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Evidence Project Final Report

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Project identification

1. Defra Project code: FA0135

2. Project title:
   Real-time PCR approach for quantitation of horse DNA and study into relevance of expression units (DNA/DNA and w/w tissue).

3. Contractor organisation(s):
   LGC Ltd
   Malcolm Burns

4. Total Defra project costs (agreed fixed price): £ 89,651

5. Project: start date ..... 8th February 2013
   end date ..... 8th June 2013

6. It is Defra's intention to publish this form.

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Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

As part of the response to the EU horse-meat issue, Defra/FSA commissioned an official UK Survey of beef products. There is a lack of guidance and scientific standardisation of how the amount of meat adulteration in a sample is expressed and sparse information in the peer reviewed literature on measurement of horse-meat relative to other species. Hence harmonised methods for the accurate quantitation of horse-meat remain to be developed.

Therefore, the aim of this project was to develop a real-time PCR approach for the quantitation of horse DNA using best measurement practice guidelines in the field of PCR and meat speciation. Additionally, a review of expressing meat adulteration in terms of w/w tissue mass and DNA:DNA copy numbers was conducted, in order to establish if guidance could be provided on the equivalence of these expression units.

A review of the current scientific literature revealed a panel of potential DNA target sequences for the detection and quantitation of horse meat in food samples using real-time PCR. These targets were assessed for their suitability as reference genes, based on published literature and experimental evidence. In line with best measurement practice associated with meat speciation and quantitation, two targets were chosen for further development and full validation. Single copy nuclear DNA targets were chosen and assays selected for the equine growth hormone (Koppel *et al*., 2011) and the myostatin gene present in mammals (Laube *et al*., 2003). Each of the assays was optimised for performance to ensure they could be run simultaneously.

Following best measurement practice with respect to molecular biology approaches and meat speciation, an approach was developed using real-time PCR that would quantitate the amount of horse DNA relative to the total amount of mammalian DNA present. Raw muscle flesh (horse-meat and beef) was sourced from reputable suppliers and authenticated as to species identity using PCR, ELISA and DNA sequencing. Following sample preparation and homogenisation, DNA was extracted from these samples and used to produce a range of DNA:DNA ad-mixtures (horse DNA: beef DNA gravimetric preparations). The same range in w/w gravimetric materials (raw horse meat in a raw beef [meat] background) were also prepared. Method validation was performed in order to qualify the fitness for purpose of the assays through the evaluation of the performance characteristics of specificity, Limit of Detection (LOD), Limit of Quantitation (LOQ), PCR efficiency, linearity, trueness (bias) and precision.

Specificity test were conducted to check the cross reactivity of the Koppel and Laube assays. Published literature stated that the Koppel assay was highly selective to horse DNA, but also exhibited some cross reactivity with mule/donkey. This was verified experimentally. However, as mule/donkey are not common meats in the UK food chain, and the undeclared presence of any meat species in food is considered non-compliant with EU labelling legislation, the assay was still considered applicable. The Laube assay targets a single copy myostation gene present in mammalian, poultry and fish species, and was considered fit for purpose.

Based on a simple serial dilution series from 100% horse DNA used as a calibrant, results indicated that both real-time PCRs assays were capable of reliably detecting a minimum of five estimated horse genome equivalents (LOD), while exhibiting good amplification curves and levels of fluorescence. The DNA:DNA ad-mixtures covered ranges of 0.1. 0.5, 1, 5, 30, 50 and 100% horse DNA in beef DNA. Linearity of calibration curves were good (mean r-squared of 0.994 for Koppel, and 0.989 for Laube) as were mean PCR efficiencies (99.6% for Koppel and 97.8% for Laube). The Koppel/Laube assay combination consistently generated good estimations of the expected percentage horse DNA present across the range of the DNA:DNA ad-mixtures compared to the horse DNA calibrant. Precision estimates were between 7 and 29 % coefficient of variation (CV), with the poorer levels of precision generally associated with low level horse DNA:DNA ad-mixtures. Relative quantitation data (mean bias varying between -30% to +10% of assigned value of calibrants) indicated that the Koppel/Laube combination was fit for purpose and suitable for further characterisation using the w/w gravimetric materials.

The assays were challenged through the range of w/w gravimetric materials prepared from the authenticated meat samples, in order to provide a more representative sample matrix. The assays demonstrated good performance characteristics, including high mean r-squared values (0.995 for Koppel and 0.997 for Laube) and mean PCR efficiencies (90.4% for Koppel and 94.6% for Laube). Precision estimates were between 5 and 27% CV, showing good comparability to the DNA:DNA ad-mixtures evaluation. Relative quantitative analyses showed reasonable agreement with the expected percentage horse DNA levels across the range of w/w gravimetric materials, with the mean bias varying between -60% to +60% depending upon level. The larger variability in these bias estimates was expected for the w/w gravimetric materials, because of the greater measurement uncertainty in preparing and sampling

from w/w gravimetric materials compared to DNA:DNA ad-mixtures.

The limit of quantitation (LOQ) was defined as the sample with the lowest relative amount of horse present, that could still be reliably quantified (95% confidence interval incorporates the assigned value). The limit of quantitation of the assay was estimated as less than 0.1% horse DNA in a background of beef DNA, and the equivalent of 0.1% gravimetric preparations of raw horse meat in a raw beef (meat) background.

The fitness for purpose of the quantitative approach was qualified through method validation and application to a range of DNA:DNA ad-mixtures and w/w gravimetrically prepared raw horse-meat in raw beef materials. When calibrants and test samples are derived from the same source, it is expected that there should be good comparability in the quantitative estimate of horse meat present in a sample when expressed using both a DNA:DNA genomic copy number approach and a w/w tissue approach. In reality, the probability of test samples and calibrants being derived from exactly the same sources will be extremely low, thus full quantitation in terms of w/w tissue mass for all possible permutations of species/tissue types cannot be achieved. This issue is attested to by other experts in the field and through previous work commissioned by the FSA. However, the assays described in this report facilitate quantitation on a DNA:DNA basis, which many experts agree should be the way forward with respect to harmonising how the relative level of different meats should be expressed in a sample, thus affording better standardisation and traceability. Additionally, the assay described in this report allows comparability to the equivalent w/w raw-horse meat in raw beef [meat] gravimetric materials. Guidance has just been provided by the EU on how to establish a threshold value for meats species present in a sample on a w/w basis (above which it is considered deliberate adulteration, and below which it is considered adventitious contamination). The approach described in this paper has been so designed to make it adaptable and effectively "future-proofed" so it can be used to determine if the level of horse DNA in a test sample is significantly above or below this equivalent w/w threshold level, and should be tested using the newly published EU guidance (EURL-AP, 2013)

In conclusion, the development and validation of a real-time PCR assay for the quantitation of horse DNA (based on a simple dilution series of 100% horse DNA) as described in this report, has shown that the assay:

1) Can accurately quantify the relative amount of horse DNA in a mixed sample, and exhibits good comparability to w/w gravimetric materials when samples and calibrants contain similar materials
2) Is subject to the same limitations inherent in all meat quantitation approaches available: that of converting DNA:DNA copy number measurements into w/w tissue measurements
3) Has significantly added value to the state-of-the-art of the science and made publically available an approach for the quantitation of horse DNA in line with best measurement practice in the field
4) Is adaptable and "future-proofed" for its potential to incorporate and fully characterise the threshold for labelling, as proposed by the recently published EU guidance (EURL-AP, 2013)

Factors that can affect the correct expression of meat species in terms of w/w tissue mass include, but are not limited to: sample composition; DNA extraction approach; real-time PCR quantification approach; choice of DNA target; level of PCR optimisation; degree of PCR inhibition/DNA degradation; and data normalisation approaches. The issue of not being able to directly compare the amount of adulteration expressed in terms of w/w tissue mass and DNA:DNA copy number is not unique to horse-meat detection and is equally applicable in all meat-speciation approaches and how PCR and related techniques are applied to meat-speciation issues in general. The issue lies with the application and interpretation of the PCR technology in these instances, as opposed to a problem with the technology itself. Greater guidance from the EU will help facilitate a more standardised approach to analysing and interpreting results from meat-speciation issues, and the work described in this report allows accurate quantitation of the relative amount of horse DNA present in a sample, and provides a solid frame-work for comparing this to the equivalent w/w of raw-horse meat in a raw beef background, as qualified by the results.

