The adaptation and validation of real-time PCR methods for the identification of exotic species, for analysis on a capillary electrophoresis chip system

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FINAL REPORT

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ABBREVIATIONS

bp	base pairs
CCFRA	Campden Chorleywood Food Research Association
CSL	Central Science Laboratory
CTAB	Cetytrimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphates
EDTA	Ethylenediaminetetraacetic acid
FU	Fluorescence units
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
LOD	Limit of detection
mCyt *b*	Mitochondrial cytochrome b gene
PCR	Polymerase chain reaction
TE	Tris-EDTA buffer
Tris	Tris(hydroxymethyl)aminomethane
VLA	Veterinary Laboratories Agency
w/w	Weight for weight

EXECUTIVE SUMMARY

The objective of this study was to adapt and validate 10 species-specific real-time PCR assays for use on the Agilent BioAnalyser. The assays, for the detection of horse, donkey, duck, deer, pheasant, turkey, chicken, pig, cow and sheep, had been developed during projects previously funded by the Food Standards Agency. The assays were to be easy to use and applicable for use by Public Analysts and other laboratories which did not have real-time PCR capability, but which did have the BioAnalyser. 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 fully met, with assays specific for the detection of all target species successfully adapted and validated for use on the BioAnalyser. The adaptation did not turn out to be as straight forward as it seemed at first, since 5 of the 10 assays needed to be redesigned. Nevertheless, these new assays were found to be specific and sensitive following optimisation. The assays were validated using a variety of matrices and were found to be able to detect all species when assessed against raw meat samples spiked at 0.5%. The assays were also found to be capable of detecting their target species in commercial products which had been processed, including salami and pate samples. However, the assays were found to be relatively insensitive for the analysis of canned samples. A limited range of canned samples (5, 10, 90 and 95% target species) where assessed and it was found that the DNA in these samples was too degraded for the detection of target species below 10%, although target species could be detected when present above 90%.

In conclusion, the project successfully adapted and validated assays for the detection of 10 meat species using the Agilent BioAnalyser, providing a comprehensive standard operating procedure to facilitate technology transfer to the Public Analysts and other laboratories.

1.0 INTRODUCTION

The identity of meat in meat food products must be accurately declared according to food labelling regulations. 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. DNA has the advantage of being a relatively stable molecule, and is more able to withstand heat and pressure processing when compared to protein, and has become the analyte of choice for meat species identification.

DNA methods have commonly been based around the use of species-specific primers in PCR followed by signal detection using gel electrophoresis (Meyer, *et al*., 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.,* 2005). 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. However, real-time PCR equipment is expensive and beyond the means of most Public Analyst laboratories. A recent innovation is the use of the capillary chip system for the analysis of amplicons, post PCR, replacing conventional gel electrophoresis (Dooley *et al*., 2005). The Food Standards Agency has embraced this technology and has successfully introduced it's use for the identification of fish species, to a number of Public Analyst laboratories. The Food standards Agency wished to build on this success by transferring other PCR based methodologies onto the chip system, thereby increasing the range and scope of assays available to the Public Analysts.

This report outlines the adaptation of 10 species-specific real-time PCR assays for use on the Agilent BioAnalyser detailing the adaptation process and the validation of each assay. The report also includes a comprehensive standard operating procedure for use by Public Analysts and other laboratories.

2.0 RESULTS AND DISCUSSION

The overarching theme for the project was to produce a set of assays which could be easily applied to the analysis of complex meat samples by the Public Analysts. The specific aim of the project was to adapt a suite of real-time PCR assays for use on the Agilent BioAnalyser. This appeared to be a relatively straight forward task since the two systems are both PCR based. However, different parameters needed to be taken into account for the two systems. For example, the amount of amplicon produced is immaterial in real-time PCR but has a large impact on the limit of detection for BioAnalyser-based assays; the size of amplicons in a multi-primer assay has no effect on detection in real-time PCR, but produces complicated electropherograms on the BioAnalyser.

The adaptation of the assays for use on the BioAnalyser involved optimisation of the assay conditions. The aim however, was to make the conditions for all BioAnalyser assays as constant as possible so that the assays could be performed in parallel, preferably in the same PCR machine. All BioAnalyser assays were transferred to using Qiagen multiplex PCR mastermix, a 2x master mix containing HotStarTaq DNA polymerase, multiplex PCR buffer, dNTP and 6mM $MgCl_2$, with generic cycling conditions (Taq activation at 94^{o}C for 15 minutes, followed by 30 or 40 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 60^{o}C for 90 seconds and extension at 72^{o}C for 90 seconds) suggested by Qiagen. Consequently, all BioAnalyser assays could be performed using one of two PCR cycling programs and a master mix which contained everything apart from primers and DNA template. However, the corollary of this was that the parameters available for optimisation were limited and effectively only included altering the concentration of the primers and/or the template, the number of cycles, or the addition of Q-sol, a propriety additive for this master mix which facilitates the amplification of difficult templates by modifying the melting behaviour of DNA.

On a few occasions, assays were found to cross amplify the DNA from non-target species. Where the assay conditions could not be amended to negate this cross amplification, assay re-design was required. The new assays were designed to conform to the generic assay conditions used for the other assays as far as possible. Amplicon size was maintained below 350bp, a maximum size of amplicon shown in a previous FSA-funded project required for efficient amplification in heat and pressure processed food items (Q01070).

The work to adapt each real-time PCR species specific assay for use on the Agilent BioAnalyser is outlined below.

2.1 Adaptation of species specific real-time PCR assays for use on the Agilent BioAnalyser.

2.1.1 Duck

The adaptation of the real-time PCR duck-specific assay for use on the Agilent BioAnalyser was relatively straight forward. Initially, the primers were assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from Mallard, Gressingham, Norfolk and French Barbury duck meat. It was found that there was good amplification of all four duck species (all at 350 FU) with peaks at the expected size (149, 147, 147 and 150bp respectively with additional peaks of 20bp and 154bp in Mallard, Gressingham and Norfolk with the latter also having a peak sized 165bp), which was slightly improved with the addition of Q-sol to the master mix where all amplified at 300 FU with an additional peak of 154bp in Mallard, Gressingham and Norfolk (Figure 1a-d). The BioAnalyser duck assay was also found to have remained specific in this format, when assessed against a range of other meat types (Table 1).

2.1.2 Horse

The adaptation of the real-time PCR horse-specific assay for use on the Agilent BioAnalyser was also relatively straight forward. Again, the primers were assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from horse meat. It was found that there was amplification of the horse DNA, with peaks at the expected size (82bp). The amplification was inefficient, with peaks heights of only 175 FU, which was only slightly improved with the addition of Q-sol to the master mix (200 FU). Amplification at this level, on 100% horse template DNA, was too low, considering the target LOD, for this assay, was 0.5% w/w. The generic cycling conditions recommended by Qiagen stipulate 30-45 cycles and 30 cycles were used as a starting point for amplification during the adaptation of these assays. Since the amplicon was present, but at a relatively low level, the number of cycles was increased to 40, which resulted in greatly improved peak heights, thereby reducing the LOD. The BioAnalyser horse assay was found to have remained specific in this format when assessed against a range of other meat types (Table 1), the increase in cycle number did not reduce the specificity of the assay.

2.1.3 Donkey

The adaptation of the real-time PCR donkey specific assay for use on the Agilent BioAnalyser was again, also relatively straight forward. As with the duck and horse assays, the primers were assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from donkey meat. It was found that there was amplification of the donkey DNA, with peaks at the expected size (128bp) of approximately 200FU, which was decreased by the addition of Q-sol to the master mix (75 FU). The concentration of the sense and anti-sense primers for the real-time PCR donkey assay, determined during project Q01083 to give the most sensitive assay in real-time PCR, are different. It was not clear whether this difference in concentration needed to be perpetuated in the BioAnalyser donkey assay: simplification of the primer concentrations would be a benefit in terms of ease of use in the future for the Public Analysts. Therefore, the use of a single primer concentration for both sense and anti-sense primers was investigated using combinations of primers at lower concentrations. It was found that there was the greatest amplification at the original primer concentrations with reduced amplification at all other combinations (Figure 1e). It was decided, therefore, to use the original primer concentrations for the optimised assay conditions.

The BioAnalyser donkey assay was also found to have remained specific in this format when assessed against a range of other meat types (Table 1).

2.1.4 Cow

As with the previous assays, the cow primers were assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from cow meat. It was found that there was good amplification of cow template DNA with a peak at the expected size (70bp), which was not improved with the addition of Q-sol to the master mix (Figure 1f). The BioAnalyser cow assay was also found to have remained specific in this format, when assessed against a range of other meat types (Table 1).

2.1.5 Pheasant

The adaptation of the real-time PCR pheasant specific assay for use on the Agilent BioAnalyser was not as straight forward as that for the real-time PCR duck, horse, donkey and cow assays. As with the previous assays, the pheasant primers were assessed for effective amplification using Qiagen Multiplex PCR master mix, generic

cycling conditions recommended by Qiagen, and template DNA purified from pheasant meat. It was found that there was amplification of the pheasant DNA, with a peak at the expected size (89bp), of approximately 400 FU: this was a very high level of amplification, indicative of an assay with very high sensitivity and an LOD lower than the target LOD of 0.5% w/w, although the primers were found to be specific for pheasant. Combinations of primers at lower concentrations were assessed for amplification efficiency, and it was found that the rate of amplification actually increased, giving a peak height of 400 FU on template DNA extracted from samples of 0.1% w/w pheasant in a meat background. Since the parameters available for optimisation were limited it was decided to effect an LOD of 0.5% w/w by dilution of the amplicons, prior to analysis on the chip (this will be covered in section 2.2).

