened to include more species of interest for the UK market. Furthermore, the inclusion of common fish species, for example haddock and whiting, would have been of greater interest than the three eel species currently included on the chip. After discussion with the manufacturer they are improving the spread of species to include more mammals, bird and fish. The chip did however correctly identify the goose sample applied to the chip as a positive control for the analysis of canned samples.

At present the system gives no indication of the level or system performance. After performing intricate experimental steps, involving many reagents and pieces of equipment, over a relatively long period of time, the analyst receives little information on the performance of the assay, usually only the species/ class presence and internal control status. This limited information presented problems when trying to become familiar with the system especially when seeking to define limits of detection and when unexpected results were returned. To overcome this the employment of a wide range of controls and standards would have been desirable, but was however, unrealistic, due to the high cost of each chip and relatively low throughput capability when compared to TaqMan or ELISA based assays.

The sensitivity of the Food Expert Id system was found to be greater than the 5% outlined in the manual and was able to detect 0.2% pork or chicken in a meat background. It was found however that the results from the system gave no indication of the level of the species identified and it was necessary to then run a confirmatory real-time assay to determine relative levels of the species identified by the chip. From the results of the chips alone it was not possible to distinguish between samples that were 0.2 and 50% pork. We would, therefore use the chip system as a general screen to inform us of the likely species present in the sample, which could then be confirmed using other methods able to give an indication of the level of the species present.

Inconsistent results were encountered, which were initially thought to relate to the timing of the fluidics station priming, prior to hybridisation. However later results disproved this theory and despite consultation with bioMerieux, the issue of negative results, for example, detection of 2% cod but not 5 or 10% cod analysed in the same batch, remain unresolved.

In conclusion, the system would be of use as an initial screen if used to analyse an unknown sample and the sensitivity appears to be lower than the 5% reported for the system. In future, if a wider range of species is included on the chip, the system may be of use in control laboratories as a first screen. The results would then be used to

inform further, more specific tests to identify classes or species identified and level of adulteration.

Table 13 Species identifiable in pure samples using the Food Expert Id detection system

Mammals	Fish	Birds
Beef	European Eel	Ostrich
Mule deer	Japanese Eel	Turkey
Reindeer	Mozambican Eel	Goose
Goat	Atlantic bonito	Guinea fowl
Sheep	Atlantic mackerel	Chicken
Pig/Boar	European Hake	
Rabbit	Atlantic cod	
European Hare	Greenland cod	
Cat	Artic char	
Human	Brook trout	
Rat	Atlantic salmon	
Mouse	Spotted tunny	
	Skipjack tuna	
	Rainbow trout	
	Sea trout	
	Tuna genus – several species: *T. albacares, T. alalunga, T. thynnus, T. obesus*	

CONCLUSION

Sensitive and specific assays have been designed and optimised for commercially important species of duck, deer, horse donkey and pheasant which have been shown to be capable of detecting their target species in heat and pressure processed commercial products. These assays are fully portable between real-time PCR and gel electrophoresis equipment. Additionally technology transfer has been facilitated by the early submission of two manuscripts to peer reviewed journals detailing the design and optimisation of the horse, donkey and duck assays and with the provision of a comprehensive set of standard operating procedures. Additionally, the FoodExpert Id system was evaluated for the identification of species in complex food products, including fish, birds and mammals.

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APPENDIX 1 MATERIALS AND METHODS

DNA Template preparation

Meat tissues

The extraction of DNA from meat tissues had previously been optimised as part of Food Standards Agency project (Q01033). 25mg of raw tissue was extracted using the GenElute Mammalian Genomic DNA kit, available from Sigma (Poole, UK), exactly according to manufacturer's instructions.

Complex samples

CTAB, Proteinase K, Wizard DNA extraction. 1g samples were incubated in 10mls CTAB buffer (2% (w/w) CTAB, 20mM EDTA, 0.1M tris base and 1.4M Sodium Chloride) containing 1mg proteinase K at 65°C. After 60 minutes the samples were allowed to cool for 5min then spun at 3939***g*** for 5min. 1.4ml of each sample supernatant was transferred to a 1.5ml microcentrifuge tube, spun at 16060***g*** for 15 minutes and 700µl of the supernatant transferred to another tube containing 600µl of chloroform. This was mixed by vortexing and spun at 16060***g*** for 5min. 350µl of the aqueous phase was transferred to a second tube containing 600µl chloroform, which was mixed by vortexing and spun as before. 250µl of the aqueous phase was processed using the Wizard Genomic DNA Purification System (Promega, Southampton, UK) method according to the manufacturer's instructions.

PCR for sequencing

The following primers were used for sequencing;
Pheasant F1CAA ATC CTC ACT GGC C, Pheasant F3CGA ATC AGG CTC AAA C, Pheasant R2GGT GGA ATG GAA TTT TG, Pheasant R4 AGT TAA GTA TTT GTC TTC TAG GGT, Wild Boar F1CAA ATC CTA ACA GGC CT, Wild Boar F2 CAC GAA ACA GGA TCC A, Wild Boar R1GGT GAA ATG GAA TTT TGT, Wild Boar R2 TTG TTT TCG ATG ATG CT, Horse F1CAA ATC TTA ACA GGC CTA TT, Horse F2 CAC GAA ACA GGA TCT AAC A, Horse/Donkey R1 GGT GGA ATG GGA TTT T, Horse/Donkey R2 TTG TTT TCG ATG GTG C, DonkeyF1CAA ATC CTA ACA GGC CTA, Donkey F2 CAC GAA ACA GGA TCC A, Duck F1CAA ATC CTC ACA GGC C,Duck F2CAA ATC CTC ACA GGC, Duck R4 AGT TAA GCA TTT TGT TTT CTA G, Fal&muntj F1CAA ATC CTC ACA GGC C,Red F1CAA ATT CTC ACA GGC CT,Roe F1CAA ATC CTC ACA GGC C, Roe&fall F8 GCT TTT CAG TAG ACA AAG C, Red F2 GCT TTT CAG TAG ATA AAG CAA , Muntj F8 GCT TTT CAG TAG ATA AAG CG, Red R1 ATA TCT TTA ATG GTA TAG TAA GGA TG, Roe R7 ATA TCT TTA ATG GTA TAG TAG GGG T, Chns&fala R7 ATA TCT TTA ATG GTG TAG TAG GGA T, Muntj&falb R7 ATA TCT TTG ATG GTG TAG TAG G, Fal R9 TCT TCA TTT TAG AAG GTT ATT CT, Red R2 TCT TCA TTT TAG GAG GTT GT, Roe R9 TCT TCA TTT TAA GAG GTT ATT TT, Sika&muntj F9 TCT TCA TTT TAG GAG GTT ATT.

The conditions for PCR used in this project are shown below (per 25 µl reaction):

Water	20.55µl
AmpliTaq Gold	0.2µl
x10 buffer	2.5 µl
10mM dNTP	0.5µl
3.5µM Forward Primer	0.125µl
3.5µM Reverse Primer	0.125 µl
Template	1µl

The reactions were assembled as above in thin walled PCR tubes (Perkin Elmer, Warrington, UK). The template was always added last. The tubes were mixed by flicking. The tubes were placed into the PCR machine (GeneAmp PCR system 9700) and after activation of AmpliTaq gold by incubation at 95°C for 10min cycled for 45 cycles of denaturation at 95°C for 1min and annealing at 72°C for 1min.

Gel electrophoresis

12µl of PCR products, mixed with 3µl loading buffer (Sigma, Poole, UK) were electrophoresed through 2% (w/v) agarose (Sigma, Poole, UK) gel in TBE (100ml 0.89M Tris borate pH 8.3 with 0.2M EDTA (Sigma, Poole, UK)) containing 5µl 10mg/ml ethidium bromide (Sigma, Poole, UK). 5µl of a 100bp PCR Molecular Ruler (BioRad, Hemel Hempstead UK) were run alongside the PCR amplicons. Submerged gel electrophoresis was performed at 100V and at room temperature until the dye front had moved approximately 2.5cm into the gel. Bands were then visualized using a Gel Documentation System (BioRad, Hemel Hempstead UK) and photographed.

Purification of PCR products

PCR amplicons, were purified after gel electrophoresis, using the QIAEX II, gel extraction kit, available from Qiagen Ltd. (Crawley, UK) exactly according to the manufacturer's instructions. The DNA was reconstituted in water and stored at -20°C.

Sequence Analysis of the mitochondrial cytochrome b gene

PCR products were labelled using the ABI-Big Dye Terminator version-2 kit available from Perkin Elmer (Warrington, UK) and subsequently analysed on an ABI-DNA sequencer PRISM-377 (Perkin Elmer, Warrington, UK).

TaqMan analysis

The reactions were assembled in thin walled PCR tubes (Perkin Elmer, Warrington, UK) using Universal Master Mix, primers and probes purchased from Sigma Genosys. The DNA template was always added last. The tubes were mixed by flicking and then placed into the TaqMan machine (Perkin Elmer, Warrington, UK Series 7700 or 9700). Generic TaqMan cycling conditions were composed of: PCR carryover eliminated using UNG enzymatic cleavage at 50°C for 2min, immediately followed by activation of Amplitaq gold by incubation at 95°C for 10min and 40 cycles of denaturation at 94°C for 15sec and annealing at 60°C for 1min.

bioMerieux FoodExpert ID

DNA extracts from samples were prepared using the NucleoSpin Food kit (Machnerey-Nagel) as recommend by Biomereaux, exactly according to the manufactures instructions. The fish extraction method from the FSA tuna survey (1999) was used to extract the DNA from some canned tuna samples. The bioMerieux Food Expert ID kit was used exactly according to the manufactures instructions. The quality of extracted DNA was tested prior to PCR by gel visualisation or if appropriate, using suitable Taqman assays. Following the PCR step successful amplification was confirmed by gel visualisation of amplicons.

Where possible control chips were run in parallel to unknown samples. When this was not possible due to a lack of chips, gel visualisation of post PCR amplicons and Taqman reactions were used to confirm the absence of contamination in blanks and the presence of amplified or amplifiable DNA in samples.