Future work should involve:

- Fully characterising the measurement uncertainty associated with the application of a threshold as proposed by the recently published EU guidance (EURL-AP, 2013). Use this method to challenge and enforce the w/w threshold for labelling of adulterant meat species in the UK.
- A Knowledge Transfer event followed by a ring-trial of the method amongst Public Analysts to evaluate the reproducibility of the method
- Application of the method to representative and commercially available food samples, to characterise and model the effects of processing and additives
- Quantitate samples regarded as positive in the original UK horse-meat Survey exercise
- Characterise and model the effect of inhibition on the method

Glossary

A260 - Absorbance at 260 nm

A260:230 - Ratio of absorbance at 260 and 230 nm

A260:280 - Ratio of absorbance at 260 and 280 nm

ACTB - Beta Actin

C_q - Cycle of quantification

CV - Coefficient of variation

DEFRA - Department for Environment, Food and Rural Affairs

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

ELISA - Enzyme Linked Immuno Sorbant Assay

EU - European Union

FSA - Food Standards Agency

gDNA - Genomic DNA

GH - Growth Hormone

GHR - Growth Hormone Receptor

LoD - Limit of Detection

LoQ – Limit of Quantitation

M – Molar

MB – Mega Base

Mg^{2+} - Magnessium ion

mL - millilitre

mM - Milli Molar

MSTN – Myostatin

NaCl – Sodium Chloride

ng - Nanograms

PCR - Polymerase Chain Reaction

qPCR - Quantitative real time Polymerase Chain Reaction

R^2 - R squared

RQ - Relative Quantification

rRNA - Ribosomal Ribonucleic Acid

SDS - Sodium Dodecyl Sulphate

SOP - Standard Operating Protocol

Std. Dev. - Standard deviation

UK - United Kingdom

w/w - Weight for Weight

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - any action resulting from the research (e.g. IP, Knowledge Exchange).

Scientific objective as set out in the contract

Objective No.	Objective Description
	Method development for the quantitation of equine DNA
01	**Current review** A current review will be conducted of approaches for the detection and quantitation of meat species using DNA.
02	**Source material and prepare samples** Source appropriate species materials and order in laboratory consumables. Extract DNA and prepare samples and standards taking into account representative w/w measurements
03	**Select DNA targets and design real-time PCR primers and probes** Select appropriate target DNA sequences to afford greatest species specificity.
04	**Set-up and validate the method** Test for specificity: theoretically (using known database sequences) and experimentally (against other meat species and appropriate food ingredients). Develop real-time PCR approach for quantitation of equine DNA in bovine DNA using calibration curves and spiked samples. Validate in terms of LOD, repeatability, trueness and specificity
05	**Report project findings from quantitative approach** The findings of the project will be disseminated and guidance provided with respect to the use of the quantitative approach
	Feasibility of establishing direct equivalence between expression units
06	**Source materials and sample preparation** Additional species tissues will be sought if necessary to complement those acquired in Objective 02. Appropriate w/w tissue samples will be prepared and DNA extracted from these. Laboratory consumables will be ordered in.
07	**Evaluation of w/w samples** Appropriate technologies (e.g. real-time PCR and digital PCR) will be used to evaluate the w/w samples in terms of DNA copy numbers and a comparison made with w/w tissue
08	**Provide guidance on equivalence of expression units** Guidance will be given on the equivalence of the expression units of w/w tissue and DNA/DNA copy number including limitations and caveats associated with the approach

2.0 The extent to which the Objectives have been met

Objective 01 – Current review

Objective 01 was completed successfully. A review of the current scientific literature was performed, and a panel of potential DNA target sequences for use in the detection and quantification of horse meat in food and feed samples using real-time PCR were sourced. The review identified a number of published real-time PCR assays whose performance warranted further evaluation.

Objective 02 – Source material and prepare sample (DNA:DNA ad-mixtures)

Objective 02 was completed successfully. Raw muscle flesh (horse-meat and beef) was sourced from a known supplier, trimmed free of surface inter-muscular fat and connective tissue and authenticated as to species type by PCR, ELISA and DNA sequencing. DNA:DNA ad-mixtures were prepared by combining known nanogram masses of DNA from authenticated raw horse-meat with DNA derived from authenticated raw beef (meat) material. Mass-based ratio preparations of 100%, 50%, 30%, 5%, 1%, 0.5% and 0.1% of raw horse-meat DNA in a raw beef-meat DNA background, were produced. 100% beef, pork, lamb, duck, mouse, donkey, human and chicken DNA reference materials were also sourced to test for cross reactivity with non specific DNA templates.

Objective 03 – Select DNA targets and design real-time PCR primers and probes

Objective 03 was completed successfully. A panel of hydrolysis probe-based quantitative real-time PCR (qPCR) assays was compiled from peer reviewed scientific literature targeting single copy nuclear genes: growth hormone (Brodmann and Moor, 2003), equine growth hormone receptor (Koppel et al., 2011) and myostatin (Laube *et al*., 2003). Mitochondrial marker sequences were excluded from this search due to quantitative limitations associated with this target type arising from tissue specific copy number variability.

Objective 04 –Method validation

Objective 04 was completed successfully. Initial evaluation identified the Koppel *et al*., (2011) equine specific and the Brodmann and Moor, (2003) / Laube *et al*., (2003) universal mammalian qPCR assays as suitable candidates for the study. Calibration curves were based on serial dilutions of 100% horse DNA. Assay performance characterisation was conducted to confirm that all assays demonstrated the minimum required PCR efficiency of 100% ± 15%, and had estimated LOD's of less than 5 genome equivalent copies for the horse specific assay, as well as confirming the appropriate target specificities. The quantitative capability of the assays was challenged using the range of DNA:DNA ad-mixtures from Objective 02, and results demonstrated the good repeatability and trueness of the method. Following on from method validation, the assay was deemed fit for purpose in terms of accurately estimating the horse DNA content relative to the total mammalian DNA content.

Objective 05 – Report project findings from quantitative approach

Objective 05 was completed successfully. A full summary of the results obtained in the validation study have been reported here and reported to Defra using this official "Evidence Project Final report", alongside an SOP (Appendix III).

Objective 06 – Source materials and sample preparation (w/w gravimetric materials)

Objective 06 was completed successfully. Raw muscle flesh (horse-meat and beef) was sourced from a known supplier, trimmed free of surface inter-muscular fat and connective tissue and authenticated as to species by PCR, ELISA and DNA sequencing. Test sample material was prepared gravimetrically by weighing the required amounts of authenticated raw horse-meat into authenticated raw beef (meat) material. Gravimetric preparations of 100%, 30%, 5%, 1%, 0.5% and 0.1% (w/w) of raw horse-meat in a raw beef (meat) background, were produced.

Objective 07 – Evaluation of w/w samples

Objective 07 was completed successfully. Gravimetric preparations of 100%, 30%, 10%, 5%, 1%, 0.5% and 0.1% (w/w) of raw horse-meat in a raw beef (meat) background, were evaluated relative to the DNA:DNA calibration curve derived from 100% horse DNA. Results exhibited good repeatability and trueness of the method as applied to the w/w gravimetric materials, based on muscle/tissue matched materials between the test samples and calibrants.

Objective 08 – Provide guidance on equivalence of expression units

Objective 08 was completed successfully. Guidance notes were compiled on the equivalence of the expression units of w/w tissue and DNA/DNA copy number which included a summary of the limitations

and caveats associated with the approach. Unless the test samples and calibrant(s) are derived from the same source, it is extremely unlikely that DNA:DNA copy number measurements can be directly equated to w/w tissue measurements. However, the assay developed as part of this project enables the quantitative analysis of the amount of horse DNA relative to the total mammalian DNA present in a test sample, provides a means of comparing this amount to the equivalent w/w tissue measurement of raw-horse mat in a raw beef [meat] background, and is "future-proofed" and adaptable in terms of complying with the latest EU guidance on establishment of threshold levels for detection of horse DNA (EURL-AP, 2013).

3.0 Materials and Methods

3.1. Objective 01 - Literature review

An initial review of the scientific literature was performed in order to identify publications referencing existing real-time PCR methods for meat species detection. The review identified a panel of DNA targets that could be employed in the design and development of additional real-time PCR assays for use in the detection and quantitation of horse meat in a beef background.

Objective 02 – Source material and prepare samples (DNA:DNA ad-mixtures)

Sourcing and authentication of materials

LGC is accredited to an international standard to produce reference materials and has produced standards of common meat species for a number of years. Meat samples were sourced from reputable suppliers inclusive of those that have the appropriate accreditation to international standards and maintained traceability records. The meat samples were surface trimmed and then prepared by removing any separable fat, gristle, etc. retaining the lean meat. The lean meat was cubed, thoroughly homogenised in a new food processor, combined and then mixed and the resultant paste stored. Similarly, beef (meat) was purchased from a reputable commercial source, and the same approach taken for preparation of beef (meat) as had been employed for the horse samples.