2.1.6 Turkey

Conversely, the adaptation of the real-time PCR turkey-specific assay for use on the Agilent BioAnalyser presented a different set of problems. Initially, the primers were assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from turkey meat. It was found that there was good amplification of turkey DNA (300FU) with a peak at the expected size (89bp), but which also cross-amplified template DNA purified from chicken, horse and cow. Despite attempts to eradicate this cross-amplification using the limited parameters available for optimisation, the primers still amplified these three species. It was therefore decided to redesign the primers using the mitochondrial cytochrome *b* (mCyt *b)* gene sequence. Primers were designed to mismatch all other non-target species at the 3' position for both the sense and anti-sense primers (Hird *et al*., 2003) (Figure 2). A primer pair was identified which, when assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen and template DNA purified from turkey meat, was found to give extremely good amplification of turkey DNA (1000FU) with a peak at the expected size (320bp) (Figure 1g). Interestingly, the peak height was found to be reduced by the addition of Q-sol to the master mix. As with the BioAnalyser pheasant assay it was decided to effect an LOD of 0.5% w/w by dilution of the amplicons, prior to analysis on the chip (this will be covered in section 2.2). The new primers were found to be immediately specific, and needed no further optimisation.

2.1.7 Pig

A similar problem was encountered during the adaptation of the real-time PCR pig-specific assay for use on the Agilent BioAnalyser as was found with the turkey assay: the pig specific primers were found to give good amplification of the target DNA (200FU) when assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from pig meat. The peak was at the expected size (70bp); however, there was cross amplification of template DNA purified from fallow deer meat. Upon analysis of the sequence, it was found that the DNA sequences for the pig sense and anti-sense primers matched the DNA sequence for the fallow deer closely (Figure 3). The pig real-time PCR assay was designed during project QO1033 and was, in fact, designed for a completely different application: assessment of the effects of heat and pressure on DNA fragmentation, and not for the identification of pig *per se*. These primers were therefore redesigned using the mCyt *b* gene sequence. Primers were designed to mismatch all other non-target species at the 3' position for both the sense and anti-sense primers (Figure 4). A primer pair was identified which, when assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from pig meat, was found to give good amplification of pig DNA (in excess of 500FU) with a peak at the expected size (225bp). Following initial screening of this primer set using the DNA from a range of species as template, it became apparent that the primers also cross amplified the non-target species muntjac, horse and fallow despite the 3' mismatches. This was unexpected since no cross amplification had occurred with species which had higher degrees of sequence homology to the pig sequence.

As with the optimisation of the other assays previously, the parameters available were limited. 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 cycling conditions. Following significant levels of truncation of sense and anti-sense primers, pig specific primer combinations were identified (Tables 2 & 3).

2.1.8 Chicken

The adaptation of the real-time PCR chicken specific assay for use on the Agilent BioAnalyser suffered a similar series of problems as the pig assay. Again this assay was designed during project Q01033 to study the effects of heat and pressure on

DNA fragmentation. Not surprisingly then, the chicken real-time PCR specific primers were found to give good amplification of the target DNA when assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from chicken meat (400 FU), with a peak at the expected size (72bp). However, this was also found to cross amplify template DNA purified from turkey, horse and cow meat. It was therefore decided to redesign the primers using the mCyt *b* gene sequence. Primers were designed to mismatch all other non-target species at the 3' position for both the sense and anti-sense primers (Figure 5). A primer pair was identified which, when assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from chicken meat, was found to give good amplification of chicken DNA (500 FU) with a peak at the expected size (132bp). Following initial screening of the chicken primer set using the DNA from a range of species as template, it became apparent that the primers also cross amplified the non-target species turkey.

As with the optimisation of the other assays previously, the parameters available were limited. Again we used the strategy outlined in Hird *et al.* (2004) to increase the specificity of the primers by truncation at the 5' position. Following significant levels of truncation of sense and anti-sense primers, specific primer combinations were identified, with a new peak size of 120bp (Tables 2 & 3).

2.1.9 Deer

The adaptation of the deer-specific real-time PCR assay presented a set of unique challenges. The real-time PCR assay was complicated, using 3 sense and 2 anti-sense primers to enable complete species coverage for the 5 target species: Roe, Fallow, Muntjac, Sika and Red deer. The assay had originally been designed during project Q01083 using TaqMan technology where the size of the amplicons plays no role in the analysis of the results. However, when these amplicons were analysed on the Agilent BioAnalyser there were peaks at 168, 180, 190 and 200bp: creating a complicated electropherogram. Additionally, the amplification of red and roe deer template DNA was found to be relatively inefficient, giving peak heights of approximately 50FU, whereas the other deer species produced peak heights of 300 FU (fallow and sika deer) and 500 FU (muntjac deer). Taking these problems together it was decided to redesign the primers using the mCyt *b* gene sequence. Primers were designed for each target deer species, using the same region of DNA, to mismatch all other non-target species at the 3' position for both the sense and anti-

sense primers (Figure 6). These 5 sets of primers were assessed, in combination, for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from deer meat. The number of primers needed to produce an effective assay was eventually reduced to using just 2 sense and 2 anti-sense primers in a single multiplex PCR (Table 2, Figure 1g-k). Amplification of deer template DNA gave peak heights ranging from 100-200FU, with no apparent cross amplification of non-target species.

2.1.10 Sheep

The design of a lamb specific assay had never been included in any of the previous FSA projects conducted at CSL. The initial work therefore, focused on an assessment of the suitability of two available real-time PCR assays specific for lamb, designed by Steve Garrett at Campden Chorleywood Food Research Association (CCFRA) and Ginny Saunders at the Veterinary Laboratories Agency (VLA), during other FSA funded projects. Both primer pairs were found to give good amplification of the target DNA when assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen and template DNA purified from lamb meat (300 FU and 400 FU for CCFRA and VLA assays respectively) with peaks at the expected sizes (132bp and 110bp for CCFRA and VLA assays respectively). However, the CCFRA primer pair was also found to cross amplify template DNA purified from horse, donkey, deer, pheasant, turkey, chicken and pig whereas the VLA assay cross amplified template DNA purified from turkey and sika deer meat. The CCFRA primer set was designed using the GAPDH gene, a genomic gene and therefore single copy, which may have reduced assay sensitivity. Additionally, upon analysis of the DNA sequence it was found that the primers for the VLA assay closely matched the sequence for sika deer (Figure 7) and, therefore, no amount of optimisation would negate amplification of sika template DNA by these primers. It was therefore decided to redesign the primers using the mCyt *b* gene sequence. Primers were designed to mismatch all other non-target species at the 3' position for both the sense and anti-sense primers (Figure 8). A primer pair was identified which, when assessed for effective amplification using Qiagen Multiplex PCR master mix, generic cycling conditions recommended by Qiagen, and template DNA purified from lamb meat, was found to give good amplification of lamb template DNA (1000 FU) with a peak at the expected size (190bp). Following initial screening of this primer set using the DNA from a range of species as template, it became

apparent that the primers also cross amplified the non-target species cow, muntjac and red deer.

Again, as with the optimisation of the previous assays, the parameters available were limited and we opted to use the strategy outlined in Hird *et al.* (2004) to increase the specificity of the primers by truncation at the 5' position. Following significant levels of truncation of both sense and antisense primers, specific primer combinations were identified when assessed against a range of species (Tables 2 & 3).

2.2 Limit of detection and multiplex analysis of amplicons

The limit of detection of the BioAnalyser assays needed to be approximately 0.5% w/w target meat species in non-target background (meat or plant species). The LOD was set at 0.5% (w/w) to ensure that detection of the target meat species would be as a result of the true presence of the target species rather than adventitious contamination. The assays all had slightly different characteristics, however it was found that by adjusting the cycling conditions, the amount of template used and/or the level of dilution prior to analysis on the chip, an LOD of 0.5% (w/w) could be effected for all assays, when a cut-off value of 50 FU was used. Moreover, the use of a dilution factor facilitated multiplexing of amplicons for analysis on the chips. For example the pheasant amplicons could be multiplexed with the chicken amplicons in a ratio of 2:3 and the deer and cow amplicons could be mixed 1:1, whilst still maintaining the LOD at 0.5% w/w. The optimised strategies used for each assay are given in Table 4. These dilution factors are pertinent to the equipment available at CSL and it is likely that they will need slight adjustment when transferred to the Public Analysts laboratories since the PCR equipment will be slightly different, however it is anticipated that only slight adjustments will be required to achieve an LOD of 0.5% w/w.

2.3 Application of BioAnalyser assays to model and commercial samples

The optimised BioAnalyser assays were used to test DNA extracted from a range of samples, including model samples composed of the target species, canned samples containing 5% or 10% w/w pig or turkey or 10% w/w chicken in a meat background and commercial samples containing unknown quantities of named species (information derived from the ingredients list) (Table 5). The assays were able to detect their target species in the majority of the samples. However, the assays were not found to be able to detect turkey, pig, cow or chicken at the 10% level in the

canned samples, although pig was detected in all samples (present at 90 and 95% w/w). These samples were produced for project Q01033, in which real-time PCR assays for pig, chicken, turkey and cow were developed. The real-time PCR assays were easily able to detect their target species in the canned samples at the 5% level and therefore the BioAnalyser assays are not as sensitive as the equivalent real-time PCR assays and are not applicable to the detection of canned meat species.