APPENDIX 2 MANUSCRIPT ACCEPTED FOR PUBLICATION IN MEAT SCIENCE.

THE DETECTION OF HORSE AND DONKEY USING REAL-TIME PCR

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ABSTRACT
We have developed real-time PCR assays specific for horse and donkey, applicable to the detection of low levels of horse or donkey meat in commercial products. Primers, designed to the mitochondrial cytochrome b gene, were 3' mismatched to closely related and other commercial species. Amplification of non-target species DNA was prevented by truncation of primers at the 5' position, thereby conferring complete specificity. Both assays were highly sensitive and detected the presence of 0.0001 % (w/w) of donkey meat or 0.001 % (w/w) of horse meat when assessed using dilutions of DNA in water. Model food samples, spiked with horse or donkey muscle and commercial products containing horse were successfully tested for the presence of horse or donkey, demonstrating the applicability of the assays to food products.

Keywords: Real-time PCR; Horse; Donkey; Detection

INTRODUCTION

Food labelling regulations require the identity of meat in meat products to be accurately labelled. This has resulted in a need for tests which will reliably identify the species of meat present in a food sample and which also must be sensitive and robust enough to be applied to complex food matrices. A range of analytical approaches have been taken to meet these demands, broadly based on detecting either protein or DNA. Of the protein based methods, immunoassay is the most widely used with several companies supplying kits for a range of species. However, proteins are denatured during heat and pressure processing, making the detection of species present in a processed sample more difficult. DNA has the advantage of being a relatively stable molecule, and is more able to withstand heat processing. DNA methods have commonly been based around the use of species-specific primers in PCR followed by signal detection using gel electrophoresis (Meyer, Hofelein, Luthy, & Candrian, 1995; Bottero *et al*., 2003; Rodriguez *et al*., 2003). More recently published reports have focused on the use of specific primers in real-time PCR using TaqMan™ technology (Brodmann & Moor, 2003; Dooley, Paine, Garrett & Brown, 2004; Mendoza-Romero *et al*. 2004; Laube *et al*. 2003; Hird *et al. in Press*). This technique utilises fluorescently labelled probes which allow signal generation to be measured in real time, thus eliminating the need

for electrophoresis, end point determination and consequently the subjective analysis of the results.
The development of tests which will reliably identify the species of meat present in food has historically focused on species of high economic importance, including pork, beef and chicken. Many different tests now exist for each of these species (Dooley *et al*., 2004, Lahiff *et al*., 2001, Laube *et al*., 2003), whereas the range of assays for the detection for less commonly used meat species, for example horse and donkey, is relatively limited.
We report the development of real-time PCR assays for horse and for donkey which are specific, sensitive and robust enough for detection of horse and donkey in complex matrices. Specific oligonucleotide primers were designed by virtue of base pair mismatch at the 3' position. Optimal primer lengths were determined to ensure that cross amplification with non-target species was eliminated. The assays were then used for the successful detection of the horse or donkey meat in complex food matrices.

MATERIALS AND METHODS

Meat and meat product samples

Donkey muscle was obtained from the Sidmouth Donkey Sanctuary, and horse muscle was obtained from B. Webster & Sons Horse Slaughterers, Eastrington, Selby UK. Samples of fresh raw meats (pig, deer, pheasant, duck, guinea fowl, grouse, pigeon, quail, cow, partridge, sheep, turkey and chicken) were obtained from local suppliers in the UK. Samples of horse sausage, horse steak, horse burger and horse salami were obtained from local suppliers in France and Italy. All samples were stored at 4 ^{o}C until DNA extraction.

DNA extraction from raw meat

DNA was extracted from raw meat using a modified version of the GenElute™ Mammalian Genomic DNA Extraction Kit (Sigma). Briefly, 2 g of tissue was finely minced with a scalpel blade, prior to incubation at 65 °C in 8 ml CTAB buffer (0.055M CTAB, 1.4M NaCl, 0.1 M Tris and 0.02 M EDTA, pH 8) containing 50 µg/ml proteinase K (Sigma). After 1 hour the samples were cooled briefly and 400 µl of the supernatant used to extract the DNA exactly according to the manufacturers instructions. The final elution step was repeated once and the eluates pooled. DNA was stored short term at 4 °C or long term at –20 ^{o}C.

DNA extraction from complex meat matrices

DNA was extracted from complex meat matrices using a modified version of the Wizard DNA extraction Kit (Promega). Briefly, 5 grams of each sample was incubated at 65°C in 10 ml CTAB buffer containing 80 µg/ml proteinase K (Sigma). After 2 hours the samples were vortexed for 10 seconds and centrifuged for 10 minutes at 4000 *g*. 1ml of the supernatant was combined with 800 µl chloroform, vortexed briefly and centrifuged for 10 minutes at 14000 *g*. 500 µl of the aqueous phase was combined with 1 ml Wizard DNA resin and incubated for 5 minutes at room temperature. The DNA was then purified exactly according to the manufacturers instructions and eluted with 100 µl Tris EDTA buffer.

Real Time PCR primer and probe set design

Primer and probe sets were designed for horse and donkey using the mitochondrial cytochrome b gene (GenBank Accession numbers X79547 and X97337 respectively) using Primer Express® software. Primers were designed to fall on a point of mismatch to either horse or donkey and other farm animal species at the 3' position, and truncated to confer specificity (Table1) (Hird, Hill & Goodier, 2003; Hird *et al*., *in Press*) and were purchased from Sigma Genosys. Probes were labelled with 5' carboxyfluorescein and 3' minor groove binding fluorescent quencher and were purchased from Applied Biosystems (UK).

Real Time PCR reaction set up

Each real-time PCR reaction, 25µL in volume, contained 1 x TaqMan™ Buffer A, 25 mM $MgCl_2$ solution, 0.625 Units AmpliTaq Gold® DNA Polymerase (Applied Biosystems) and 0.5µM of each deoxynucleotide triphosphate (Sigma). Optimised primer and probe concentrations are shown in Table 1. DNA template was diluted 1:4 DNA:water and 5 µl of either DNA or water, as a negative control, were added per reaction. Reactions were assembled in Axygen thin walled 96 well plates with optical caps and run on ABI Prism 7700 or 9700 sequence detection systems with the following thermal cycling protocol: 50 ^{o}C for 2 min, 95 ^{o}C for 10 min followed by 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 1 min.

RESULTS AND DISCUSSION

Assay Development

Primer design

There is a large body of literature and commercial tests available for the identification of economically important meats. However, the range of assays available for the detection of less commonly used meat species, for example horse and donkey, is relatively limited. The objective of this study was to design species-specific real-time PCR primer and probe sets for horse and donkey, which would provide the basis of rapid and robust assays, applicable to the detection of horse or donkey meat in commercial products.

The differentiation of horse from donkey is challenging since they are closely related species and have a high degree of sequence homology. Nevertheless, differentiation is desirable because whilst horse is consumed in some countries, donkey is not. There are many published reports outlining detection of commercially important species using real-time PCR (Lahiff, Glennon, Lyng, Smith, Shilton & Maher 2002; Laube *et al.,*2003; Sawyer, Wood, Shanahan, Gout & McDowell, 2003) however, recent PCR studies have focused upon the detection of multiple species within a single assay since real-time PCR is relatively expensive compared to standard PCR with gel based visualisation of amplicons. These studies have utilised probes common to many mammalian species and specific primers or multiprimer assays using a common sense and species-specific antisense primers (Walker, Hughes, Anders, Shewale, Sinha, & Batzer, 2003; Mendoza-Romero, *et al*. 2004; Dooley, *et al*. 2004; Brodmann & Moor, 2003). Although these systems were cost effective these strategies did not result in complete specificity for the target species. Recently the Food Standards Agency of the UK used a commercial method to conduct a survey for the undeclared presence of horse and donkey meat in imported salami (Food Standards Agency, 2003). This survey used a PCR based approach which utilised a PCR primer set which would detect both horse and donkey in conjunction with a primer set which would detect horse alone, and the presence of donkey was determined by difference. Whilst this two tier approach would detect the presence of equine meat in a sample it would not detect the presence of donkey directly. Single species-specific assays allow the unequivocal identification of the origins of meat compared to a non-discriminatory test with cross amplification of non-target species. We therefore designed separate primer and probe sets for each species using the mitochondrial cytochrome b gene. Sequence data from the NCBI database for horse, donkey and other commercially popular species were aligned and areas of mismatch identified (Figure 1). Using Primer Express® software, primer and probe sets were designed to have mismatches to all other species at the 3' position of either, or where possible, both, sense and antisense primers (Hird *et al*., 2003). Independent primer and probe sets for horse and donkey maximised design flexibility for specificity and allowed amplicons to be less than 150 base pairs.

Primer Truncation & Specificity

Following initial screening of the horse and donkey primer and probe sets using the DNA from a range of species as template it became apparent that they cross amplified species other than the target species, despite the 3' mismatches (Table 2). The horse primer and probe set amplified cow and donkey template DNA, whilst the donkey primer and probe set amplified horse template DNA. The pattern of cross amplification was related to the degree of sequence homology between the species. The horse primer and probe set had only one base difference in the sense primer, two base differences in the probe, and four base differences in the antisense primer when compared to the cow sequence. Similarly, the donkey primer and probe set had only three base differences in the sense primer, one base difference in the probe, and three base differences in the antisense primer when compared to the horse sequence. This type of inappropriate amplification has been observed in previous studies (Hird *et al*., *in press*) and stems from the degree of sequence homology between the target and cross amplifying species, as well as the apparent discrepancy between the calculated melting temperature of the primers and the annealing temperature of TaqMan™ cycling conditions. We used the strategy outlined in Hird *et al.* (in press) to increase the specificity of the primers by truncation at the 5' position, a strategy which reduces the melting temperature of the primer, thereby reducing the temperature differential between primer melting temperature and the TaqMan™ cycling conditions. Following significant levels of truncation of sense and

antisense primers in both the horse and donkey sets, specific primer combinations were identified (Figure 1 & Table 2). No cross-species amplification was observed using template DNA isolated from pig, deer, pheasant, duck, rabbit, guinea fowl, grouse, pigeon, quail, cow, partridge, sheep, turkey, chicken, horse, and donkey, for the truncated horse and donkey primer sets thus demonstrating specificity.

