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 methods that permit species identification 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 *only is qualitative.*

The species present in canned meat samples can be determined using this methodology but the assays are 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
FU – Fluorescence Units

5. PRINCIPLE OF THE METHOD

DNA is extracted from known meat samples. DNA extracts then are subjected to 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.
Ethanol
Hydrochloric acid (Fisher, 7647-01-0)
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.
Rnase-free water

6.3 Solutions, standards and reference materials

Hydrochloric acid 0.1 M (see section 11.1.1)
Neutracon 5% (v/v, see section 11.1.2)
Lyophilised primers (desalted) (Sigma) diluted to 20 μM (see section 11.2).
TE Buffer
Proteinase K

6.4 Commercial kits

Biokit - DNA extraction kit (speciation) (Tepnel, 901040N)
Multiplex PCR Kit (Qiagen®, 206143)
Agilent DNA series II LabChip kit 1000 (Agilent, 5067-1504)

6.5 Plasticware

200 μl thin walled PCR tubes (Thistle Scientific, PCR-0208-C) or semi-skirted plates (Thistle Scientific, PCR-0208-C, PCR-96M2-HS-C)
200 μl cap strips (Thistle Scientific, PCR-02CP-C)
50 ml sterile centrifuge tubes (Corning, 430290)
Plastic Bijoux (Sterilin, 129A)
1.5 ml Microcentrifuge tubes (autoclaved) (Starlab, S1615)
5 ml tips (autoclaved) (Anachem, F161370)
Axygen low retention microcentrifuge tubes – 1.5 ml and 0.5 ml (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 1000 μl Barrier filter tips (sterile) (Starlab, S1121-3810, S1120-1810, S1126-7810, S1120-8810)
250 ml plastic wash bottles (Fisher Scientific, BTS-520030Y)

6.6 Equipment

Agilent 2100 BioAnalyser
PCR machine
Autoclave capable of reaching and maintaining 121^{o}C, 1.1 Bar, for 15 minutes
Balance capable of weighing 2g
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. Erlab Biocap DNA/RNA)
or other designated DNA free area
Hybridisation oven (Hybaid HBSNSR110) or waterbath, preheated to 65 °C ± 2.0 °C
Disposable scalpels with plastic handle (Swann-Morton, 0511)
Microcentrifuge (Stratagene 400551)
Microcentrifuge tubes, 1.5ml (Starlab S1615-5500)
PCR workstation (e.g. 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
Magnetic beads, magnetic rack, magnetic stand

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 using the Tepnel DNA extraction kit (speciation).

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 % (v/v) 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, reagents should be aliquotted. Small aliquots of reagents should be used and fresh batches should be started frequently. Tips should be pre-rinsed once when pipetting liquids. 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 2 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.
B. Add 5 ml of tissue extraction solution 1 and 20 µl proteinase K (10 mg/ml) to each tube. Mix each sample thoroughly by vortexing for 6 seconds to produce a slurry. Add 5 ml of tissue extraction solution 1 and 20 µl proteinase K (10 mg/ml) to an empty centrifuge tube labelled 'extraction blank' and treat the same as the samples.
C. Incubate the samples at 65°C ± 5.0°C for 1 hour in a Hybaid oven or a waterbath with constant movement.
D. Transfer 1 ml of each sample into a clean microcentrifuge tube and label with sample contents. Cool samples on ice for 5 minutes. Then add 54 µl of tissue extraction solution 2, invert to mix. If lumps are present, flick base of tube several times to loosen them.
E. Return samples to the ice for a further 10 minutes to facilitate protein precipitation.
F. Centrifuge samples at >8000 ***g*** for 5 minutes.
G. Prepare magnetic beads by agitating them until they are in suspension. Dispense 50 µl of beads into a clean microcentrifuge tube for each sample. Immobilise the beads by placing the tubes in the magnetic stand, then remove the liquid using a pipette.
H. Move the microcentrifuge tubes to the row of holes furthest away from the magnets in the magnetic rack. Add 400 µl from the sample supernatant to the magnetic beads.
I. Add 400 µl of DNA binding solution to the magnetic bead/sample lysate mixture. Flick the base of each tube to ensure the beads are completely suspended.
J. Incubate the samples at room temperature for 5 minutes. Flick the base of each tube every minute.
K. Immobilise the beads by placing the tubes against the magnets in the magnetic stand. Invert the stand several times to wash the beads.
L. Leave the tubes by the magnets for 2 minutes before carefully removing and discarding the supernatant fluid.
M. Move the microcentrifuge tubes so that they are not directly next to the magnets and wash the bead/DNA by resuspending in 500 µl 75% (v/v) ethanol. Flick the tube to mix then immobilise the beads by placing the tube in the magnetic rack next to the magnets. Remove all excess ethanol by pipetting.
N. Repeat the above step.
O. Incubate the samples with lids open for 5 minutes at 65 °C or air dry at room temperature for 15 minutes to remove any remaining ethanol.
P. If any residual solvent remains after incubation remove using a pipette.
Q. Add 100 µl of TE buffer, flick each tube gently to resuspend the beads and incubate at 65 C for 10 minutes. Flick the tubes gently after 5 minutes and after 10 minutes.
R. Centrifuge the tubes for 5 seconds in the microcentrifuge.
S. Immobilise the beads in the magnetic rack, pipette the supernatant into a clean microcentrifuge tube.
T. Repeat steps Q to S using a further 100 µl of TE buffer. Combine the two eluates.
U. Place the final eluted samples next to the magnets and leave for 2 minutes then cap the tube. The supernatant should be clear and pellet-free, if a black