The assays worked well on the commercial samples, and in the main, detected the species which were designated on the product label. The deer assay failed to detect any deer in one of the venison salami samples, however the actual species of deer used was not stipulated on the label and could therefore have been a species not covered by the deer assay. The cured venison sample was found to contain only cow, with no detection of venison by the deer assay. In this case there could have been a straight substitution of venison with cow. Chicken was not detected in the pheasant pate, although both pheasant and duck were detected. It is not known how much chicken was actually contained in this sample however, which could have been below the LOD of the assay.

2.4 Analysis of operator blind samples

Operator blind samples were prepared and analysed by different analysts. The results are given in Table 6. There was just one instance where the sample was incorrectly identified: sample 8 was found to contain chicken, although the amount in the sample was below the LOD for the assay at 0.25% w/w, the peak height of 70FU clearly indicated the presence of chicken in the sample, although the peak height was relatively close to the 50FU cut-off for detection.

The analysis of the electrophoretograms is the most subjective part of the whole procedure, although the application of a cut-off for detection at 50FU reduces this subjectivity. The analysis of the electrophoretograms was further assessed using 7 different analysts at CSL, who were not familiar with the BioAnalyser, to analyse the electrophoretograms from 8 samples, with varying peak heights. It was found that these untrained analysts could easily distinguish between peaks above and below the cut-off threshold and when given a list of the peak sizes were able to correctly identify the species present (Table 7). Only one sample was called incorrectly by one analyst: sample 3, where the analyst correctly identified the presence of turkey and duck, but attributed the peak at 120bp to cow, instead of chicken. This appears to be an error since cow has a peak at 70bp and they correctly identified all other peaks.

3.0 CONCLUSION

Sensitive and specific assays have been adapted and validated for the detection of the commercially important meat species horse, donkey, duck, deer, pheasant, turkey, chicken, pig, cow and lamb, using the Agilent BioAnalyser. The assays were validated using a variety of matrices and were found to be able to detect all species when assessed against raw meat samples spiked at 0.5%. The assays were also found to be capable of detecting their target species in commercial products which had been processed, including salami and pate samples. However, the assays were found to be relatively insensitive for the analysis of canned samples. A limited range of canned samples (5, 10, 90 and 95% target species) where assessed and it was found that the DNA in these samples was too degraded for the detection of target species below 10%, although target species could be detected when present above 90%.

In conclusion, the project successfully adapted and validated assays for the detection of 10 meat species using the Agilent BioAnalyser, providing a comprehensive standard operating procedure to facilitate technology transfer to the Public Analysts and other laboratories.

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Table 1. Range of species used to determine specificity for BioAnalyser species-specific assays

Species	Species scientific name
Muscovy duck	*Cairina moschate*
Mallard, Aylesbury and Gressingham duck	*Anas platyrhynchos*
Turkey	*Meleagris gallopavo*
Chicken	*Gallus gallus*
Pig	*Sus scrofa*
Cow	*Bos Taurus*
Sheep	*Ovis aries*
Pheasant	*Phasianus colchicus*
Horse	*Equus caballus*
Donkey	*Equus asinus*
Red deer	*Cervus elaphus*
Fallow deer	*Cervus dama*
Roe Deer	*Capreolus capreolus*
Muntjac deer	*Muntiacus muntjak*
Sika deer	*Cervus Nippon*

Table 2 Final primer sequences for each species-specific BioAnalyser assay

Assay	**Primer name**	**Sequence 5' – 3'**	**Size bp**
Chicken	Ch sense Ch anti-sense	GGCTCCTACCTCTACAAG CCTCAGAATGATATTTGG	120
Turkey	T sense T anti-sense	TCAGGCTCAAACAATCCTCTT TGAAGGAAGGGGATAAGGAGA	320
Pheasant	Ph sense Ph anti-sense	TCGGACGCGGCCTCTAT TGAGTGTGAGGAGTAGGACG	89
Duck	Du1 sense Du2 sense	TCCTACCTGTATAAAGAAACTTGAAAT TCCTACCTGTACAAAGAAACCTGAAAT	147, 149 & 150
	Du3 anti-sense Du4 anti-sense	GGGCTGAAAATAAGTTGGTAATT GGGCTGAGAACAGGTTGGTAATT	
Horse	H sense H anti-sense	GAAGCATAATATTCCGG TTAGTGTCAGTAAGTCTGCC	82
Donkey	Do sense Do anti-sense	CCTTATCCTTTCCATCT GTAAGTCTGCTACTAAGAGTCAGAAC	128
Deer	V1 sense V2 sense	TCTTAGGCATCTTACTTCTAGTACTCT TATTCTAGGTGTCCTACTTCTAATTCTCT	94, 96 & 102
	V3 anti-sense V4 anti-sense	CTGGGGTATAGTTGTCTGGA TGTAGTTGTCGGGGTCTCCA	
Cow	Co sense Co anti-sense	CACGAAACAGGCTCC TGGAATGGGATTTTGTC	70
Pig	Po sense Po anti-sense	CTATTCATCCACGTAGGC AGATTCATTCTACGAGGTC	225
Sheep	Sh sense Sh anti-sense	ATCCTCCTATTTGCGAC AAATCGGGTGAGGGTA	190

Table 3 Effect of primer truncation on assay specificity

Assay	Primer truncation*		Target species FU	Cross amplifying species & FU
	Sense	Anti-sense		
Pig	0	0	>200	horse (>50 FU) muntjac (>300 FU) fallow deer (>200 FU)
	-2	-3	>200	muntjac (>100 FU)
	-2	-4	>200	none
Chicken	0	0		turkey (FU)
	-7	-2	>300	turkey (>40 FU)
	-7	-3	>150	none
Sheep	0	0	600	cow (80 FU) muntjac (300 FU) red deer (300 FU)
	-3	-3	300	muntjac (150 FU) red deer (100 FU)
	-5	-3	300	muntjac (>20 FU) red deer (>50 FU)
	-6	-4	>200	none
	-7	-4	>200	none

*Negative figures indicate the number of bases removed from the ‘5 end of the original primer.

0 indicates full length primer

Table 4. Parameters to effect an LOD of 0.5% w/w for each assay

Assay	µl template/PCR reaction	Number of PCR cycles	Dilution factor prior to analysis
Horse	5	40	x2
Donkey	6	40	no dilution
Duck	5	30	x6
Deer	5	40	x2
Pheasant	5	30	x2.5
Turkey	5	30	x6
Chicken	5	40	x1.6
Pig	5	30	x1.5
Cow	5	30	x2
Sheep	5	40	x3

Table 5 Analysis of model and commercial samples using the optimised BioAnalyser species-specific assays

Sample type	Sample number/ type	chicken	pig	turkey	cow	donkey	pheasant	sheep	duck	deer	horse
Canned	5% turkey 95% pig		✓	×							
	10% turkey 90% pig		✓	×							
	95% turkey 5% pig		×	✓							
	90% turkey 10% pig		×	✓							
	90% pig 10% chicken	×	✓								
	90% pig 10% cow		✓		×						
Commercial samples	venison salami 1				×					✓	
	venison salami 2		✓							×	
	horse salami 1		✓								✓
	horse salami 2		✓								✓
	game and stuffing pie		✓							✓	
	raw beef sausages				✓						
	minced beef and onion				✓						
	pheasant pate	×					✓		✓		
	duck pate		✓								
	cured venison				✓						
	pork salami		✓								

✓ denotes correct detection, ✓ denotes incorrect detection, × denotes incorrect absence of detection

Table 6. Results of the analysis of operator blind samples

Sample composition	chicken	pig	turkey	cow	donkey	pheasant	sheep	duck	deer	horse
0.5% lamb, 0.5% duck, 99% deer							✓	✓	✓	
99% chicken, 0.5% lamb, 0.5% duck	✓						✓	✓		
7% horse & 7% donkey					✓					✓
2.5% turkey, 2.5% cow, 95% pig		✓	✓	✓						
0.05% pheasant, 0.5% fallow deer & 99.45% pig		✓							✓	
1.25% pig, 50% horse & 48.75% cow		✓		✓						✓
1% pheasant, 1% horse, 4% duck, 6% deer, 1% donkey & 87% pig		✓			✓	✓		✓	✓	✓
0.25% sheep, 0.25% duck, 0.25% chicken 0.25% beef & 99% pig	✓	✓								

✓ denotes correct detection, ✓ denotes incorrect detection

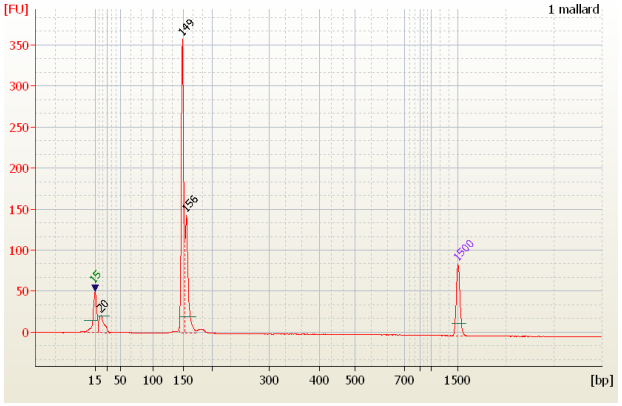
Table 7 Results of data analysis exercise by 7 analysts