Limit of detection.

Horse meat is regularly consumed in many European countries including France and Italy, whilst the consumption of donkey is very limited. In Britain, there is an aversion to horse and donkey meat being used for human consumption and the presence of horse or donkey meat, at any level, would not be tolerated. Consequently, for widespread practical use, these assays were designed to be highly sensitive to enable the detection of trace amounts of horse or donkey meat in commercial products. The limit of detection for both primer and probe sets was determined using ten fold dilutions of horse or donkey DNA diluted in water.

The limit of detection for the horse primer and probe set extended to a 1:500,000 dilution (average Ct 35.68) (Figure 2a), whereas the limit of detection for the donkey primer and probe set extended to a 1:5,000,000 dilution (average Ct 34.87) (Figure 2b). The dilutions relate to the detection of 0.0001% (w/w) donkey meat or 0.001% (w/w) horse meat in a sample. Both assays are therefore highly sensitive despite the fact that primer truncation for the horse assay led to a reduction in reaction efficiency. Although these limits of detection were calculated from dilutions of DNA in water, with detection at these orders of magnitude, the assays could be used as a basis for enforcing accurate product labelling and provide reassurance to consumers concerned about even the smallest levels of contamination of horse or donkey meat in products.

Application of assay to model samples

The optimised horse and donkey assays were used to test DNA extracted from test meat samples containing high levels (48% w/w) of either fat (beef lard), carbohydrate (soya flour), or protein (pork) spiked with 4, 2 and 1% (w/w) of either donkey or horse muscle and prepared in a meat muscle background. Both primer sets successfully detected the spikes at all three concentrations in all three matrices, returning comparatively low Ct values (<26) as shown in Figure 3a and 3b. The higher Ct values returned from the high carbohydrate samples are probably caused by enzyme activity within the soya flour matrix causing degradation of the target DNA. Finally, DNA was extracted from commercial products of horse sausage, horse steak, horse burgers and horse salami and assayed using the horse specific primer and probe set. The horse specific assay successfully detected the presence of horse DNA in all samples, whereas the donkey specific assay did not detect donkey DNA in these samples. Equivalent donkey samples could not be tested since commercial products which contain donkey were not available. No gross matrix effects were found in the successful testing of the matrix samples and commercial products, indicating the applicability of the assays to detection in complex food matrices.

Conclusions

Real-time PCR assays for the detection of horse and donkey species have been developed. The optimised assays are specific, highly sensitive, applicable to complex food matrices and, for the horse assay, has been successfully tested on commercial samples. These are the first of their kind to be published and are ready for use by analysts.

Acknowledgments

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Table1. Primer and probe sequences and optimised concentrations, for horse & donkey assays

Species	Oligonucleotide	Sequence (5'-3')	Tm[1]	Optimised concentration (nM)	Amplicon Size (bp)
Horse	Sense primer	GAA GCA TAA TAT TCC GG	52.7	900	69
	Antisense primer	TTA GTG TCA GTA AGT CTG CC	54.4	900	
	Probe	FAM-TCT CAG CCA ATG CGT-MGB		50	
Donkey	Sense primer	CCT TAT CCT TTC CAT CT	50.9	900	119
	Antisense primer	GTA AGT CTA CTA CTG AGA GTC AGA AC	57.7	300	
	Probe	FAM-AAT CCT AGC ACT CAT CC-MGB		50	

[1]Sigma Genosys Tm.

Table 2. Effect of truncation of horse and donkey primers on assay specificity

Primer and probe Set	Primer truncation Sense	Antisense	Horse DNA Ct	Donkey DNA Ct	Cattle DNA Ct
Horse	0	0	15.88	37.45	25.25
	0	-5	15.81	34.85	32.59
	0	-8	19.98	N	N
	-4	-8	19.28	N	N
	-6	-8	19.01	N	N
Donkey	0	0	20.77	17.35	N
	-7	-7	34.79	17.00	N
	-8	-7	N	16.99	N
	-9	-7	N	16.24	N

Negative figures indicate the number of bases removed form the ‘5 end of the original primer.
0 indicates full length primer, N indicates no amplification signal could be detected after 40 cycles.

Figure 1. Alignment of horse, donkey, cow, pig, sheep and chicken cytochrome b sequence, showing position of horse and donkey full length and truncated sense primers.

a) Horse sense primer

```
           A
           ------------------------------------------------------\
                       B
                       ------------------------------------------\
Horse      a a c a a c g a a g c a t a a t a t t c c g g c c
Donkey     . . . . . . . . . . . . . . . . g . . . . . a . .
Cow        . . . . . . . . . . . . . . . . . . . . . . a . .
Pig        . . . . . . . . . . . . . . . . . . . t . . a . .
Sheep      . g . . . . . g . . . . . . . . . . . . . . a . .
Chicken    . . . . . . . . . c a . . . . c c . . . . . a . .
```

b) Donkey sense primer

```
           A
           ----------------------------------------------------\
                             B
                             ----------------------------------\
Donkey     t g t a t t a g c c c t t a t c c t t t c c a t c t t
Horse      c . . . . . . . . . . . a . . . . . c . . . . . . c .
Cow        a . . . c . . . . . . . a g c . t . c . . t . . . c .
Pig        a . . g c . . . . t . . a . . a g c c . . . . . . c .
Sheep      a . . c c . c . . . . . a . . . . . c . . a . . . c .
Chicken    . . . . c . . . . . . . a g c a g c c . . a g . . c .
```

A - indicates the full length primer sequence, B – indicates the truncated primer sequence.

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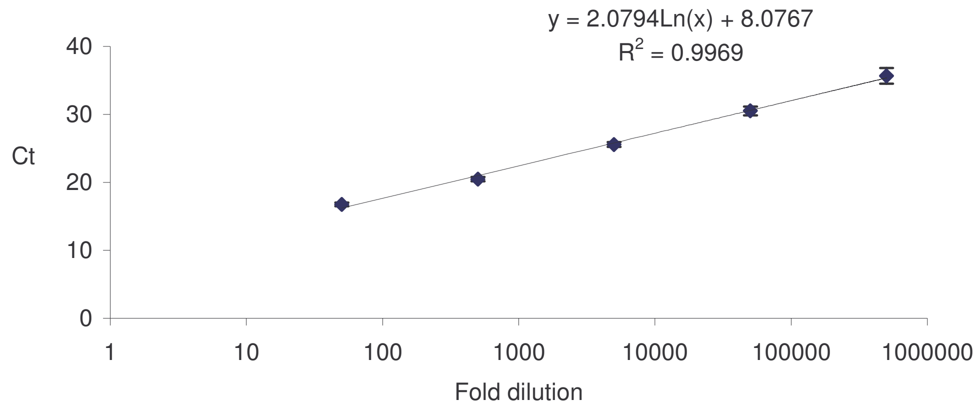
Figure 2. Limit of detection of horse and donkey assays. 10 fold dilution series of horse and donkey DNA in water related to Ct value.

a) horse



b) donkey

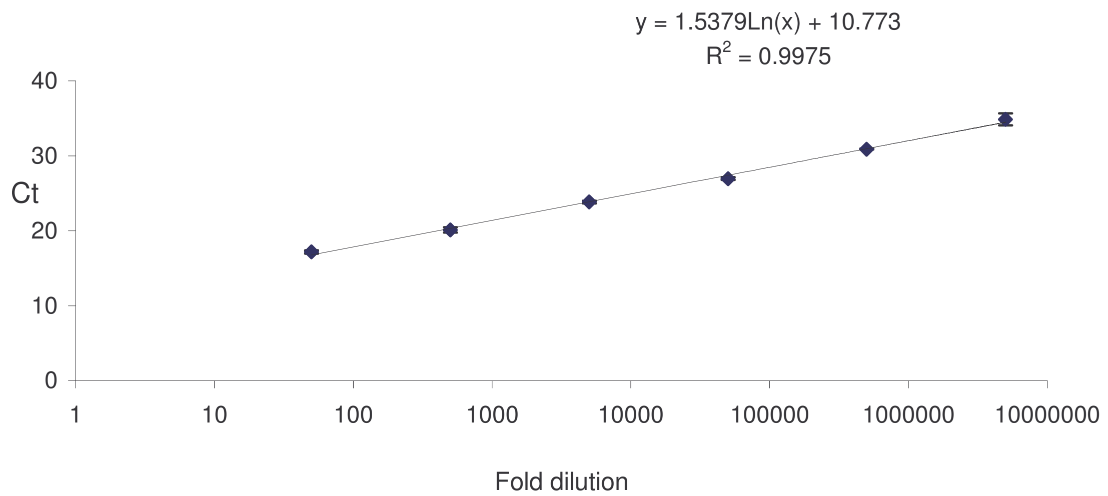
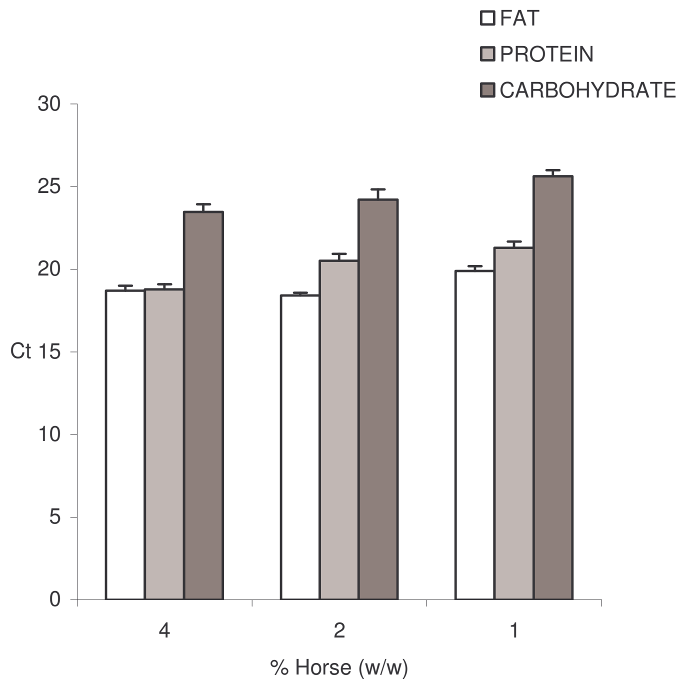
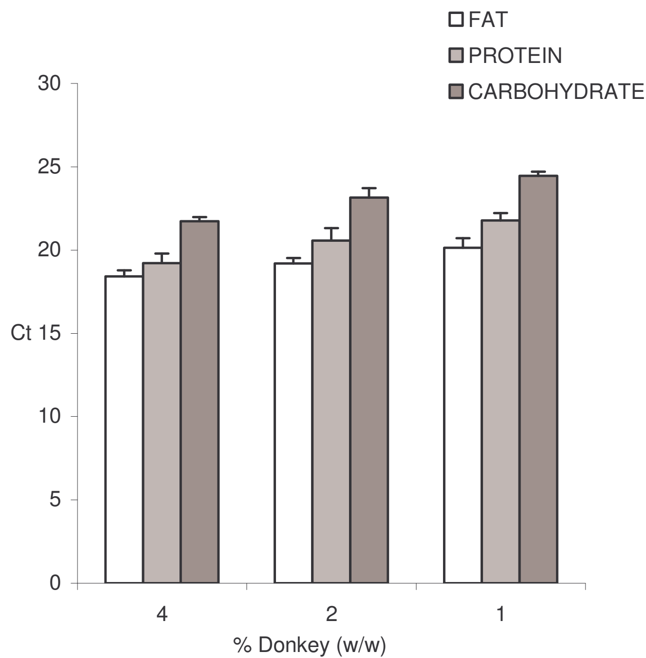