DNA extraction

DNA extraction was performed on 1 g tissue samples using the silica-based Kleargene DNA extraction method (KBioscience, UK). The extraction process comprised sample homogenisation followed by incubation, lysis of cellular components in an SDS buffer with proteinase K and binding of the isolated DNA to positively charged silica beads. Multiple washing stages were used to clean the DNA which was eluted in 0.5/1 ml of elution buffer. DNA yield (A260) and quality characteristics (A260:230 and A260:280) were determined using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, UK).

Authenticity testing

Species identify was authenticated in-house with using real-time PCR, ELISA and DNA sequencing:

Immunoassay analysis - Commercially available sandwich Enzyme Linked Immuno Sorbent Assays (ELISA) meat speciation kits were used. The meat samples were extracted using 0.15M NaCl; where necessary the sample extracts were subjected to a heating step (95-100^{o}C for 15 minutes) before testing. Meat extracts were added to duplicate wells of a pre-coated microtitre plate supplied with each kit and the assay was carried out in accordance with the manufacturer's protocol (ELISA-TEK® Cooked meat species assays)

DNA analysis - DNA was analysed by Sanger DNA sequencing. Two mitochondrial genes (12S rRNA and cytochrome b) were used for species specific identification of each joint of meat. All samples were identified as the correct species by alignment using DNA sequence databases. Additionally, samples were further validated based on the amplification of species specific genomic targets using PCR assays specific for horse and beef. Only the DNA product specific for the correct meat material being tested for was found in each case.

Preparation of DNA:DNA ad-mixtures

DNA:DNA ad-mixtures were prepared using horse and beef DNA derived from authenticated muscle tissue purified using the Kleargene DNA extraction technique (KBioscience, UK) as described above. DNA extracts were quantified by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, UK) and used to prepare DNA:DNA ad-mixes (50 ng total) at a range of mass-based ratios: 0.1%, 0.5%, 1%, 5%, 30%, 50% and 100% horse gDNA in beef gDNA.

Objective 03 – Select DNA targets and design real-time PCR primers and probes

A relative quantitative-based approach was developed to determine horse DNA content through the comparative analyses of horse/beef samples using a horse specific qPCR assay (horse DNA only) and universal mammalian assay (total DNA). Published hydrolysis probe-based qPCR assays developed by

Koppel *et al*., (2011) (horse/donkey/mule growth hormone receptor specific), Brodmann and Moor, (2003) (mammalian growth hormone gene specific) and Laube *et al*., (2003) (mammalian, poultry and fish myostatin gene) were optimised and then evaluated to determine basic performance characteristics.

Objective 04 – Method validation

The mammalian specific assays were designed to operate under standard 2-step real-time PCR cycling conditions (10 min/ 95^{o}C; 15 s/ 95^{o}C, 1 min/ 60^{o}C, 45 cycles) whilst the Koppel horse specific qPCR assay operated under modified conditions (62^{o}C rather than 60^{o}C). The Koppel assay was optimised to work under the standard conditions in order to simplify the method development process. Whilst the published assays provided results qualifying the specificity associated with each of the tests, it was decided to further confirm this experimentally, so the optimised assays were challenged with a panel of genomic DNAs (horse, donkey, beef, pork, lamb, duck, mouse, human and chicken) to ensure appropriate specificity levels and standard curve-based analyses to estimate repeatability, Limit of Detection (LOD) and PCR efficiencies.

Calibration curves were produced based on serial dilutions of 100% horse DNA. Validation of the relative quantitation-based method was initially performed using DNA:DNA ad-mixtures comparing the discriminatory potential of the Koppel horse/Brodmann and Koppel horse/Laube assay combinations. Triplicate qPCR plates were undertaken comprising a 20,480 to ~1.25 horse genome equivalents (assuming a haploid genome size of 2474.93 MB) 8 point standard curve, 100%, 50%, 30%, 10%, 5%, 1%, 0.5% and 0.1% DNA:DNA horse in beef ad-mixtures and appropriate controls per target assay at a triplicate PCR technical replicate level. Assay performance-based metrics such as PCR efficiency, precision and LOD were derived from the validation work (Table 1).

Performance Metric	Methodology
PCR Efficiency	Assay specific mean PCR efficiency from triplicate plates: **% E = 100 x (-1 + 10$^{-(1/\text{calibration curve slope})}$)**
Limit of Detection (LOD)	Pooled assay specific calibrant data from triplicate plates to identify calibrant with lowest copy number that could still be detected with at least 95% probability
Limit of Quantitation (LOQ)	Pooled assay specific DNA:DNA ad-mixture and % w/w data from triplicate plates to identify test sample with lowest relative amount of horse DNA present that could be reliably quantified (estimated mean value ± 95% confidence interval incorporates assigned value of calibrant).
Precision	Assay specific mean percentage coefficient of variation from triplicate plates: **% CV = 100 x (standard deviation/mean)**
Bias	Assay specific mean bias from triplicate plates: **Bias = estimated value/assigned value**

Table 1. Performance metrics employed to characterise qPCR assays and associated methodology.

Horse gDNA content was determined by standard curve analyses and normalized to total mammalian gDNA content to generate a percentage ratio.

$$\text{Estimated \% horse DNA content} = \frac{\text{Standard curve derived horse genome equivalent copy number}}{\text{Standard curve derived total mammalian genome equivalent copy number}} \times 100$$

Objective 05 – Report findings from quantitative approach

The results of the quantitative approach have been summarised in this final report form, and an SOP provided in Appendix III.

Objective 06 – Source materials and sample preparation (w/w gravimetric materials)

Weight for weight test samples

Raw horse muscle flesh and mixtures of raw lean horse in beef were produced. The w/w materials were

prepared gravimetrically by weighing the required amounts of the authenticated raw horse-meat into the authenticated raw beef (meat) and placed in 50mL screw-cap sample pots. The units were prepared individually by weighing authenticated meats into sample pots. When stored under the recommended conditions (-20 +/- 5) ^{o}C, the materials are expected to remain stable. Tests were carried out at LGC to check the effect of one additional freeze-thaw cycle and no significant change was observed on analyses. Gravimetric preparations of 100%, 30%, 5%, 1%, 0.5% and 0.1% (w/w) of raw horse-meat in a raw beef (meat) background, were prepared. Samples were prepared individually and gravimetrically by weighing out each meat material. The uncertainty of the weighings was estimated as approximately 2% at the 95% confidence level (k=2). Assigned values represent nominal values subject to no more than a 10% relative margin of error. Examples of the measurement statements associated with the raw horse-meat and w/w raw horse-meat in raw beef (meat) materials are provided in Appendices I and II. DNA was extracted from these samples as described previously in Objective 2.

Objective 07 – Evaluation of w/w samples

Final validation of the relative quantitation-based method was performed using w/w gravimetric materials and the Koppel horse/Laube assay combination. Triplicate qPCR plates were undertaken comprising 7 point standard curve (Koppel assay, 20,480 horse to ~ 5 genome equivalent copies), 6 point standard curve (Laube assay, 20,480 horse to ~ 20 genome equivalent copies), 100%, 30%, 5%, 1%, 0.5% and 0.1% w/w horse in beef tissue ad-mixture DNA extracts with appropriate controls per target assay at a triplicate technical replication level. Horse gDNA content was determined by standard curve analyses and normalized to total mammalian gDNA content to generate a percentage ratio. Assay performance-based metrics including PCR efficiency and LOD were also derived.

Supplementary work beyond the scope of the current project was conducted to examine inhibitory effects. A preliminary study investigating the potential impact of matrix effects upon relative quantitative performance was undertaken using ethylenediaminetetraacetic acid (EDTA) which is a well characterized PCR inhibitor. A panel of DNA:DNA horse in beef ad-mixtures containing 0 mM (control), 3 mM (inhibitory effect anticipated) or 6 mM (total inhibition anticipated, equals Mg^{2+} levels within master mix) EDTA final reaction concentration were analysed using the standard Koppel/Laube method.

Objective 08 – Provide guidance on equivalence of expression units

The feasibility of establishing direct equivalence between expression units of DNA copy numbers to w/w tissue equivalents was further investigated by a review of the relevant scientific literature. This would complement results from Objective 7 where the same species/tissues types were used between test samples and calibrants for the evaluation of the w/w gravimetric materials.

4.0 Results

Objective 01 – Review of current quantitative meat detection approaches using DNA

A number of publications exist detailing the detection of commercially important species using real-time PCR (Lahiff *et al*., 2002, Laube *et al*., 2003, Sawyer *et al*., 2003). In general, it is agreed amongst experts that the use of mitochondrial DNA allows more sensitive assays to be developed because of the abundance of the mitochondrial genome in cells (Hird *et al*., 2005b; Holzhauser *et al.,* 2006). However, because of the variability in the number of mitochondrial copies amongst species, and even between tissue types within the same species, mitochondrial DNA should generally not be used for quantitative purposes (Rodriguez *et al*., 2003) and articles published in the recent scientific literature (Laube *et al*., 2003, 2007a, 2007b) favour the use of single copy nuclear DNA targets (Ballin *et al*., 2009).

