FOOD STANDARDS AGENCY

STANDARD OPERATING PROCEDURE (SOP) 002

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STANDARD OPERATING PROCEDURE FOR THE GENETIC IDENTIFICATION OF COMMON AND EXOTIC MEAT SPECIES USING THE AGILENT 2100 BIOANALYSER CAPILLARY ELECTROPHERESIS SYSTEM

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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 qualitative 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 common and 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 common and 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 and is qualitative only.

The species present in canned meat samples can be determined using this methodology however it was found that the assays were relatively insensitive for the analysis of canned samples when compared to other processed samples. Thus, the method would need to be adapted to reflect the reduced sensitivity for the analysis of canned products and the results would not be comparable to those of other matrices. For this reason the analysis of canned samples is not covered by this SOP.

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. Analysis of the PCR amplicons using an Agilent Bioanalyser indicates 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)
Decon Neutrcon (Fisher, D/0027/21)

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) diluted to 20μM (see section 11.1).

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 e.g. 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 $\pm$ 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 electrophoresis 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 species 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 for each species to be tested for 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
   Note the duck and deer PCR reactions use 2 sets of primers.
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. To increase the throughput of this methodology, amplicons from different species specific PCR are mixed together for analysis, according to the table in Appendix 12.4.

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 PCR products for each meat sample according to appendix 12.4
C. Switch the Bioanlayser 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. CALCULATIONS AND DATA ANALYSIS

9.1 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.
E. A single PCR amplicon should be produced for each of the assays apart from the deer and duck assays which could produce 3 amplicons if multiple deer and duck species are present.
F. The mixing of the amplicons will result in multiple peaks, each of a diagnostic size according to the tables in appendices 12.1 and 12.4, for samples of mixed composition. For example, a sample which contained 50% (w/w) pig and 50% (w/w) sheep, would give a profile containing 2 peaks at 190 and 225bp, when analysed with the pig and sheep species specific PCR according to the protocol, indicating the presence of both in this sample.
G. The marker peaks should be well resolved from sample peaks and be at least 3 FU higher than baseline readings.
H. Any peaks seen with a FU value of <50 should be ignored as this level falls below our limit of detection (LOD).
I. The smallest amplicon length is for cow at 70bp in size and the largest is turkey 320bp. Any peaks seen smaller or larger than these (+/- 10% according to Agilent Technologies sizing accuracy for the DNA 1000 Assay) should be ignored.

9.2 Limit of Detection

The 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.

10 QUALITY ASSURANCE

10.1 Extracting DNA from meat products

10.1.1 Temperature checks

The temperature of the Hybaid oven and heating block should be monitored 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.

10.1.2 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.

10.2 Running PCR assays

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

10.2.1 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.

10.2.2 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.

10.2.3 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. If the positive controls do not produce a band or produce a band not of the expected size, the analysis should be repeated.

10.3 Running samples on Agilent Bioanalyser

10.3.1 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

10.3.2 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).

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.

C. 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

In the DNA free cabinet label microcentrifuge tubes as 1 x TE.
Dilute 0.05ml buffer (100x) in 4.95ml sterile water in 50ml centrifuge tube and aliquot 0.6ml into the labelled microcentrifuge tubes.
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)

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

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
Dispense the prepared solution into wash bottles as required.

11.1.6 5% Neutracon

Add 25 ml of Neutracon to a 500ml glass bottle containing 475 ml of deionised water and mix by inversion

Dispense the prepared solution into wash bottles as required.

11.2 Primer preparation

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).

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.

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

$$\text{Volume of water required} = \frac{481 \times 50}{6345} = 3.790\text{ml}$$

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.

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°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 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.

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.

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

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