Figure 3. Detection of horse and donkey in model food samples of high fat, carbohydrate or protein, containing horse and donkey spiked at 4, 2, and 1% levels. Error bars represent 1S.D.

a) horse



b) donkey



APPENDIX 3 MANUSCRIPT ACCEPTED FOR PUBLICATION IN EUROPEAN FOOD RESEARCH AND TECHNOLOGY.

THE DETECTION OF COMMERCIAL DUCK SPECIES IN FOOD USING A SINGLE PROBE - MULTIPLE SPECIES SPECIFIC PRIMER REAL-TIME PCR ASSAY.

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ABSTRACT

A real-time PCR assay for the simultaneous detection of Mallard and Muscovy duck is described. Species-specific primers were designed for Mallard or Muscovy duck using the mitochondrial cytochrome *b* gene sequence. These primer sets were multiplexed with a single duck probe to produce a simple, rapid and robust real-time PCR assay. This assay was shown to be specific for duck compared to a wide range commercially important meat species and was used for the successful detection of duck meat in complex food matrices. This is the first report of an assay that will detect all species of commercially available duck in commercial products using real-time PCR.

Keywords: real-time PCR; duck; Muscovy; Mallard; detection

INTRODUCTION

For many years duck meat has been one of the more expensive commercially available meat types, consumption of which was reserved for special occasions. There has however, been a recent move towards large-scale production of duck using modern husbandry techniques which has resulted in a greater availability of duck and a proliferation of processed products containing duck. Additonally, the range of duck species used commercially has been extended from mainly Mallard ducks (*Anas platyrhynchos)* to include the subspecies Gressingham and Allesbury, and Muscovy ducks *(Cairina moschate)*.

There is now a need for a sensitive and robust assay for the detection of all species of commercial duck in heat and pressure processed products. Heat and pressure processing are known to denature protein and fragment DNA, however DNA based analyses remain the best option for detection since DNA survives heat and pressure processing relatively intact when compared to proteins [1]. There are few reports on the successful detection of duck species in commercial products. Partis *et al* (2000) used PCR-RFLP for the detection of duck, however they found that pork preferentially amplified over all other species such that the method was not applicable for mixtures of meats [2]. Cavalo *et al* (2001) outline a method using RAPD for duck detection but found that similar bands were produced by amplification of both duck and pork making interpretation of the banding patterns subjective [3]. Both of these reports did however demonstrate the utility of a DNA based approach and more recent reports have outlined DNA based methods based upon species specific PCR systems which circumvent co-

amplification in mixtures [4, 5, 6]. Rodriguez *et al* (2003a & b) used conventional PCR for the detection of Mule duck, a cross between Mallard and Muscovy duck (*Anas platyrhynchos x Cairina moschate*), using separate primer sets for two genes: 12S ribosomal RNA and α-actin genes in foie gras [4, 5]. Whereas Rodriguez *et al* (2004) developed a real-time PCR assay for the detection of Mule duck. These assays were based on Mallard DNA sequence and would not detect wild type Muscovy duck [6].

We report the development of a real-time PCR assay for the detection of both Mallard and Muscovy duck. Species-specific primers were designed using the mitochondrial cytochrome *b* gene, which when multiplexed, were used for the successful detection of duck meat in complex food matrices which had been heat and pressure processed.

MATERIALS AND METHODS

Meat and meat product samples

Samples of fresh raw meat from Mallard, Gressingham, Allesbury and Muscovy ducks, pig, deer, pheasant, duck, goose, guinea fowl, grouse, pigeon, quail, cow, partridge, sheep, turkey, chicken and commercial products were obtained from local suppliers in the UK. All samples were stored at 4 ^{o}C until DNA extraction.

DNA extraction from raw meat

DNA was extracted from raw meat using a modified version of the GenElute™ Mammalian Genomic DNA Extraction Kit (Sigma). Briefly, 2 g of tissue was finely minced with a scalpel blade, prior to incubation at 65 °C in 8 ml CTAB buffer (0.055 M CTAB, 1.4M NaCl, 0.1 M Tris and 0.02 M EDTA, pH 8) containing 50 µg/ml proteinase K (Sigma). After 1 hour the samples were cooled briefly and 400 µl of the supernatant used to extract the DNA exactly according to the manufacturers instructions. The final elution step was repeated once and the eluates pooled. DNA was stored short term at 4 °C or long term at –20 ^{o}C.

DNA extraction from complex meat matrices

DNA was extracted from complex meat matrices using a modified version of the Wizard DNA extraction Kit (Promega). Briefly, 5 grams of each sample was incubated at 65°C in 10 ml CTAB buffer containing 80 µg/ml proteinase K (Sigma). After 2 hours the samples were vortexed for 10 seconds and centrifuged for 10 minutes at 4000 *g*. 1ml of the supernatant was combined with 800 µl chloroform, vortexed briefly and centrifuged for 10 minutes at 14000 *g*. 500 µl of the aqueous phase was combined with 1 ml Wizard DNA resin and incubated for 5 minutes at room temperature. The DNA was then purified exactly according to the manufacturers instructions and eluted with 100 µl Tris EDTA buffer.

Real Time PCR primer and probe set design

Mallard and Muscovy duck specific primers and probe sets were designed using the mitochondrial cytochrome b gene DNA sequence (GenBank Accession numbers AF059081 and LO8385 respectively) using Primer Express® software (Applied Biosystems) (Table 1). Primers were designed to mismatch all other commercially important species at the 3' position [7] and were purchased from Sigma Genosys. The probe was labelled with 5' carboxyfluorescein (FAM) and 3' minor groove binding fluorescent quencher (MGB) and was purchased from Applied Biosystems.

Real Time PCR reaction set up

Each real-time PCR reaction contained 1 x TaqMan™ Buffer A, 25 mM $MgCl_2$ solution, 0.625 Units AmpliTaq Gold® DNA Polymerase (Applied Biosystems) and 0.5µM of each deoxynucleotide triphosphate (Sigma) in 25µL. Optimised primer and probe concentrations are shown in Table 1. DNA template was diluted 1:4 DNA:water and 5 µl of either DNA or water, as a negative control, was added per reaction. Reactions were assembled in Axygen thin walled 96 well plates with optical caps and run on ABI Prism 7700 or 9700 sequence detection systems with the following thermal cycling protocol: 50 ^{o}C for 2 min, 95 ^{o}C for 10 min followed by 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 1 min.

RESULTS AND DISCUSSION

Primer design

There are two commercially important species of duck: Mallard and Muscovy. These species are of the same family but diverge at the genus level and it is widely considered that all domestic and commercially important ducks species, with the exception of the Muscovy, have been bred from the Mallard.

Comparison of the mitochondrial cytochrome b gene sequences for these species (GenBank accession numbers AF059081 and LO8385 respectively) revealed that although closely related there is significant sequence heterogeneity between the species (Figure 1). We designed an assay that would identify both duck species using a probe common to all duck species combined with species-specific primers. Sequence data from the NCBI database for Mallard and Muscovy ducks and other commercially popular species were aligned and areas of mismatch identified. Using Primer Express® software, primer sets were designed to have mismatches to all other species at the 3' position of both sense and antisense primers for Mallard and Muscovy ducks. The probe was designed to the Muscovy sequence and had only a single base mismatch for the Mallard sequence (Figure 1).

Efficacy and Specificity

The aim of the study was to develop an assay which would identify the presence of duck in commercial products. The identification of the type of duck was less important than the detection of duck *per se*. We therefore developed the primer sets for Mallard and Muscovy separately and then multiplexed them to form a duck group assay.

The Mallard and Muscovy assays were found to be highly sensitive on their matching template (Ct values 14.68 and 13.48 for Mallard and Muscovy DNA template using Muscovy and Mallerd assays respectively). After primer limitation and multiplexing, the duck group assay was compared to the single assays for amplification efficiency on either Mallard or Muscovy duck template DNA (Figure 2). There was no significant difference in the Ct values of the single or multiplexed assays for either template. The single assays were found to cross amplify the alternate template with lower sensitivity (Ct values 28.27 and 30.09 for Muscovy and Mallard DNA template respectively). Cross amplification at these levels equates to approximately 10,000 fold less amplification efficiency on the alternate template compared to the matching template. However, since there is a common probe for both primer sets, the cross amplification of the alternate template presented no problem for the sensitivity of the assay.