The quantitative work conducted in the current study, therefore focused on an evaluation of the quantitative potential with respect to existing real-time PCR assays that used nuclear DNA as a target. Assays selected for evaluation targeted the horse growth hormone receptor gene (GHR) (Koppel *et al*., 2011), mammalian growth hormone gene (GH) (Brodmann *et al*., 2003), and the myostatin gene (MSTN) present in mammals, poultry and fish (Laube *et a*l., 2003). Koppel *et al* (2011) have justified the use of the GHR gene as an equine specific target on the basis of the high levels of assay specificity predicted for the target *in silico.* Conversely, Brodmann and Moor (2003) have advocated the use of the GH gene as a target for the identification of mammalian species in general on the basis of the high degree of sequence conservation observed with this gene between species. As an alternative to GH, Laube *et al* (2003) suggested the use of MSTN as a target for detection of other non-equine species owing to the fact that the degree of sequence conservation for MSTN existing between mammals also extends to poultry.

Objective 02 – Source material and prepare samples (DNA:DNA ad-mixtures)

Material was sourced and prepared as explained in the Materials and Methods section.

Objective 03 – Select DNA targets and design real-time PCR primers and probes

The relative quantitative-based approach employed within this project necessitated the identification and optimisation of horse specific and universal mammalian real-time PCR assays. Published hydrolysis probe-based assays were identified with the appropriate performance characteristics and subsequently validated. The selected assays comprised:

- Koppel *et al*., (2011) assay targeting the equine growth hormone receptor (Koppel)
- Brodmann and Moor (2003) assay targeting the mammalian growth hormone gene (Brodmann)
- Laube *et al*., (2003) assay targeting the mammalian, poultry and fish myostatin gene (Laube)

Objective 04 – Method validation

Calibration curves were based on serial dilutions of the 100% horse DNA. Initial evaluation work was performed to establish general qPCR assay performance characteristics including sensitivity and specificity. All qPCRs were capable of detecting a minimum of 5 estimated horse genome equivalents (a LOD of 5 copies could be detected with at least 95% probability) and demonstrated good amplification curves/fluorescence levels. Specificity testing showed that the Koppel horse assay exhibits some cross-reactivity with the closely related domesticated donkey species (*Equus asinus asinus*), as described in the original paper (Koppel *et al*., 2011), whilst the universal mammalian assays correctly detected mammalian gDNA template (plus poultry template for the Laube assay).

Following this evaluation work, two approaches were investigated for the relative quantitation (RQ) of horse DNA utilising the Koppel *et al*. (2011) horse specific qPCR assay in combination with either the Brodmann and Moor (2003) or Laube *et al*., (2003) universal mammalian qPCR assays.

A panel of DNA:DNA ad-mixtures were used to challenge the Koppel/Brodmann and Koppel/Laube RQ methods and determine key performance characteristics including LOD, precision and bias. Initial validation work demonstrated that the individual qPCR assays successfully detected at least 5 estimated genome equivalents of horse DNA calibrant and generated calibration curves characterised by $R^2 > 0.980$ and PCR efficiency > 88% (see Figures 1 & 2, Tables 2 & 3).

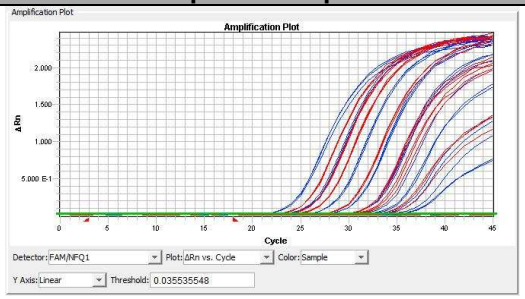
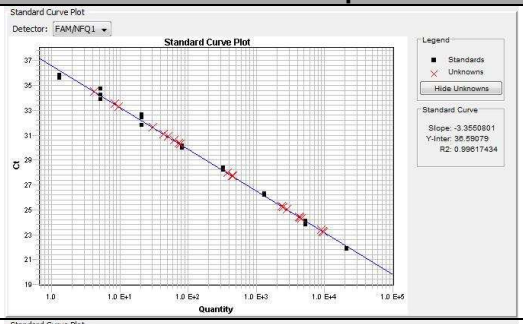
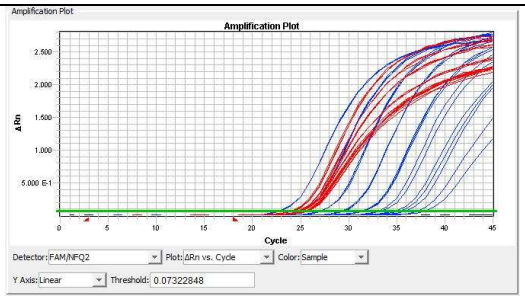
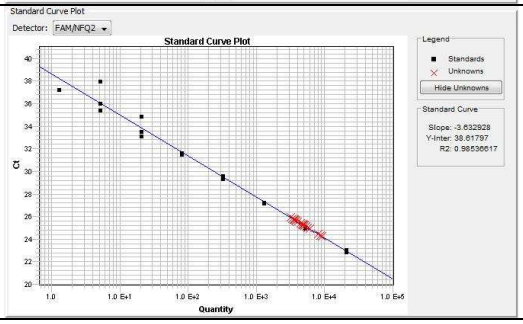
Assay	Amplification plots	Calibration curve plots
Koppel Horse		
Brodmann		

Figure 1. Example of Koppel/Brodmann relative quantitation method qPCR data. The plots were based on an 8 point serial dilution series of 100% horse DNA (20,480 to ~1.25 horse genome equivalents); 100%, 50%, 30%, 5%, 1%, 0.5% and 0.1% DNA:DNA horse in beef ad-mixtures and appropriate controls per target assay at a triplicate technical replicate level. Amplification plots displayed as calibrant (blue) and test sample (red). Calibration curve components displayed as calibrant (black squares) and test samples(red crosses).

Assay	R^2	Intercept	Slope	PCR Efficiency
Koppel Horse	0.997	36.820	-3.429	95.7 %
Brodmann	0.993	38.579	-3.626	88.7 %

Table 2. DNA:DNA ad-mixtures - Koppel/Brodmann RQ method performance data. Performance metrics generated based on triplicate reactions within one plate (n=1) comprising a 7 point serial dilution series of 100% horse DNA (20,480 to ~5 horse genome equivalents).

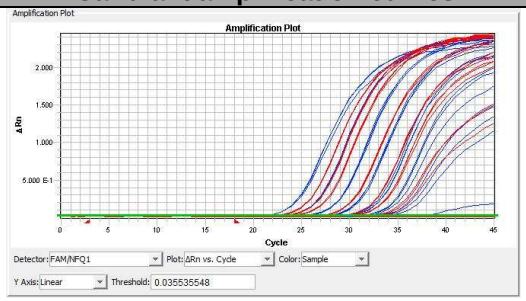
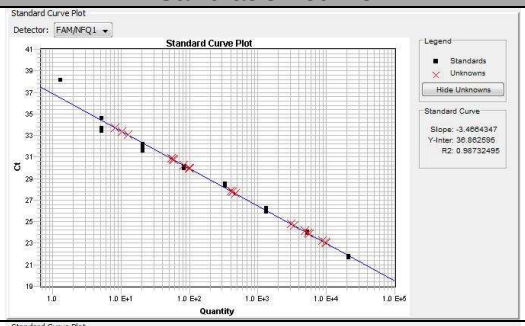
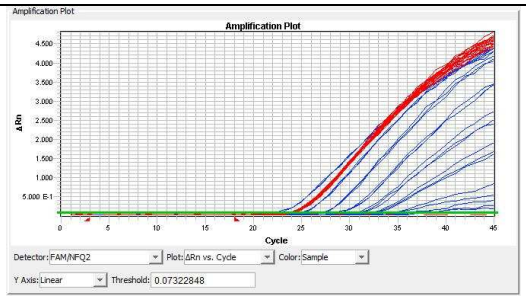
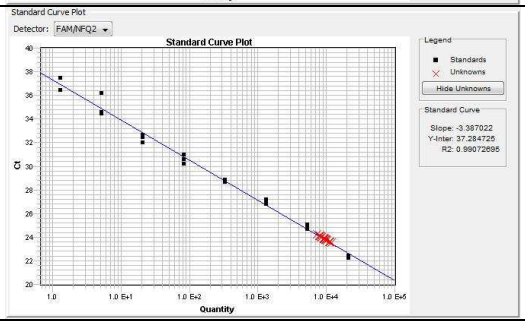
Assay	Calibrant amplification curves	Calibration curve
Koppel Horse		
Laube		

Figure 2. Example of Koppel/Laube relative quantitation method qPCR data. The plots were based on an 8 point serial dilution series of 100% horse DNA (20,480 to ~1.25 horse genome equivalents); 100%, 50%, 30%, 5%, 1%, 0.5% and 0.1% DNA:DNA horse in beef ad-mixtures and appropriate controls per target assay at a triplicate technical replicate level. Amplification plots displayed as calibrant (blue) and test (red) sample types. Calibration curve components displayed as calibrant (black squares) and test (red crosses) sample types.