Sample number and composition	Analyst						
	1	2	3	4	5	6	7
1 5% cow & 1% fallow deer	✓	✓	✓	✓	✓	✓	✓
2 7% donkey	✓	✓	✓	✓	✓	✓	✓
3 0.1% pheasant, 1% duck, 5% chicken and 5% turkey	✓	✓	✓	×	✓	✓	✓
4 10% donkey	✓	✓	✓	✓	✓	✓	✓
5 0.5% pig & 0.25% cow	✓	✓	✓	✓	✓	✓	✓
6 0.5% donkey	✓	✓	✓	✓	✓	✓	✓
7 0.1% fallow deer	✓	✓	✓	✓	✓	✓	✓
8 0.1% sheep	✓	✓	✓	✓	✓	✓	✓

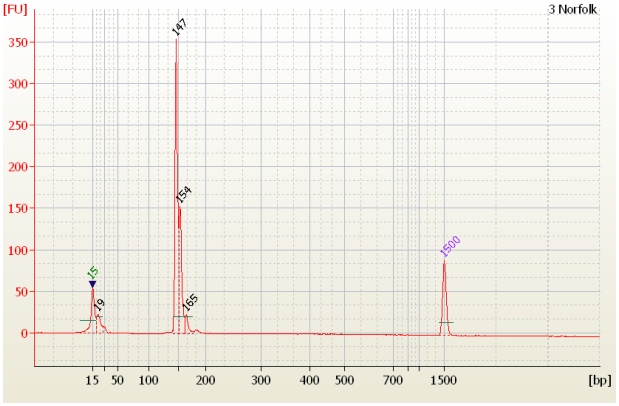
✓ denotes correct interpretation, × denotes incorrect interpretation

Figure 1. Electropheretograms of peaks for BioAnalyser assays

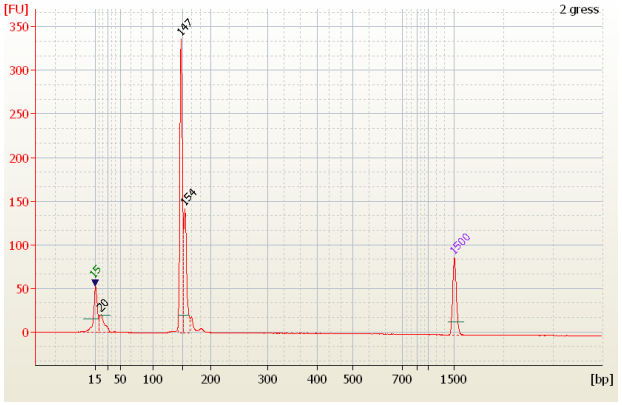
a) Duck assay using Gressingham duck template DNA



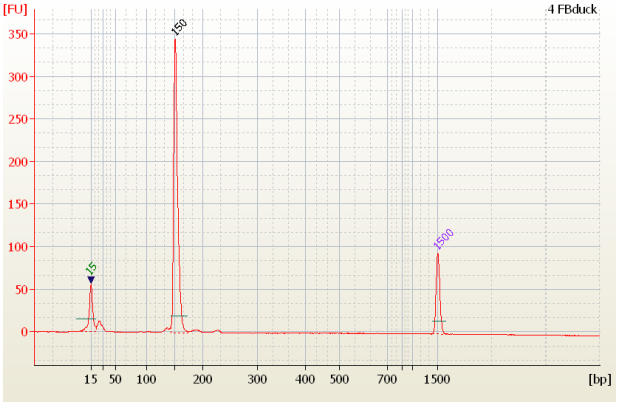
b) Duck assay using Norfolk duck template DNA



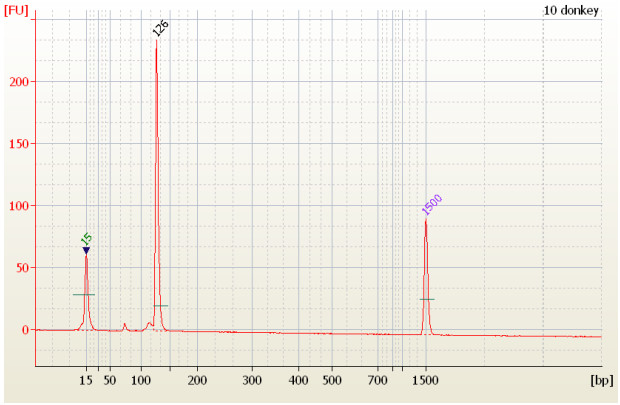
c) Duck assay using mallard duck template DNA



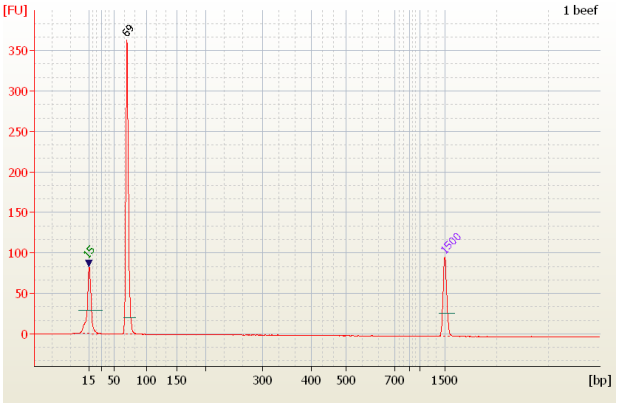
d) Duck assay using French barbary duck template DNA



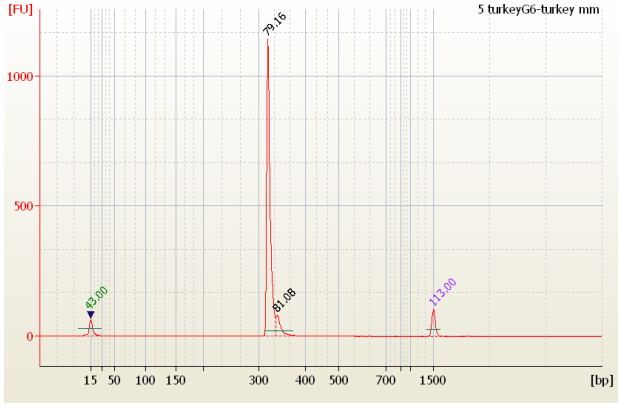
e) Donkey assay using donkey template DNA



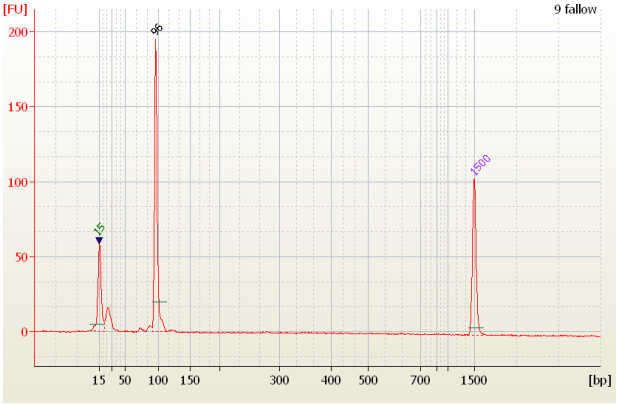
f) Cow assay using cow template DNA



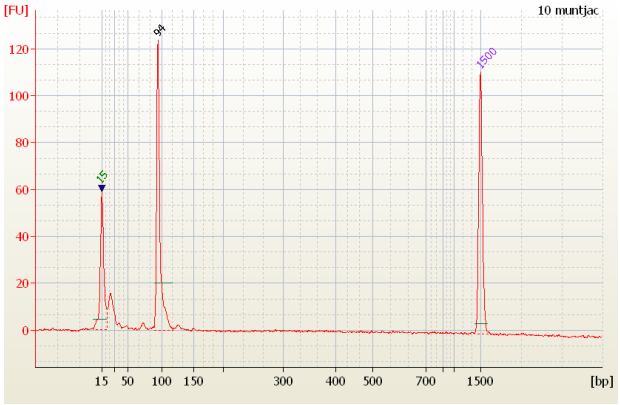
g) Turkey assay using turkey template DNA



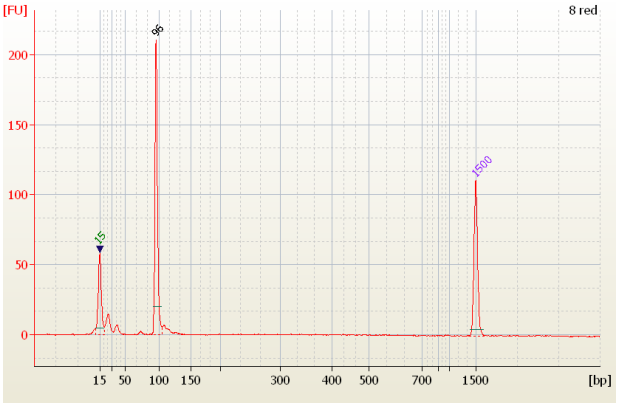
g) Deer assay using fallow deer template DNA