The specificity of the duck group assay was then assessed using DNA from a wide variety of species, including pheasant, chicken, partridge, goose, guinea fowl, pigeon, quail, grouse, turkey, cow, pig, horse, donkey, red deer and sheep, and two varieties of duck originating from the Mallard: Gressingham and Allesbury (Table 2). It was found that there was no cross amplification of non-duck species (Ct value of 40 equates to no amplification), but good amplification of all types of duck, demonstrating complete specificity of the duck group assay for duck DNA.

Duck group assay efficiency and limit of detection.

Efficiency and limit of detection for the duck group assay were determined using ten fold dilutions of Mallard duck DNA diluted in water. Primer and probe sets which are working at 100% efficiency will require approximately an additional 3.3 cycles (3.3 Ct) for a 10 fold dilution of template. The limit of detection is the DNA dilution where Ct values are still reproducibly produced, but beyond which the assay fails. The detection of duck for the duck group assay extended to a 1,000,000 fold dilution of the duck DNA in water (average Ct 33.65) (Figure 3). Additionally, over this range of dilutions the response was linear, and the slope of the Ct values plotted against the log of the DNA dilution was -3.5, indicating an assay working at 106% efficiency. The limit of detection relates to the detection of 0.0001% (w/w) duck meat in a sample. Although this limit of detection was calculated from dilutions of DNA in water, with detection at these orders of magnitude, the assays could be used as a basis for enforcing accurate product labelling and provide reassurance to consumers concerned about even the smallest levels of contamination of duck meat in products.

Application of assay to commercial samples

The duck group assay was then used to test DNA extracted from a range of commercial samples where either duck or game had been included in the ingredients list (Table 3). Duck was successfully detected in all samples. In samples of roasted and smoked duck and of game casserole, the DNA did not show evidence of significant fragmentation by low Ct values. Similarly, a low Ct value was returned for duck pate which contained not only duck but pork, pork fat and milk. Higher Ct values were returned for samples which were highly processed or long life: the cat foods. The level of duck in these samples (4%) was only quoted as a minimum since the labelling requirements for pet foods are different than those for human consumption, however we would expect a Ct value of approximately 15 for a sample containing 4% duck. The greater Ct values returned for these samples indicated a high degree of DNA fragmentation. An anomaly for the pet food results was given by the duck biscuits, which returned a relatively low Ct value. This indicated that the duck meat in the biscuit was not as highly processed as in the other pet foods analysed. The presence of duck was therefore detected in all samples analysed where duck had been listed in the ingredients. Additionally, there was specific detection of duck in complex food matrices, even in the presence of pork, a confounding factor for RFLP and RAPD based assays [2, 3].

Conclusions

A real-time PCR assay for the detection of duck has been developed. The optimised assay is specific, highly sensitive and applicable to complex food matrices. This is the first real-time PCR based assay for the detection of commercial duck species to be published and as such, can now be recommended to food control laboratories.

Acknowledgments

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Figure 1. Alignment of Muscovy duck, Mallard duck, pheasant, chicken, and partridge mitochondrial cytochrome b sequence, showing position of specific Muscovey and Mallard primers and the common probe.

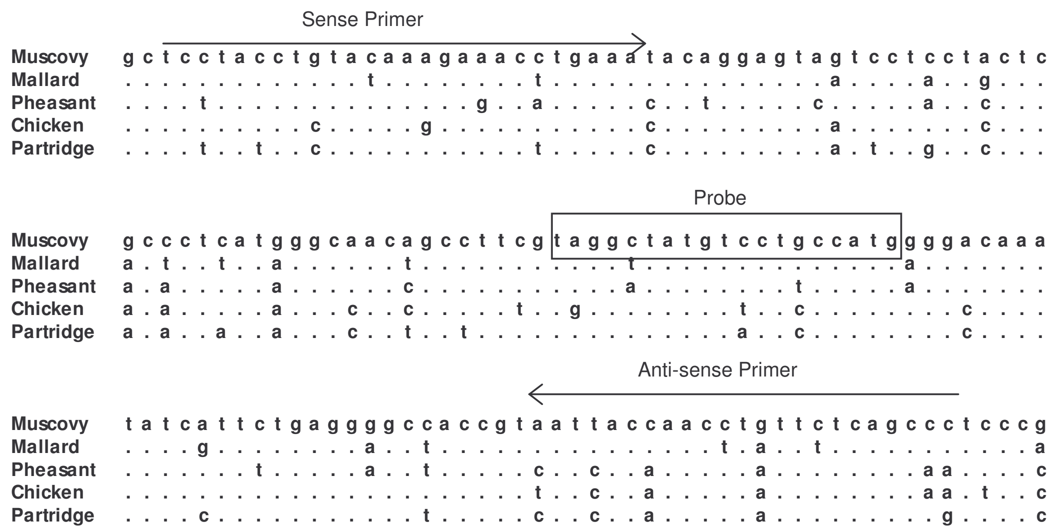

Figure 2. Effect of multiplexing on sensitivity of duck group real-time PCR assay. The Mallard and Muscovy assays were used as single assays or multiplexed on the same DNA extracts from Mallard and Muscovy duck meat.

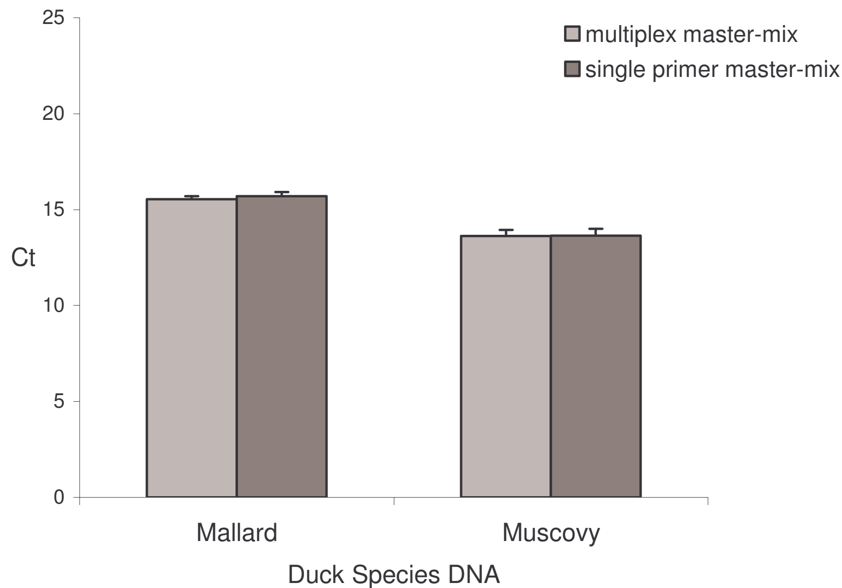

Figure 3. Duck group assay on fold dilution series of duck template (DNA diluted in water, fold dilutions A 10, B 100, C 1,000, D 10,000, E 100,000, F 1,000,000).

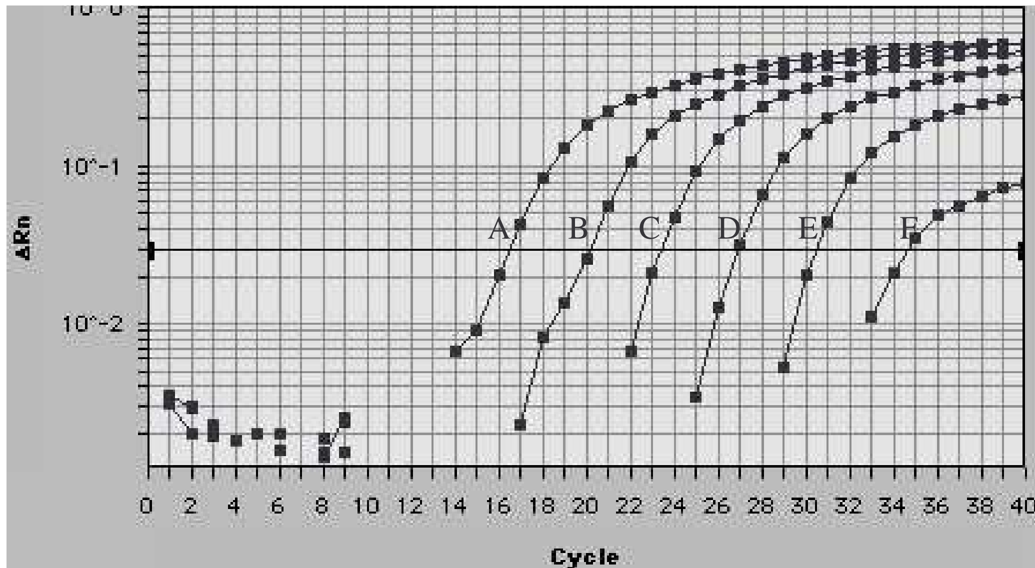

Table 1 Primer and probe sequences and optimised concentrations, for duck group real-time PCR assay.

Species	Oligonucleotic	Sequence (5'-3')	Optimised concentration (nM)
Mallard	Sense primer	TCC TAC CTG TAT AAA GAA ACT TGA AAT	300
	Antisense primer	GGG CTG AAA ATA AGT TGG TAA TT	300
	Probe	FAM-TAG GCT ATG TCC TGC CAT G -MGB	50
Muscovy	Sense primer	TCC TAC CTG TAC AAA GAA ACC TGA AAT	300
	Antisense primer	GGG CTG AGA ACA GGT TGG TAA T	300

Table 2. Specificity of duck group real-time PCR assay on DNA template from a variety of species.

Species	Species scientific name	Average Ct value ± SD
Muscovy duck	*Anas platyrhynchos*	13.63 ± 0.32
Mallard duck	*Cairina moschate*	15.54 ± 0.16
Allesbury	*Cairina moschate*	16.07 ± 0.20
Gressingham	*Cairina moschate*	15.18 ± 0.10
Turkey	*Meleagris gallopavo*	40.00 ± 0.00
Chicken	*Gallus gallus*	40.00 ± 0.00
Pig	*Sus scrofa*	40.00 ± 0.00
Cow	*Bos Taurus*	40.00 ± 0.00
Sheep	*Ovis aries*	40.00 ± 0.00
Goose	*Anser anser*	40.00 ± 0.00
Pheasant	*Phasianus colchicus*	40.00 ± 0.00
Partridge	*Alectoris rufa*	40.00 ± 0.00
Pigeon	*Columba livia*	40.00 ± 0.00
Guinea fowl	*Numida meleagris*	40.00 ± 0.00
Grouse	*Lagopus lagopus*	40.00 ± 0.00
Horse	*Equus caballus*	40.00 ± 0.00
Donkey	*Equus asinus*	40.00 ± 0.00
Red deer	*Cervus elaphus*	40.00 ± 0.00
Quail	*Coturnix coturnix*	40.00 ± 0.00

Table 3. Real-time PCR analysis of commercial products using the duck group assay.