Assay	Mean R^2	Mean Intercept	Mean Slope	Mean PCR Efficiency
Koppel Horse	0.994	36.440	-3.331	99.6 %
Laube	0.989	37.246	-3.375	97.8 %

Table 3. DNA:DNA ad-mixtures - Koppel/Laube RQ method performance data. Performance metrics generated based on triplicate reactions within three replicate plates (n=3) comprising a 7 point serial dilution series of 100% horse DNA (20,480 to ~5 horse genome equivalents) per target assay.

Calibration curves were typically based on 7 point serial dilution series from 100% horse DNA, ranging from approximately 20,480 to 5 horse genome equivalents. In all cases, the last calibration point with a nominal copy number of 5 was reliably detected on all occasions. The eighth serial dilution consisting of approximately 1.25 horse genome equivalents was detected on less than 95% of occasions, and was omitted from the calibration curve. Therefore the LOD of the Koppel/Brodmann and Koppel/Laube assays was estimated to be approximately 5 horse genome equivalents.

Relative quantitative analyses of the DNA:DNA ad-mixtures by both methods identified clear differences in calculated percentage horse DNA content whereby the Koppel/Brodmann method over estimated percentage horse content proportional to beef DNA content (Table 4). This observation supported earlier findings and suggests that the Broadmann and Moor assay preferentially amplifies horse DNA (bias values above 2) as compared to beef DNA, and impacts the suitability of this method for relative quantitation applications. Therefore, the Koppel/Brodmann method validation study was not continued and was removed from subsequent work, and is thus represented by data from just one replicate plate in this

report.

In comparison the Koppel/Laube method consistently generated good estimations of the expected percentage horse DNA levels across a wide range of ad-mixtures as shown by the low associated bias values (see Table 5). Precision estimates were found to vary between 7 and 29 % CV with poorer levels of precision generally associated with low level horse ad-mixtures, as expected. The RQ data demonstrated that this method was fit for purpose and suitable for further characterisation using weight/weight gravimetric preparations.

DNA:DNA ad-mixture	Estimated Horse content (Brodmann)	Bias
0.1% Horse	0.22%	2.2
0.5% Horse	1.32%	2.6
1% Horse	2.06%	2.1
5% Horse	9.45%	1.9
30% Horse	49.07%	1.6
50% Horse	72.34%	1.4
100% Horse	103.85%	1.0

Table 4. DNA:DNA ad-mixtures - Koppel/Brodmann relative quantitation method data, RQ data from a single replicate plate (n=1) based on a seven point calibration curve of 100% horse DNA (20,480 to ~5 horse genome equivalents) with DNA:DNA horse in beef ad-mixture test samples (100%, 50%, 30%, 5%, 1%, 0.5% and 0.1%) per target assay at a triplicate technical replicate level.

DNA:DNA ad-mixture	Estimated Mean Horse content (Laube)	% CV	Mean Bias
0.1% Horse	0.08%	23.5	0.7
0.5% Horse	0.46%	16.6	0.9
1% Horse	0.86%	9.2	0.9
5% Horse	4.74%	11.1	0.9
30% Horse	31.89%	11.8	1.1
50% Horse	54.10%	29.4	1.1
100% Horse	95.40%	7.0	1.0

Table 5. DNA:DNA ad-mixtures - Koppel/Laube relative quantitation method data, RQ data from three replicate plates (n=3) based on a seven point calibration curve of 100% horse DNA (20,480 to ~5 horse genome equivalents) with DNA:DNA horse in beef ad-mixture test samples (100%, 50%, 30%, 5%, 1%, 0.5% and 0.1%) per target assay at a triplicate technical replicate level.

The limit of quantitation (LOQ) for the Koppel/Laube assay was estimated as less than 0.1% on a DNA:DNA basis, because the estimated mean value and associated 95% confidence interval of the lowest test sample analysed (0.1% Horse) incorporated the true assigned value of 0.1% horse DNA.

Objective 05 – Report findings from quantitative approach

The results of the quantitative approach have been summarised in this final report form, and an SOP provided in Appendix III.

Objective 06 – Source materials and sample preparation (w/w gravimetric materials)

Materials were sourced and prepared as explained in the Materials and Methods section.

Objective 07 – Evaluation of w/w samples

DNA:DNA ad-mixtures provide a model system with inherently lower levels of variability unlike the more commonly used weight/weight based gravimetric materials, which would also be more representative of

samples that laboratories would have to extract and analyse. A study similar to that conducted with DNA:DNA admixtures was performed with DNA extracted from the range of percentage w/w horse in beef gravimetric materials. Comparable R^2 (~ 0.99) and PCR (> 90%) efficiency values were found to those observed in the DNA:DNA ad-mixture study (see Table 6)

Assay	Mean R^2	Mean Intercept	Mean Slope	Mean PCR Efficiency
Koppel Horse	0.995	38.496	-3.577	90.4%
Laube	0.997	37.924	-3.458	94.6%

Table 6. w/w gravimetric materials - Koppel/Laube assay performance data. Performance metrics generated from three replicate plates (n=3) comprising 6 point (Laube) and 7 point (Koppel) serial dilution series of 100% horse DNA (20,480 to ~ 20 (Laube) or 5 (Koppel) horse genome equivalents) per target assay at a triplicate technical replicate level.

Relative quantitative analyses of the w/w gravimetric materials showed good comparisons between the estimated and expected percentage horse DNA levels across the range of the gravimetric materials, as demonstrated by the bias estimates (see Table 7). Precision estimates were found to vary between 7 and 25 % CV with poorer levels of precision generally exhibited with the lower level w/w gravimetric materials.

w/w gravimetric materials	Estimated Mean Horse content (Laube)	% CV	Mean Bias
0.1% Horse	0.17%	25.8	1.7
0.5% Horse	0.53%	7.8	1.1
1% Horse	0.86%	15.7	0.8
5% Horse	2.15%	6.4	0.4
30% Horse	33.91%	7.7	1.1
100% Horse	106.65%	1.5	1.1

Table 7. w/w gravimetric ad-mixtures - Koppel/Laube assay relative quantitation data. RQ data from three replicate plates (n=3) based on a 6 point (Laube) and 7 point (Koppel) calibration curve of 100% horse DNA (20,480 to ~ 20 (Laube) or 5 (Koppel) horse genome equivalents) with w/w horse in beef ad-mixture test samples (100%, 30%, 10%, 5%, 1%, 0.5% and 0.1%) per target assay at a triplicate technical replicate level. Assigned values for the gravimetric materials represent nominal values subject to no more than a 10% relative margin of error.

The limit of quantitation (LOQ) of the Koppel/Laube assay was estimated as less than 0.1% w/w (raw horse meat in a raw beef [meat] background), based on the estimated mean value and associated 95% confidence interval of the lowest test sample analysed (0.1% Horse) incorporating the true assigned value of 0.1% horse DNA.

Further analysis was performed to normalise estimated horse content to the 100% horse sample and hence reduce sources of variability (see Table 8). This normalisation strategy had a limited impact on results as shown by similar calculated mean % horse content, %CV and mean bias values.

w/w gravimetric materials	Estimated Mean Horse content (Laube)	% CV	Mean Bias
0.1% Horse	0.16%	27.0	1.6
0.5% Horse	0.51%	4.7	1.0
1% Horse	0.71%	16.7	0.7
5% Horse	2.02%	7.5	0.4
30% Horse	31.81%	8.6	1.1
100% Horse	n/a	n/a	n/a

Table 8. w/w gravimetric ad-mixtures - Koppel/Laube assay relative quantitation data normalised to 100% horse ad-mixture. RQ data from three replicate plates (n=3) based on 6 point (Laube) and 7 point (Koppel) calibration curve of 100% horse DNA (20,480 to ~ 20 (Laube) or 5 (Koppel) horse genome equivalents) with w/w horse in beef ad-mixture test samples (100%, 30%, 10%, 5%, 1%, 0.5% and 0.1%) per target assay at a triplicate technical replicate level. Assigned values for the gravimetric materials represent nominal values subject to no more than a 10% relative margin of error.