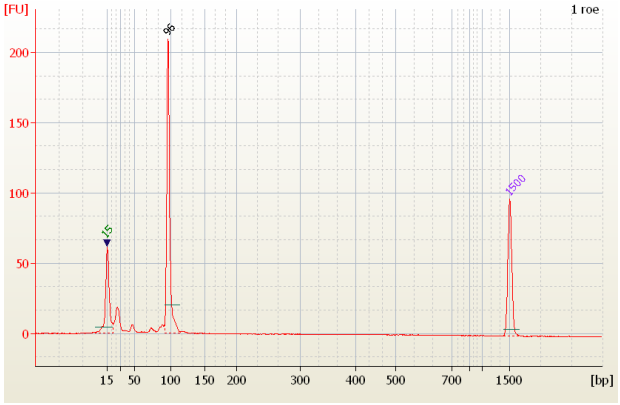
h) Deer assay using muntjac deer template DNA



i) Deer assay using red deer template DNA



j) Deer assay using roe deer template DNA



k) Deer assay using sika deer template DNA

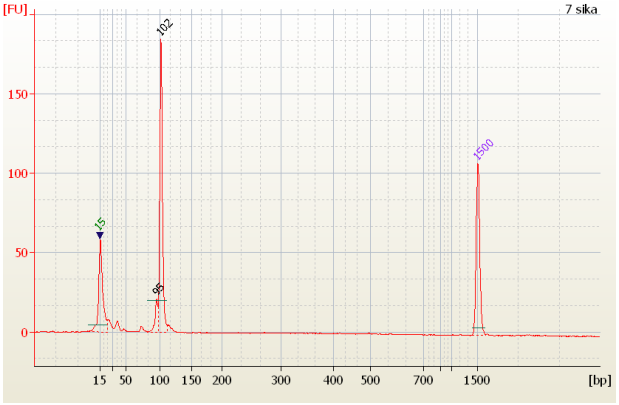


Figure 2. Alignment of turkey, chicken, cow, deer, donkey, duck, horse, pheasant, pig and sheep mitochondrial cytochrome b gene sequence, showing position of BioAnalyser turkey sense and anti-sense primers.

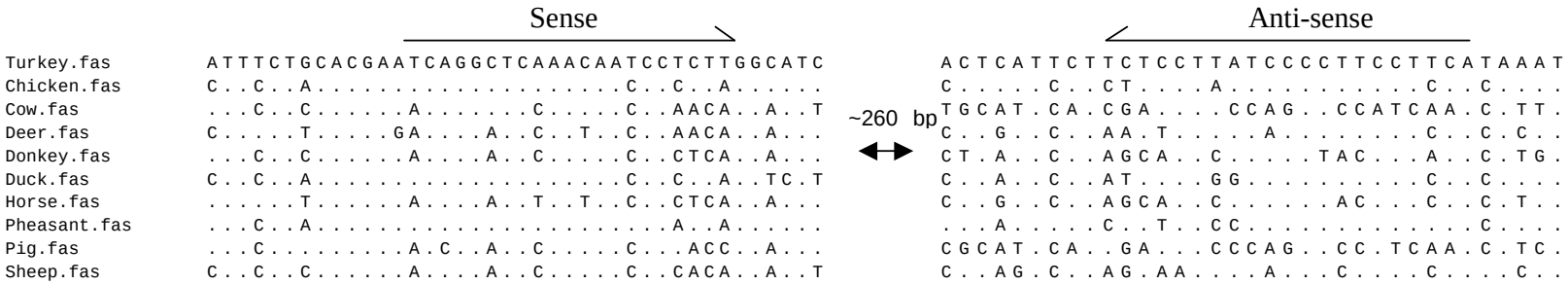


Figure 3. Alignment of pig and fallow deer mitochondrial cytochrome b gene sequence, showing position of real-time PCR pig sense and anti-sense primers.

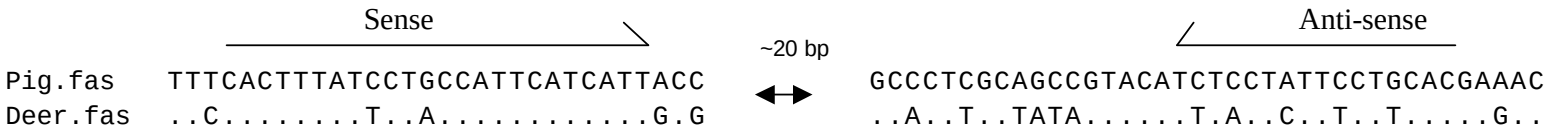


Figure 4. Alignment of pig, turkey, chicken, cow, deer, donkey, duck, horse, pheasant and sheep mitochondrial cytochrome b gene sequence, showing position of BioAnalyser pig sense and anti-sense primers.

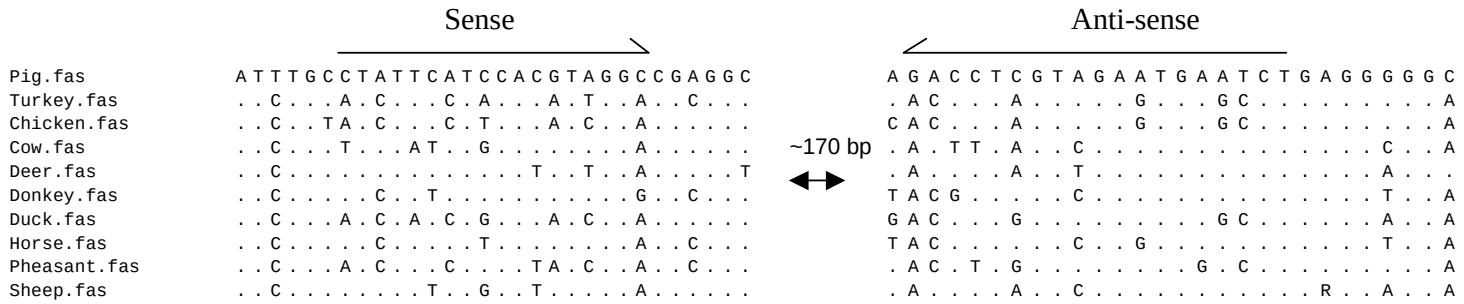


Figure 5. Alignment of chicken, cow, deer, donkey, duck, horse, pheasant, pig, sheep and turkey mitochondrial cytochrome b gene sequence, showing position of BioAnalyser chicken sense and anti-sense primers.

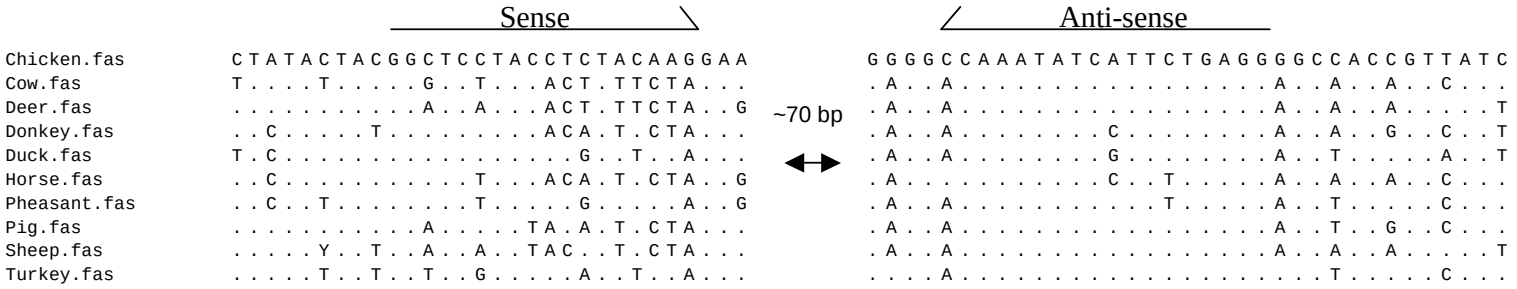
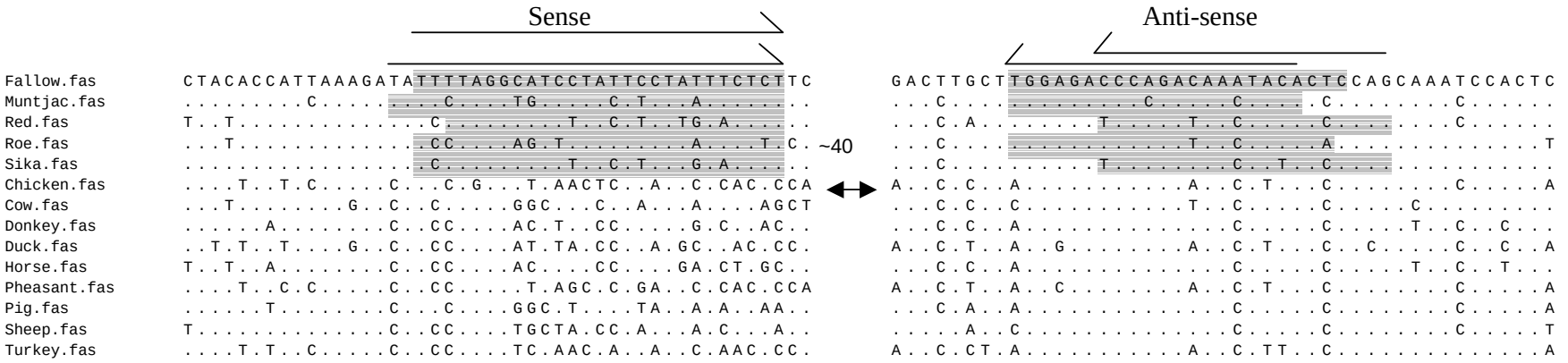


Figure 6. Alignment of fallow deer, muntjac deer, red deer, roe deer, sika deer, chicken, cow, donkey, duck, horse, pheasant, pig, sheep and turkey mitochondrial cytochrome b gene sequence, showing position of BioAnalyser deer sense and anti-sense primers. Shaded areas show original primers designed for the 5 species, arrows show the two sense and 2 anti-sense primers used in the deer specific BioAnalyser assay.