pellet is seen, carefully remove the supernatant and place in another clean Eppendorf tube.

V. Store DNA extracts at 4 °C.

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. Simultaneously irradiate a 96 well plate and the plate cover with UV for 20 minutes in the UV crosslinker. At the same time irradiate the DNA-free workstation and the PCR workstation for at least 30 minutes.
B. Each species-specific PCR run must include positive and negative controls that are run alongside the test samples. To facilitate this, 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 the labelled tubes in a rack.
D. Defrost the primers and Qiagen Multiplex PCR reagents. Ensure that they are homogenous by flicking the tubes and centrifuge on pulse mode for 20 seconds.
E. Defrost the DNA from the meat samples. Ensure that they are homogenous by flicking the tubes. Centrifuge on pulse for 20 seconds. Dilute the samples 1:4, DNA:water, mix by flicking and centrifuge on pulse for 20 seconds. Dilute the positive control DNA appropriately.
F. Make up a working master mix solution for each species to be tested using the components as given in appendix 12.2. This preparation should be done in the 1.5 mL Axygen microcentrifuge tubes. The amounts shown are for each reaction. The number of reactions to be prepared may be calculated thus:
   [(no. of samples to be tested + 1 negative control + 1 positive control) x2] +3 extra
   Note that the duck and deer PCR reactions use multiple primers.
G. Perform all the remaining steps in the PCR workstation.
H. Place the PCR reaction plate onto the 96 well base plate.
I. Aliquot 20 µL 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 this step is complete, cover the plate with the plate cover and set to one side.
K. Add 5 µL of positive control, or 5 µL water as negative control or 5 µL diluted sample DNA 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 the caps or sealing film 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 (25 μL) 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 30 minutes 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 as detailed in the DNA 1000 kit guide.
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 on the computer attached to the Bioanalyser.
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 10 seconds. Open the lid and remove the electrode cleaner, wait for another 10 seconds 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 30 minutes) immediately remove the chip and replace with the electrode cleaner chip containing 350 μL Rnase-free water. Close the lid and leave for 10 seconds. Open the lid and remove the electrode cleaner, wait another 10 seconds 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 in 2100 expert software. Select the electropherogram tab to view the peaks.
   Note: 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 samples should be re-analysed using a fresh chip.
C. Ensure that any unused wells (containing water instead of sample) have no peaks present other than the size markers.
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 present in each of the assays apart from the deer and duck assays where up to 3 amplicons may be present if meat from multiple deer and duck species is present in the samples.
F. If the samples are of mixed composition 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 example, a sample which contained 50% (w/w) duck and 50% (w/w) pheasant would give a profile containing 2 peaks (150bp and 91bp).
G. The marker peaks should be well resolved from sample peaks and be at least 3 FU higher than baseline readings. If this is not the case the chip should be re-run.
H. Any peaks seen with a FU value of <50 should be ignored as this level falls below the limit of detection (LOD).
I. The smallest amplicon length is for cow (70bp) and the largest is for turkey (320bp). Any peaks that are smaller or larger than these values (+/- 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 1% (w/w) 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 1% (w/w), with a cut-off value of 50 FU.
The LOD has been determined using raw meat and therefore is likely to be at least equal or higher to 1% (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, preferably using 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/or drift is detected, the 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 and 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. 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 that contamination has occurred and the PCR batch is invalid. All samples must be prepared again and 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 prepared again and 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% (w/w) 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 of at least 50 fluorescence units.
Both marker peaks are well resolved from the sample peaks