Supplementary work beyond the scope of the current project was undertaken to investigate the impact of PCR inhibition upon RQ performance using EDTA as a well characterised model inhibitor. Initial evaluations showed that EDTA has a differential impact upon the Koppel and Laube qPCR assays (Table 9). The Laube assay was found to be more susceptible to the effect of EDTA as shown by the increased estimate of percentage horse content and associated bias. These differing susceptibilities to inhibition could strongly impact on relative percentage horse content determinations as illustrated by the over estimation of horse DNA content within the 100% Horse (3 mM EDTA) samples. Diluting the EDTA supplemented samples (100% Horse 3mM or 6 mM EDTA) provides a potential strategy to minimise the effect of PCR inhibitors as demonstrated by the lower biases associated with the diluted samples. It is recommended that the potential impact of inhibitors and how to account for these in the analyses should be further examined, as these inhibitory effects are likely to impact upon all quantitative approaches for meat speciation analysis and are not confined to the assays described in this report.

Sample	Estimated Mean Horse content (Laube)	% CV	Mean Bias
100% Horse (Reference)	117.7%	12.4	1.2
100% Horse (3 mM EDTA)	627.4%	17.1	6.3
1:4 Dilution 100% Horse (3 mM EDTA)	124.9%	36.1	1.2
100% Horse (6 mM EDTA)	Total inhibition	-	-
1:4 Dilution 100% Horse (6 mM EDTA)	109.7%	2.7	1.1

Table 9. Investigating the impact of EDTA inhibition on the Koppel/Laube relative quantitation method. RQ data from three replicate plates (n=3) based on 100% horse DNA six point calibration curve (20,480 to 20 copies) with 100% w/w horse ad-mixture DNA extract supplemented with 0 mM, 3 mM or 6 mM (final concentration) EDTA and diluted appropriately per target assay at a duplicate technical replicate level.

Objective 08 – Provide guidance on equivalence of expression units

Guidance on equivalence of expression units

While identification of undeclared meat in products by DNA methods can be relatively straightforward (detected or not detected with an associated LOD) accurate quantification of adulterant meat content is not routine. The conversion of DNA measurements (target copy number or mass) to the corresponding meat content values (w/w) is analytically complex (Hird *et al*., 2005a; Ballin *et al*., 2009). Recent reviews have suggested that the quantitative capability of meat speciation approaches can be best facilitated through using calibration curves based on expressions of genome:genome equivalents from single copy targets (Ballin *et al*., 2009). It has been suggested that quantitative species determination should be based on qPCR analysis and the results expressed in terms of genome/genome equivalents and not on w/w. Standardisation and best practice guidelines have already been established for this type of approach, and

a number of DNA based, certified reference materials are available (Ballin *et al*., 2009). Despite the reservations raised in the literature to the use of w/w expressions, current legislative guidelines remain adherent to the reporting of meat adulteration on a w/w basis. The following list is not exhaustive, but highlights those factors which can affect the quantitative determination of meat sample content on w/w basis, and which should be taken into account when reporting the results on any analysis:

1. Sample composition, processing and storage conditions;
2. DNA extraction/DNA extractability;
3. Quantification approaches and choice of assay target;
4. PCR efficiency, PCR inhibition / DNA degradation;
5. Data normalisation.

The above points have been described in detail elsewhere (Laube *et al*., 2007a; Ballin *et al*., 2009) but are graphically represented in Figure 3, and summarised in the following sections.

Figure 3. Factors affecting the determination of meat species and reporting on a meat content (w/w) basis.

Sample composition

Sample composition can significantly influence the determination of the constituent content of a sample by DNA based methods. Modern food processing practices commonly include the addition of minced meats which can originate from multiple tissue types as well as from different animal species. Additional compounds may be included to enhance a product's characteristics (appearance, shelf life) and can include derivatives from plants and other sources. The resulting product may not be of a homogeneous composition and may affect a sample's composition. In some instances product additives may co-purify with the extracted DNA and inhibit downstream processes including PCR. Best practice guidelines advocate that DNA quality should always be evaluated prior to being employed in downstream applications. Spectrophotometry and the use of PCR internal controls are examples of quality control

approaches routinely employed for this purpose. Where contamination or PCR inhibition is observed, steps should be taken to re-purify samples, for example through ethanol mediated DNA precipitation or using a number of commercially available PCR purification kits for these purposes.

DNA extraction

Processed food is frequently comprised of a mix of different ingredients. The target DNA species may therefore be present in low amounts, and dependent upon a sample's treatment this may be of a degraded or fragmented condition. A wide number of DNA extraction procedures are now available, either from the scientific literature or in kit format from commercial providers. It is advisable to evaluate more than one method as each may offer advantages or disadvantages with use for different sample types. For example, column based methods may not recover smaller DNA fragments whereas solvent based methods can favour co-purification of PCR inhibitors. Good molecular biology practice should be adhered to in order to ascertain if the DNA is of the right quality and sufficient quantity. This can be achieved through spectrophotometric quality metrics, as well as assessing PCR performance, and testing internal amplification controls.

Real-time PCR quantification approaches

Different real-time PCR approaches exist for the quantification of DNA targets. An approach frequently adopted for quantitation of meat content is one that is often referred to as "absolute" quantitation (ABI Bulletin 2, Pfaffl, 2004). Such an approach involves the use of calibration curves based on the difference in C_q values obtained from the use of serially diluted reference material. However, the use of calibration standards is only valid where species and tissue composition of the standard and test sample are matched exactly - which is not normally the case. In the absence of such an exact match, best measurement practice guidelines advocate the use of the best available standard materials (e.g. DNA extracted from control materials consisting of muscle tissue derived from authenticated meat species) in which to compare and standardise responses from. The use of a genome equivalent based approach for quantitation has been suggested as the best way to harmonise and standardise responses (Ballin *et al*., 2009).

Choice of PCR assay target

Dependent upon purpose (e.g. detection or quantitation), different kinds of DNA are used in PCR assays (Figure 4). PCR amplification of mitochondrial based targets has been extensively used in species determination (Herbert *et al*., 2003). All cell types contain multiple copies of mitochondria organelles which allow PCR methods to be developed which have low limits of detection (LOD) (Hird, Chisholm, and Brown, 2005b). However, the number of mitochondria can vary significantly between tissues (Rodriguez *et al*., 2003), species, and even with age, thus forestalling their full application for any quantitative methods (Hird *et al*., 2004). Current scientific literature (Laube *et al*., 2003, 2007a, 2007b; Ballin *et al*., 2009) favours the use of single copy nuclear DNA targets for its quantitative potential in terms of genome/genome equivalents, but with a compromised LOD compared to methods based on mitochondrial DNA.