NB shaded areas represent coverage of redesigned 5 sense and 5 anti-sense primers, arrows represent final 4 primer positions

Figure 7. Alignment of sheep and sika deer mitochondrial cytochrome b gene sequence, showing position of VLA PCR sheep sense and anti-sense primers.

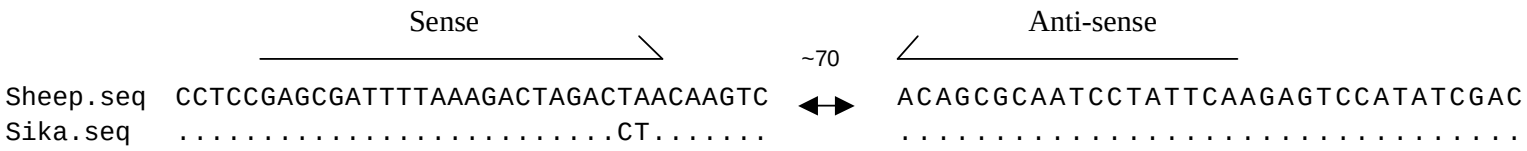
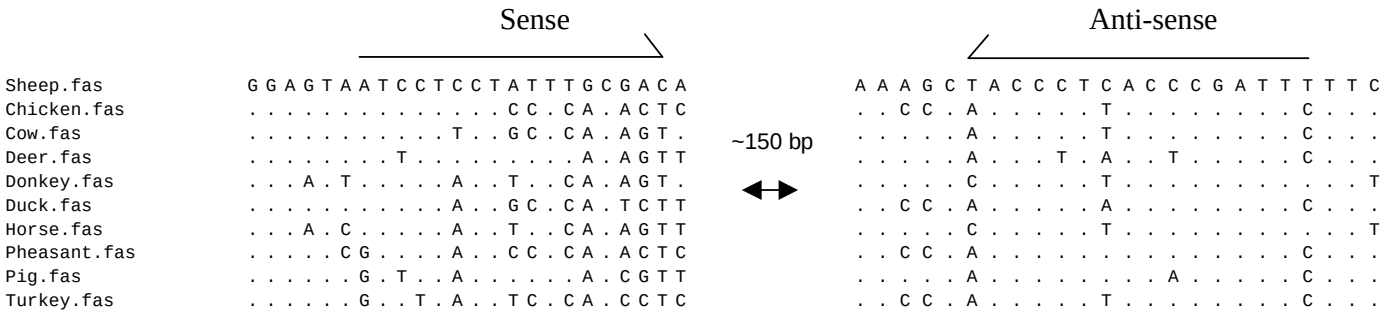


Figure 8. Alignment of sheep, chicken, cow, deer, donkey, duck, horse, pheasant, pig and turkey mitochondrial cytochrome b gene sequence, showing position of BioAnalyser sheep assay sense and anti-sense primers.



APPENDIX 1 MATERIALS AND METHODS

DNA Template preparation

Two 5g aliquots from each sample were incubated in 10mls CTAB buffer (2% (w/w) CTAB, 20mM EDTA, 0.1M tris base and 1.4M Sodium Chloride) containing 0.04ml proteinase K (20mg/ml) at 65^{o}C. After 120 minutes the samples were vortexed briefly and then centrifuged at 4500***g*** for 10min. 1ml of each sample supernatant was then transferred to a 2ml microcentrifuge tube containing 0.8ml chloroform and the microcentrifuge tubes vortexed prior to centrifugation at 16060***g*** for 10 minutes. 0.5ml of the supernatant was then transferred to a fresh microcentrifuge tube containing 1ml wizard resin which was then processed using the Wizard Genomic DNA Purification System (Promega, Southampton, UK) method according to the manufacturer's instructions.

PCR

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

Sheep/Pig

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Sh or Po sense primer (20µM)	0.125
Sh or Po anti-sense primer(20µM)	0.125
Water	7.25

Turkey

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Q-Solution, 5x	2.5
T sense primer (20µM)	0.125
T anti-sense primer(20µM)	0.125
Water	4.75

Pheasant/Chicken/Cow

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Ph, Ch or Co sense primer (20µM)	0.375
Ph, Ch or Co anti-sense primer(20µM)	0.375
Water	6.75

Horse

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Q-Solution, 5x	2.5
H sense primer (20µM)	1.125
H anti-sense primer (20µM)	1.125
Water	2.75

Donkey

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Do sense primer (20µM)	1.125
Do anti-sense primer (20µM)	0.375
Water	6

Duck

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Q-Solution, 5x	2.5
Du1sense primer (20µM)	0.375
Du2 anti-sense primer (20µM)	0.375
Du3 sense primer (20µM)	0.375
Du4 anti-sense primer (20µM)	0.375
Water	3.5

Deer

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
V1 sense primer (20µM)	0.2
V3 anti-sense primer (20µM)	0.2
V2 sense primer (20µM)	0.2
V4 anti-sense primer (20µM)	0.2
Water	6.7

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 and after activation of hotstart taq DNA polymerase by incubation at 95^{o}C for 15min cycled for between 30 and 40 cycles of denaturation at 94^{o}C for 0.5min,

annealing at 62^{o}C for 1.5mins and extension at 72^{o}C for 1.5mins, with a final extension at 72^{o}C for 10mins.

Amplicon electrophoresis using the Agilent BioAnalyser

Samples, diluted according to the table below, were analysed on the Agilent BioAnalyser, using DNA 1000 series II chips, exactly according to the instructions in the Agilent DNA 1000 Kit Guide.

Optimum dilutions of post PCR reaction mix to effect an LOD of 0.5% w/w

Species	Ratio
Donkey	no dilution (run neat)
Deer: cow	1:1
Pig: sheep	2:1
Turkey: duck: horse	1:1:4
Pheasant: chicken	2:3

FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP) 1

Version 1.0, March 2008

STANDARD OPERATING PROCEDURE FOR ANALYSIS OF DNA FROM MEAT USING A CAPILLARY ELECTROPHERESIS CHIP SYSTEM

Prepared by Hez Hird, C.S.L. **Date** 28th March 2008

CONTENTS

1. HISTORY / BACKGROUND

1.1 Background

The correct labelling of food products in the United Kingdom is subject to UK and EU regulation. To ensure adherence to these regulations, enforcement systems that possess species identification methods are necessary. The methods set out in this document were developed as part of the Food Standard Agency project Q01107. The aim of this project was to adapt and validate species-specific real-time PCR assays for use on the Agilent BioAnalyser that would allow the accurate detection of horse, donkey, duck, deer, pheasant, turkey, chicken, pig, cow and sheep in meat products.

2. PURPOSE

The purpose of this SOP is to provide methods for the detection of exotic meat species for use by public analysts that have the capacity to perform PCR with detection of products on an Agilent Bioanalyser capillary electropheresis chip system. The specific meat assays have been adapted from previously validated real time PCR methods for the detection of exotic meat species.

3. SCOPE

The methods covered in this document relate to the analysis of DNA from meat products to determine the species of meat present; specifically the species identification of horse, donkey, duck, deer, pheasant, turkey, chicken, pig, cow and sheep. The assay has been designed to work with raw, boiled, autoclaved and processed mixed samples.

4. DEFINITIONS AND ABBREVIATIONS

PCR – Polymerase chain reaction
NTC – No template control
EU – European union
UK –United Kingdom
DNA – Deoxyribulose nucleic acid
LOD – Limit of detection
COSHH – Control of substances hazardous to health
MW – Molecular weight
SOP- Standard operating procedure

5. PRINCIPLE OF THE METHOD

DNA is extracted from known meat samples. DNA extracts are then tested by conventional PCR using species-specific primers and probe sets. Analysis of the PCR amplicons using an Agilent Bioanalyser indicate the presence or absence of horse, donkey, duck, deer, pheasant, turkey, chicken, pig, cow and sheep.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

Unless otherwise stated, all chemicals should be of molecular biology grade.
Hexadecyl-trimethyl-ammonium-bromide (CTAB)
Chloroform - min 99% (Sigma, C2432)
Ethylenediaminetetraacetic acid (EDTA) (Sigma, E5134)
Ethanol
Hydrochloric acid (Fisher, 7647-01-0)
Isopropanol – min 99% (Sigma, I-9516)
Sodium chloride (Sigma, S3014)
Proteinase K 100mg (Sigma, P2308)
Sodium dodecyl sulphate (SDS) (Sigma, L4509)
Tris-EDTA (TE) buffer, 100x concentrate (Sigma, T-9285)
Tris(hydroxymethyl)aminomethane (Sigma, T6791)

6.2 Water

Unless otherwise stated, sterile molecular biology grade water (Sigma W-4502) should be used.
Deionised water refers to water that has undergone reverse osmosis.