If the results obtained contravene these conditions, the fault needs to be investigated and a number of measures taken including repeating the chip run and/or repeating the PCR step.

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 Hydrochloric Acid (0.1M)

Measure 991.38ml of deionised water in a measuring cylinder and 8.62ml of concentrated hydrochloric acid using a pipette and slowly add the acid to the water in a 1 litre glass bottle, mix by inversion. Dispense the prepared solution into labelled wash bottles as required.

11.1.2 Neutracon (5% v/v)

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 labelled 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 that the sequence delivered matches 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
20 μM primer solutions:

$$(\mu g \times 50)/MW = vol\ (ml)$$

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 and μg = 481
Volume of water required = (481 x 50)/6345 = 3.790 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 20 μM and leave to rehydrate at 37°C for 30 minutes 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 bottle and add the required volume of water to give a 20 μM 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 Du3 anti-sense Du4 anti-sense	TCCTACCTGTATAAAGAAACTTGAAAT TCCTACCTGTACAAAGAAACCTGAAAT GGGCTGAAAATAAGTTGGTAATT GGGCTGAGAACAGGTTGGTAATT	149 &150
Horse	H sense H anti-sense	GAAGCATAATATTCCGG TTAGTGTCAGTAAGTCTGCC	82
Donkey	Do sense Do anti-sense	CCCTTATCCTTTCCATCT TAGTAAGTCTGCTACTAAGAGTCAGAAC	128
Deer	V1 sense V2 sense V3 sense V4 anti-sense V5 anti-sense	TCTTAGGCATCTTACTTCTAGTACTCT TATTCTAGGTGTCCTACTTCTAATTCTCT TCCTAGGAGTTCTATTCCTAATTCTTT CTGGGGTATAGTTGTCTGGA TGTAGTTGTCGGGGTCTCCA	95, 97, 98, 104
Cow	Co sense Co anti-sense	CACGAAACAGGCTCC TGGAATGGGATTTTGTC	70
Pig	Po sense Po anti-sense	CTATTCATCCACGTAGGC AGATTCATTCTACGAGGTC	231
Sheep	Sh sense Sh anti-sense	ATCCTCCTATTTGCGAC AAATCGGGTGAGGGTA	190

12.2 Mastermix composition

Turkey/Sheep/Pig

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

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	2.75
H sense primer (20µM)	1.125
H anti-sense primer (20µM)	1.125
Water	2.5

Donkey

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
Do sense primer (20µM)	1.125
Do anti-sense primer (20µM)	1.125
Water	5.25

Duck

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
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	6

Deer

Components	x1 (µl)
2x QIAGEN Multiplex Master Mix	12.5
V1 sense primer (20µM)	0.75
V2 sense primer (20µM)	0.75
V3 sense (20µM)	0.75
V4 anti-sense primer (20µM)	0.75
V5 anti-sense primer (20µM)	0.75
Water	3.75

12.3 PCR cycling parameters

Step	Function	Temperature	Time
1	Initial activation step	95°C	15minutess
2 (30 - 45 cycles*)	Denaturation	94°C	30 seconds
3	Annealing	60°C	90 seconds
4	Extension	72°C	90 seconds
5	Final extension	72°C	10 minutes

*Use 30 cycles for pig, pheasant and duck assays
Use 40 cycles for sheep, turkey and donkey assays
Use 45 cycles for deer, cow, chicken and horse assays

12.4 Preparation of amplicons for analysis on BioAnalyser

Species	Ratio
Sheep:Horse:Donkey:water	12:4:3:5
Turkey:Chicken:Cow	1:1:1
Duck:Pheasant:water	1:3:2
Pig	neat
Deer	neat

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