Product	Declared meat species or animal content	Average Ct value ± SD
Roasted duck in plum sauce	Roasted duck	12.03 ± 0.09
Smoked duck slices	Smoked Barbary duck	11.13 ± 0.09
Duck pate	19% Pork, pork fat, 19% duck liver, milk, 11% chicken liver, duck fat	12.09 ± 0.07
Whiskers duck cat food (pouch)	Meat and animal derivatives including min 4% duck	30.67 ± 0.59
Felix duck and heart cat food (tin)	Min 4% duck, min 4% heart, fish and fish derivatives	30.07 ± 1.81
Sainsburys duck and chicken cat food (tin)	Min 4% duck, min 4% chicken	36.20 ± 1.48
Friskies duck cat biscuits	Min 4% duck	19.81 ± 0.14
Game casserole	Raw pheasant, partridge, Mallard duck, venison, pigeon and rabbit	11.35 ± 0.14
Friskies game, turkey and vegetable cat food (pouch)	Min 4% game, 4% turkey	33.40 ± 0.97
Whiskers game cat food (pouch)	Meat and animal derivatives including min 4% game	29.87 ± 0.53
Whiskers poultry cat food (pouch)	Meat and animal derivatives including min 4% poultry	29.99 ± 0.78

APPENDIX 4 STANDARD OPERATING PROCEDURES FOR THE IDENTIFICATION OF HORSE, DONKEY, DUCK, VENSION OR PHESANT.

APPENDIX 4.1 FOOD STANDARDS AGENCY: STANDARD OPERATING PROCEDURE FOR QUALITY CONTROL FOR ANALYSIS OF MEAT SAMPLES USING REAL-TIME (TAQMAN™) PCR

The real-time PCR process must be controlled to ensure accurate and reproducible results which are easily interpretable providing an unequivocal answer. Appropriate controls to be run along side samples are outlined below.

DNA Extraction Negative Control

An extraction blank must be run with every batch of DNA extracts. This should be a tube of buffer taken through the extraction protocol at the same time as the DNA extracts. This should then be assessed using the real-time PCR assay appropriate for the matrix extracted with the extraction blank. An extraction negative showing Ct<38±1 means the PCR batch is invalid and all samples must be re-amplified. If, after re-amplification, a Ct<38±1 is still obtained, the extraction batch is invalid and all samples must be re-extracted.

PCR Negative Controls

A PCR negative must be used as a method control with every set of samples amplified at the same time. For a PCR negative, use sterile Milli-Q water in place of the sample DNA when setting up the PCR plate.

The purpose of the PCR negative is to identify if contamination has occurred during the PCR procedure. The PCR negative is usually manipulated last at each stage of the process, to pick up any possible source of contamination.

Both the extraction negative and PCR negative should show no PCR amplification (Ct>38±1). A Ct<38±1 indicates contamination has occurred and the PCR batch is invalid. All samples must be re-amplified.

PCR Positive Controls

PCR positive controls must be run for each mastermix and should be template DNA matched to the primer and probe sets in the mastermix. Positive controls must return Ct values below 30, if the results are above 30 then the results are invalid and the Real-time PCR must be repeated.

PCR Inhibition Controls

To determine the presence and level of PCR inhibitors, an inhibition assay must be run on the samples prior to real-time PCR analysis, the presence of PCR inhibitors could lead to a false negative result. The assays should be run on samples at 10, 30 and 100 fold dilutions and the lowest dilution which shows no inhibitors should be used in rea-time PCR

The limit of detection

The limit of detection for these assays will be set at 0.1% of target species in a background of another species. This is equivalent to obtaining a Ct value which is around 9.9 Ct units less than the positive control. To apply this cut-off value, the positive control should be run alongside the samples to be assessed and any sample returning a Ct value more than 9.9 Ct values greater than the positive control would be deemed to be below the limit of detection.

APPENDIX 4.2 FOOD STANDARDS AGENCY: STANDARD OPERATING PROCEDURE FOR PREPARATION OF REAGENTS REQUIRED FOR ANALYSIS OF MEAT PRODUCTS

Procedural variation

No procedural variation is permitted and all steps must be done exactly as described. If for any reason a procedural variation is made then it must documented in full and the appropriate authority notified.

CTAB BUFFER

Introduction

Cetyl trimethyl ammonium bromide (CTAB) buffer is a protein denaturing detergent used in the isolation and precipitation of DNA. All reagents are to be of molecular biology grade.

Safety

Safety glasses, laboratory coats and disposable gloves to be worn at all times.

Reagents

CTAB (Sigma H6269)
NaCl (Sigma S3014)
Tris base (Sigma T6791)
Ethylenediaminetetraacetic acid (EDTA) (Sigma E5134)
6M Hydrochloric acid (dilute stock concentrated Hydrochloric acid (Fisher H1150PB17) 1:1with deionised water).
Standard buffer solutions pH 7 and 9.
Deionised water

Equipment

pH meter
Magnetic stirrer and flea
Autoclave
2l beaker
1l volumetric flask
100ml bottles

Method

1. Place 20g CTAB, 82g NaCl, 12.11g Tris base and 7.5g EDTA into a 2l beaker with 500ml of deionised water. Place the solution on a magnetic stirrer and use a flea to stir until the all the reagents have dissolved (usually overnight).
2. Check the calibration of the pH meter with the standard buffer solutions pH7 and 9.
3. Adjust the pH of the CTAB solution with 6M HCl to pH 8.0 using the pH meter.

4. Transfer the solution to a volumetric flask and adjust the volume to 1 litre with deionised water.
5. Aliquot the CTAB buffer into smaller volumes (eg 100ml bottles).
6. Autoclave the CTAB buffer at 121°C for 15min. When cool, label with the date of preparation and date of disposal (6 months from date of preparation). Store at room temperature.

80% (v/v) ISOPROPANOL

Safety

Safety glasses, laboratory coats and disposable gloves to be worn at all times.

Reagents

Isopropanol – molecular biology grade, min 99% (Sigma I-9516)
Sterile molecular biology grade water (Sigma W-4502)

Equipment

100ml measuring cylinder
100ml bottles

Method

1. Add 80ml of isopropanol to 20ml of sterile water using a 100ml measuring cylinder.
2. Store in a bottle in a solvent cupboard.

1 x TE BUFFER

Safety

Safety glasses, laboratory coats and disposable gloves to be worn at all times.

Reagents

Tris-EDTA buffer, 100x concentrate molecular biology grade (Sigma T-9285).
Sterile molecular biology grade water (Sigma W-4502)

Equipment

1.5 ml Microcentrifuge tubes (autoclaved) (Starlab S1615)
P5000 Gilson pipette fitted with aerosol barrier filter (Anachem F161280)
P1000 and P200 Gilson pipettes or equivalent for example M1000, M100 Micoman positive displacement pipettes
5ml tips (autoclaved) (Anachem F161370)
1ml and 200µl Barrier filter tips (sterile) (Starlab S1126-7810, S1120-8810)
UV crosslinker (UVP CL1000)

50ml sterile centrifuge tubes (Corning 430290)
DNA free cabinet or other designated DNA free area

Method

1 In DNA free cabinet label microcentrifuge tubes as 1 x TE.
2 Dilute 0.05ml buffer (100x) in 4.95ml sterile water in 50ml centrifuge tube and aliquot 600µl into the labelled microcentrifuge tubes.
3 UV irradiate with the lids off in the UV cross linker for 10min. Close lids and store.

PROTEINASE K 20mg/ml

Safety

Safety glasses, laboratory coats and disposable gloves to be worn at all times.

Reagents

Proteinase K 100mg (Sigma P2308).
Sterile molecular biology grade water (Sigma W-4502)

Equipment

1.5 ml Microcentrifuge tubes (autoclaved) (Starlab S1615)
P5000 Gilson pipette fitted with aerosol barrier filter (Anachem F161280)
P1000 and P200 Gilson pipettes or equivalent for example M1000, M100 Micoman positive displacement pipettes
5ml tips (autoclaved) (Anachem F161370)
1 ml and 200 µl Barrier filter tips (sterile) (Starlab S1126-7810, S1120-8810)

Method

1 Add 5ml sterile water to the Proteinase K 100mg container and mix by inversion until dissolved, to make a 20mg/ml solution.

2 Dispense the prepared solution in 200 µl aliquots into labelled microcentrifuge tubes and store at –20°C. These solutions have an estimated shelf life of 12 months at –20°C, and should only be freeze thawed once.

PRIMERS AND PROBES

Introduction

Oligonucleotide primers and fluorogenic probes are used in the Taqman assay.

Safety

Gloves, laboratory coats and disposable safety glasses to be worn at all times.

Reagents

Lyophilised primers (desalted) (Sigma)
Dual-labelled fluorogenic probes (HPLC purified) (Sigma)
Sterile molecular biology grade water (Sigma W-4502)

Equipment

DNA Free cabinet
P1000 and P200 Gilson pipettes
1 ml and 200 µl Barrier filter tips (sterile) (Starlab S1126-7810, S1120-8810)
Axigen low retention microcentrifuge tubes – 1.5ml and 0.5ml (autoclaved) (Thistle Scientific cat. No. MCT-175-L-C; MCT-060-L-C)
Plastic Bijoux (Sterilin 129A)

Method

PRIMERS

1 Purchase lyophilised oligonucleotide primers (stable indefinitely at –20°C). All dilutions of the primers are carried out in a DNA Free cabinet. Order replacement primers when the last vial of lyophilised primer is opened.