6.3 Solutions, standards and reference materials

Extraction Buffer (see section 11.1.1)
80% (v/v) Isopropanol (see section 11.1.2)
1 x TE buffer (see section 11.1.3)
Proteinase K - 20mg/ml (see section 11.1.4)
Hydrochloric acid 0.1M (see section 11.1.5)
Neutracon 5% (see section 11.1.6)

Buffer solutions pH 7 and pH 10 (BDH 33274 2D, 33276 2H)
Lyophilised primers (desalted) (Sigma) see section 12.2

6.4 Commercial kits

Wizard® DNA Clean-up System (Promega, A7280)
Multiplex PCR Kit (Qiagen®, 206143)
Agilent DNA series II LabChip kit 1000 (Agilent, 5067-1504)

6.5 Plasticware

200µl thin walled PCR tubes (Thistle Scientific, PCR-0208-C) or semi-skirted plates (Thistle Scientific, PCR-0208-C, PCR-96M2-HS-C)
200µl cap strips (Thistle Scientific, PCR-02CP-C)
50ml sterile centrifuge tubes (Corning, 430290)
Plastic Bijoux (Sterilin, 129A)
1.5 ml Microcentrifuge tubes (autoclaved) (Starlab, S1615)
5ml tips (autoclaved) (Anachem, F161370)
Axygen low retention microcentrifuge tubes – 1.5ml and 0.5ml (Thistle Scientific, MCT-175-L-C; MCT-060-L-C) (these should be autoclaved at 121^{o}C and 1.1 bar pressure for 15 minutes, before use)
10, 20, 200 and 1000µl Barrier filter tips (sterile) (Starlab, S1121-3810, S1120-1810, S1126-7810, S1120-8810)
250 ml plastic wash bottles (Fisher Scientific, BTS-520030Y)

6.6 Glassware

100ml glass bottles with lids
100ml volumetric measuring cylinder
500ml glass bottles with lids
1L plastic beaker

6.7 Equipment

Agilent BioAnalyser
PCR machine
Autoclave capable of reaching and maintaining 121^{o}C, 1.1 Bar, for 15 minutes
Balance capable of weighing up to 1kg

Base plate (TS-328-96-AS/5)
Cap installing tool (ABI 4330015) or alternatively semi-automated Laboratory Plate Sealer (ABgene, AB-1443)
Centrifuge (Heraeus Labofuge 400R)
DNA free cabinet eg PCR workstation (erlab Biocap DNA/RNA)
or other designated DNA free area
Heating block set to 65°C+/-5°C (able to hold 1.5 ml microcentriguge tubes)
Hybridisation oven (Hybaid HBSNSR110) or waterbath, preheated to 65°C ± 2.0°C
Magnetic stirrer and flea
Disposable scalpels with plastic handle (Swann-Morton, 0511)
Microcentrifuge (Stratagene 400551)
PCR workstation (erlab Biocap DNA/RNA)
P2, P20, P200 and P1000 Gilson positive displacement pipettes or equivalent (for example M1000, M100, M25 or M10 Microman pipettes)
P5000 Gilson pipette fitted with aerosol barrier filter (Anachem F161280)
pH meter capable of measuring pH values between 2 and 14.
Ultraviolet cross-linker (UVP, CL–1000), or UV light source designed for decontamination of PCR plasticware
VacMan Vacuum manifold (Promega A7231)

7. SAFETY

Safety glasses, laboratory coats and disposable gloves to be worn at all times. Consult laboratory COSHH assessment for specific instructions prior to undertaking this method

8. PROCEDURES

8.1 Extracting DNA from meat products

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

8.1.1 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 this SOP. If the work area is contaminated by a sample the area should be treated with 5 % Neutracon 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 the stock bottle.

8.1.2 Method

A. 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 the letters A or B (samples are analysed in duplicate). Change gloves between samples. Add 10ml of Extraction buffer and 40µl proteinase K (20mg/ml) to each tube. Mix each sample thoroughly by vortexing for 6 seconds to produce a slurry. Add 10mls of Extraction buffer and 40µl proteinase K (20mg/ml) to an empty centrifuge tube labelled 'extraction blank' and treat the same as the samples.
B. Incubate the samples at 65°C ± 5.0°C for 2 hours in a Hybaid oven or a waterbath
C. Place 1 x TE buffer in the Heating block set to 65°C+/-5°C.
D. Change gloves. Label 1 x 2ml microcentrifuge tube and 3 x 1.5ml microcentrifuge tubes with the sample number and the letter A, and a replicate set of tubes with the letter B. Add 800µl of chloroform to the 2ml tubes. Re-suspend the Wizard® kit resin by shaking (do not vortex) and add 1ml of resin to one of the 1.5ml microcentrifuge tubes for each sample.
E. After incubation of the samples at 65°C (step B), 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 800µl chloroform. Vortex for 3 sec and centrifuge at 16060***g*** (if not possible due to centrifuge model, the centrifugal force should be no less than 13,000***g***) for 10 mins. Change gloves.

F. 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.

G. 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.

H. 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.

I. 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.

J. Change gloves. Remove the syringe barrel and transfer each minicolumn to a labelled microcentrifuge tube with the cap removed. Centrifuge the minicolumn at 16060***g*** (if not possible due to centrifuge model, the centrifugal force should be no less than 13,000***g***) to remove any residual isopropanol.

K. Change gloves. Transfer the minicolumn to a labelled 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.

L. Centrifuge the minicolumn at 16060***g*** (if not possible due to centrifuge model, G force should be no less than 13,000***g***) for 2 minutes to elute the bound DNA.

M. 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, but **the DNA must only be thawed once**.

8.2 Analysis of meat samples using an Agilent BioAnalyser

This procedure describes the method used to analyse DNA extracts for the presence or absence of duck, pheasant, chicken, turkey, sheep, cow, pig, deer, horse and donkey using an Agilent Bioanalyser capillary electropheresis chip system.

8.2.1 Method - PCR

A. Irradiate, at the same time, a 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.
B. Each PCR run must include positive and negative controls run alongside the test samples. Construct a template detailing the location of the duplicate wells of each sample and controls on the plate.
C. 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.
D. Defrost the primers and Qiagen Multiplex PCR reagents, mix by flicking and centrifuge on pulse for 20sec and place on ice.
E. Defrost the DNA from the meat samples and mix by flicking. Centrifuge on pulse for 20 sec and place on ice. Dilute the samples 1:4, DNA : water, mix by flicking and centrifuge on pulse for 20 sec. Dilute the positive control DNA appropriately.
F. Make up a working master mix solution in the 1.5 mL Axygen microcentrifuge tubes using components as given in appendix 12.2. 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

G. Perform all remaining steps in the DNA workstation.
H. Place the PCR reaction plate onto the 96 well base plate.
I. Using a pipette aliquot 20µL of the master mix into all the required wells of the PCR plate. Cover the rest of the plate with the plate cover to avoid inadvertently contaminating any open wells.
J. When complete, cover with the plate cover and set to one side.
K. Add 5µL of positive control, water as negative control or diluted sample DNA with a pipette to duplicate wells on the PCR plate. While doing this, cover

the rest of the plate with the cover so that you are not working over open wells.

L. Put caps onto the wells and press the caps down using the cap-installing tool until the wells no longer 'crackle'. Alternatively, if using optical heat sealing film, place the film on to the plate and seal using the heat sealing machine following manufacturers instructions.
M. 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.
N. Put the plate into a PCR machine in the correct orientation, and close the machine lid.
O. Open the saved cycling programme required.
P. Check that the cycling parameters (appendix 12.3) and reaction volume (25µL) settings are correct and start the run.

8.2.2 Method – Agilent Bioanalyser

The Agilent 2100 Bioanalyser is a capillary electrophoretic system, which is used to separate and size PCR products. Different sized DNA products require different chip assays according to the expected product size range. For this SOP, the DNA series II labchip 1000 should be used.

A. Allow all reagents to equilibrate to room temperature for 30mins before use. Protect the dye concentrate from light while bringing it to room temperature. Fresh gel-dye mix should be prepared after 10 chips or after 4 weeks when necessary.
B. Prepare the amplicons by mixing according to appendix 12.4
C. Switch the Bioanlyser on at the back of the machine and wait for the green light on the front of the machine to remain static. Turn the computer attached to the Bioanalyser on.
D. Double click on the '2100 expert' icon on the desktop.
E. Click on Assay selection and select the DNA1000 Series II assay.
F. Slowly fill one of the wells of the electrode cleaner with 350µL Rnase-free water. Open the lid and place electrode cleaner in the BioAnalyser. Close the lid and leave for 10secs. Open the lid and remove the electrode cleaner,

wait for another 10secs to allow the water on the electrodes to evaporate before closing the lid.

G. Load the gel-dye mix, samples and ladder onto the chip as indicated in the Agilent DNA 1000 Kit Guide.
H. Open the lid of the Bioanalyser, carefully place the chip in the correct orientation, close the lid slowly and click on the start button as soon as it is illuminated.
I. At the end of the run (approximately 30mins) immediately remove the chip and replace with the electrode cleaner chip containing 350µL Rnase-free water. Close the lid and leave for 10secs. Open the lid and remove the electrode cleaner, wait another 10secs to allow the water on the electrodes to evaporate before closing the lid.

9 Quality Assurance

9.1 Extracting DNA from meat products

Temperature checks

The temperature of the Hybaid oven and heating block should be monitiored while in use via an *in situ* thermometer or temperature probe to ensure that the temperature limits stated in the procedure are not exceeded. The temperature of the Hybaid oven and heating block should be measured and recorded weekly using a thermometer or temperature probe to establish the relationship between the set temperature and the temperature reached. If any difference is not constant and drift is detected the piece of equipment should be taken out of use for repair or replacement.

DNA Extraction Negative Control

A DNA extraction negative control is prepared with every set of samples extracted at the same time. **To pick up contamination, the negative control is manipulated last at each stage of the process** and is treated exactly the same way as all other samples. It is then analysed in the same way as the other samples using the species specific PCR assays.

9.2 Running PCR assays

Each assay must be adequately controlled by the inclusion of a range of positive and negative controls as outlined below.