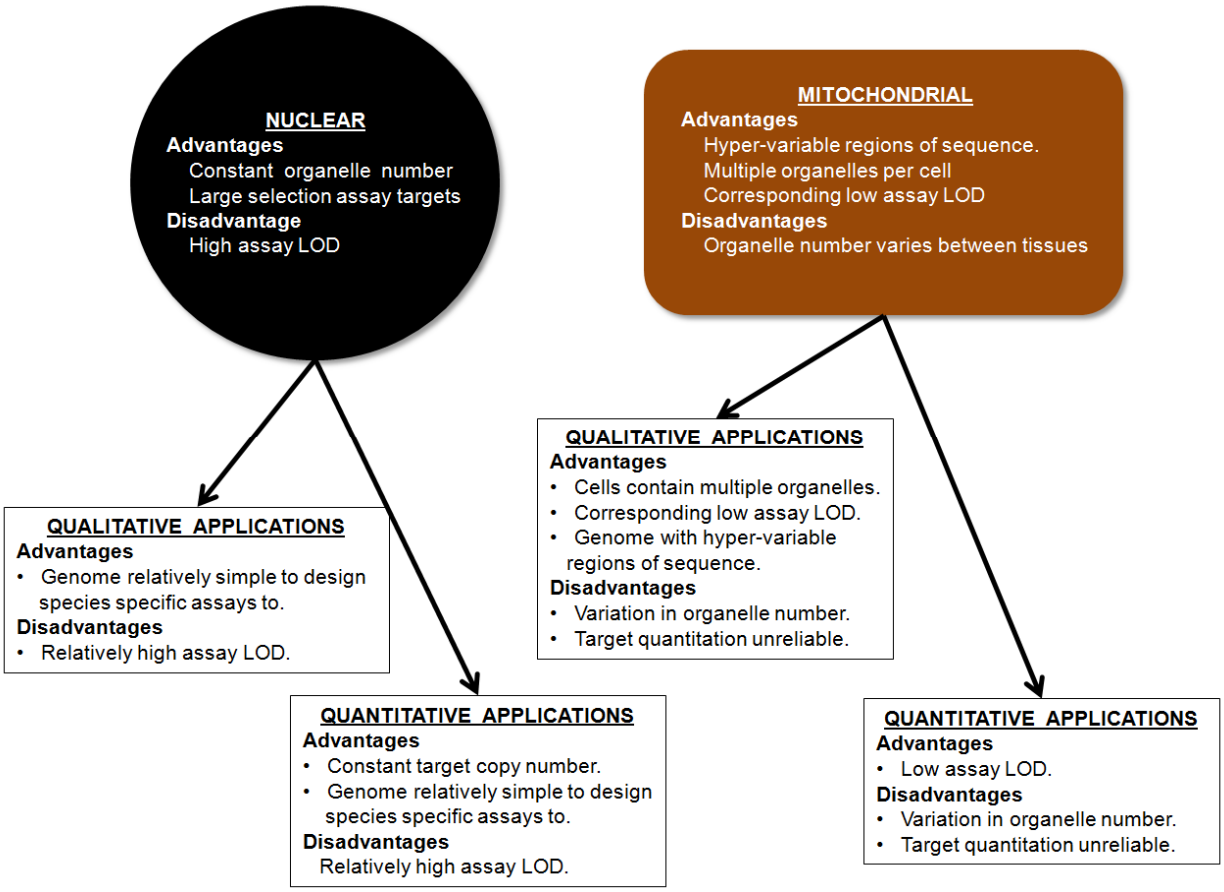


Figure 4. Advantages and disadvantages of different real-time PCR assay targets in qualitative and quantitative determination. Adapted from Ballin *et al*., 2009.

PCR assay optimisation

Optimisation of PCR assay conditions is essential to ensure satisfactory PCR efficiency. ABI User Bulletin 2 describes a method for determining the optimum primer and probe concentrations when inputting a fixed quantity of template. Employing an un-optimised PCR assay can result in an over or under quantification of the meat content.

PCR assay efficiency

It is important to establish that sample and calibrant are amplified with similar efficiencies, since unequal efficiencies lead to biased results. PCR efficiencies can be influenced by: the presence of PCR inhibitors; low template concentration; non-specific priming (presence of fragmented target); non-optimised assay (limiting reagents, particularly in multiplexed reactions). Detection of PCR inhibition can be assessed with the use of an internal control as described by Laube *et al.,* 2007b. The ABI bulletin 2 advises that the difference in slope employed to determine PCR efficiency should be less than 0.1 for assays to be comparable and usable for normalising data.

Assessment of PCR inhibition/DNA degradation

DNA degradation can result from sample processing (heat or shearing) or incorrect storage. A decrease in amplifiable DNA results in a relative increase of the mean C_q. Similarly, the presence of PCR inhibitors in the sample can influence the result in a similar way. To distinguish between PCR inhibition and DNA degradation, a duplex assay analysis should be performed which incorporates both a normalisation gene target plus some form of spiked in control. For DNA degradation the C_q value of the normalisation target will increase while the value for the spiked in control will remain constant. In the case of inhibition both values will increase. An appropriate correction factor, such as that described by Pfaffl (2004) can be employed to compensate if this is the case

Data normalisation

The normalisation strategy employed to correct for any induced measurement bias can have a significant impact on the data. Normalisation must be performed if sample treatment (processing) or the degree of DNA degradation present is suspected to be different between the test samples and the calibration standards employed. Appropriate normalisation factors can be determined by amplifying samples containing known amounts of DNA and comparing them to the amplification measured for DNA present in

a non-degraded control. The differences obtained in the results can then be used to derive an appropriate normalisation factor such as that described by Pfaffl (2004).

Summary

Many scientific experts in the field generally agree that the quantitative capability of meat speciation approaches can be best facilitated through using calibration curves based on expressions of genome:genome equivalents from single copy targets. Despite these reservations, current EU legislative guidelines remain adherent to the reporting of meat adulteration on a w/w basis.

Accurate quantification of adulterant meat content is not a straightforward process, and the conversion of DNA measurements (target copy number or mass) to the corresponding meat content values (w/w) can be prone to error. This is because there are a number of factors that can affect the expression of the amount of meat adulteration, and its conversion from DNA:DNA copies to w/w tissue. These factors include, but are not limited to, sample composition; DNA extraction approach; real-time PCR quantification approach; choice of DNA target; level of PCR optimisation; degree of PCR inhibition/DNA degradation; and data normalisation approaches. The impact and effect of each these factors cannot be predicted with certainty, and they all have the potential to bias the conversion of DNA:DNA copies to w/w tissue, either negatively or positively dependent upon the case being investigated.

Thus guidance has been provided on all the factors an analyst must consider when undertaking a conversion from target copy numbers to meat content (w/w) whilst recognising that it is impossible to achieve direct equivalence unless, as stated by best measurement practice guidelines that dictate that better accuracy can be afforded when the calibrants and test samples are of the same species, tissue, and ingredient type. The feasibility and likelihood of this occurring or being achieved in practice may be very low, so it is extremely important to stipulate the units of measurement used on the calibration line for estimating the meat content of a test sample, and how these units were derived.

5.0 Discussion

As part of the work described in this report, a current review of molecular approaches for the detection of horse DNA was conducted and identified a panel of potential DNA target sequences for use in the quantitation of horse meat in food samples using real-time PCR methodologies. These biomarkers included single copy targets (e.g. growth hormone gene) suited to quantitative analysis and multiple copy targets (e.g. mitochondrial cytochrome b and 12s rRNA genes) that enhance detection sensitivity at the cost of quantitative potential. Because the number of copies of mitochondrial DNA can vary between species, and between tissue types within a species, it was decided to focus on single copy nuclear DNA targets in order to afford the greatest potential for quantitation, in line with other expert views in the field (Ballin *et al*., 2009). Three assays were chosen for this purpose: Koppel *et al*., (2011) assay targeting the equine growth hormone receptor; Brodmann and Moor (2003) assay targeting the mammalian growth hormone gene and Laube *et al*., (2003) assay targeting the myostatin gene present in mammalian, poultry, and fish species.

A comprehensive strategy was implemented for the sourcing and preparation of test samples. Materials were sourced from reputable suppliers adhering to appropriate quality systems, and lean muscle tissue was used in the preparation of the standards and gravimetric materials. Species identity was confirmed through using multiple molecular approaches (PCR, ELISA and DNA sequencing) and measurement traceability was ensured throughout the process. Tissue type composition, sample homogeneity and sampling approach are closely interconnected and a good understanding of these issues is central to the accurate analyses of meat samples. The materials prepared for this study represent model samples with inherently low variability due to high muscle tissue content, good mixing procedures and relatively large sampling quantities. However, real-world samples are typically much more heterogeneous and derived from multiple meat tissue types which will affect levels of amplifiable template and expand the general uncertainty surrounding measurements.

This report describes the development of a real-time PCR approach for the quantitation of horse DNA, based on a simple dilution series of 100% horse DNA. The results have provided evidence that a relative quantitation based approach can accurately measure the amount of horse DNA present in a sample relative to the amount of beef DNA. When there is similarity in species, tissue type and ingredients between test samples and calibrants, this quantitation can also be extended to w/w gravimetric materials with acceptable levels of precision and bias. The qPCR assay performance characteristics associated with the Laube and Koppel qPCR assays were found to be suited to quantitative measurements as demonstrated by > 90% PCR efficiencies, LODs of around 5 genome equivalents, good dynamic range and low C_q standard deviations. Whilst the Koppel assay was known to exhibit some cross-reactivity with mule/donkey, both of these species are not common meat types, and the undeclared presence of horse/mule/donkey in a food sample is also non-compliant with the relevant EU legislation on food

labelling.

Inherently lower measurement variability is observed with DNA:DNA samples as compared to the w/w gravimetric materials due to issues such as sampling and sample homogeneity. Poorer bias and precision estimates are typically seen with the lower range of % w/w gravimetric materials, providing supportive evidence for this. It is known that 'real-world' samples would result in higher levels of measurement uncertainty due to problems such as sample heterogeneity and the complexity of matrices.

Assessing the impact of matrix interferents on quantitative performance is particularly important due to the complex nature of food matrices and the potential carryover of PCR inhibitors from the sample of extraction technique. There are a number of strategies available to help identify/minimise the impact of PCR inhibition, and these include the addition of positive internal controls, reference to standardised samples or simply monitoring performance metrics such as PCR efficiency. The impact of EDTA upon the horse RQ assay performance was illustrated by complete inhibition in the case of high concentrations (false negative) to a characteristic right shift in C_q value for lower EDTA levels (under estimating template levels). However, the impact of EDTA on assay performance was found to vary between the Koppel and Laube qPCR assays and therefore affect quantitative measurements. This observation highlights one of the limitations associated with any quantitative PCR approach, and is not confined to just the approaches described in this report. It is recommended that the effect of inhibitors co-extracted with test samples be further explored as additional work, in order to take into account, model, and explore the feasibility of mitigating the effect of PCR inhibition on PCR approaches involved in testing for food authenticity and adulteration.