2 On arrival confirm the sequence of the primers on the vial with the sequence ordered. The supplier's name, batch number and the date must be recorded and batch number allotted to the primer. This information, together with the datasheet supplied with the primer should be kept in a 'Taqman primers and probe' folder.

3 Use the following formula to calculate the amount of water to be used to prepare the primer solutions of 20µM.

$$\frac{\mu g \times 50}{MW} = \text{vol (ml)}$$

µg = the amount of primer in the tube (information provided by supplier)
MW = the molecular weight of the primer (information provided by the supplier)

e.g. MW=6345
ug = 481
Volume of water required $= \frac{481 \times 50}{6345} = 3.790$ml

4 Centrifuge the tubes containing the lyophilized primer for 10 seconds to collect the powder at the bottom of the tube.

5 In a DNA Free cabinet label Axigen tubes with the primer name and the sense or antisense designation. Add the required amount of sterile water to the lyophilised primers to prepare a solution of 20µM and leave to rehydrate at 37°C for 30mins or 4°C overnight. In the event that the tube is not large enough for the volume of water required, add 1ml of water and rehydrate. At the end of the rehydration period, transfer the primer to a bijoux and add the required volume of water to give a 20µM solution.

6 Dispense the prepared primer in 130 µl aliquots into labelled Axigen tubes and store at –20°C. These solutions have an estimated shelf life of 1 year at –20°C, and should only be freeze thawed once.

7 All information concerning preparation of primers should also be recorded in a laboratory notebook.

PROBES

1 Purchase Fluorogenic probes either lyophilised or in solution.

2 On arrival confirm the sequence of the probe on the vial with the sequence ordered. The supplier's name, batch number and the date must be recorded and batch number allotted to the probe. This information, together with the datasheet supplied with the probe should be kept in a 'Taqman primers and probe' folder. All dilutions of the probe are carried out in a DNA Free cabinet.

3 If the probe is supplied in solution go to step 6, if the probe is lyophilised use the following formula to calculate the amount of water to be used to prepare the probe solutions of 5 µM.

$$\frac{\mu g \times 200}{MW} = \text{vol (ml)}$$

µg = the amount of probe in the tube (information provided by supplier)
MW = the molecular weight of the probe (information provided by the supplier)

e.g. MW=6345
ug = 185

$$\text{Volume of water required} = \frac{185 \times 200}{6345} = 5.831 \text{ ml}$$

4 Centrifuge the tubes containing the lyophilized probe for 10 seconds to collect the powder at the bottom of the tube.

5 In the DNA Free safety cabinet label Axigen tubes with the probe name. Add the required amount of sterile water to the lyophilised probe to prepare a solution of 5µM and leave to rehydrate at 37°C for 30 mins or

4°C overnight. In the event that the tube is not large enough for the volume of water required, add 1ml of water and rehydrate. At the end of the rehydration period, transfer the probe to a bijoux and add the required volume of water to give a 5 µM solution. Go to step 7.

6 Add the required amount of sterile water to dilute the probe to a solution of 5µM, and mix well by inversion. In the event that the tube is not large enough for the volume of water required, add 1ml of water and mix well by inversion, transfer the probe to a bijoux and add the required volume of water to give a 5 µM solution. Go to step 7.

7 Dispense the prepared probe in 110 µl aliquots into labelled Axigen tubes and store at –20°C. These solutions have an estimated shelf life of 1 year at –20°C, and should only be freeze thawed once.

All information concerning preparation of probes should also be recorded in a lab notebook.

APPENDIX 4.3 FOOD STANDARDS AGENCY: STANDARD OPERATING PROCEDURE FOR EXTRACTING DNA FROM MEAT PRODUCTS

Introductions

This SOP describes the method used to extract DNA from samples containing meat.

Scope

The extracted DNA can be tested for the presence/absence of meat species.

Safety

Safety glasses, disposable gloves and laboratory coats to be worn at all times.
Chloroform should be used in a fume hood and waste chloroform should be discarded using local methods for the safe disposal of organic solvents.

Other Relevant SOPs

Preparation of reagents

Reagents

CTAB extraction Buffer (see SOP Preparation of Reagents)
Chloroform - molecular biology grade, min 99% (Sigma C2432)
Wizard® DNA Clean-up System (Promega A7280)
80% (v/v) Isopropanol (see SOP Preparation of Reagents)
1 x TE buffer (see SOP Preparation of Reagents)
Proteinase K - 20mg/ml (see SOP Preparation of Reagents)

Equipment

Sterile spatulas
50 ml centrifuge tubes - sterile (Corning 430290)
1.5 ml Microcentrifuge tubes (autoclaved) (Starlab S1615)
2 ml Microcentrifuge tubes (autoclaved) (Alpha Laboratories LT2198R)
Axygen low retention microcentrifuge tubes – 1.5 ml (autoclaved) (Thistle Scientific Cat. No. MCT-175-L-C)
P1000 and P200 Gilson Pipettes or equivalent (for example M 1000, M100, microman positive displacement pipettes)
P5000 Gilson pipette fitted with aerosol barrier filter (Anachem F161280)
5ml tips (autoclaved) (Anachem F161370)
1 ml and 200 µl Barrier filter tips (sterile) (Starlab S1126-7810, S1120-8810)
Hybridisation oven (Hybaid HBSNSR110) or waterbath, preheated to 65°C ± 2.0°C
Centrifuge (Heraeus Labofuge 400R)
VacMan Vacuum manifold (Promega A7231)
Heating block set to 80°C+/-5°C (able to hold 1.5 ml microcentriguge tubes)

Precautions

Good molecular biology techniques should be used throughout this protocol.

Care should be taken not to cause cross-contamination between samples. Line the space where the sample is to be handled with a sheet of paper towel. The paper towel should be replaced each time a new sample is handled. Gloves should also be changed each time a new sample is handled and at stages stated in the SOP. If the work area is contaminated by a sample the area should be treated with 5 % Decon followed by 0.1 M HCl before progressing any further.

Microcentrifuge tubes should be opened using two hands (do not 'pop' open), touching the tab of the lid only and not the collar.
Use separate tips to add the buffers to different samples.
Pipettes should never be put into bottles beyond the tip, if necessary aliquot reagents instead.
Small aliquots of reagents should be used and fresh batches should be started frequently.
When pipetting liquids, tips should be pre-rinsed once. When pipetting volatile liquids (such as chloroform) tips should be pre-rinsed twice and reverse-mode pipetting should be used. When using barrier filter tips a greater time than usual should be allowed between take up of liquid and removal from stock bottle.

Procedural Variation
No procedural variation is permitted and all steps must be done exactly as described. If for any reason a procedural variation is made then it must documented in full and the appropriate authority notified.

Method

1. Using a sterile spatula weigh 5 g (±0.1 g) of homogenised meat sample into each of two 50 ml centrifuge tubes labelled with the sample number and A or B (samples are analysed in duplicate). Change gloves between samples. Add 10ml CTAB extraction buffer and 40 µl proteinase K (20 mg/ml) to the samples. Mix each sample thoroughly by vortexing for 6 seconds to produce a slurry. Add 10mls of CTAB to an empty centrifuge tube labelled 'extraction blank' and treat the same as the samples.

2. Incubate the samples at 65°C ± 2.0°C for 2 hours in a Hybaid oven or a waterbath

3. Place 1 x TE buffer in the Heating block set to 65°C+/-5°C

4. Change gloves. Label two 2 ml microcentrifuge tubes and one 1.5ml microcentrifuge tube with the sample number and A, and a replicate set of tubes with B. Add 800µl of chloroform to one of the 2 ml tubes. Re-suspend the Wizard® kit resin by shaking (do not vortex) and add 1ml of resin to the other 2ml microcentrifuge tubes.

5. After incubation at 65°C, vortex the sample briefly then centrifuge at between 3,000 and 5,000***g*** for 10 minutes. Transfer 1000µl sample supernatant to the labelled 2ml microcentrifuge tube containing 800 µl chloroform. Vortex for 3 sec and centrifuge at 16060***g*** (if not possible due to centrifuge model, G force should be no less than 13,000***g***) for 10 min. Change gloves.

6. Transfer 500µl of the supernatant, ensuring that the interface is not disturbed, to the microcentrifuge tube containing 1ml of Wizard® resin. Mix gently by inverting several times– do not vortex. Incubate at room temperature for 5 minutes. Change gloves.

7. For each sample, prepare one Wizard® minicolumn which should be handled carefully to minimise contact with the top and bottom ends. Label the column with the sample number and attach the syringe barrel provided to the luer-lock extension of each minicolumn. Attach each minicolumn/syringe barrel assembly to the vacuum manifold.

8. Mix samples gently by inverting several times. Pipette the resin/aqueous phase into the syringe barrels. Open each tap and apply the vacuum to draw the resin/aqueous phase into the minicolumn. When all the resin/aqueous phase has been drawn into the column close each tap.

9. Wash each minicolumn by adding 2ml +/- 0.1ml of 80% isopropanol to the syringe barrel and re-apply the vacuum to draw the solution through the minicolumn. Close each tap as soon as all the solution has been drawn through. Once all the samples are complete re-open all the taps and apply the vacuum for a further 20 sec before closing again and turning off the vacuum manifold.

10. Change gloves. Remove the syringe barrel and transfer each minicolumn to a labelled microcentrifuge tube with the cap removed. Centrifuge the minicolumn at 10,000 ***g*** for 2 minutes to remove any residual isopropanol.

11. Change gloves. Transfer the minicolumn to a labeled 1.5 ml low retention microcentrifuge tube. Add 100 µl of TE buffer (previously heated to 65°C +/- 5°C) to the minicolummn and wait for 1 minute.

12. Centrifuge the minicolumn at 10,000 ***g*** for 45 sec to elute the bound DNA.

13. Change gloves. Remove and discard the minicolumn from the tube which contains the eluted DNA. The DNA can be stored at 4°C for up to 2 weeks during analysis or stored at -20°C until required, noting that the DNA must only be thawed once.