No Template Control (NTC)

No template controls (NTCs) are used to determine if contamination has occurred during the PCR procedure. NTCs are prepared with every set of samples amplified at the same time. NTCs contain sterile molecular grade water in place of the sample DNA. To pick up contamination, the NTCs are manipulated last at each stage of the process. NTCs should show no PCR amplification. Amplification in the NTC indicates contamination has occurred and the PCR batch is invalid. All samples must be re-amplified.

DNA Extraction Negative Control

A DNA extraction negative control showing amplification means the PCR batch is invalid and all samples must be re-amplified. If, after re-amplification, the DNA extraction control is still positive, the extraction batch is invalid and all samples must be re-extracted.

PCR Positive Controls

A PCR positive control is prepared from DNA extracted from a 100% single species sample. PCR positive controls should be extracted and diluted in a similar manner to the test samples. PCR positive controls must be analysed with each PCR batch.

9.3 Running samples on Agilent Bioanalyser

DNA 1000 Ladder

To check that the chip has run correctly look at the ladder in the electropherogram. The major features of a successful ladder run are:

13 peaks for DNA 1000 ladder

All peaks are well resolved

Flat baseline

Correct identification of both marker peaks

Sample peaks

The major features of a successful DNA sample run are:

All sample peaks appear between the lower and upper marker peaks

Flat baseline

Baseline readings at least 50 fluorescence units.

Both marker peaks are well resolved from sample

If the results obtained contravene these conditions, repeat the PCR.

The assays have been tested against each of the other meat samples only. Extensive testing on other species has not been performed and the possibility of misidentification of other species cannot be ruled out. For definitive species identification, PCR products should be sequenced and analysed by comparison to a reference sequence database (e.g. EMBL, GenBank).

10. CALCULATIONS AND DATA ANALYSIS

10.1 interpretation of the Bioanalyser results.

A. After completion of the Bioanalyser run the results must be assessed under the Data context and selecting the electropherogram tab. The computer analysis of each well is carried out in real time and can be viewed during the run.
B. The ladder should contain 13 peaks and resemble the picture found in the Agilent DNA 1000 Kit Guide. If there is a problem with the ladder the chip should be repeated.
C. Ensure that any un-used wells (containing water instead of sample) have no peak other than the markers, present.
D. Sample wells should contain a peak of the required size for the species analysed (see appendix 12.1) in between 2 marker peaks.

Limit of Detection

The limit of detection (LOD) for these assays is approximately 0.5% of raw target species in a background of other meat species. The LOD has been determined using model samples composed of each species spiked into a meat background at 0.5% w/w, with a cut-off value of 50FU.
The LOD has been determined using raw meat and therefore is likely to be at least equal or higher to 0.5% w/w for samples which have been heat and/or pressure processed.

11. RELATED PROCEDURES

11.1 Preparation of reagents required for analysis of MEAT products

11.1.1 CTAB extraction Buffer

CTAB extraction buffer is a protein denaturing detergent used in the isolation and precipitation of DNA. All reagents are to be of molecular biology grade.

A. Weigh out the following into a 3 litre plastic beaker

20g CTAB

81.8g NaCl (1.4M)

12.1g TRIS (100mM)

7.4g EDTA (20mM)

B. Add 700ml sterile water and a magnetic flea, place on a magnetic stirrer and leave to mix overnight or until the all ingredients have dissolved.

A. Adjust pH using diluted (1M) HCl to pH 8. Fill up to 1 litre in a volumetric flask. Pour the buffer into a 1 litre glass flask and autoclave (121°C, 1.1 Bar for 15min. When cool, label with the date of preparation and date of disposal (6 months from date of preparation). Store at room temperature.

11.1.2 80% (v/v) Isopropanol

Add 80ml of isopropanol to 20ml of water using a 100ml measuring cylinder.

Store in a glass bottle in a solvent cupboard.

11.1.3 1xTE Buffer

A. In the DNA free cabinet label microcentrifuge tubes as 1 x TE.
B. Dilute 0.05ml buffer (100x) in 4.95ml sterile water in 50ml centrifuge tube and aliquot 0.6ml into the labelled microcentrifuge tubes.
C. UV irradiate the tubes with the lids off in the UV cross linker for 10min. Close lids and store at room temperature.

11.1.4 Proteinase K (20mg/ml)

A. Add 5ml sterile water to the Proteinase K 100mg container and mix by inversion until dissolved.

B. Dispense the prepared solution in 1mL 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.

11.1.5 Hydrochloric Acid (0.1M)

A. Slowly add 8.62 ml of concentrated hydrochloric acid to a 1 litre glass bottle containing 991.38 ml of deionised water and mix by inversion
B. Dispense the prepared solution into wash bottles as required.

11.1.6 5% Neutracon

A. Add 25 ml of Neutracon to a 500ml glass bottle containing 475 ml of deionised water and mix by inversion
B. Dispense the prepared solution into wash bottles as required.

11.2 Primer preparation

A. 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 (see appendix 12.1 for sequence).
B. When the primers arrive confirm their sequence with the sequence ordered. The supplier's name and the date must be recorded and a batch number allotted to the primer. This information, together with the datasheet supplied with the primer should be kept in a 'primers and probe' folder.
C. 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 = $\frac{481 \times 50}{6345}$ = 3.790ml

D. Centrifuge the tubes containing the lyophilized primer at between 3,000 and 5,000*g* for 10 seconds to collect the powder at the bottom of the tube.

E. In a DNA-free cabinet label Axygen 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^{o}C for 30mins or 4^{o}C overnight. In the event that the tube is not large enough for the volume of water required, add 1ml of water and rehydrate as above. At the end of the rehydration period, transfer the solution to a bijoux and add the required volume of water to give a 20μM solution.

F. Dispense the solution in 130 μl aliquots into labelled Axygen 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.

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

12. APPENDICES

12.1 Primer sequences

Assay	Primer name	Sequence 5' – 3'	Size bp
Chicken	Ch sense Ch anti-sense	GGCTCCTACCTCTACAAG CCTCAGAATGATATTTGG	120
Turkey	T sense T anti-sense	TCAGGCTCAAACAATCCTCTT TGAAGGAAGGGGATAAGGAGA	320
Pheasant	Ph sense Ph anti-sense	TCGGACGCGGCCTCTAT TGAGTGTGAGGAGTAGGACG	89
Duck	Du1 sense Du2 sense	TCCTACCTGTATAAAGAAACTTGAAAT TCCTACCTGTACAAAGAAACCTGAAAT	147, 149 &150
	Du3 anti-sense Du4 anti-sense	GGGCTGAAAATAAGTTGGTAATT GGGCTGAGAACAGGTTGGTAATT	
Horse	H sense H anti-sense	GAAGCATAATATTCCGG TTAGTGTCAGTAAGTCTGCC	82
Donkey	Do sense Do anti-sense	CCTTATCCTTTCCATCT GTAAGTCTGCTACTAAGAGTCAGAAC	128
Deer	V1 sense V2 sense	TCTTAGGCATCTTACTTCTAGTACTCT TATTCTAGGTGTCCTACTTCTAATTCTCT	94, 96 & 102
	V3 anti-sense V4 anti-sense	CTGGGGTATAGTTGTCTGGA TGTAGTTGTCGGGGTCTCCA	
Cow	Co sense Co anti-sense	CACGAAACAGGCTCC TGGAATGGGATTTTGTC	70
Pig	Po sense Po anti-sense	CTATTCATCCACGTAGGC AGATTCATTCTACGAGGTC	225
Sheep	Sh sense Sh anti-sense	ATCCTCCTATTTGCGAC AAATCGGGTGAGGGTA	190

12.2 Mastermix composition

Sheep/Pig

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Sh or Po sense primer (20µM)	0.125
Sh or Po anti-sense primer(20µM)	0.125
Water	7.25

Turkey

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Q-Solution, 5x	2.5
T sense primer (20µM)	0.125
T anti-sense primer(20µM)	0.125
Water	4.75

Pheasant/Chicken/Cow

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Ph, Ch or Co sense primer (20µM)	0.375
Ph, Ch or Co anti-sense primer(20µM)	0.375
Water	6.75

Horse

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Q-Solution, 5x	2.5
H sense primer (20µM)	1.125
H anti-sense primer (20µM)	1.125
Water	2.75

Donkey

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Do sense primer (20µM)	1.125
Do anti-sense primer (20µM)	0.375
Water	6

Duck

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Q-Solution, 5x	2.5
Du1sense primer (20µM)	0.375
Du2 anti-sense primer (20µM)	0.375
Du3 sense primer (20µM)	0.375
Du4 anti-sense primer (20µM)	0.375
Water	3.5

Deer

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
V1 sense primer (20µM)	0.2
V3 anti-sense primer (20µM)	0.2
V2 sense primer (20µM)	0.2
V4 anti-sense primer (20µM)	0.2
Water	6.7

12.3 PCR cycling parameters

Step	Function	Temperature	Time
1	Initial activation step	95°C	15mins
2 (30 - 40 cycles*)	Denaturation	94°C	30secs
3	Annealing	60°C	90secs
4	Extension	72°C	90secs
5	Final extension	72°C	10mins

*use 30 cycles for turkey, pig, pheasant, cow and duck assays

40 cycles for deer, chicken, sheep, horse and donkey assays

12.4 Preparation of amplicons for analysis on BioAnalyser

Species	Ratio
Donkey	no dilution (run neat)
Deer: cow	1:1
Pig: sheep	2:1
Turkey: duck: horse	1:1:4
Pheasant: chicken	2:3