As supported by general agreement of experts in the field and demonstrated through work previous to the current project, there is no direct level of equivalence for expressing the amount of meat adulteration present in terms of DNA:DNA copy numbers compared to w/w tissue mass, unless the test samples and calibrants are of exactly the same nature. The likelihood of this occurring in a test sample case is minimal. There are a number of factors that can affect the correct conversion of the meat adulteration expressed as DNA:DNA copies into w/w tissue mass, including sample composition; DNA extraction approach; real-time PCR quantification approach; choice of DNA target; level of PCR optimisation; degree of PCR inhibition/DNA degradation; and data normalisation approaches. The project has reviewed the current state of scientific developments and has provided guidance on a non exhaustive list of the factors that can affect quantitation of meat species on a w/w tissue basis that an analyst must be aware of when reporting quantitative sample results for meat authenticity.

Greater standardisation and guidance at an EU level with respect to the expression of the amount of meat adulteration in a sample will help provide a framework for more meaningful results and discussions to be had. The provision of a real-time PCR approach for the quantitation of horse DNA in the public domain, as described in this report, provides a solid foundation to help underpin and accurately measure the amount of horse DNA present relative to total mammalian DNA in a test sample. This accuracy in estimating the horse content of a sample can also be extended to w/w gravimetric materials when there is at least a partial match between species and tissue type present in test samples and calibrant. Finally, the EU has recently published guidance on the establishment of a threshold for the labelling of meat species, above which the presence of any undeclared meat species will be assumed to be due to deliberate adulteration (EURL-AP. 2013). The development and validation of an approach for the quantitation of horse DNA, as described in this report, has been so designed so as to be adaptable and "future-proofed" to accommodate and be compliant with the establishment of this threshold (be this on a DNA:DNA or a w/w tissue basis), and should be further tested using the (EURL-AP, 2013) guidance on threshold settings.

6.0 Main implications

The recent incident of the undeclared presence of horse-meat being detected in food products destined for human consumption, has emphasised both the need for the development of accurate analytical approaches for the detection of meat products, as well as the requirement for harmonised guidance on the expression of the amount of meat adulteration in a sample. Additionally, there is sparse information available in the peer reviewed literature regarding the accurate measurement of horse-meat relative to other species.

Therefore the current project has significantly added value to the current state of the art of the science by making available an approach for the detection and quantitation of horse DNA using an easily accessible assay. Given the recent EU wide issues of horse-meat being found in beef products intended for human consumption, the application of the assay is extremely topical and useful.

The work described in the current report has shown that accurate estimates of the amount of horse DNA relative to beef DNA can be made using a simple serial dilution series derived from 100% horse DNA, as qualified through validation of the performance characteristic of bias and precision. When the calibrant and test samples have at least a partial match in terms of species, tissue and ingredient composition, the

quantitative capabilities can also be extended to w/w gravimetric materials. In reality, the chances of test samples and calibrant being derived from the same sources will be extremely rare, thus full quantitation in terms of w/w tissue mass for all types of species/tissue composition cannot be achieved. This issue is attested to by other experts in the field and previous work commissioned by the FSA. However, the assay described in this report facilitates quantitation on a DNA:DNA basis, which many experts agree should be the way forward with respect to standardising how the relative level of different meats should be expressed in a sample, thus affording better standardisation and traceability. Additionally, the assay described in this report allows comparability to the equivalent w/w raw-horse meat in raw beef (meat) gravimetric materials. At the time of writing this report, EU guidance has been published (EURL-AP, 2013) regarding establishing a threshold value for meat species being present in a sample on a w/w basis (above which it is considered deliberate adulteration, and below which it is considered adventitious contamination). The approach described in this report has been so designed as to be adaptable enough to accommodate this threshold, and further testing should be conducted to apply the EU guidance to this quantitative approach to ensure that it can be used to determine if the level of horse DNA in a test sample is significantly above or below the equivalent w/w threshold level.

Experts in the field generally agree that it is impossible to cover all permutations in sample types and sample preparation to ever facilitate a direct conversion between w/w tissue mass and DNA:DNA copy numbers, when expressing the amount of meat adulteration present in a sample. However, this limitation is not unique to horse-meat detection and is equally applicable in all meat-speciation approaches and how PCR derived techniques are applied to meat-speciation issues in general. The issue does not lie with the technique itself, but more with the application and interpretation of the PCR technology and the sampling and extraction techniques. Greater guidance from the EU will help facilitate a more standardised approach to analysing and interpreting results from meat-speciation issues, and the work described in this report allows accurate quantitation of the relative amount of horse DNA present in a sample, and provides a solid frame-work for comparing this to the equivalent w/w of raw-horse meat in a raw beef background. Additionally, this report has provided guidance on a non exhaustive list of the factors that can affect quantitation of meat species on a w/w tissue basis that an analyst must be aware of when reporting quantitative sample results for meat authenticity.

The development and validation of a real-time PCR approach for the quantitation of horse DNA, as described in this report, will allow food companies to make decisions on their supply chain based on accurate results which will help to identify the source of the adulterant. Regulators will also be able to confidently enforce labelling laws in cases where this method identifies a non-compliant result. Having a fully quantitative method for the determination of horse DNA in beef based meat products will help regulators to enforce this UK/EU legislation and enable honest traders to robustly defend their food supply chain.

7.0 Possible future work

There are four main areas suggested for future work:

1) The EU has recently published guidance on the establishment of a threshold for labelling meat species (EURL-AP, 2103), above which the presence of an undeclared meat species is considered deliberate adulteration, and below which it is considered as adventitious contamination. In line with other current EU guidance on meat speciation, this threshold value is still expressed in terms of w/w tissue mass. The assay described in this report has been so designed to be adaptable and “future-proofed” in terms of its applicability to accommodate the different measurement scales. A gravimetric material can be rapidly and accurately prepared at LGC at the EU threshold level using authenticated material, and this can be used to challenge the calibration curve, and the measurement uncertainty in and around this value fully characterised. Any test sample can then be evaluated and compared to the measurement response and measurement uncertainty from the threshold sample, and the results can be characterised as being significantly above or below the threshold based on the equivalent w/w raw-horse meat in a raw beef (meat).

2) The work described in this paper has generated an SOP that can be used by any analytical laboratory. Whilst the repeatability of the approach has been validated and qualified at LGC, the reproducibility of the assay should be confirmed through a ring-trial of the approach amongst Official Control Laboratories and other stakeholders using different real-time PCR machines. A Knowledge Transfer event needs to be organised in order to disseminate best measurement practice guidelines to Public Analysts. Using the authenticated samples and gravimetric w/w materials prepared at LGC, these can then be sent to Public Analysts as blind samples and participants asked to evaluate the relative horse DNA content of the test samples. Results will be collated at LGC and a report given to Defra/FSA in order to qualify the fitness for purpose of the approach through its application by other analytical laboratories.

3) Proof of principle of the approach for the accurate quantitation of horse DNA using the real-time PCR

approach has been provided in this report. This was facilitated using model systems including DNA:DNA ad-mixtures and lean muscle tissue used to prepare w/w gravimetric materials of raw horse-meat in a raw beef (meat) background. The approach allows the instrument measurement response from a test sample to be equated to the equivalent response from a w/w raw-horse meat in a raw beef (meat) gravimetric material. It will thus be useful to apply the approach to a representative range of mixed tissue type w/w ad-mixtures and real-life commercial samples (e.g. mice-meat; beef burgers; lasagnes; stock-cubes) identified as containing horse-meat as part of the original UK horse-meat survey of beef products. Additionally, work should be conducted to characterise and model the effects of processing by procuring bulk quantities of horse and beef meat and subject the meats to different processing conditions. It would also be beneficial to evaluate the effect of different levels of additives and cooking preparations.

4) Inhibition through the presence of impurities co-extracted with the DNA template is a common issue that can affect the measurement response of any PCR approach employed for analytical work. Preliminary studies associated with the assay described in this report has shown the potential detrimental effect inhibitors can have on the quantitative performance of a PCR assay. Further work to model the likely effect of inhibitors, and ways to control and mitigate such effects in the interpretation of results, should be examined in order to assess the likely impact, and to help provide solutions to this problem that can affect the application of any molecular biology approach.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

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Appendices

Appendix I – Statement of measurement - raw horse-meat material (7220)

Appendix II – Statement of measurement – raw horse-meat in beef (7241)

Appendix III - SOP