APPENDIX 4.4 FOOD STANDARDS AGENCY: STANDARD OPERATING PROCEDURE FOR ANALYSIS OF PCR INHIBITORS IN MEAT SAMPLES USING REAL-TIME (TAQMAN™) PCR

The level and presence of PCR inhibitors in the DNA extracts should be assessed using an appropriate assay. The CSL uses the Applied Biosystem kit exactly according to the manufacturers instructions. Dilution of DNA are assessed for the presence of inhibitors at 10, 30 and 100 fold dilutions in water. The lowest dilution, found to be free from PCR inhibitors is subsequently used in real-time PCR assays.

Amplification should occur in the water wells. These Ct values should be averaged and regarded as the 'no inhibitor present' results, to which the DNA samples are compared. Inhibitors are present if the efficiency of the reaction is reduced (flatter curve in exponential plot) and/ or the Ct values are higher by 3 S.D. or higher than 0.5 Ct or higher than 103% compared to the 'no inhibitor present' result.

APPENDIX 4.5 FOOD STANDARDS AGENCY: STANDARD OPERATING PROCEDURE FOR ANALYSIS OF MEAT SAMPLES USING REAL-TIME (TAQMAN™) PCR

Introduction

Real-time (TaqMan™) PCR is used for a variety of applications. This SOP describes how to set up all reactions irrespective of the primers to be used.

Precautions

To limit contamination of pre-PCR samples with DNA, especially amplified DNA, special precautions must be taken. Laboratory coats solely for use in the PCR laboratory must be worn instead of normal laboratory coats.
The PCR mastermix must be prepared in a DNA free cabinet or DNA free designated area.
DNA must be aliquoted in the PCR workstation and at no time should DNA be placed in the DNA free cabinet or DNA free designated area.
Barrier or positive displacement tips must be used for all steps and pipettes must not be swapped between the PCR workstation and the DNA free cabinet or DNA free designated area.

Reagents

Molecular biology grade sterile water (Sigma W-4502)
2x Taqman Universal PCR Master Mix (Applied Biosystems 4304437)
Primers and probes (aliquoted and stored frozen, see SOP Preparation of reagents)
DNA samples (including DNA extracted from control material)- to be kept separate from PCR reagents

Equipment

Base plate (E2396)
UV cross-linker (UVP CL -1000), or UV light source designed for decontamination
DNA free cabinet
PCR workstation (erlab Biocap DNA/RNA)
10, 20, 200 and 1000µl Barrier filter tips (sterile) (Starlab S1121-3810, S1120-1810, S1126-7810, S1120-8810)
1.5 ml Microcentrifuge tubes (autoclaved) (Starlab S1615)
200µl thin walled PCR tubes or semi-skirted plates (Thistle Scientific Cat. No. PCR-0208-C, PCR-96M2-HS-C)
Optical lids (Thistle Scientific Cat. No. PCR-2CP-RT-C)
TaqMan (ABI 7700 or 9700)
P2, P20, P200 and P1000 Gilson pipettes or equivalent (for example M1000, M100, M25 or M10 Microman positive displacement pipettes)
Cap installing tool (ABI 4330015)
Microcentrifuge (Stratagene 400551)
Axigen low retention microcentrifuge tubes - 1.5ml (autoclaved) (Thistle Scientific Cat. No. MCT-175-L-C)

Method

1. Irradiate a TaqMan 96 well plate and the plate cover with UV for 20 min in the UV crosslinker.At the same time irradiate the DNA workstation for at least 30 min.

2. Each TaqMan run must include positive and negative controls run alongside the unknown samples. Construct a template, detailing the location of the duplicate wells of each sample and controls on the TaqMan plate.

3. Label one Axygen microcentrifuge tube for each sample for DNA dilution plus one tube for each mastermix to be prepared. Place labelled tubes in a rack.

4. Defrost the primers, probes and TaqMan core reagents, mix by flicking and centrifuge on pulse for 20sec and place on ice.

5. Defrost the DNA from the meat samples and mix by flicking. Centrifuge on pulse for 20 sec and place on ice. Dilute the sample DNA in water according to the results of the inhibition test (Standard operating procedure for the Internal positive control assessment of inhibition within the Polymerase Chain Reaction using real time PCR), mix by flicking and centrifuge on pulse for 20 sec. Dilute the positive control DNA appropriately

6. Make up a working master mix solution in the 1.5 mL Axigen microcentrifuge tubes. Examples are given in Appendix I. The amounts shown are for each reaction, the number of reactions to be prepared may be calculated thus:

[(number of samples to be tested + 1 negative control + 1 positive control)x2] +3 extra -this equation may need be altered to take in to account any extra controls from Steve's SOP addition.

Perform all remaining steps in the DNA workstation.

7. Place the TaqMan reaction plate onto the 96 well base plate.

8. Using a pipette aliquot 20µL of the master mix into all the required wells of the TaqMan plate (as defined in the plate layout template).Cover the rest of the plate with the plate cover to avoid inadvertently contaminating any open wells.

9. When complete, cover with the plate cover and set to one side.

10. Add 5µL of positive control, water as negative control or sample DNA with a pipette to quadruplicate wells on the TaqMan plate according to the plate

layout template. While doing this cover the rest of the plate with the cover so that you are not working over open wells. Put caps onto the wells.

11. When all samples and control DNA have been aliquoted, press the caps down using the cap-installing tool until the wells no longer ‘crackle’.

12. Mix the plate at least 5 times by inversion then flick down to make sure that samples are in the working master mix solutions and all the solution is in the bottom of the plate.

13. Put the plate into the TaqMan machine in the correct orientation, tighten the machine lid.

14. Open the Sequence Detector Software. Under FILE, choose NEW PLATE. Toggle between SHOW SETUP and SHOW ANALYSIS to designate all wells FAM POSITIVE and to insert the correct cycling parameters (see Appendix II) and sample volume.

15. Press RUN to start the machine.

Calculation and expression of results

1. After the completion of the run, designate the used wells as ‘FAM’ positive and unused wells as ‘not in use’. In log ΔRn (TaqMan operators manual) change the upper baseline limit to one Ct below where the earliest curve begins to exponentially increase and move the Ct threshold to the exponential part of the amplification curve.

2. Press the update button to ensure changes affect Ct values.

3. Export the results into the Excel® spreadsheet.

4. Remove any data points where amplification has failed. If the water blank gives a positive result the plate must be rejected and rerun.

Addendum I to Appendix 4.5 Mastermix composition

Specificity	Components	x1 (µl)
Deer group assay	2x Universal PCR Master mix	12.500
	Red Sense primer (20µM) (forward)	0.375
	Red Antisense primer (20µM) (reverse)	0.375
	Sika Sense primer (20µM) (forward)	0.375
	Fallow Roe Sense primer (20µM) (forward)	0.375
	Fallow Roe Antisense primer (20µM)	0.375
	Muntjac Sense primer (20µM)	1.125
	Muntjac Antisense primer (20µM)	1.125
	Probe (5µM)	1.000
	Water	2.375
Duck group assay	2x Universal PCR Master mix	12.500
	Mallard Sense primer (20µM)	0.375
	Mallard Antisense primer (20µM)	0.375
	Muscovy Sense primer (20µM)	0.375
	Muscovy Antisense primer (20µM)	0.375
	Probe (5µM stock)	1.000
	Water	5.000
Pheasant	2x Universal PCR Master mix	12.500
	Phesasant Sense primer (20µM)	0.375
	Pheasant Antisense primer (20µM)	0.375
	Probe (5µM stock)	1.000
	Water	5.75
Horse	2x Universal PCR Master mix	12.500
	Horse Sense primer (20µM)	1.125
	Horse Antisense primer (20µM)	1.125
	Probe (5µM stock)	1.000
	Water	4.250
Donkey	2x Universal PCR Master mix	12.500
	Donkey Sense primer (20µM)	1.125
	Donkey Antisense primer (20µM)	0.375
	Probe (5µM stock)	1.000
	Water	5.000

Addendum 2 to Appendix 4.5 TaqMan cycling parameters

Step	Function	Temperature	Time
1	AmpErase UNG reaction	50°C	2 min
2	Initial denaturation	95°C	10 min
3 (40 cycles)	Denaturation	95°C	15 sec
	Annealing/extension	60°C	1 min

Addendum 3 to Appendix 4.5 Primer and probe sequences

Oligonucleotide	Sequence 5' – 3'
Red Sense primer	ATC CTT ATT ATA CCA TTA AAG ATA TCT TAG GCA T
Red Antisense primer	GCA AAT AGG AAA TAT CAT TCA GGT TTA ATA
Sika Sense primer	CCA TCC TTA CTA TAC TAT TAA AGA T
Fallow Roe Sense primer	CCT ACT ACA CCA TTA AAG AT
Fallow Roe Antisense primer	GTT GGG AAT TGA TCG TAG GAT T
Muntjac Sense primer	CCT ACT ACA CCA TCA AAG AT
Muntjac Antisense primer	GTT AGG GAT AGA TCG TAG AAT T
Deer probe	FAM-CAG CAA ATC CAC-MGB
Mallard Sense primer	TCC TAC CTG TAT AAA GAA ACT TGA AAT
Mallard Antisense primer	GGG CTG AAA ATA AGT TGG TAA TT
Muscovy Sense primer	TCC TAC CTG TAC AAA GAA ACC TGA AAT
Muscovy Antisense primer	GGG CTG AGA ACA GGT TGG TAA TT
Duck Probe	FAM-TAG GCT ATG TCC TGC CAT G -MGB
Phesasant Sense primer	TCG GAC GCG GCC TCT AT
Pheasant Antisense primer	TGA GTG TGA GGA GTA GGA CG
Pheasant Probe	FAM-TTACCTGTACAAAGAGACATGA -MGB
Horse Sense primer	GAA GCA TAA TAT TCC GG
Horse Antisense primer	TTA GTG TCA GTA AGT CTG CC
Horse Probe	FAM-TCT CAG CCA ATG CGT-MGB
Donkey Sense primer	CCT TAT CCT TTC CAT CT
Donkey Antisense primer	GTA AGT CTG CTA CTA AGA GTC AGA AC
Donkey Probe	FAM-AAT CCT AGC ACT CAT CC-MGB